

# Glycine receptor $\alpha 3$ and $\alpha 2$ subunits mediate tonic and exogenous agonist-induced currents in forebrain

Lindsay M. McCracken<sup>a,1</sup>, Daniel C. Lowes<sup>b,1</sup>, Michael C. Salling<sup>a</sup>, Cyndel Carreau-Vollmer<sup>a</sup>, Naomi N. Odean<sup>a</sup>, Yuri A. Blednov<sup>c</sup>, Heinrich Betz<sup>d</sup>, R. Adron Harris<sup>c</sup>, and Neil L. Harrison<sup>a,b,2</sup>

<sup>a</sup>Department of Anesthesiology, Columbia University College of Physicians and Surgeons, New York, NY 10032; <sup>b</sup>Department of Pharmacology, Columbia University College of Physicians and Surgeons, New York, NY 10032; <sup>c</sup>The Waggoner Center for Alcohol and Addiction Research, The University of Texas at Austin, Austin, TX 78712; and <sup>d</sup>Max Planck Institute for Medical Research, 69120 Heidelberg, Germany

Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved July 17, 2017 (received for review March 14, 2017)

Neuronal inhibition can occur via synaptic mechanisms or through tonic activation of extrasynaptic receptors. In spinal cord, glycine mediates synaptic inhibition through the activation of heteromeric glycine receptors (GlyRs) composed primarily of  $\alpha 1$  and  $\beta$  subunits. Inhibitory GlyRs are also found throughout the brain, where GlyR  $\alpha 2$  and  $\alpha 3$  subunit expression exceeds that of  $\alpha 1$ , particularly in forebrain structures, and coassembly of these  $\alpha$  subunits with the  $\beta$  subunit appears to occur to a lesser extent than in spinal cord. Here, we analyzed GlyR currents in several regions of the adolescent mouse forebrain (striatum, prefrontal cortex, hippocampus, amygdala, and bed nucleus of the stria terminalis). Our results show ubiquitous expression of GlyRs that mediate large-amplitude currents in response to exogenously applied glycine in these forebrain structures. Additionally, tonic inward currents were also detected, but only in the striatum, hippocampus, and prefrontal cortex (PFC). These tonic currents were sensitive to both strychnine and picrotoxin, indicating that they are mediated by extrasynaptic homomeric GlyRs. Recordings from mice deficient in the GlyR  $\alpha 3$  subunit (*Gla3*<sup>-/-</sup>) revealed a lack of tonic GlyR currents in the striatum and the PFC. In *Gla2*<sup>-Y</sup> animals, GlyR tonic currents were preserved; however, the amplitudes of current responses to exogenous glycine were significantly reduced. We conclude that functional  $\alpha 2$  and  $\alpha 3$  GlyRs are present in various regions of the forebrain and that  $\alpha 3$  GlyRs specifically participate in tonic inhibition in the striatum and PFC. Our findings suggest roles for glycine in regulating neuronal excitability in the forebrain.

glycine receptor | tonic inhibition | Cys-loop receptor | strychnine | alpha subunits

GABA and glycine are the major inhibitory neurotransmitters in the mammalian CNS. The classical form of neuronal inhibition results from the brief release of these transmitters at synapses, resulting in a transient hyperpolarization and/or conductance increase that serves to decrease action potential generation. Synaptic inhibition is essential to the function of local circuits and is involved in both the timing and synchronization of oscillations within neuronal networks (1, 2). Tonic inhibition is a second form that results from continuous activation of extrasynaptic receptors (3). Tonic inhibition serves to regulate the excitability of individual neurons and can alter both threshold and gain in response to excitatory inputs (4, 5). GABAergic tonic inhibition in the brain has been well documented and is mediated by a distinct class of GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) in regions such as the striatum (6–8), hippocampus (9, 10), thalamus (11, 12), and cerebellum (13).

In the spinal cord, both GABA and glycine mediate synaptic inhibition. Glycine was first identified as the inhibitory transmitter at the Renshaw cell–motoneuron synapse, where it produces inhibition via strychnine-sensitive glycine receptors (GlyRs) (14–17). GlyRs also mediate synaptic inhibition in the auditory brainstem (18–20) and extrasynaptic inhibition in the dorsal raphe (21). There have been a number of reports of glycine responses and expression of strychnine-sensitive GlyRs in higher brain regions (22–27). However, the nature and function of these receptors have remained obscure.

Synaptic GlyRs are heteropentamers consisting of different  $\alpha$  subunits ( $\alpha 1$ – $\alpha 4$ ) coassembled with the  $\beta$  subunit (28), which is obligatory for synaptic localization due to its tight interaction with the anchoring protein gephyrin (29). GlyR  $\alpha$  subunits exist in many higher brain regions (30) and may include populations of homopentameric GlyRs expressed in the absence of  $\beta$  subunits (31, 32).

Although the physiology of the glycinergic system is not thoroughly defined in the mammalian brain, several studies have linked alterations in GlyR genes to human diseases. For example, polymorphisms resulting in single amino acid mutations in both GlyR  $\alpha$  and  $\beta$  subunits have been identified in human hyperkplexia (startle disease) (33). In addition, differential splicing of the human GlyR  $\alpha 3$  subunit gene (*Gla3*) and changes in the expression of high-affinity (RNA-edited) GlyR  $\alpha 2$  and  $\alpha 3$  subunits have been observed in cases of intractable temporal lobe epilepsy (32, 34). More recently, the *Gla3* gene has been genetically linked to alcoholism (35), and *Gla3*<sup>-/-</sup> mice have been found to show increased ethanol preference in a two-bottle choice model, while *Gla2*<sup>-Y</sup> animals display the opposite behavior (36). Together these findings highlight the importance of better understanding the in vivo functions of inhibitory GlyRs in the brain to unravel their roles in pathophysiological and behavioral processes.

Given the widespread distribution of GlyR  $\alpha 2$  and  $\alpha 3$  mRNAs in the forebrain (37), we tested the hypothesis that these subunits form functional GlyRs that might be responsible for inhibitory

## Significance

Neuronal inhibition in the CNS occurs via two mechanisms. Synaptic inhibition is transient and localized; extrasynaptic inhibition is continuous and diffuse. Glycine receptor (GlyR)-mediated synaptic inhibition in spinal cord was established decades ago; however, GlyR physiology in higher brain regions remains obscure. We show that functional GlyRs are expressed in forebrain structures and mediate tonic and exogenous glycine-induced currents. Unlike synaptic  $\alpha 1\beta$  GlyRs in the spinal cord, forebrain GlyRs are primarily composed of  $\alpha 2$  or  $\alpha 3$  subunits. We identified distinct roles for these subunits by showing that  $\alpha 3$  subunits mediate tonic GlyR currents, whereas  $\alpha 2$  subunits mediate exogenously activated GlyR currents. Our findings demonstrate mechanisms of neuronal modulation by glycine in the forebrain due to subunit-specific contributions of GlyRs.

Author contributions: L.M.M., D.C.L., M.C.S., Y.A.B., H.B., R.A.H., and N.L.H. designed research; L.M.M., D.C.L., M.C.S., C.C.-V., and N.N.O. performed research; L.M.M., D.C.L., M.C.S., C.C.-V., and N.N.O. analyzed data; and L.M.M., D.C.L., M.C.S., H.B., R.A.H., and N.L.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

<sup>1</sup>L.M.M. and D.C.L. contributed equally to this work.

<sup>2</sup>To whom correspondence should be addressed. Email: nh2298@cumc.columbia.edu.

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1703839114/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1703839114/-DCSupplemental).

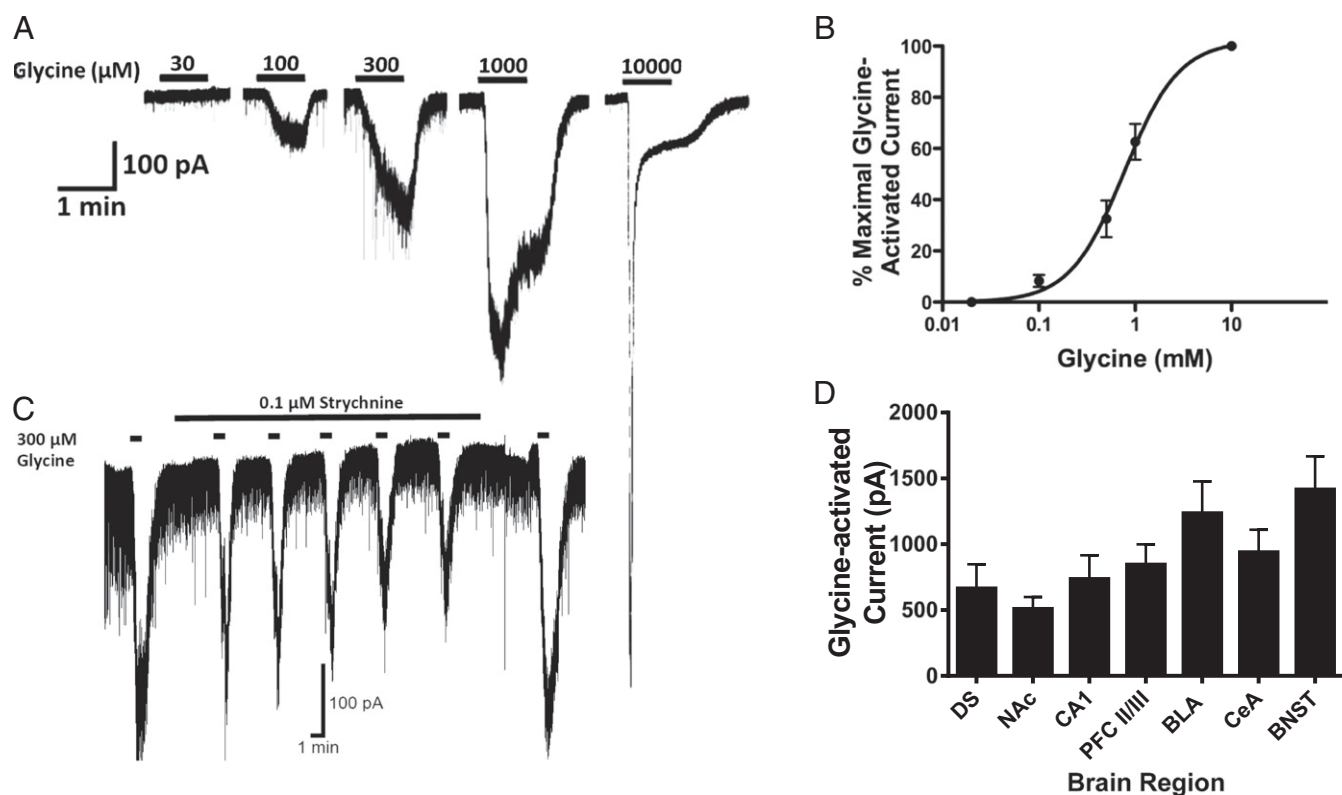
tonic currents. We show that functional strychnine-sensitive GlyRs are widely expressed in the adolescent and young adult mouse brain and that they mediate both large-amplitude responses to exogenous glycine and tonic currents of 10–20 pA amplitude that are similar to tonic GABA currents. The use of *Gla2*<sup>-Y</sup> and *Gla3*<sup>-/-</sup> strains of mice allowed us to identify the involvement of these particular  $\alpha$  subunits in endogenous and exogenous responses to glycine in the forebrain. Our findings suggest roles for distinct GlyR isoforms in mediating the effects of glycine on neuronal excitability in higher brain regions.

## Results

**Demonstration of Exogenously Activated GlyR Currents in Various Brain Regions.** To confirm the presence of functional GlyRs in brain, we measured whole-cell glycine-activated currents *in vitro* in various regions of the forebrain in adolescent and young adult mice between postnatal days 21 and 50 (P21–P50) using an intracellular solution with increased chloride concentration to facilitate the detection of chloride currents. While the expression of GlyR subunits is known to change, especially during the early postnatal period (28, 37), we observed no significant differences in glycine responses over the age range studied. Fig. 1*A* shows the currents elicited by a series of glycine applications (30  $\mu$ M–10 mM) in medium spiny neurons (MSNs) in the nucleus accumbens (NAc), and Fig. 1*B* demonstrates that these glycine-activated currents are concentration-dependent. In addition, we confirmed that the currents were mediated by strychnine-sensitive GlyRs and not by activation of other neuro-

transmitter receptors. Fig. 1*C* shows that the currents elicited by 300  $\mu$ M glycine were reduced by 100 nM strychnine, which is a GlyR-specific antagonist with little or no known effects at other receptors at this concentration (38). Furthermore, neither 20  $\mu$ M gabazine nor 50  $\mu$ M D(-)-2-amino-5-phosphonopentanoic acid (AP5) affected the amplitude of currents elicited by either 300  $\mu$ M or 1 mM glycine, eliminating the possibility that the currents measured were due to glycine action at either GABA<sub>A</sub> or NMDA receptors (Fig. S1). Notably, the effects of both glycine and low concentrations of strychnine were completely reversible in these experiments.

We also investigated GlyR currents in a number of other brain regions, including the dorsal striatum, hippocampus, prefrontal cortex (PFC), amygdala, and bed nucleus of the stria terminalis (BNST). Whole-cell currents were first measured in response to an application of 1 mM glycine. Fig. 1*D* shows that, in addition to the NAc, large-amplitude (1–2 nA) GlyR currents were detected in all the other brain regions studied. Glycine concentration–response curves were generated for each region, and values for the maximal glycine-activated currents ( $I_{max}$ ) and glycine EC<sub>50</sub> are shown in Table 1. It should be noted here that the glycine EC<sub>50</sub> values presented in Table 1 are larger than those determined for recombinant GlyRs in heterologous expression systems, such as *Xenopus* oocytes. We attribute this to the presence of diffusional barriers and the expression of functional glycine transporters (GlyTs) in slice preparations. Hence the glycine concentrations actually present at the receptor sites are likely to be much lower than those applied with the perfusion medium. Consistent with



**Fig. 1.** Exogenous glycine-activated GlyR currents in the forebrain. (*A* and *B*) Whole-cell currents elicited by exogenous glycine applications (30  $\mu$ M–10 mM) were recorded from MSNs *in vitro* in the NAc (*A*), and glycine concentration–response curves were generated (*B*), confirming the presence of measurable glycine-activated currents. (*C*) To demonstrate that the currents measured in *A* and *B* were due to glycine action at strychnine-sensitive GlyRs and not other neurotransmitter receptors, whole-cell currents elicited by glycine were first measured in the absence and then in the presence of a concurrent application of the GlyR-specific antagonist strychnine (100 nM). The amplitude of current produced by applications of 300  $\mu$ M glycine was reduced in the presence of strychnine. (*D*) Currents elicited by 1 mM glycine were measured in several brain regions. Glycine-activated currents were present in the dorsolateral striatum (DS), NAc, hippocampus (CA1), layer II/III of the medial PFC, basolateral amygdala (BLA), central nucleus of the amygdala (CeA), and the BNST. Data represent means  $\pm$  SEM ( $n = 8$ –26 neurons from 7–11 mice).

**Table 1. Glycine EC<sub>50</sub> and maximal currents**

Brain region	Glycine EC <sub>50</sub> , $\mu$ M	I <sub>max</sub> , pA
Amygdala	892 $\pm$ 24	706 $\pm$ 181
Hippocampus	418 $\pm$ 81	1,126 $\pm$ 274
BNST	1,011 $\pm$ 91	2,264 $\pm$ 503
Dorsal striatum	598 $\pm$ 21	1,050 $\pm$ 155
NAC	748 $\pm$ 32	1,334 $\pm$ 326
PFC	674 $\pm$ 32	1,063 $\pm$ 102

Glycine responses in different regions of the mouse forebrain. I<sub>max</sub> and EC<sub>50</sub> values were determined from concentration–response curves obtained by neuronal recordings in each of the brain regions indicated. Data represent mean  $\pm$  SEM ( $n = 5$ –12 neurons from three to five mice).

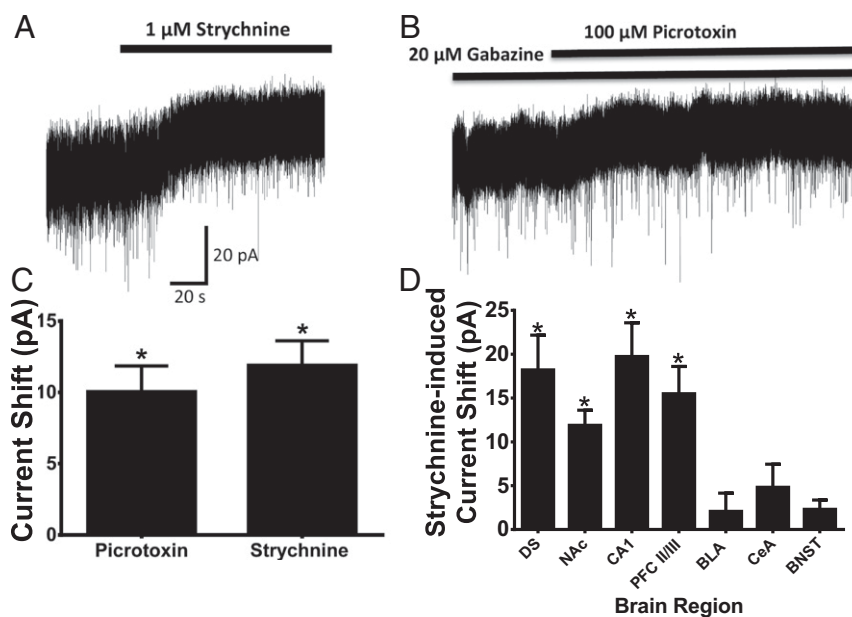
this view, our recordings in the presence of the GlyT inhibitor sarcosine demonstrate that, in the absence of externally applied glycine, GlyT inhibition results in quite large “tonic” currents, presumably by allowing local glycine concentrations to rise to levels sufficient for receptor activation (see Fig. S4).

**Identification of Endogenously Activated Tonic GlyR Currents.** To identify tonic GlyR currents in brain, we measured the shift from baseline holding current in response to the GlyR antagonist strychnine. In the dorsal striatum, NAc (Fig. 2 *A* and *C*), PFC (layer II/III), and hippocampus (CA1) 1  $\mu$ M strychnine produced significant shifts in the holding current (Fig. 2*D*). In contrast to the GABA<sub>A</sub> and AMPA receptor antagonists gabazine and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX), strychnine did not affect the mean amplitude or frequency of postsynaptic potentials (Fig. S2). We therefore conclude that these

shifts in the holding current in response to strychnine were due to the blockade of extrasynaptic GlyRs. In contrast, strychnine had no significant effect on the holding current of neurons in the basolateral amygdala or central nucleus of the amygdala or in the BNST (Fig. 2*D*), indicating the absence of tonic GlyR currents in these regions despite robust responses to applied glycine (Fig. 1*D*). Interestingly, the amplitude of the tonic currents observed in different brain regions had a significant negative correlation with the EC<sub>50</sub> values estimated for applied glycine [ $r(4) = -0.96$ ,  $P = 0.0028$ ], which suggests that a common mechanism may contribute to regional differences in agonist potency and tonic activation of GlyRs.

Although strychnine has been reported to inhibit  $\alpha 7$  nicotinic acetylcholine receptors (nAChRs) at micromolar concentrations (39), 1  $\mu$ M strychnine produced significantly larger shifts in the holding current of NAc neurons than 10 or 100 nM concentrations of the  $\alpha 7$  nAChR-specific inhibitor methyllycaconitine; this is consistent with the strychnine-induced shifts in the holding current being due to the block of a GlyR-mediated conductance (Fig. S3). Furthermore, preapplication of the glycine transporter inhibitor sarcosine (500  $\mu$ M) enhanced the strychnine-sensitive current, supporting our conclusion that these strychnine-induced shifts in the holding current are mediated by glycine acting at GlyRs (Fig. S4).

To determine if the tonic currents were due to activation of homo- or heteromeric GlyRs, we measured the effects of picrotoxin on the whole-cell baseline holding currents of MSNs in the NAc. Picrotoxin is ineffective as a channel blocker at GlyR  $\alpha\beta$  heteromers at concentrations  $\leq 100$   $\mu$ M (40) but is a non-competitive antagonist at GABA<sub>A</sub>Rs and homomeric GlyRs. To isolate the effects of picrotoxin at GlyRs, picrotoxin was applied



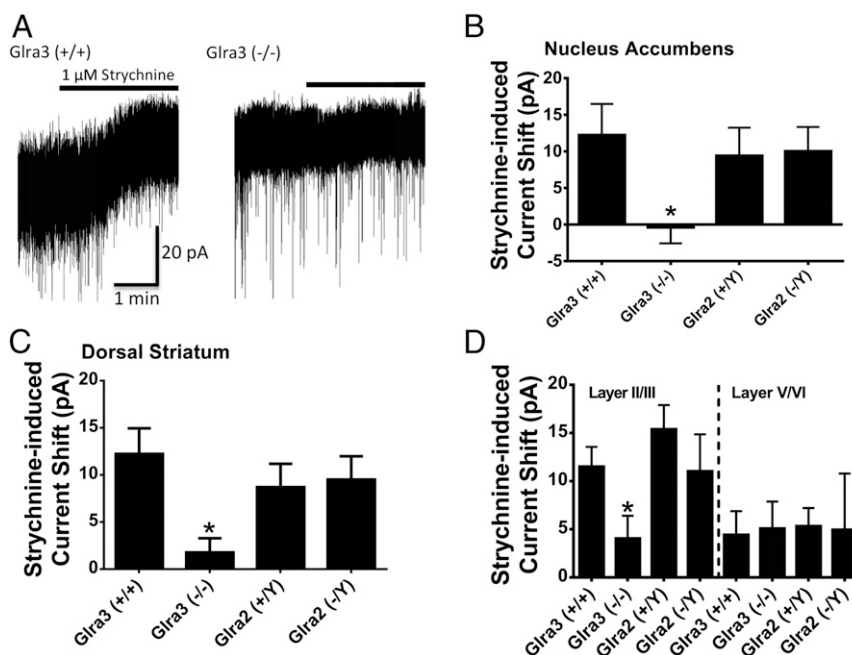
**Fig. 2.** Endogenous tonically activated GlyR currents in brain. (*A*) To identify tonic GlyR currents in MSNs in the nucleus accumbens, the effects of 1  $\mu$ M strychnine on baseline holding currents were measured. (*B*) To determine whether tonic GlyR currents are due to activation of  $\alpha$  homomers, the effects of 100  $\mu$ M picrotoxin (in the presence of gabazine) on baseline holding currents were determined. (*C*) Strychnine induced significant shifts in the holding current of MSNs [ $t(42) = 6.872$ ,  $P < 0.0001$ ], indicating the presence of a tonic GlyR conductance. Similarly, picrotoxin applications induced significant shifts in holding currents [ $t(5) = 5.52$ ,  $P = 0.003$ ]. The amplitudes of the current shifts induced by strychnine and picrotoxin were not significantly different from each other [ $t(47) = 0.39$ ,  $P = 0.70$ ];  $n = 6$ –43 neurons from 3–18 mice. (*D*) To identify tonic GlyR currents in each of the brain regions in which exogenous glycine-activated currents were detected, the effects of 1  $\mu$ M strychnine on baseline holding currents were measured. Significant strychnine-induced shifts in holding current were detected in the dorsolateral striatum (DS) [ $t(17) = 4.59$ ,  $P = 0.0003$ ], NAc [ $t(42) = 6.87$ ,  $P < 0.0001$ ], hippocampus (CA1) [ $t(6) = 5.12$ ,  $P = 0.0022$ ], and PFC (layer II/III) [ $t(11) = 4.97$ ,  $P = 0.0004$ ], indicating the presence of tonic GlyR currents in these regions. In contrast, there was no effect of strychnine on the holding current of neurons recorded from the basolateral amygdala (BLA) [ $t(9) = 1.00$ ,  $P = 0.34$ ], central nucleus of the amygdala (CeA) [ $t(6) = 1.82$ ,  $P = 0.12$ ], or BNST [ $t(10) = 2.15$ ,  $P = 0.06$ ], indicating the absence of tonic GlyR currents in these regions. Data are presented as means  $\pm$  SEM ( $n = 7$ –43 neurons from 5–18 mice); \* $P < 0.05$ .

concurrently with the GABA<sub>A</sub>R-specific antagonist gabazine, as has been done previously (41). While it has been suggested that gabazine is unable to unmask tonic GABA<sub>A</sub>R-mediated currents, we have previously used this concentration to reveal tonic GABA<sub>A</sub>R conductance in the thalamus (42). Here, we found that applications of picrotoxin in combination with gabazine produced significant shifts in the holding currents of MSNs (Fig. 2*B*). Significant shifts in the holding currents of MSNs were detected in response to applications of 100  $\mu$ M picrotoxin, and the amplitudes of the picrotoxin- and strychnine-induced shifts in holding current were not significantly different (Fig. 2*B* and *C*), suggesting that these tonic GlyR currents are mediated by homomeric receptors composed of  $\alpha$  subunits. Thus, it appears that tonic currents in MSNs can be generated by both GABA<sub>A</sub>Rs (6) and homomeric GlyRs.

The endogenous tonic GlyR currents we identified may contribute to the regulation of neuronal excitability. To investigate the role of these tonic currents, we performed current-clamp excitability experiments on MSNs in the NAC before and after the administration of 1  $\mu$ M strychnine. Because the equilibrium potential for chloride,  $E_{Cl}$ , is depolarized relative to the resting membrane potential (RMP) in MSNs, our high internal  $[Cl^-]$  intracellular solution to some extent mimics the normal physiological condition in these neurons. While the input resistance did not significantly change with the application of strychnine, strychnine hyperpolarized the RMP and increased the rheobase of neurons (Fig. S5), both changes being consistent with a decrease in excit-

ability upon addition of strychnine. These results suggest that tonic activation of GlyRs can regulate the excitability of neurons and show that a tonic glycine conductance can actually enhance MSN excitability at membrane potentials close to the RMP.

***Gla3*<sup>-/-</sup> Mice Lack Tonic GlyR Currents in the Forebrain.** Following the identification of tonic GlyR currents in the forebrain, a major goal of our study was to ascertain the  $\alpha$  subunit composition of the GlyRs involved. To accomplish this, whole-cell recordings were performed in brain slices from two lines of GlyR  $\alpha$  subunit-deficient mice, *Gla2*<sup>-Y</sup> and *Gla3*<sup>-/-</sup>, and their WT littermates. **Striatum.** In both *Gla2*<sup>+Y</sup> and *Gla2*<sup>-Y</sup> mice, MSNs in the NAC exhibited tonic GlyR currents, and the amplitude of these currents was not significantly different between genotypes. In contrast, applications of strychnine produced shifts in holding current in MSNs from *Gla3*<sup>+/+</sup> mice, but this effect was nearly abolished in MSNs from *Gla3*<sup>-/-</sup> mice, indicating that  $\alpha 3$ , but not  $\alpha 2$ , subunits are necessary for tonic GlyR currents in the NAC (Fig. 3*A* and *B*). Whole-cell recordings in dorsal striatum revealed the presence of tonic GlyR currents in both *Gla2*<sup>+Y</sup> and *Gla2*<sup>-Y</sup> mice, and the amplitude of the currents was not significantly different between WT and KO mice (Fig. 3*C*). In contrast, strychnine had no effect on the holding current of MSNs in the dorsal striatum from *Gla3*<sup>-/-</sup> mice but produced shifts in the holding current of MSNs from *Gla3*<sup>+/+</sup> mice (Fig. 3*C*), indicating that tonic GlyR currents in dorsal striatum are mediated by  $\alpha 3$  subunit-containing GlyRs.



**Fig. 3.** Tonic GlyR currents in forebrain are mediated by  $\alpha 3$  subunit-containing GlyRs. The effects of strychnine on baseline holding currents were measured and compared in *Gla2*<sup>-Y</sup> and *Gla3*<sup>-/-</sup> mice and their WT littermates. Whole-cell recordings were performed in the striatum and PFC, regions in which significant tonic GlyR currents were identified in C57/BL6J mice. (A) Sample tracings demonstrating the effects of 1  $\mu$ M strychnine on the holding currents of MSNs in the NAC of *Gla3*<sup>+/+</sup> (Left) and *Gla3*<sup>-/-</sup> (Right) mice. (B and C) Strychnine-induced shifts in the holding currents of MSNs in the NAC ( $n = 4$ –10 cells from three to seven mice) (B) and dorsal striatum ( $n = 6$ –9 cells from four to six mice) (C) of *Gla3*<sup>+/+</sup> mice, indicating the presence of a tonic GlyR current [NAC:  $t(14) = 2.93$ ,  $P = 0.011$ ; dorsal striatum:  $t(11) = 3.58$ ,  $P = 0.004$ ]; however, no significant shift was observed in *Gla3*<sup>-/-</sup> mice. Strychnine-induced shifts in holding current were detected in MSNs in the NAC (B) and dorsal striatum (C) of both *Gla2*<sup>+Y</sup> and *Gla2*<sup>-Y</sup> mice. The amplitude of the tonic GlyR current was not significantly different between the two genotypes in either region of the striatum [NAC:  $t(10) = 0.12$ ,  $P = 0.91$ ; dorsal striatum:  $t(16) = 0.24$ ,  $P = 0.81$ ]. (D) In the medial prefrontal cortex, applications of 1  $\mu$ M strychnine produced shifts in the holding currents of layer II/III pyramidal neurons in *Gla3*<sup>+/+</sup> mice; however, as in the striatum, this effect of strychnine was absent in neurons from *Gla3*<sup>-/-</sup> mice [ $t(15) = 2.40$ ,  $P = 0.029$ ]. GlyR tonic currents were detected in neurons recorded from both *Gla2*<sup>+Y</sup> and *Gla2*<sup>-Y</sup> mice, and the amplitude of the strychnine-induced currents was not significantly different between the genotypes [ $t(15) = 0.97$ ,  $P = 0.35$ ]. In contrast to layer II/III, there were no significant effects of strychnine on the baseline holding currents of pyramidal neurons in layer V/VI in any of the mouse genotypes tested, suggesting the lack of an appreciable tonic GlyR current in this cortical layer. Data represent means  $\pm$  SEM ( $n = 6$ –9 neurons from three to seven mice; \* $P < 0.05$  compared with WT littermates).

**PFC.** Applications of strychnine produced significant shifts in the baseline holding currents of pyramidal neurons in layer II/III of the PFC in *Gla2<sup>+Y</sup>*, *Gla2<sup>-Y</sup>*, and *Gla3<sup>+/+</sup>* mice and confirmed the presence of tonic GlyR currents (Fig. 3D). The effects of strychnine on the holding current of pyramidal neurons were strongly reduced in *Gla3<sup>-/-</sup>* mice, indicating that, as in the striatum, tonic GlyR currents in layer II/III of the PFC are mediated by  $\alpha 3$  subunit-containing GlyRs. Notably, there was no difference in the effect of strychnine on the holding current of layer V/VI pyramidal neurons between the mouse genotypes examined, suggesting that there is not an appreciable tonic GlyR conductance in this area of the PFC.

#### ***Gla2<sup>-Y</sup>* Mice Have Reduced Exogenous Glycine-Activated Currents.**

In addition to examining differences in tonic GlyR currents in *Gla2<sup>-Y</sup>* and *Gla3<sup>-/-</sup>* and WT mice, we measured whole-cell currents in response to exogenous applications of glycine.

**Striatum.** Fig. 4A and B shows significantly reduced amplitudes of glycine-induced currents in MSNs of NAc from *Gla2<sup>-Y</sup>* compared with *Gla2<sup>+Y</sup>* mice. In contrast, the amplitude of glycine-activated currents in MSNs was not significantly different between *Gla3<sup>+/+</sup>* and *Gla3<sup>-/-</sup>* mice. These results indicate that  $\alpha 2$  subunit-containing GlyRs mediate the large-amplitude currents elicited by exogenous glycine in the ventral striatum.

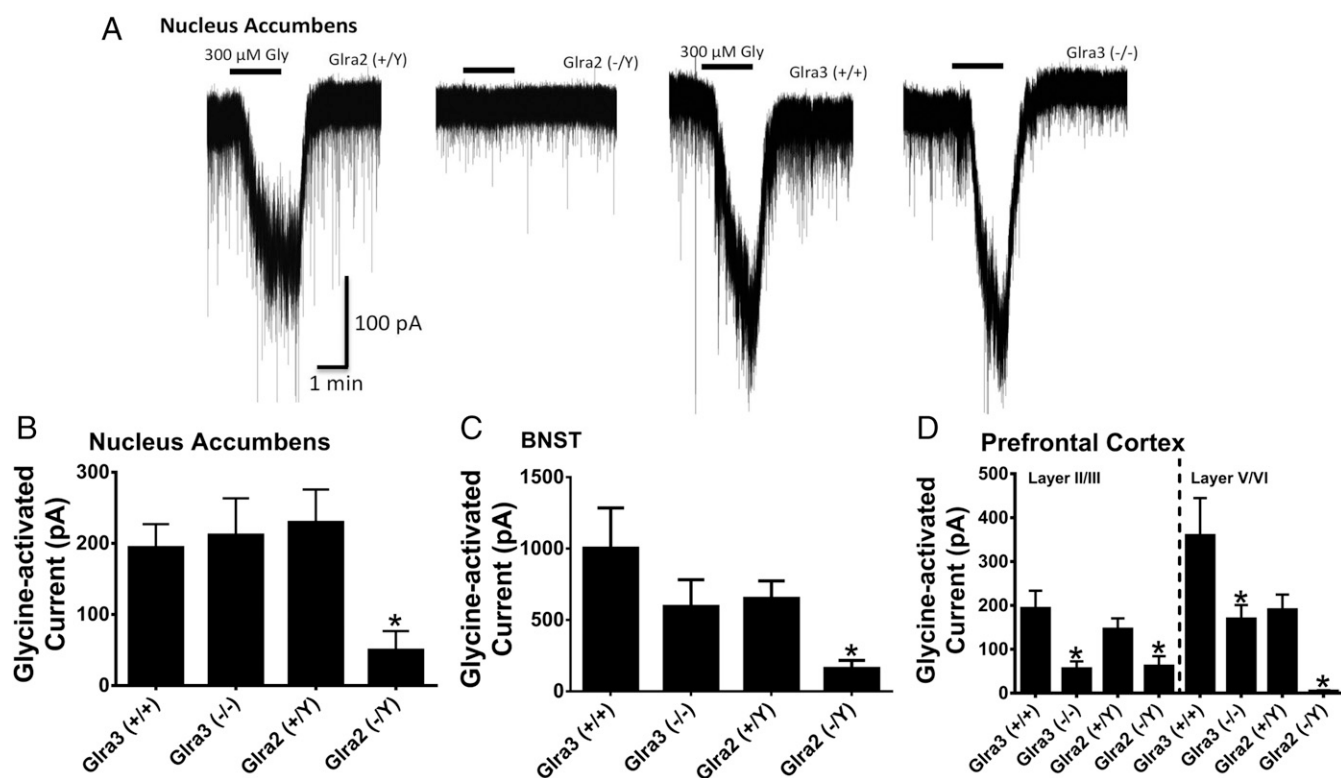
**BNST.** As in the striatum, the current amplitudes recorded in response to 300  $\mu$ M glycine from neurons in the dorsal and ventral

BNST of *Gla2<sup>-Y</sup>* mice were significantly reduced compared with those recorded from *Gla2<sup>+Y</sup>* mice (Fig. 4C). In contrast, the difference between the current amplitudes recorded in *Gla3<sup>+/+</sup>* and *Gla3<sup>-/-</sup>* mice was not statistically significant (Fig. 4C), suggesting that the glycine-activated currents in the BNST are also primarily mediated by  $\alpha 2$  subunit-containing GlyRs.

**PFC.** Consistent with our findings in the striatum and BNST, the current responses in cortical pyramidal neurons elicited by exogenous application of 300  $\mu$ M glycine were significantly reduced in layer II/III and essentially abolished in layer V/VI in *Gla2<sup>-Y</sup>* mice compared with *Gla2<sup>+Y</sup>* mice (Fig. 4D). However, in contrast to the results obtained in the striatum, the amplitudes of these currents were also significantly reduced in neurons from both cortical layers of *Gla3<sup>-/-</sup>* mice compared with those from WT littermates (Fig. 4D). Thus, it is possible that both  $\alpha 2$  and  $\alpha 3$  GlyRs contribute to the large-amplitude glycine-activated currents in the PFC.

#### **Discussion**

Strychnine-sensitive GlyRs, expressed throughout the brain and spinal cord, are chloride-conducting Cys-loop receptors with an established role in mediating inhibitory neurotransmission in spinal cord (43) and a less well-defined role in the physiology of higher brain regions. In the present study, we first confirmed the presence of specific GlyR-mediated currents in the PFC, striatum,



**Fig. 4.** Large-amplitude glycine-activated currents are mediated by  $\alpha 2$  subunit-containing GlyRs in brain. (A) Examples of whole-cell GlyR currents elicited by exogenous applications of glycine recorded from MSNs in the NAc of *Gla2<sup>-Y</sup>* and *Gla3<sup>-/-</sup>* and WT mice. (B) Glycine (300  $\mu$ M) produced currents of significantly reduced amplitude in MSNs in the NAc of *Gla2<sup>-Y</sup>* compared with *Gla2<sup>+Y</sup>* mice [ $t(17) = 3.03$ ,  $P = 0.007$ ]. In contrast, the amplitude of current in MSNs elicited by 300  $\mu$ M glycine was not significantly different between *Gla3<sup>+/+</sup>* and *Gla3<sup>-/-</sup>* mice [ $t(19) = 0.29$ ,  $P = 0.77$ ];  $n = 8$ –11 cells from three to four mice. (C) Although no tonic GlyR currents were detected in the BNST, GlyR currents were measured in response to exogenous applications of glycine. As in the striatum, the amplitude of current in BNST neurons in response to 300  $\mu$ M glycine was significantly reduced in *Gla2<sup>-Y</sup>* compared with *Gla2<sup>+Y</sup>* mice [ $t(21) = 2.67$ ,  $P = 0.014$ ] but was not significantly different between neurons recorded from *Gla3<sup>+/+</sup>* and *Gla3<sup>-/-</sup>* mice [ $t(7) = 0.95$ ,  $P = 0.37$ ];  $*P < 0.05$ ;  $n = 4$ –12 cells from two to four mice. (D) Similarly, in the PFC, the amplitude of glycine-activated currents in *Gla2<sup>-Y</sup>* mice was significantly reduced compared with *Gla2<sup>+Y</sup>* mice in pyramidal neurons in both layer II/III [ $t(30) = 2.48$ ,  $P = 0.019$ ] and layer V/VI [ $t(20) = 4.38$ ,  $P = 0.0003$ ]; however, unlike in striatum and BNST, the amplitude of glycine-activated currents was also significantly reduced in *Gla3<sup>-/-</sup>* compared with *Gla3<sup>+/+</sup>* mice in both layer II/III [ $t(28) = 3.42$ ,  $P = 0.0019$ ] and layer V/VI [ $t(26) = 2.41$ ,  $P = 0.024$ ];  $*P < 0.05$ ;  $n = 9$ –17 cells from three to five mice. Data are presented as means  $\pm$  SEM.

hippocampus, amygdala, and BNST of adolescent mice. Our results are consistent with earlier electrophysiological reports of glycine-activated currents in brain (22, 24–27, 44), as well as studies of GlyR mRNA and protein expression, which demonstrate that four ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\beta$ ) of the five known GlyR subunits are expressed in the mammalian brain (30, 31, 36, 37, 45, 46).

We identified endogenously activated tonic GlyR currents in a subset of the brain regions in which exogenous glycine-activated currents were measured. Strychnine produced significant shifts in the holding currents of neurons in the PFC (layer II/III), striatum, and hippocampus, indicating the presence of tonically activated GlyRs. Isolation of these currents in the PFC, dorsal striatum, and hippocampus confirms previous reports of GlyR-mediated tonic currents in these regions (23, 27, 41, 47); in addition, we found evidence for the presence of a tonic GlyR conductance in the NAc. Tonic currents were not observed in the basolateral amygdala, central nucleus of the amygdala, or BNST despite the presence of large-amplitude currents activated by exogenously applied glycine. Notably, the tonic GlyR currents in the striatum were blocked by low concentrations of picrotoxin, indicating that they are mediated by homomeric receptors containing only  $\alpha$  subunits. Homomeric GlyRs are thought to be extrasynaptically localized, since they lack the GlyR  $\beta$  subunit required for binding to the postsynaptic gephyrin scaffold (28, 29).

A major goal of this study was to determine the  $\alpha$  subunit composition of tonically activated GlyRs in forebrain. Based on evidence of  $\alpha 2$  and  $\alpha 3$  subunit expression in the brain regions tested and the proposed role of these particular subunits in tonic inhibition (32), we examined the hypothesis that  $\alpha 2$  and/or  $\alpha 3$  subunit-containing GlyRs mediate tonic currents. Tonic GlyR currents were absent in whole-cell recordings in the PFC (layer II/III) and striatum (dorsolateral striatum and NAc) of mice lacking  $\alpha 3$  GlyR subunits but were present not only in WT controls but also in GlyR  $\alpha 2$ -deficient mutant mice. Together these results show that tonically activated GlyRs in forebrain depend on  $\alpha 3$  subunits.

Unlike the GlyRs expressed in spinal cord, which are predominantly synaptic  $\alpha 1\beta$  heteromers, studies of GlyR subunit mRNA and receptor membrane expression indicate an equal or greater abundance of  $\alpha 2$  and  $\alpha 3$  compared with  $\alpha 1$  subunits in some higher brain regions (30, 37, 45, 46), particularly in the forebrain. Recent evidence suggests that coassembly of the  $\beta$  subunit, which is required for GlyR synaptic localization, with  $\alpha$  subunits occurs to a lesser extent in the forebrain than in spinal cord and hindbrain (31); thus, homomeric GlyRs may be preferentially formed in this brain region. Our demonstration of picrotoxin-sensitive tonic GlyR currents in the NAc is consistent with this view and provides evidence for the existence of functional GlyR  $\alpha$  homomers in this region of the forebrain.

GABA<sub>A</sub>Rs (i.e.,  $\alpha 4/\delta$  or  $\alpha 5\beta 3\gamma 2$ ) known to mediate tonic inhibition in the brain have been found to exhibit high agonist sensitivity and reduced receptor desensitization (3, 10, 13, 48, 49). GlyR  $\alpha 3$  transcripts have been shown to undergo RNA editing, which also results in receptors with significantly increased glycine sensitivity and reduced desensitization (50–52). Whether such RNA-edited GlyR  $\alpha 3$  transcripts contribute to increased GlyR agonist sensitivity and larger tonic current amplitudes in vivo is unclear. While we found that tonically activated GlyRs are composed of the  $\alpha 3$  subunit, RNA-edited  $\alpha 3$  transcripts are rather rare, and our acute slice preparations yielded a glycine EC<sub>50</sub> much higher than that observed upon heterologous overexpression of the RNA-edited  $\alpha 3$  GlyR (50). Hence we propose that the tonic currents seen in our experiments are carried by unedited  $\alpha 3$  GlyRs.

Recent work in the dorsal raphé nucleus has revealed a large tonic GlyR conductance (~45 pA), which, based on preliminary pharmacological measures, was proposed to be mediated by extrasynaptic  $\alpha 1$  subunit-containing GlyRs expressed in this brain region (21). In the same study, no tonic GlyR currents were

seen in the NAc. Although the amplitude of the tonic GlyR currents that we measured were of considerably smaller magnitude (~10–20 pA) than those in the dorsal raphé, 100 nM or 1  $\mu$ M strychnine (Figs. 1C and 2A) produced significant shifts in the holding current of MSNs, confirming the presence of tonic currents in our study. The small amplitude of these currents likely reflects lower expression levels, as the overall relative abundance of GlyRs in the NAc is lower than in the dorsal raphé, with nearly 40% of accumbal GlyRs being located outside the synapse (21). Furthermore, the different findings might be attributed to longer-duration applications of low-nanomolar concentrations of strychnine being required to reveal the tonic GlyR conductance that we observed. Maguire et al. (21) applied strychnine for <5 min, whereas in our study ~10-min applications of strychnine were required to reveal the shifts in holding current. Alternatively, the possibility of sex differences in GlyR tonic currents in the NAc exists, as Maguire et al. (21) performed recordings in both female and male mice, whereas only males were included in our study.

A comparison of the brain regions exhibiting GlyR tonic currents with those that show GABA<sub>A</sub>R-mediated tonic currents (6–9, 11, 12, 53) suggests that GABAergic and glycinergic tonic inhibition can coexist (i.e., PFC, striatum, hippocampus). Our data demonstrating the presence of both GABA<sub>A</sub>R- and GlyR-mediated tonic currents in accumbal MSNs further support this concept. These findings raise the possibility that the excitability of individual neurons and neural networks in the brain may potentially be regulated by a combination of GABA<sub>A</sub>R and GlyR activation.

Activation of GlyRs by exogenous applications of glycine produced large-amplitude currents in neurons in all the brain regions in which we detected tonic GlyR currents (PFC II/III, striatum, and hippocampus) as well as in the amygdala and BNST, where tonic GlyR currents were absent. Our results indicate that  $\alpha 2$  subunit-containing GlyRs mediate large-amplitude glycine-activated currents in brain. More specifically, measurement of GlyR currents in *Gla2*<sup>-Y</sup> and *Gla3*<sup>-/-</sup> mice revealed that these currents were nearly eliminated in *Gla2*<sup>-Y</sup> mice; in contrast, the amplitude of glycine-activated currents was not significantly different between *Gla3*<sup>-/-</sup> and WT mice. This result was consistent across all the brain regions tested, with the exception of the PFC, in which exogenous glycine responses were decreased but not eliminated in *Gla3*<sup>-/-</sup> mice, indicating that large-amplitude  $\alpha 2$ -mediated currents in several brain regions exist independently of small-amplitude  $\alpha 3$ -mediated tonic GlyR currents. Evidence of large  $\alpha 2$ -dependent currents in our study correlate well with studies of GlyR subunit mRNA and protein expression, which identify  $\alpha 2$  as the predominant  $\alpha$  subunit in the forebrain (30, 37, 45, 46).

Our results conflict with earlier research in the prenatal cortex (54). In the cited study, cortical neurons from *Gla2*<sup>-Y</sup> mice were found to be unresponsive to glycine administration at day E17 but showed large glycine-induced currents at P7. This difference was ascribed to compensation by increased expression of *Gla1* and *Gla3* at postnatal ages (54). By contrast, our work demonstrates that  $\alpha 2$  subunit-containing GlyRs contribute significantly to currents produced by glycine application in the mouse brain during adolescence and early adulthood, as glycine-activated currents were consistently lower in *Gla2*<sup>-Y</sup> mice relative to *Gla2*<sup>+Y</sup> mice, a comparison not made previously (54). Together, our results reveal two functionally distinct populations of GlyRs in the mouse forebrain: one that produces large-amplitude currents and contains  $\alpha 2$  subunits and a second one that, upon tonic activation, produces small-amplitude currents and is comprised of  $\alpha 3$  subunits.

Here, we used exogenous applications of glycine to activate GlyRs expressed in different forebrain regions. However, other amino acids found in the CNS, such as taurine and  $\beta$ -alanine,

also gate GlyRs but act as partial agonists (55). GlyR  $\alpha 2$ -mediated currents in response to taurine have been implicated in the differentiation of cortical and retinal (56) neurons; however, the analysis of *Gla2<sup>-Y</sup>* mice failed to reveal retinal or cortical abnormalities (54). Notably, the GlyR  $\alpha$  subunits expressed in brain respond differently to taurine and  $\beta$ -alanine, such that these amino acids have little to no efficacy at homomeric  $\alpha 2$  or  $\alpha 3$  GlyRs but do activate  $\alpha 1$  GlyRs, albeit with less efficacy and potency than glycine (57, 58). Our data showing that  $\alpha 3$  GlyRs mediate tonic currents in brain suggest that glycine itself probably serves as the main endogenous GlyR ligand in the forebrain. Low micromolar concentrations of ambient glycine are present in the cerebrospinal fluid (59), and there are multiple sources for extracellular glycine, including glycinergic neurotransmission (60), synaptic corelease with glutamate or GABA (61–64), and release from astrocytes (65, 66). Further studies are necessary to determine the specific sources of glycine responsible for the tonic activation of GlyRs.

In addition to functioning as a full agonist at strychnine-sensitive GlyRs, glycine also acts as a coagonist with glutamate to activate NMDA receptors, and it was recently proposed that glycine functions as coagonist specifically at extrasynaptic NMDA receptors, whereas D-serine constitutes the coagonist of synaptic NMDA receptors (67). These results, in combination with our present findings, highlight the diverse mechanisms by which glycine may be involved in regulating neuronal excitability in the mammalian CNS.

In conclusion, our recordings have identified small-amplitude endogenously activated tonic GlyR currents in multiple forebrain regions that are mediated by  $\alpha 3$  subunit-containing GlyRs. In addition, we demonstrate the presence of large-amplitude GlyR  $\alpha 2$ -mediated glycine currents throughout many brain regions, including some in which tonic GlyR currents are absent. Overall, our findings highlight the heterogeneity in the subunit expression and function of GlyRs expressed in the CNS and provide evidence for a role of glycine in facilitating neuronal inhibition in the forebrain.

## Materials and Methods

**GlyR $\alpha$  Subunit KO Mice.** Experimental procedures were performed under the guidelines set forth in the NIH *Guide for the Care and Use of Laboratory Animals* (68) and with the approval of the Institutional Animal Care and Use Committee of Columbia University. *Gla2* and *Gla3* KO mice generated previously (54, 69) had been back-crossed to the C57/B6J strain at the Animal Resource Center of The University of Texas at Austin. All KO mice used in the present study were then bred at Columbia University. Heterozygous *Gla3* breeding pairs were used to produce litters containing both KO mice and WT littermate controls (*Gla3<sup>-/-</sup>* and *Gla3<sup>+/+</sup>*). The mouse *Gla2* gene is located on the X chromosome, and because electrophysiological recordings were performed on brains from male mice only, an alternate breeding strategy was required. To produce male *Gla2* KO and WT littermate controls (*Gla2<sup>+Y</sup>* and *Gla2<sup>-Y</sup>*), female *Gla2<sup>+/-</sup>* and male *Gla2<sup>+Y</sup>* mice were mated to generate the mice used in our study. Mouse PCR genotyping was performed off-site (GeneTyper).

## Brain Slice Electrophysiology.

**Brain slice preparation.** For all electrophysiology recordings, P21–P50 male mice (C57/B6J, *Gla2<sup>+Y</sup>*, *Gla2<sup>-Y</sup>*, *Gla3<sup>+/+</sup>*, and *Gla3<sup>-/-</sup>*) were used. Before decapitation and brain removal, mice were lightly anesthetized with sevoflurane; after brain removal the tissue was submerged in ice-cold artificial CSF (aCSF; 124 mM NaCl, 2.5 mM KCl, 26 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 2 mM MgSO<sub>4</sub>, 10 mM glucose) for slicing. Brains were sectioned into 300- $\mu$ m-thick slices using a vibratome (VT1000S; Leica). Slices were incubated in carbogenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) aCSF at 32 °C for 30 min and thereafter at room temperature for an additional 30 min before recording.

**Electrophysiological recordings.** After incubation, slices were transferred to a recording chamber, secured with a tissue harp, and continuously perfused with aCSF via a gravity-based perfusion system. Neurons in each of the brain regions of interest (dorsal striatum, NAc, CA1 hippocampus, PFC, basolateral and central nucleus of the amygdala, and the ventral and BNST) were visually identified using an Olympus BX51WI microscope equipped with differential interference contrast-IR optics. Whole-cell patch-clamp recordings were performed using a Multiclamp 700B amplifier and a 1440A Digidata digitizer (Molecular Devices),

interfaced to a computer equipped with Clampex 10 software (Molecular Devices). Borosilicate glass electrodes with resistances of 3–7 M $\Omega$  were filled with internal solution for recordings. The internal solution contained 145 mM KCl, 10 mM EGTA, 5 mM MgATP, 0.2 mM NaGTP, and 10 mM Hepes (pH 7.25). In all brain regions investigated, neurons were voltage-clamped at  $-70$  mV except for MSNs of the NAc and dorsal striatum, which were voltage-clamped at  $-80$  mV, which is approximately the resting membrane potential of these neurons. Data were acquired at a rate of 10 kHz and were filtered at 1 kHz. Throughout all recordings, access resistance was monitored, and a priori exclusion criteria were established such that cells with a series resistance of  $>25$  M $\Omega$  were not included in the data analysis, nor were cells for which the series resistance changed  $>30\%$  during the experiment. Recordings were performed at room temperature (22 °C).

**Exogenously activated GlyR currents.** Neurons were voltage-clamped, and currents elicited by an application of 1 mM exogenous glycine were measured at peak amplitude. Glycine was applied for  $\sim 60$  s or until a peak current was reached and then was washed off to demonstrate reversibility. Concentration–response curves for glycine were generated in each brain region studied using a series of exogenous applications of glycine (10  $\mu$ M–10 mM). Each concentration of glycine was applied for  $\approx 60$  s or until a peak current was reached; applications were separated by 5-min recovery periods. The largest current amplitude was defined as maximal (I<sub>max</sub>), and relative current amplitudes elicited by the remaining concentrations of glycine were determined. Competition experiments were performed using glycine and the GlyR-specific antagonist strychnine. Specifically, applications of 300  $\mu$ M glycine (determined from the glycine concentration–response curve to approximately correspond to the EC<sub>40</sub>) were applied until currents of consistent amplitude were recorded. Once a stable response was established, several additional applications of the same concentration of glycine were applied concurrently with a continuous application of 100 nM strychnine. After a 5- to 15-min recovery period following removal of strychnine, 300  $\mu$ M glycine was reapplied in the absence of strychnine; 3-min washout periods separated each application of glycine. To further demonstrate that the glycine-activated currents measured were due to glycine action at GlyRs and not due to activation of other ligand-gated ion channels (i.e., GABA<sub>A</sub> or NMDA receptors), we also measured the currents elicited by applications of 300  $\mu$ M and 1 mM glycine in the presence of 20  $\mu$ M gabazine and 50  $\mu$ M AP5.

**Identification of tonic GlyR currents.** To detect the presence of tonically activated GlyR currents in brain, neurons were voltage-clamped, and changes in baseline current were measured in response to applications of 1  $\mu$ M strychnine. Strychnine was applied for either 3–5 min (1  $\mu$ M) or  $\sim 10$  min (100 nM), and strychnine-induced baseline current shifts were calculated as the difference in holding current before and during drug application (30- to 60-s epochs). We also measured changes in the baseline holding current in response to picrotoxin, alone or concurrently with gabazine to isolate its effects specifically at GlyRs. Gabazine (20  $\mu$ M) was first applied alone for  $\sim 90$  s and then was concurrently applied with 100  $\mu$ M picrotoxin for 3–5 min. Picrotoxin-induced shifts in current were calculated as the difference in holding current before and during drug application (30- to 60-s segments). To measure the contribution of  $\alpha 7$  nAChRs to the strychnine-induced current, methyllycaconitine (10 nM or 100 nM) was applied for 2 min, and the methyllycaconitine-induced baseline current shifts were calculated as the difference in holding current before and during drug application (30-s epochs). Excitability experiments were performed in current-clamp mode. Ten-picoampere steps from  $-30$  pA to  $+30$  pA were applied to measure RMP and input resistance, and 2-pA steps from  $-10$  pA were applied to determine rheobase.

**Data Analysis.** Analyses of electrophysiological data were performed using AxoGraph and Clampfit 10 (Molecular Devices) software. Statistical analyses were conducted using GraphPad Prism software. Student's *t* test and one-way ANOVA were used to determine statistically significant effects;  $P < 0.05$  was considered statistically significant. One-sample *t* tests were used to determine whether shifts in the holding current were significantly different from zero. One-sample nonlinear regression analyses, using a modified Hill equation, were performed to calculate EC<sub>50</sub> values and Hill coefficients for glycine concentration–response curves.

**Drugs and Chemicals.** All drugs and chemicals used in this study were obtained from either Sigma-Aldrich or Tocris.

**ACKNOWLEDGMENTS.** We thank Dr. Maureen McCall for generously providing the *Gla2* mice. This work was supported by NIH Grants R01AA19801 (to N.L.H.) and R0106399 (to R.A.H.); by Integrative Neuroscience Initiative on Alcoholism-West Consortium Grants AA013520 (to R.A.H. and Y.A.B.), F32AA022554 (to L.M.M.), F32AA022028 (to M.C.S.), and T32DA016224 (to L.M.M. and M.C.S.); and by the Max Planck Society (H.B.).

- Klausberger T, et al. (2004) Spike timing of dendrite-targeting bistratified cells during hippocampal network oscillations in vivo. *Nat Neurosci* 7:41–47.
- Klausberger T, Somogyi P (2008) Neuronal diversity and temporal dynamics: The unity of hippocampal circuit operations. *Science* 321:53–57.
- Farrant M, Nusser Z (2005) Variations on an inhibitory theme: Phasic and tonic activation of GABA(A) receptors. *Nat Rev Neurosci* 6:215–229.
- Mitchell SJ, Silver RA (2003) Shunting inhibition modulates neuronal gain during synaptic excitation. *Neuron* 38:433–445.
- Semyanov A, Walker MC, Kullmann DM, Silver RA (2004) Tonically active GABA A receptors: Modulating gain and maintaining the tone. *Trends Neurosci* 27:262–269.
- Ade KK, Janssen MJ, Ortinski PI, Vicini S (2008) Differential tonic GABA conductances in striatal medium spiny neurons. *J Neurosci* 28:1185–1197.
- Janssen MJ, Ade KK, Fu Z, Vicini S (2009) Dopamine modulation of GABA tonic conductance in striatal output neurons. *J Neurosci* 29:5116–5126.
- Janssen MJ, Yasuda RP, Vicini S (2011) GABA(A) receptor  $\beta 3$  subunit expression regulates tonic current in developing striatopallidal medium spiny neurons. *Front Cell Neurosci* 5:15.
- Caraiscos VB, et al. (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* 101:3662–3667.
- Yeung JY, et al. (2003) Tonically activated GABA(A) receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. *Mol Pharmacol* 63: 2–8.
- Jia F, et al. (2005) An extrasynaptic GABA(A) receptor mediates tonic inhibition in thalamic VB neurons. *J Neurophysiol* 94:4491–4501.
- Chandra D, et al. (2006) GABA(A) receptor alpha 4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. *Proc Natl Acad Sci USA* 103:15230–15235.
- Brickley SG, Cull-Candy SG, Farrant M (1999) Single-channel properties of synaptic and extrasynaptic GABA(A) receptors suggest differential targeting of receptor subtypes. *J Neurosci* 19:2960–2973.
- Eccles JC, Fatt P, Koketsu K (1954) Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurons. *J Physiol* 126:524–562.
- Aprison MH, Werman R (1965) The distribution of glycine in cat spinal cord and roots. *Life Sci* 4:2075–2083.
- Graham LT, Jr, Shank RP, Werman R, Aprison MH (1967) Distribution of some synaptic transmitter suspects in cat spinal cord: Glutamic acid, aspartic acid, gamma-aminobutyric acid, glycine and glutamine. *J Neurochem* 14:465–472.
- Curtis DR, Watkins JC (1960) The excitation and depression of spinal neurones by structurally related amino acids. *J Neurochem* 6:117–141.
- Turecek R, Trussell LO (2001) Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. *Nature* 411:587–590.
- Lu T, Rubio ME, Trussell LO (2008) Glycinergic transmission shaped by the corelease of GABA in a mammalian auditory synapse. *Neuron* 57:524–535.
- Balakrishnan V, Kuo SP, Roberts PD, Trussell LO (2009) Slow glycinergic transmission mediated by transmitter pooling. *Nat Neurosci* 12:286–294.
- Maguire EP, et al. (2014) Extrasynaptic glycine receptors of rodent dorsal raphe serotonergic neurons: A sensitive target for ethanol. *Neuropsychopharmacology* 39: 1232–1244.
- Lu Y, Ye JH (2011) Glycine-activated chloride currents of neurons freshly isolated from the prefrontal cortex of young rats. *Brain Res* 1393:17–22.
- Adermark L, Clarke RB, Ericson M, Söderpalm B (2011) Subregion-specific modulation of excitatory input and dopaminergic output in the striatum by tonically activated glycine and GABA(A) receptors. *Front Syst Neurosci* 5:85.
- Sergeeva OA (1998) Comparison of glycine- and GABA-evoked currents in two types of neurons isolated from the rat striatum. *Neurosci Lett* 243:9–12.
- Sergeeva OA, Haas HL (2001) Expression and function of glycine receptors in striatal cholinergic interneurons from rat and mouse. *Neuroscience* 104:1043–1055.
- McCool BA, Botting SK (2000) Characterization of strychnine-sensitive glycine receptors in acutely isolated adult rat basolateral amygdala neurons. *Brain Res* 859: 341–351.
- Chattipakorn SC, McMahon LL (2002) Pharmacological characterization of glycine-gated chloride currents recorded in rat hippocampal slices. *J Neurophysiol* 87:1515–1525.
- Dutertre S, Becker CM, Betz H (2012) Inhibitory glycine receptors: An update. *J Biol Chem* 287:40216–40223.
- Meyer G, Kirsch J, Betz H, Langosch D (1995) Identification of a gephyrin binding motif on the glycine receptor beta subunit. *Neuron* 15:563–572.
- Jonsson S, Kerekes N, Hyytiä P, Ericson M, Söderpalm B (2009) Glycine receptor expression in the forebrain of male AA/ANA rats. *Brain Res* 1305(Suppl):S27–S36.
- Weltzien F, Puller C, O'Sullivan GA, Paarmann I, Betz H (2012) Distribution of the glycine receptor  $\beta$ -subunit in the mouse CNS as revealed by a novel monoclonal antibody. *J Comp Neurol* 520:3962–3981.
- Eichler SA, et al. (2009) Splice-specific roles of glycine receptor alpha3 in the hippocampus. *Eur J Neurosci* 30:1077–1091.
- Bode A, Lynch JW (2014) The impact of human hyperekplexia mutations on glycine receptor structure and function. *Mol Brain* 7:2.
- Eichler SA, et al. (2008) Glycinergic tonic inhibition of hippocampal neurons with depolarizing GABAergic transmission elicits histopathological signs of temporal lobe epilepsy. *J Cell Mol Med* 12:2848–2866.
- Han S, Gelernter J, Krantzler HR, Yang BZ (2013) Ordered subset linkage analysis based on admixture proportion identifies new linkage evidence for alcohol dependence in African-Americans. *Hum Genet* 132:397–403.
- Blednov YA, et al. (2015) Glycine receptors containing  $\alpha 2$  or  $\alpha 3$  subunits regulate specific ethanol-mediated behaviors. *J Pharmacol Exp Ther* 353:181–191.
- Malosio ML, Marqu e-Pouey B, Kuhse J, Betz H (1991) Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *EMBO J* 10: 2401–2409.
- Brams M, et al. (2011) A structural and mutagenic blueprint for molecular recognition of strychnine and d-tubocurarine by different cys-loop receptors. *PLoS Biol* 9: e1001034.
- Matsubayashi H, Alkondon M, Pereira EF, Swanson KL, Albuquerque EX (1998) Strychnine: A potent competitive antagonist of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal neurons. *J Pharmacol Exp Ther* 284: 904–913.
- Pribilla I, Takagi T, Langosch D, Bormann J, Betz H (1994) The atypical M2 segment of the beta subunit confers picrotoxinin resistance to inhibitory glycine receptor channels. *EMBO J* 13:1493.
- Salling MC, Harrison NL (2014) Strychnine-sensitive glycine receptors on pyramidal neurons in layers II/III of the mouse prefrontal cortex are tonically activated. *J Neurophysiol* 112:1169–1178.
- Jia F, Goldstein PA, Harrison NL (2009) The modulation of synaptic GABA(A) receptors in the thalamus by eszopiclone and zolpidem. *J Pharmacol Exp Ther* 328:1000–1006.
- Callister RJ, Graham BA (2010) Early history of glycine receptor biology in mammalian spinal cord circuits. *Front Mol Neurosci* 3:13.
- McCool BA, Farroni JS (2001) Subunit composition of strychnine-sensitive glycine receptors expressed by adult rat basolateral amygdala neurons. *Eur J Neurosci* 14: 1082–1090.
- Delaney AJ, Esmaili A, Sedlak PL, Lynch JW, Sah P (2010) Differential expression of glycine receptor subunits in the rat basolateral and central amygdala. *Neurosci Lett* 469:237–242.
- Jonsson S, et al. (2012) Changes in glycine receptor subunit expression in forebrain regions of the Wistar rat over development. *Brain Res* 1446:12–21.
- Zhang LH, Gong N, Fei D, Xu L, Xu TL (2008) Glycine uptake regulates hippocampal network activity via glycine receptor-mediated tonic inhibition. *Neuropsychopharmacology* 33: 701–711.
- Mody I, Pearce RA (2004) Diversity of inhibitory neurotransmission through GABA(A) receptors. *Trends Neurosci* 27:569–575.
- Nusser Z, Mody I (2002) Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J Neurophysiol* 87:2624–2628.
- Meier JC, et al. (2005) RNA editing produces glycine receptor alpha3(P185L), resulting in high agonist potency. *Nat Neurosci* 8:736–744.
- Legendre P, F rster B, J ttner R, Meier JC (2009) Glycine receptors caught between genome and proteome—Functional implications of RNA editing and splicing. *Front Mol Neurosci* 2:23.
- McCracken LM, Trudell JR, McCracken ML, Harris RA (2013) Zinc-dependent modulation of  $\alpha 2$ - and  $\alpha 3$ -glycine receptor subunits by ethanol. *Alcohol Clin Exp Res* 37:2002–2011.
- Badanich KA, Mulholland PJ, Beckley JT, Trantham-Davidson H, Woodward JJ (2013) Ethanol reduces neuronal excitability of lateral orbitofrontal cortex neurons via a glycine receptor dependent mechanism. *Neuropsychopharmacology* 38:1176–1188.
- Young-Pearse TL, Ivic L, Kriegstein AR, Cepko CL (2006) Characterization of mice with targeted deletion of glycine receptor alpha 2. *Mol Cell Biol* 26:5728–5734.
- Lape R, Colquhoun D, Sivilotti LG (2008) On the nature of partial agonism in the nicotinic receptor superfamily. *Nature* 454:722–727.
- Young TL, Cepko CL (2004) A role for ligand-gated ion channels in rod photoreceptor development. *Neuron* 41:867–879.
- Kuhse J, Schmieden V, Betz H (1990) A single amino acid exchange alters the pharmacology of neonatal rat glycine receptor subunit. *Neuron* 5:867–873.
- Schmieden V, Kuhse J, Betz H (1992) Agonist pharmacology of neonatal and adult glycine receptor alpha subunits: Identification of amino acid residues involved in taurine activation. *EMBO J* 11:2025–2032.
- Nagy K, et al. (2010) Alterations in brain extracellular dopamine and glycine levels following combined administration of the glycine transporter type-1 inhibitor Org-24461 and risperidone. *Neurochem Res* 35:2096–2106.
- Zeilhofer HU, et al. (2005) Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. *J Comp Neurol* 482: 123–141.
- Muller E, et al. (2013) Vesicular storage of glycine in glutamatergic terminals in mouse hippocampus. *Neuroscience* 242:110–127.
- Romei C, Raiteri M, Raiteri L (2012) GABA transporters mediate glycine release from cerebellum nerve endings: Roles of Ca(2+) channels, mitochondrial Na(+)/Ca(2+) exchangers, vesicular GABA/glycine transporters and anion channels. *Neurochem Int* 61: 133–140.
- Simat M, Parpan F, Fritschy JM (2007) Heterogeneity of glycinergic and gabaergic interneurons in the granule cell layer of mouse cerebellum. *J Comp Neurol* 500:71–83.
- Crook J, Hendrickson A, Robinson FR (2006) Co-localization of glycine and gaba immunoreactivity in interneurons in Macaca monkey cerebellar cortex. *Neuroscience* 141:1951–1959.
- Harsing LG, Jr, Matyus P (2013) Mechanisms of glycine release, which build up synaptic and extrasynaptic glycine levels: The role of synaptic and non-synaptic glycine transporters. *Brain Res Bull* 93:110–119.
- Shibasaki K, Hosoi N, Kaneko R, Tominaga M, Yamada K (2017) Glycine release from astrocytes via functional reversal of GlyT1. *J Neurochem* 140:395–403.
- Papouin T, et al. (2012) Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists. *Cell* 150:633–646.
- National Research Council (2011) *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington, DC), 8th Ed.
- Harvey RJ, et al. (2004) GlyR alpha3: An essential target for spinal PGE2-mediated inflammatory pain sensitization. *Science* 304:884–887.