Microinjection of Glycine into the Ventral Tegmental Area Selectively Decreases Ethanol Consumption

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ABSTRACT

The mechanisms of ethanol addiction are not completely understood. The mesolimbic dopaminergic system is involved in many drug-related behaviors, including ethanol self-administration. The dopaminergic neurons in this system originate in the ventral tegmental area (VTA) and are under the control of GABAergic transmission. Our previous *in vitro* electrophysiological data indicate that glycine receptors (GlyRs) exist on the GABAergic terminals, which make synapses on VTA dopaminergic neurons, and activation of these GlyRs reduces GABAergic transmission and increases the activity of VTA dopaminergic neurons. In the current study, we tested the hypothesis that the activation of the presynaptic GlyRs in the VTA might interfere with ethanol self-administration. Glycine and strychnine, the selective antagonist of GlyRs, were injected, either alone or in combination, into the VTA of rats. Ethanol self-administration

by rats was evaluated by using three different drinking models: intermittent access, continuous access, and operant self-administration. We found that the infusion of glycine into the VTA selectively reduced the intake of ethanol but not sucrose or water in rats chronically exposed to ethanol under the intermittent-access and continuous-access procedures and decreased lever-press responding for ethanol under an operant self-administration procedure. The effects of glycine probably were mediated by strychnine-sensitive GlyRs, because the coinjection of glycine and strychnine reduced neither ethanol intake in the home cages nor lever-press responding for ethanol in the operant chambers. Thus, GlyRs in the VTA may play a critical role in ethanol self-administration in animals chronically exposed to ethanol. Therefore, drugs targeting GlyRs may be beneficial for alcoholics.

Introduction

The mechanisms of ethanol addiction are not completely understood. It is currently believed that the mesocorticolimbic dopaminergic (DA) system, originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAc), is involved in many drug-related behaviors (Gatto et al., 1994; Debanne et al., 1996; Koob and Le Moal, 1997; Rodd et al., 2004; Barak et al., 2011). Many studies indicate that ethanol self-administration might be correlated with changes in the activity of VTA DA neurons. For exam-

ple, acute ethanol exposure increased the firing rate of VTA DA neurons (Gessa et al., 1985; Brodie et al., 1990, 1999; Bunney et al., 2000; Appel et al., 2003), and ethanol self-administration increased dopamine levels in the NAc (Gonzales and Weiss, 1998). In contrast, acute withdrawal from chronic ethanol exposure reduced VTA DA neuron firing rate and dopamine levels in the NAc (Diana et al., 1993; Weiss et al., 1996; Bailey et al., 1998, 2001; Shen, 2003), and self-administration of ethanol after withdrawal reinstated and maintained dopamine levels in the NAc (Weiss et al., 1996).

Furthermore, the output of midbrain DA neurons is normally constrained by powerful GABA-mediated synaptic inhibition (Johnson and North, 1992b; Tepper et al., 1998). Blocking VTA GABA_A receptors both *in vivo* and *in vitro* strongly increases DA cell firing (Yim and Mogenson, 1980; Johnson and North, 1992a; Xiao et al., 2007), and the dopamine levels in the NAc (Westerink et al., 1996; Ikemoto et al., 1997), but reduced ethanol self-administration (Nowak et al., 1998). Acute withdrawal from chronic ethanol exposure in-

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ABBREVIATIONS: DA, dopaminergic; GlyR, glycine receptor; NAc, nucleus accumbens; STR, strychnine; VTA, ventral tegmental area; 10E, 10% ethanol + 90% water (v/v); RM ANOVA, repeated-measures analysis of variance; FR, fixed ratio; n.s., not significant; SNc, substantia nigra pars compacta; Gly5, glycine at dose of 5 μ M; Gly50, glycine at dose of 50 μ M.

creased the activity of VTA GABAergic neurons (Gallegos et al., 1999), which may contribute to reduced DA neuronal activity.

Immunological, molecular, and electrophysiological studies have revealed that functional glycine receptors (GlyRs) are widely distributed throughout the mammalian central nervous system, including the mesocorticolimbic DA system (Betz, 1991; Ye et al., 1998, 2004; Zheng and Johnson, 2001; Jonsson et al., 2009; Lu and Ye, 2011). The DA system is under the control of several neurotransmitter systems, including the GABA_A receptors and GlyRs (Ye et al., 2001; Molander et al., 2005). A previous microdialysis study has demonstrated that GlyRs in the NAc are involved in both the regulation of basic dopamine levels and NAc dopamine output in response to ethanol (Molander et al., 2005). Our previous *in vitro* electrophysiological study has indicated that GlyRs also exist on the GABAergic terminals, which make synapses on the VTA DA neurons. Activation of these presynaptic GlyRs reduces GABAergic transmission and increases VTA DA neuron firing (Ye et al., 2004). Because GABAergic transmission onto VTA DA neurons plays a critical role in both the control of the activity of the DA neurons and ethanol-induced excitation of DA neurons (Gallegos et al., 1999; Xiao et al., 2007; Xiao and Ye, 2008), in the current study we tested the hypothesis that the activation of the presynaptic GlyRs might interfere with voluntary alcohol consumption behavior.

Materials and Methods

Animals and Housing. Adult, male Long-Evans rats (Harlan, Indianapolis, IN) were individually housed in ventilated Plexiglas cages in a climate-controlled room (20–22°C). The rats were allowed to acclimatize to the individual housing conditions and handling before the start of the experiments with unlimited access to food and water. All rats were kept on a 12-h light/dark cycle: lights on at 2:45 AM for the group under the intermittent-access, two-bottle choice drinking paradigm and lights on at 7:00 AM for the groups that underwent the continuous-access, two-bottle choice drinking paradigm and operant self-administration training. Food and water were available *ad libitum*, except for short periods during initial training in the operant self-administration paradigm, as outlined below. All experiments were performed in accordance with the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and approved by the Institutional Animal Care and Use Committees of the University of Medicine and Dentistry of New Jersey and the Gallo Center at the University of California at San Francisco.

Intermittent-Access to 20% Ethanol Two-Bottle Choice Drinking Procedure. The intermittent-access to 20% ethanol drinking procedure was similar to that described previously (Simms et al., 2008; Li et al., 2010, 2011). In brief, 38 2-month-old Long-Evans rats (255 ± 9 g) were given 24-h concurrent access to one bottle of 20% (v/v) ethanol in tap water and one bottle of water, starting at 3:00 PM on Monday. After 24 h, the ethanol bottle was replaced with a second water bottle that was available for the next 24 h. This pattern was repeated on Wednesdays and Fridays. On all other days the rats had unlimited access to two bottles of water. In each ethanol drinking session, the placement of the ethanol bottle was alternated to control for side preferences. The amount of ethanol or water consumed was determined by weighing the bottles before access and after 24 h of access. Animals were weighed weekly. Ethanol consumption was determined by calculating grams of alcohol consumed per kilogram of body weight. Preference for ethanol was calculated as the amount of ethanol consumed as a percentage of

the total fluid consumption: % preference = ml of ethanol/(ml of ethanol + ml of water) $\times$ 100%. Rats were implanted with guide cannulae into the VTA ($n = 30$; 12 rats were tested with glycine; 10 rats were tested with strychnine and glycine; 8 rats were tested for locomotor activity) or the substantia nigra pars compacta (SNc) ($n = 8$) (see below for surgical method) after a stable baseline drinking level of the 20% (v/v) ethanol solution was achieved (5.7 ± 0.23 g/kg/24 h, 18 ethanol exposures, 6 weeks). Glycine and vehicle were administered as described below.

As described in our recent report (Li et al., 2011), we took blood samples 30 min after the start of the dark cycle in a separate group of rats maintained on an identical protocol and found that alcohol levels (range 26–249 mg/dl; $n = 8$) were significantly correlated with oral alcohol consumption (alcohol consumption 0.20–1.68 g/kg/30 min; $p < 0.01$; $r^2 = 0.714$).

Intermittent-Access to 5% Sucrose Two-Bottle Choice Drinking Procedure. To determine whether the alterations in drinking were alcohol-specific, a separate group of rats ($n = 8$) was implanted with cannulae into the VTA. After recovery from the surgery for more than 1 week, these rats were trained to drink sucrose solution under intermittent access to 5% sucrose by using a two-bottle choice drinking procedure, similar to that for alcohol drinking. Drug administration began after the rats had maintained stable baseline drinking levels for 2 weeks.

Measurements of Locomotor Activity. To test the effect of glycine on locomotor activity in rats, on the test days glycine (5 μ M) or vehicle was given approximately 10 min before the rats were placed in the locomotor testing chambers (TruScan Photobeam Activity Monitors, $16 \times 16 \times 16$ inches; Coulbourn Instruments, Whitehall, PA) for 60-min sessions. Total travel distance (centimeters) was recorded automatically by using TruScan 2.0 software as described previously (Liu et al., 2010) in the first hour and repeated at the 24th hour after microinjection. Rats were allowed to access ethanol and water during the second to 23rd hours on the days of locomotor assessments.

Continuous-Access, Two-Bottle Choice Drinking Procedure. After acclimating to the home-cage environment for 1 week, 24 Long-Evans rats in the home cage were given 3 days of access to a single bottle of ethanol (10% v/v; 10E) as the only fluid available. From that point forward, all subjects received *ad libitum* water in the home cage. Next, animals were allowed free choice between two bottles, a sucrose-ethanol solution (10% sucrose in 10E, w/v) in tap water and tap water for 20 days. The sucrose-ethanol solution was replaced with 10E, and the exposure was continued for 14 more days. The consumption of and the preference for ethanol were determined in a manner similar to that under the intermittent-access drinking paradigm described above. To control for side preference, left-right positions of the tubes were switched daily. After 2 weeks of 10E drinking in home cages, rats were implanted with guide cannulae into the VTA and divided into two groups: one group ($n = 14$) continued to drink 10% ethanol under the continuous-access paradigm, and the other group ($n = 10$) received operant self-administration training.

Operant Self-Administration after Continuous Access to Ethanol. The conditioning chambers were 30 cm wide and 29 cm high and contained within larger sound-attenuating chambers. Two levers were located against the right wall, 7 cm from the floor and 1 cm from the right or left edge of the right wall. A 2.5-cm white stimulus light was located above each lever. A rectangular recess (3 cm in diameter) was located between the two levers, 3 cm above the floor. Syringe pumps delivered fluid into a fluid receptacle within this recess (ethanol port). A house light, located on the right wall 14 cm from the floor, was on for the duration of each behavioral session. In addition, the operant chambers contained infrared head poke detectors that recorded how many times an animal's head entered the ethanol reward port. All behavioral equipment (MED Associates, St. Albans, VT) was computer-controlled via software (MED Associ-

ates) that also recorded the responses and reinforcer deliveries during behavioral sessions.

One week after recovery from surgery, one group of subjects ($n = 10$) under continuous access to ethanol received one overnight (12–14 h) session with 0.1 ml of 10E available on a fixed-ratio 1 (FR1) schedule after responses on the active lever. After shaping, subjects began daily 60-min sessions, 5 days a week. One week later, the response requirement was increased to a FR3 schedule. Drug testing began after 3 weeks of responding for 10E on a FR3 schedule.

Stereotaxic Surgery and Postsurgical Care. Surgery was performed on rats that were under anesthesia with isoflurane (Baxter, Deerfield, IL) or ketamine/xylazine (80 mg/5 mg/kg i.p.). Bilateral guide cannulae (C235G-2.0, 26 gauge; Plastics One, Roanoke, VA) were aimed dorsal to the VTA (6.00 mm posterior to bregma, ± 0.75 mm mediolateral, 7.5 mm ventral to the skull surface) or the SNc (5.7 mm posterior to bregma, ± 2.4 mm mediolateral, 7.0 mm ventral to the skull surface), according to Paxinos and Watson (2007). We chose to target the posterior part of the VTA because it is implicated in the rewarding processes and reinforcing effects of ethanol (Rodd-Henricks et al., 2000; Rodd et al., 2004; Ikemoto, 2007). One week after recovery, subjects were returned to ethanol (intermittent or continuous access) or 5% sucrose (intermittent access) or received operant self-administration training. Microinjections began when ethanol (sucrose) intake or responding were stable. Among rats that were under intermittent access to 20% ethanol paradigm two in the group tested with glycine and one in the group tested with strychnine and glycine died after surgery.

Before microinjection, animals were taken from the colony, brought to the experimental room, and handled for 5 min per day until the experimental day. During this phase, animals became accustomed to the experimenter, the experimental room, and the manipulation procedure with a total of four to five sessions to decrease the stress of, and habituate subjects to, the microinjection procedures.

Microinjection Procedure. Drugs, including glycine and strychnine (purchased from Sigma, St. Louis, MO), or vehicle (saline) were administered through a 28-gauge internal cannulae (Plastics One) connected to a Hamilton 1.0- μ l syringe driven by a syringe pump (Harvard Instruments, South Natick, MA). All rats in the glycine experimental groups (intermittent access to ethanol or sucrose) received each of the five treatments (vehicle and 1, 5, 10, and 50 μ M glycine). The order of treatments was partially counterbalanced such that vehicle was given first, followed by injections of drug, with doses administered in random order. These concentrations of glycine were chosen because previous *in vitro* electrophysiological evidence indicates that glycine at these concentrations alters DA neuronal activities (Ye et al., 2004). All rats in the continuous-access experiments received each of the three treatments (vehicle and 5 and 50 μ M glycine) in a counterbalanced order, using a Latin square design. Rats in the operant self-administration experiment received each of the five microinjections (vehicle, 5 μ M glycine and/or 20 μ M strychnine, or 50 μ M glycine), in a counterbalanced order, using a Latin square design. There was a minimum of 7 days between successive drug tests. The treatments in the following week were reversed so that all rats received treatment with both the vehicle and glycine.

On the test days, microinjections were given approximately 10 min before the access to ethanol or sucrose. Obstructors were gently removed and injectors were inserted bilaterally to a depth of 1 mm beyond the end of the guide cannulae. Saline vehicle control or a drug doses in a total volume of 0.5 μ l was infused over 1 min into the VTA of gently restrained rats via internal cannulae extending 1.0 mm beyond the guide cannulae tip. The injectors were left in place for an additional 60 s to allow for diffusion. After removal of the injectors, new sterile obstructors were inserted.

The mean body weight was 255 ± 9 g in the initial phase of the experiments when rats were approximately 2 months old, 456 ± 10 g at the first glycine test session when rats were approximately 3½

months old, and 527 ± 15 g at the last test session when rats were approximately 5 months old. All the rats looked healthy and progressively gained weight over the duration of the protocol.

Histological Verification of Cannulae Placements. After completion of the experimental sessions, each rat was given an overdose of ketamine-xylazine mixture and transcardially perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and then transferred to a solution containing 20% sucrose in 4% paraformaldehyde for 3 days. Brains were frozen, cut into 30- μ m sections, and stained with cresyl violet to verify probe and cannulae placement. Data from rats with injection sites located outside of the VTA or SNc were not used in the analysis.

Chemicals. Glycine, strychnine, and all command salts were obtained from Sigma. Ethanol (95%, prepared from grain) was from Pharmco (Brookfield, CT) and stored in glass bottles.

Statistical Analysis. Drinking data during 24-h access periods and locomotor activity during the 60-min sessions were subjected to a one-way or two-way repeated-measures analysis of variance (RM ANOVA) to extract the significant main effects and interactions with Bonferroni post hoc comparisons. Lever press and reward port entry data from ethanol self-administration in the operant chambers were analyzed by using two-way RM ANOVA on lever type (active versus inactive) and treatment and one-way RM ANOVA on port entry. Statistical significance was declared at $p < 0.05$.

Results

Intra-VTA Infusion of Glycine Selectively Reduces Alcohol Intake in Rats that Chronically Consume High Amounts of Ethanol (Intermittent Access to 20% Ethanol). The effect of intra-VTA microinjection of glycine (1, 5, 10, and 50 μ M) on voluntary ethanol intake was evaluated initially by using the intermittent-access, two-bottle choice drinking paradigm. Rats were given unlimited access to one bottle of ethanol (20%) and one bottle of water for 24 h but only on alternate days (see *Materials and Methods*). Two of 10 animals in this experiment showing cannulae placements outside of the posterior VTA were excluded from the statistical analysis. The cannulae tip placements for the rats ($n = 8$) included in this experiment are depicted in Fig. 1A. Average baseline data (mean $\pm$ S.E.M.; $n = 8$) for the session preceding the test of glycine and vehicle (normal saline) on ethanol intake are shown in Table 1. There was an overall main effect on ethanol consumption at all time points examined (1 h, $F_{4,28} = 3.29$, $p < 0.05$; 2 h, $F_{4,28} = 4.07$, $p = 0.01$; 24 h, $F_{4,28} = 7.02$, $p < 0.001$). Post hoc analysis revealed that intra-VTA injection of 5 μ M glycine significantly lowered ethanol consumption over a period of 1 h compared with the vehicle-treated group ($p < 0.05$; Fig. 2A). At 2 and 24 h, both 5 and 10 μ M glycine significantly decreased ethanol consumption (Fig. 2, B and C; $p < 0.05$). However, neither 1 nor 50 μ M glycine significantly altered ethanol consumption at all time points examined. Glycine at all doses did not alter water consumption, the preference for ethanol at 1 and 2 h (1 h, water intake, $F_{4,28} = 0.95$, n.s., preference for ethanol, $F_{4,28} = 2.15$, n.s.; 2 h, water intake, $F_{4,28} = 0.95$, n.s., preference for ethanol, $F_{4,28} = 1.74$ n.s.; data not shown) and water consumption at 24 h ($F_{4,28} = 1.59$; $p > 0.05$; Table 2). However, 5 and 10 μ M glycine significantly decreased the preference for ethanol ($F_{4,28} = 4.51$; $p = 0.006$; Table 2). The amount of ethanol consumed between 48 and 72 h after intra-VTA infusion of 5 and 10 μ M glycine did not differ from that consumed after

Fig. 1. Diagram of coronal sections showing injector placements from individual rats with accurate bilateral placements in the posterior VTA and SNc. A, solid circles represent injector sites of animals that were under the intermittent-access to 20% ethanol paradigm, tested with glycine; solid triangles represent injector sites of animals that were under the intermittent-access to 20% ethanol paradigm, tested with strychnine and glycine; solid squares represent injector sites of animals that were trained to consume sucrose (intermittent access to 5% sucrose) tested with glycine; solid rhombuses represent injector sites of animals that were under the intermittent-access to 20% ethanol paradigm, tested for locomotor activity. B, empty circles represent injector sites of animals that were under continuous access to 10% ethanol tested with glycine; empty triangles represent animals trained in operant self-administration of ethanol tested with glycine and STR; empty rhombuses represent injector sites of animals that were under the intermittent-access to 20% ethanol paradigm, with cannulae implanted into SNc. Numbers indicate the distance from the anterior to bregma [adapted from Paxinos and Watson (2007)].

TABLE 1

Ethanol consumption during the drinking session immediately before microinjection administration (baseline)

Results are mean $\pm$ S.E.M.

Treatment	Intermittent Access to Ethanol	Continuous Access to Ethanol
	<i>g/kg/24 h</i>	
VTA		
Vehicle	6.08 $\pm$ 0.69	2.25 $\pm$ 0.39
Glycine 1 μ M	6.39 $\pm$ 1.40	
Glycine 5 μ M	6.10 $\pm$ 0.79	2.09 $\pm$ 0.24
Glycine 10 μ M	6.20 $\pm$ 0.82	
Glycine 50 μ M	5.70 $\pm$ 1.02	2.27 $\pm$ 0.34
Substantia nigra		
Vehicle	5.38 $\pm$ 0.75	
Glycine 5 μ M	5.73 $\pm$ 0.86	

vehicle treatment ($F_{2,14} = 0.14$; n.s.; Table 2), indicating no rebound increase in ethanol consumption after glycine treatment.

The above result indicates that intra-VTA injection of glycine regulates ethanol consumption. To determine whether this effect was selective for ethanol, in a separate group of rats ($n = 8$) we measured the intake of a natural reward (5% sucrose) by using the intermittent-access, two-bottle choice drinking paradigm. One of eight animals in this experiment showing cannulae placements outside of the posterior VTA was excluded from the statistical analysis. The cannulae tip placements for the rats ($n = 7$) included in this experiment are depicted in Fig. 1A. Intra-VTA infusion of glycine at all doses (1, 5, 10, and 50 μ M) failed to significantly alter the intake of a 5% sucrose solution ($F_{4,24} = 2.26$; $p = 0.093$; Fig. 2D) or water consumption ($p > 0.05$) (data not shown).

To determine whether the reduction in ethanol drinking was accompanied by motor impairments, we tested the effect of glycine (5 μ M), which effectively reduced alcohol drinking, on locomotor activity in rats that were under the intermittent-access, two-bottle choice drinking paradigm. Locomotor data were analyzed at the first 60 min postinjection. Histo-

logical verification found that injection sites of two of eight animals (Fig. 1A) were outside of the posterior VTA and these subjects were excluded from the statistical analysis. Glycine (5 μ M) did not significantly alter locomotor activity across the 60-min session (Fig. 2E). Two-way ANOVA showed a significant increase in the cumulative distance traveled over time ($F_{5,50} = 14.86$; $p < 0.001$) and a significant decrease in the distance traveled in 10-min intervals ($F_{5,50} = 41.19$; $p < 0.001$) with no significant main effect of glycine treatment or treatment $\times$ time interaction. In addition, we tested the effect of glycine (at 50 μ M) on locomotor activity and found that intra-VTA infusion of 50 μ M did not affect locomotor activity in the ethanol-naïve rats (data not shown).

To determine whether the effect of glycine is site-specific, we injected glycine into the neighboring midbrain DA region, the SNc. Histological verification found that the cannulae tip placements for all animals in this experiment were in the SNc (Fig. 1B). Intra-SNc perfusions of glycine (5 μ M) had no significant effect on the intake of ethanol ($F_{1,6} = 0.49$; $p > 0.05$) (Fig. 3A), preference for ethanol (Fig. 3C), or intake of water (Fig. 3B).

Strychnine-Sensitive GlyRs Mediate the Effects of Glycine. To determine whether strychnine-sensitive GlyRs are responsible for the effect of glycine on ethanol consumption, 5 μ M strychnine, a selective antagonist of GlyRs, and a mixture containing 5 μ M glycine and 5 μ M strychnine were injected into the VTA. Histological verification showed that in two of nine animals the cannulae placements were outside of the posterior VTA, so those two animals were excluded from the statistical analysis. The tip cannulae placements for the rats included in this experiment are depicted in Fig. 1A. As illustrated in Fig. 4, although 5 μ M strychnine alone did not significantly alter ethanol intake, the addition of strychnine to 5 μ M glycine eliminated the reduction in ethanol intake induced by 5 μ M glycine. Examination of ethanol intake revealed a main effect of treatment ($F_{3,18} = 5.46$; $p = 0.008$).

Intermittent access to 20% ethanol or 5% sucrose

Fig. 2. Intra-VTA glycine selectively reduces ethanol consumption in rats chronically consuming high amounts of ethanol (intermittent access to 20% ethanol) without affecting spontaneous motor activity. Glycine (1–50 μ M) was administered 10 min before the start of the drinking session or locomotor test. A, intra-VTA infusion of glycine at the dose of 5 μ M, but not the high dose, significantly decreased ethanol consumption at 1 h after the onset of drinking ($n = 8$). B and C, both 5 and 10 μ M doses decreased ethanol consumption at 2 (B) and 24 (C) h after the onset of drinking ($n = 8$). D, all doses of glycine ($n = 7$) did not significantly affect sucrose consumption, using the intermittent-access to 5% sucrose in the two-bottle choice drinking paradigm. E, total distance traveled (cumulative cm) across the first one 60-min session after intra-VTA infusion of 5 μ M glycine; inset, the time course of the distance traveled during the 60 min after the microinjection of glycine (5 μ M) or vehicle ($n = 6$). The values are expressed as mean $\pm$ S.E.M. (one-way or two-way RM ANOVA followed by Bonferroni post hoc test). *, $p < 0.05$; **, $p < 0.01$ compared with vehicle.

TABLE 2

The effects of intra-VTA microinjection of glycine on water intake and ethanol preference in rats that consumed high amounts of ethanol (intermittent access to 20% ethanol)

Values are expressed as mean $\pm$ S.E.M. One-way RM ANOVA followed by Bonferroni post hoc test ($n = 8$).

Treatment	Intermittent Access to 20% Ethanol			
	Ethanol Solution	Water Intake	Preference for Ethanol	Ethanol Intake
	ml/24 h		%	g/kg/48–72 h
Vehicle	17.6 $\pm$ 1.7	19.2 $\pm$ 3.9	51.5 $\pm$ 8.2	5.8 $\pm$ 0.8
Glycine 1 μ M	17.1 $\pm$ 2.3	18.4 $\pm$ 3.6	49.8 $\pm$ 8.9	
Glycine 5 μ M	8.6 $\pm$ 1.7*	30.4 $\pm$ 2.4	21.7 $\pm$ 9.3*	5.5 $\pm$ 0.6
Glycine 10 μ M	8.6 $\pm$ 1.3*	27.7 $\pm$ 3.8	23.8 $\pm$ 2.9*	5.3 $\pm$ 0.7
Glycine 50 μ M	16.4 $\pm$ 2.3	22.9 $\pm$ 6.1	46.9 $\pm$ 8.8	

* $P < 0.05$.

Intra-VTA Infusion of Glycine Reduces Ethanol Intake in Rats that Chronically Consume Low to Moderate Amounts of Ethanol (Continuous Access to 10% Ethanol).

To determine whether intra-VTA glycine could also be effective on rats under different procedures, rats in a separate group were trained to drink ethanol under the continuous-access procedure (see *Materials and Methods*), under which rats consumed low to moderate amounts of ethanol. When rats maintained a stable baseline of ethanol consumption (Table 1), the effects of intra-VTA injection of glycine (5 and 50 μ M) on ethanol intake were tested. Histological verification showed that cannulae placements of 2 of 12 animals were outside of the posterior VTA; these subjects were excluded from the statistical analysis. Figure 1B shows the

cannulae tip placements for all animals used in this experiment. Ethanol intake (gram/kilogram) is presented in Fig. 5. One-way RM ANOVA revealed significant main effects of treatment ($F_{2,18} = 5.05$, $p < 0.05$ at 1 h; $F_{2,18} = 13.03$, $p < 0.001$ at 2 h; and $F_{2,18} = 8.34$, $p = 0.003$ at 24 h after injections). Post hoc analysis revealed that intra-VTA injection of 5 but not 50 μ M glycine significantly decreased ethanol consumption over a period of 1 h relative to that consumed after pretreatment with vehicle ($p < 0.05$). It is noteworthy that at 2 and 24 h both tested doses of glycine significantly decreased ethanol consumption ($p < 0.01$; Fig. 5 B and C). Intra-VTA glycine did not alter water consumption ($F_{2,16} = 0.57$; $p > 0.05$; Table 3), similar to findings in the intermittent-access paradigm. Although

Fig. 3. Effect of intra-VTA microinjection of glycine on ethanol consumption is brain region-specific. Intrastantia nigra injections of glycine (5 μ M) do not affect ethanol consumption (A), water intake (B), or preference for ethanol (C) under the intermittent-access 20% ethanol in two-bottle choice drinking paradigm. The values are expressed as mean $\pm$ S.E.M. ($n = 8$; one-way RM ANOVA).

Fig. 4. Strychnine, a GlyR antagonist, eliminates glycine (5 μ M)-induced reduction in ethanol consumption. Intra-VTA infusions of glycine and/or strychnine were given 10 min before the start of the drinking session (intermittent access to 20% ethanol). The values are expressed as mean ethanol intake $\pm$ S.E.M. (one-way RM ANOVA followed by Bonferroni post hoc test). *, $p < 0.01$ compared with vehicle; #, $p < 0.05$ compared with Glycine + STR. $n = 7$ animals in each group.

intra-VTA glycine produced a tendency to decrease the preference for ethanol under the continuous-access drinking procedure, this was not statistically significant change ($F_{2,16} = 0.97$; $p > 0.05$; Table 3).

Intra-VTA Infusion of Glycine Robustly Reduces Operant Ethanol Self-Administration. We next tested the effect of intra-VTA glycine on ethanol seeking by using an

Fig. 5. Intra-VTA glycine significantly decreases ethanol consumption in rats that chronically consume low to moderate amounts of ethanol (continuous access to 10% ethanol). Glycine (5 or 50 μ M) was administered 10 min before the start of the drinking session. A, the microinjection of glycine at the dose of 5 μ M, but not the high dose, into the VTA significantly decreased ethanol consumption at 1 h after the onset of drinking. B and C, both doses decreased ethanol consumption at 2 (B) and 24 (C) h after the onset of drinking. The values are expressed as mean $\pm$ S.E.M. (one-way RM ANOVA followed by Bonferroni post hoc test; $n = 10$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with vehicle.

TABLE 3

The effects of intra-VTA microinjection of glycine on water intake and ethanol preference in rats that consumed low to moderate amounts of ethanol (continuous access to 10% ethanol)

Values are expressed as mean $\pm$ S.E.M. One-way RM ANOVA followed by Bonferroni post hoc test ($n = 10$).

Treatment	Continuous Access to 10% Ethanol		
	Ethanol Solution	Water Intake	Preference for Ethanol
	ml/24 h		%
Vehicle	11.8 $\pm$ 1.7	18.0 $\pm$ 1.8	39.5 $\pm$ 5.3
Glycine 5 μ M	6.6 $\pm$ 1.6**	16.7 $\pm$ 1.7	27.7 $\pm$ 6.2
Glycine 50 μ M	7.1 $\pm$ 1.1**	18.0 $\pm$ 1.0	26.6 $\pm$ 4.1

** $p < 0.01$ compared with vehicle.

operant self-administration model. In this model, the delivery of the ethanol (10%) reward was contingent on responses on the active lever under an FR3 schedule (see *Materials and Methods*). No reward was received if the rats pressed the

Fig. 6. Intra-VTA microinjection of glycine decreases ethanol seeking. Glycine and/or strychnine were administered 10 min before the start of the session. Microinjection of glycine at 5 μ M, but not at 50 μ M, decreased the number of active lever presses (A) but not entries into the ethanol port (B). Infusion of strychnine with glycine into the VTA blocked glycine-induced decreases at the active lever. Values are mean $\pm$ S.E.M. (one-way or two-way RM ANOVA followed by Bonferroni post hoc test; $n = 10$ per group). *, $p < 0.05$, compared with vehicle; #, $p < 0.001$, compared with Gly5 + STR.

inactive lever, and the event was merely recorded as a measure of nonspecific behavioral activity. When the rats had maintained a stable level of responding over 20 sessions (3 weeks), vehicle, glycine (5 and 50 μ M), and glycine (5 μ M) and/or strychnine (20 μ M) were administered 10 min before the session. Histological verification showed that the cannulae tip placements for all animals used in this experiment were in the posterior VTA (Fig. 1B). Figure 6 depicts operant responding for ethanol by rats after injection of glycine and/or strychnine into the VTA. Two-way RM ANOVA revealed significant main effects of drug treatment ($F_{4,43} = 4.38$; $p = 0.004$) and lever ($F_{1,43} = 254.76$; $p < 0.001$) with a significant interaction between the two main factors ($F_{4,43} = 3.12$; $p < 0.05$). Post hoc analysis revealed that pretreatment with glycine at the low dose of 5 μ M (Gly5), but not the high dose (50 μ M, Gly50), decreased the number of active lever presses ($p < 0.05$) compared with vehicle injection. There were no significant changes for inactive lever responding at any tested dose. Furthermore, infusion of strychnine (20 μ M) with glycine (5 μ M, Gly5+STR) into the VTA blocked the glycine-induced decreases at the active lever ($p < 0.001$, compared with 5 μ M glycine). Although the low dose of glycine (5 μ M) also produced a tendency to decrease entries into the ethanol port, this was not a statistically significant change ($p = 0.082$; Fig. 6B).

Discussion

In the present study, we demonstrated that infusion of glycine into the posterior VTA of Long-Evans rats selectively reduced ethanol self-administration in three different models, intermittent access, continuous access, and operant self-

administration. By contrast, intra-VTA glycine did not alter either sucrose or water consumption. The effects of glycine probably were mediated by strychnine-sensitive GlyRs, because coinjection of glycine and strychnine reduced neither ethanol intake in the home cage nor responding for ethanol in the operant chamber. This finding indicates that VTA GlyRs play a critical role in modulating ethanol self-administration. Our data suggest that the reduction in ethanol self-administration induced by intra-VTA injection of glycine probably is mediated by glycine-induced disinhibition of VTA DA neurons.

Remarkably, a single infusion of 5 or 10 μ M glycine into the VTA reduced free alcohol intake in rats that chronically consumed high amounts of ethanol by almost half in the home cage at the 24-h time point. The mechanism underlying such a potent and long-lasting effect of glycine is probably complex and may involve synaptic plasticity. It warrants further investigation. Our finding is in general agreement with a previous study that showed that intra-NAc glycine induced an increase in DA levels in the NAc and was associated with a reduced ethanol consumption in a subgroup of Wistar rats (Molander et al., 2005). It is possible that elevated DA levels in the NAc would produce reward, and therefore the animals would not need to drink as much ethanol in order to achieve the desired effect, thereby resulting in decreased ethanol intake and preference.

In contrast to 5 μ M glycine, 50 μ M glycine had no effect on ethanol self-administration both in rats that chronically consume high amounts of ethanol (intermittent access to 20% ethanol) and in the operant chambers, as well as at home cages at the 1-h time point in rats under the continuous-access to 10% ethanol drinking procedure. Although the mechanism causing this difference requires further investigation, our interpretation is that whereas 5 μ M glycine selectively activates the presynaptic GlyRs on the GABAergic terminals that make synapses to the VTA DA neurons, 50 μ M glycine may activate both presynaptic GlyRs, as well as postsynaptic GlyRs on VTA DA neurons. Activation of the postsynaptic GlyRs may counteract the effect of activation of presynaptic GlyRs. This possibility is supported by our previously published in vitro electrophysiological data, which show that presynaptic GlyRs are more sensitive to glycine. Whereas the presynaptic GlyRs on the GABAergic terminals have an EC_{50} of ~ 1.5 μ M (Ye et al., 2004), the postsynaptic GlyRs have an EC_{50} of ~ 25 μ M (Ye, 2000). Activation of the presynaptic GlyRs on the GABAergic terminals will reduce GABA release and activate VTA DA neurons by disinhibition, whereas activation of the postsynaptic GlyRs on the VTA DA neurons will reduce the excitability of VTA DA neurons. The net outcome will be determined by the balance between these two opposite effects. When the activation of presynaptic GlyRs dominates, VTA DA neurons are activated. Conversely, when the activation of postsynaptic GlyR dominates, VTA DA neurons are inhibited. Another interpretation is that VTA GABAergic neurons are more sensitive to glycine than VTA DA neurons (Zheng and Johnson, 2001). Thus, glycine at low concentrations will inhibit GABA neurons more than the DA neurons, which may lead to the excitation of DA neurons due to disinhibition. This study focuses on presynaptic GlyRs and therefore we focused on 1, 5, and 10 μ M glycine, which is known to predominantly work on the presynaptic site.

It is unclear why the GlyRs on GABAergic neurons or presynaptic axons in the VTA are more sensitive to glycine than GlyRs on DA neurons. Factors such as receptor density, subunit composition, and structural difference all are possible contributors. A high density of presynaptic GlyRs may account for the fact that low concentrations ($\leq 3 \mu\text{M}$) of glycine alters spontaneous inhibitory postsynaptic current frequency without affecting spontaneous inhibitory postsynaptic current amplitude (an indication of a presynaptic effect without eliciting significant postsynaptic responses), as shown in our previous published work (Ye et al., 2004). Another possible explanation may be their greater sensitivity to glycine, probably owing to a different subunit composition. According to expression studies, α -homomeric GlyRs may be more sensitive than $\alpha\beta$ -heteromeric GlyRs (Bormann et al., 1993; Handford et al., 1996). Differential presynaptic and postsynaptic actions of picrotoxin indicate different properties of presynaptic and postsynaptic GlyRs in the spinal cord (Jeong et al., 2003).

It is noteworthy that although $5 \mu\text{M}$ but not $50 \mu\text{M}$ glycine reduced lever-press behavior in the operant chamber and heavy alcohol intake in home cages in rats under the intermittent-access to 20% ethanol drinking procedure, $50 \mu\text{M}$ glycine did significantly reduce free ethanol intake in home cages by almost half at the 2- and 24-h time points in rats under the continuous-access to 10% ethanol drinking procedure. This may imply that there are changes in the synapses onto VTA DA neurons induced by the repeated cycles of ethanol drinking and withdrawal in the intermittent-access and operant self-administration procedures that differ from those induced by the continuous-access paradigm.

Regardless of the underlying mechanism, the current study reports a previous unrecognized robust and long-lasting effect of VTA glycine on alcohol drinking. These data provide new insight into glycine's effects on the brain reward pathways and could lead to the development of new therapies against alcohol abuse.

Authorship Contributions

Participated in research design: Li, Nie, Janak, and Ye.

Conducted experiments: Li, Nie, Bian, and Dave.

Performed data analysis: Li, Nie, Janak, and Ye.

Wrote or contributed to the writing of the manuscript: Li, Nie, Janak, and Ye.

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