Targeting Glycine Reuptake in Alcohol Seeking and Relapse

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ABSTRACT

It has recently been demonstrated that pharmacological blockade of the glycine transporter 1 (GlyT1) reduced alcohol intake and relapse in rats. The aim of the present study was to further explore the role of GlyT1 in alcohol relapse-like behavior. For this purpose we used three different GlyT1 blockers—SSR504734, A-1246399, and RO4993850—and tested their effect on alcohol-seeking and relapse-like consumption. Two behavioral models, the alcohol deprivation effect model and the cue-induced reinstatement model, were used. Our data show that all three

GlyT1 blockers reduce relapse-like alcohol consumption and cause either minimal or no side effects, measured as changes in home-cage activity, water intake, and body weight. In the reinstatement test, GlyT1 blockers completely abolished alcohol-seeking responses. Furthermore, we tested other drug/cue associations and found that cocaine-seeking responses were also abolished by GlyT1 blockade. Our data confirm that GlyT1 can be used as a target to develop novel anticraving and antirelapse drugs.

Introduction

Approved pharmacological treatments for alcohol use disorder are limited in their effectiveness, and new drug targets are warranted. The glycinergic system, because of its interaction with several alcohol-induced effects, is one of the tentative targets, and indeed, there is increasing evidence that targeting the glycinergic system may interfere with alcohol consumption and alcohol relapse (Molander et al., 2005; 2007; Vengeliene et al., 2010; Lidö et al., 2012, 2017). This is not surprising, as it is known that the activity of the dopaminergic reinforcement system is largely under the control of several neurotransmitter systems that are targeted by alcohol directly, including strychnine-sensitive glycine receptors (GlyRs) and N-methyl-D-aspartate receptors (NMDARs), which also have a glycine-binding site (Molander and Söderpalm, 2005; Vengeliene et al., 2008; Spanagel, 2009; Jonsson et al., 2014; Clarke et al., 2015; Söderpalm et al., 2017).

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One key target to modulate glycinergic activity is the glycine transporter 1 (GlyT1). Genetic inactivation or pharmacological blockade of GlyT1 was shown to result in both glycinergic overinhibition and a facilitation of NMDAR responses via the glycine-binding site (Chen et al., 2003; Gomeza et al., 2003). Blockade of the GlyT1 by Org-25935 reduced both baseline and relapse-like alcohol consumption (Molander et al., 2007; Vengeliene et al., 2010; Lidö et al., 2017). This blocker acts mainly through GlyT1 and is known to significantly raise extracellular glycine levels after its systemic administration (Lidö et al., 2009). However, preclinical findings with Org-25935 were not confirmed in a recent clinical study. A multicenter, randomized, double-blind, placebo-controlled clinical trial of Org-25935 showed no benefit of this treatment over placebo in the prevention of heavy drinking and no effect on any relapse-related parameters (de Bejczy et al., 2014). This raised a doubt whether targeting GlyT1 would be beneficial to treat drug addiction, and how well preclinical findings translate into the clinical setting. On the other hand, the failure of the clinical trial may have been caused by such factors as inadequate dosing or not sufficient power [see de Bejczy et al. (2014) for the discussion of further limitations of the clinical trial].

Up to now, Org-25935 was tested in two preclinical studies, both of which used voluntary alcohol consuming rats under either baseline or relapse-like conditions (Molander et al.,

ABBREVIATIONS: A-1246399, N-(2-((7S,8R)-7-(azetidin-1-yl)-8-benzyl-5,6,7,8-tetrahydronaphthalen-2-yl)oxy)ethyl)-1-methyl-1H-imidazole-4-sulfonamide; ADE, alcohol deprivation effect; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; CS, conditioned stimulus; FR, fixed ratio; GlyR, glycine receptor; GlyT1, glycine transporter 1; NMDAR, N-methyl-D-aspartate receptors; Org-25935, 2-((1R,2S)-6-methoxy-1-phenyl-1,2,3,4-tetrahydronaphthalen-2-yl)methyl-methylamino)acetic acid; RO4993850, [5-methylsulfonyl-2-[(1S)-2,2,2-trifluoro-1-methyl-ethoxy]phenyl]-[3-(trifluoromethyl)-5,7-dihydropyrido[3,4-b]pyridin-6-yl]methanone; S, stimulus; SSR504734, 2-chloro-N-[(S)-phenyl-[(2S)-piperidin-2-yl]methyl]-3-(trifluoromethyl)benzamide.

2007; Vengeliene et al., 2010; Lidö et al., 2017). Clearly, more information should be obtained by preclinical research to provide more comprehensive analysis of this pharmacological target. Therefore, the aim of this study was to investigate whether alcohol-seeking and relapse-like behavior could be modified by different GlyT1 blockers. For this purpose we performed a dose-response study of three GlyT1 blockers: SSR504734 (Depoortere et al., 2005), A-1246399, and RO4993850 (Kohli et al., 2016). To study the effect of the GlyT1 blockade, two behavioral models, the alcohol deprivation effect (ADE) model and the cue-induced reinstatement model, were used. In the ADE model, relapse-like drinking is measured as a robust temporal increase in voluntary alcohol intake after a period of deprivation. Following repeated deprivation phases, alcohol drinking behavior becomes compulsive (Vengeliene et al., 2013, 2014), similar to a relapse situation in alcoholic patients. Preclinical studies have shown that subacute treatment with GlyT1 blocker Org-25935 or with NMDAR antagonists, attenuated the expression of ADE (Vengeliene et al., 2005, 2010).

In the reinstatement model the reacquisition of responding in an operant task is examined by re-exposing rats to environmental stimuli associated with alcohol (De Wit and Stewart, 1981; Sanchis-Segura and Spanagel, 2006; Bossert et al., 2013). Several NMDAR antagonists have been shown to attenuate cue-induced alcohol-seeking but frequently also caused an impairment in motor behavior (Bäckström and Hyttiä, 2004; Bachteler et al., 2005; Spanagel, 2009; Holmes et al., 2013). The most effective way to reduce drug-seeking behavior though this receptor seems to be by targeting its glycine binding site (Bäckström and Hyttiä, 2004, 2006). Up to date, compounds that interfere with GlyRs or GlyT1 have not been tested on cue-induced reinstatement of alcohol- or cocaine-seeking behavior.

Our working hypothesis was that GlyT1 is a viable target to treat alcohol craving and relapse and that testing GlyT1 blockers with unrelated basic chemical structures in the ADE and reinstatement models would support this hypothesis. We also tested the side-effect profile of three GlyT1 blockers in animals that had long-term excessive alcohol consumption. And finally, we tested whether GlyT1 is a selective target to treat alcohol addiction or whether other drugs of abuse would be also affected by GlyT1 blockade.

Methods

Animals

Ninety-five 2-month-old male Wistar rats (from our own breeding colony at the CIMH, Mannheim, Germany) were used for the ADE experiments, thirty-three 2-month-old male Wistar rats (Harlan Laboratories, Venray, Netherlands) were used for ethanol cue-induced reinstatement experiments and fifteen 2-month-old male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were used for cocaine cue-induced reinstatement experiments. All animals were housed individually in standard rat cages (Ehret, Emmendingen, Germany) under a 12-hour artificial light-dark cycle. Standard laboratory rat food (Ssniff Spezialdiäten GmbH, Soest, Germany) and tap water were provided ad libitum throughout the experimental period (unless stated otherwise). All experimental procedures are approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe, Karlsruhe, Germany) and were carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Drugs

Alcohol drinking solutions were prepared from 96% ethanol (Sigma-Aldrich, Taufkirchen, Germany) and then diluted with tap water. Cocaine hydrochloride (Sigma-Aldrich) was dissolved in sterile saline. SSR504734 (generously provided by AbbVie, Ludwigshafen, Germany) was dissolved in water for injections (aqua ad iniectionem; Braun, Melsungen AG, Germany). A-1246399 (generously provided by AbbVie) was dissolved in polyethylene glycol 400 (PEG 400; Sigma-Aldrich Co., St. Louis, MO) and then diluted with injection water to a final PEG 400 concentration of 20%. SSR504734 and A-1246399 were freshly prepared and injected intraperitoneally in a volume of 3 ml/kg. RO4993850 was generously provided by F. Hoffmann-La Roche (Basel, Switzerland) as ready-to-use material and administered orally in a volume of 5 ml/kg.

Long-Term Voluntary Alcohol Consumption with Repeated Deprivation Phases

After 2 weeks of habituation to the animal room, rats were given ad libitum access to tap water and to 5, 10, and 20% ethanol solutions (v/v) as well. Drinking of alcohol and water was monitored daily/weekly by weighing bottles. From these data, water consumption (milliliters per kilogram of body weight per day; ml/kg per day) and alcohol consumption (grams of pure alcohol per kilogram of body weight per day; g/kg per day) was calculated. The first 2-week deprivation period was introduced after 8 weeks of continuous alcohol availability. After the deprivation period, rats were given access to alcohol again and four to six more deprivation periods were introduced in a random manner. The long-term voluntary alcohol drinking procedure including all deprivation phases lasted for approximately 1 year.

Relapse-Like Drinking (Pharmacological Studies I). To study the effects of drug treatment on the expression of the ADE, rats were divided into groups ($n = 7-8$ per group) in such way that the mean baseline total alcohol intake was approximately the same in each group. Baseline drinking was monitored daily for 1 week. After the last day of baseline measurement, the ethanol bottles were removed from the cages leaving the animals with free access to food and water for 2 weeks. Thereafter, the first eight groups of animals were subjected to a total of five intraperitoneal injections (once every 12 hours) of either vehicle or SSR504734 (1, 3, and 10 mg/kg) or A-1246399 (3, 10, and 30 mg/kg). The other four groups received three administrations by mouth (once daily) of either vehicle or RO4993850 (3, 10, and 30 mg/kg). The doses of SSR504734 were selected according to the previously published research (Depoortere et al., 2005). The doses of A-1246399 were selected on the basis of glycine increase in the rat prefrontal cortex as measured by microdialysis yielding respectively an enhancement over the baseline of 1.3- and 1.8-fold at 3 and 10 mg/kg i.p., respectively. These doses significantly exceeded the doses associated with half-maximal target occupancy (1.3 mg/kg i.p. in rats, assessed by an ex vivo binding assay) and half-maximal NMDAR activation (0.8 mg/kg i.p. in mice; assessed by the reversal of L-687,417-induced hyperlocomotion). The doses of RO4993850 were selected on the basis of glycine increase in rat striatum as measured by microdialysis yielding respectively an enhancement over the baseline of 1.5-, 1.8-, and 2.1-fold. The highest dose gave an increase corresponding to the maximal effect induced in the cerebrospinal fluid of healthy volunteers (Hofmann et al., 2016). The alcohol bottles were reintroduced after the second drug administration and the occurrence of an ADE was determined. Total ethanol (g/kg per day) and water intake (ml/kg per day) were measured daily for a subsequent week.

Home-Cage Locomotor Activity Measurements by the E-Motion System. To test for any sedative effects resulting from the drug treatment, home-cage locomotor activity was monitored by use of an infrared sensor connected to a recording and data-storing system (Mouse-E-Motion; Infra-e-motion, Henstedt-Ulzburg, Germany). A Mouse-E-Motion device was placed above each cage (30 cm from the

bottom) so that the rat could be detected at any position inside the cage. The device was sampling every second whether the rat was moving or not. The sensor could detect body movement of the rat of at least 1.5 cm from one sample point to the successive one. The data measured by each Mouse-E-Motion device were downloaded into a personal computer and processed with Microsoft Excel. Monitoring of locomotor activity started 4 days before drug treatment procedure and was continued for four more post-treatment days. The percentage of each rat's locomotor activity during and after treatment days was calculated by using the "before treatment" activity data as a reference.

Cue-Induced Reinstatement of Alcohol Seeking

Operant Alcohol Self-Administration Apparatus. Cue-induced reinstatement of alcohol seeking was carried out in operant chambers (MED Associates Inc., St. Albans, VT) enclosed in ventilated sound-attenuating cubicles. The chambers were equipped with a response lever on each side panel of the chamber. Responses at the active lever activated a syringe pump that delivered a ~30- μ l drop of fluid into a liquid receptacle next to it. Responses at the inactive lever were recorded but had no programmed consequences. A light stimulus (house light) was mounted above the left and right response levers of the self-administration chamber. An IBM-compatible computer controlled the delivery of fluids, presentation of stimuli, and data recording.

Alcohol Self-Administration Conditioning and Extinction Phase. All animal training and testing sessions were performed during the dark phase of their light/dark cycle. Animals were trained to self-administer either 10% (v/v) ethanol or water in daily 30-minute sessions using a fixed-ratio 1 (FR 1) schedule. The purpose of the conditioning phase was to train the animals to discriminate between the availability of ethanol (reinforcement) and water (nonreinforcement). Discriminative stimuli predicting ethanol or water availability were presented during each ethanol or water self-administration session. An orange flavor extract served as the contextual stimulus (S+) for ethanol, whereas water availability was signaled by a lemon grass extract (S-). These olfactory stimuli were generated by depositing a few drops of the respective extract into the bedding of the operant chamber before each session. In addition, each lever press resulting in ethanol delivery was accompanied by a 5-second blinking-light conditioned stimulus (CS+), whereas a 5-second constant-light stimulus (CS-) was presented with water delivery. The 5-second period served as a "time-out," during which responses were recorded but not reinforced. At the end of each session, the bedding of the chamber was changed and trays were thoroughly cleaned. During the first 2 days of conditioning, animals were kept fluid deprived for 20 hours per day. Subsequently, alcohol and water sessions were conducted without fluid deprivation in a random manner until the animals received a total of 10 alcohol and 10 water sessions.

After completing the conditioning phase, rats were subjected to daily 30-minute extinction sessions for 5 consecutive days, which in total was sufficient to reach reduced response rates approximating the extinction criterion of 20% of the last conditioning sessions. Extinction sessions began by extending the levers without presenting olfactory discriminative stimuli. Responses at the previously active lever activated the syringe pump, without resulting in the delivery of either alcohol or water or the presentation of response-contingent cues (stimulus blinking-light or constant-light).

Ethanol Cue-Induced Reinstatement (Pharmacological Studies II). Reinstatement testing began 3 days after the final extinction session. In these tests, rats were exposed to the same conditions as during the conditioning phase, except that the liquids (ethanol or water) were not made available. Sessions were initiated by the extension of both levers and the presentation of either the ethanol-(S+) or water-(S-) associated discriminative stimuli. Responses at the

active lever were followed by the activation of the syringe pump and the presentation of the CS+ in the S+ condition or the CS- in the S- condition. Half of animals were tested under the S+/CS+ condition on day 1 and under the S-/CS- condition on day 2. Conditions were reversed for the other half of animals. The number of responses on both the active (i.e., ethanol-associated lever for S+/CS+ condition and water-associated lever for S-/CS- condition) and inactive lever (i.e., water-associated lever for S+/CS+ condition and ethanol-associated lever for S-/CS- condition) was recorded throughout the experiment.

To test the effect of SSR504734 and A-1246399 on the cue-induced reinstatement of alcohol seeking, animals were divided into four groups on the basis of their performance during the last conditioning and extinction sessions ($n = 8-9$ per group). For the test, two groups received either 10 mg/kg of SSR504734 or 30 mg/kg of A-1246399, whereas the other two groups of animals were injected with the respective vehicle. Drug administration was performed 30 minutes before the reinstatement test procedure. Please note that lower doses of these compounds were not effective in the previous ADE test and were excluded from the further study, and the research contract for studying RO4993850 did not cover the cue-induced reinstatement test.

Cue-Induced Reinstatement of Cocaine-Seeking

To test if GlyT1 blockade only affects alcohol-related effects, or whether the long-term effects of other drugs of abuse such as cocaine are also affected, a separate cohort of rats in the cocaine cue-induced reinstatement model.

Catheter Implantation. Rats were anesthetized with 4% isoflurane and maintained anesthetized with ~2% isoflurane during the entire surgery. A catheter composed of a Micro-Renathane tube (internal diameter: 0.58 mm, external diameter: 0.94 mm; Bilaney Consultants, Düsseldorf, Germany) was implanted into the right jugular vein, and a compatible back-mount (Bilaney Consultants) was passed under the skin and protruded in the mid-scapular region. Rats were given 9-14 days recovery before cocaine self-administration sessions began. Catheters were flushed daily with a heparinized solution (100 IU/ml) containing 1 mg/ml of enrofloxacin (Baytril, Bayer HealthCare LLC Animal Health Division, Shawnee Mission, KS).

Self-Administration Apparatus. Cue-induced reinstatement of cocaine seeking was carried out in operant chambers (Imetronic, Pessac, France) enclosed in ventilated sound-attenuating cubicles. Two nose-poke holes were located on the opposite walls of the chambers, 5 cm above the grid floor. Nose-poke responses were recorded by the interruption of a photo-beam projected across the hole. Poking in one (active) hole resulted in the delivery of 40 μ l (0.26 mg) of cocaine over a period of 2-seconds. Poking in the other (inactive) hole was recorded but had no programmed consequences. A white cue light was located 9.5 cm above the active hole, and a blue cue light was on the opposite wall 33 cm above the grid floor. A sound generator ("beep," 3 kHz, 60 dB) was located on the back wall 40 cm from the grid floor. Experiments were controlled and data collected with Windows-compatible SK_AA software.

Cocaine Self-Administration Conditioning and Extinction Phase. All animal training and testing sessions were performed during the dark phase of their light/dark cycle. Rats were trained to self-administer cocaine in 2-hour daily sessions until stable responding was established. During the first three sessions animals were trained under a fixed ratio 3 (FR 3) schedule of reinforcement. FR 5 schedule was used for the remainder of the conditioning phase. Cocaine availability was signaled by the blue cue light, which was constantly on during the session. Following the required number of nose-pokes in the active hole, the white cue light was illuminated and a beep-sound was generated; after 1 second the infusion pump was activated. The white cue light and beep-sound was maintained for 4 seconds in total. Cocaine infusion was followed by a 40-second

time-out period during which nose-poking at either hole had no scheduled consequences.

After completing the conditioning phase, rats entered extinction training, during which nose-poking did not result in either the administration of cocaine or the presentation of response-contingent cues. Extinction lasted for 17 sessions, which in total was sufficient to reach reduced response rates approximating the extinction criterion of 20% of the last conditioning sessions.

Cocaine Cue-Induced Reinstatement (Pharmacological Studies III). Reinstatement testing was performed on the next day after the final extinction session. In this test, rats were exposed to the same conditions as during the conditioning phase, except that the cocaine was not made available. Operant chamber was constantly illuminated by the blue cue light that served as a contextual stimulus for cocaine availability. Following the required number of nose-pokes in the active hole, the white cue light was illuminated and a beep-sound was generated; after 1 second the infusion pump was activated. The number of nose-poking in both the active and inactive holes was recorded throughout the test.

To test the effect of SSR504734 and A-1246399 on the cue-induced reinstatement of cocaine seeking, animals were divided into two groups on the basis of their performance during the last conditioning and extinction sessions ($n = 6-8$ per group). For the test, one group of animals was injected with the vehicle, and the other one received 10 mg/kg of SSR504734. Two weeks later, rats were assigned to new treatment groups according to a Latin square design. One group of rats was injected with 30 mg/kg of A-1246399 and another received the respective vehicle. Drug administration was performed 30 minutes before the reinstatement test.

Statistical Analysis

Data obtained from ADE measurements (total alcohol intake, water intake) and locomotor activity was analyzed using a two-way analysis of variance (ANOVA) with repeated measures (factors were: treatment group and day). Locomotor activity data were analyzed using recordings of 12-hour postinjection intervals that corresponded to the animals' active phase. Data analysis regarding the effects of treatment on the change in the rat body weight was performed using a one-way ANOVA (factor was: treatment group). Data obtained from the cue-induced alcohol- and cocaine-seeking experiments was analyzed by use of a three-way ANOVA with repeated measures [factors were: treatment group, lever-responses/nose-pokes (active vs. inactive), and session (extinction vs. reinstatement)]. Whenever significant differences were found, Student Newman-Keuls post hoc tests were performed. The chosen level of significance was $P < 0.05$.

Results

SSR504734, A-1246399, and RO4993850 Reduced Relapse-Like Drinking. During the drug testing, the relapse-like drinking in vehicle-treated animal groups was not different from the previous deprivation phases (data not shown). A two-way ANOVA with repeated measures demonstrated an overall increase in alcohol intake after a deprivation phase compared with baseline drinking [factor day: $F(6,168) = 69.3$, $P < 0.0001$; $F(6,162) = 53.3$, $P < 0.0001$ and $F(6,168) = 8.2$, $P < 0.0001$ for SSR504734, A-1246399, and RO4993850 treatment groups, respectively]. Further, analysis of data revealed that all three treatments—SSR504734, A-1246399, and RO4993850—significantly reduced alcohol intake during postabstinence days compared with that of vehicle-treated animals [factor treatment group $\times$ day interaction effect: $F(18,168) = 3.1$, $P < 0.0001$; $F(18,162) = 3.3$, $P < 0.0001$ and $F(18,168) = 4.3$, $P < 0.0001$ for SSR504734, A-1246399, and RO4993850 treatment groups, respectively]. Reduced ethanol

intake in rats treated with either GlyT1 blocker was short-lasting and could be measured only during the first postabstinence week (data on drinking during subsequent postabstinence weeks are not shown). The following post hoc test showed that treatment of rats with the highest dose (10 mg/kg) of SSR504734 abolished relapse-like alcohol consumption (Fig. 1A). The highest dose (30 mg/kg) of A-1246399 treatment did not completely eliminate ADE. Ethanol intake was increased on the first postabstinence day compared with the baseline consumption (Fig. 1C). Once-daily administration of any RO4993850 dose completely abolished ADE, and the highest dose (30 mg/kg) of RO4993850 reduced alcohol intake below baseline consumption (Fig. 1E). Water intake in SSR504734-, A-1246399-, and RO4993850-treated rats was increased/tended to be higher during treatment days compared with that of vehicle-treated animals [factor treatment group $\times$ day interaction effect: $P = 0.29$; $F(18,162) = 2.7$, $P < 0.001$ and $P = 0.06$ for SSR504734, A-1246399, and RO4993850 treatment groups, respectively], demonstrating that all treatments selectively affected alcohol consumption (Fig. 1, B, D, and F).

Analysis of locomotor activity data revealed that there was a general reduction in home-cage activity in all animal groups, which was probably caused by alcohol intoxication during the first postabstinence drinking days [factor day: $F(6,168) = 38.7$, $P < 0.0001$; $F(6,162) = 69.7$, $P < 0.0001$ and $F(6,168) = 48.2$, $P < 0.0001$ for SSR504734, A-1246399, and RO4993850 treatment groups, respectively] (Fig. 2). A two-way ANOVA with repeated measures revealed that treatment with SSR504734 had no significant effect on locomotor activity of animals (factor treatment group and treatment group $\times$ day interaction effect: $P = 0.19$ and $P = 0.14$, respectively) (Fig. 2A). However, the highest dose of A-1246399 significantly reduced home-cage activity of animals [factor treatment group $\times$ day interaction effect: $F(6,162) = 5.1$, $P < 0.0001$] (Fig. 2B). RO4993850 reduced locomotor activity as well; however, this activity change was significant only after the first drug administration [factor treatment group and treatment group $\times$ day interaction effect: $F(3,28) = 3.3$, $P < 0.05$ and $P = 0.08$, respectively] (Fig. 2C).

Treatment of rats with the highest dose of either SSR504734 or A-1246399 caused small ($\sim 1\%$) but significant loss of body weight [factor treatment group: $F(3,28) = 3.5$, $P < 0.05$ and $F(3,27) = 27.1$, $P < 0.0001$]. Animal's body weight did not change significantly during treatment with RO4993850, indicating that food intake and/or metabolism was not affected (factor treatment group: $P = 0.19$).

SSR504734 and A-1246399 Reduced Cue-Induced Reinstatement of Alcohol-Seeking Behavior. Rats reached 90 ± 6 ethanol-associated lever responses (S+/CS+ condition) and 22 ± 2 water-associated lever responses (S-/CS- condition) by the end of the conditioning phase. Lever presses progressively decreased over five extinction sessions to 9.3 ± 0.6 and 7.1 ± 0.4 for the previously ethanol-reinforced and water-reinforced lever, respectively.

Three-way ANOVA with repeated measures showed that the number of lever presses increased during the ethanol cue-induced reinstatement test compared with that during the last extinction sessions [factor session: $F(1,28) = 14.0$, $P < 0.001$ and $F(1,30) = 11.6$, $P < 0.01$ for SSR504734 and A-1246399 treatment groups, respectively]. This was mostly driven by increased responding on the active lever in the vehicle-treated rat group. Significant reduction in lever responding during the

Fig. 1. Intake of total ethanol, calculated in grams of pure alcohol per kilogram of body weight per day (A, C, and E), and water, calculated in milliliters per kilogram of body weight per day (B, D, and F), in (A and B) vehicle, 1 mg/kg of SSR504734, 3 mg/kg of SSR504734, and 10 mg/kg of SSR504734 ($n = 8$ per treatment condition), (C and D) vehicle, 3 mg/kg of A-1246399, 10 mg/kg of A-1246399, and 30 mg/kg of A-1246399 ($n = 7-8$ per treatment condition), and (E and F) vehicle, 3 mg/kg of RO4993850, 10 mg/kg of RO4993850, and 30 mg/kg of RO4993850 ($n = 8$ per treatment condition)-treated rats before and after a deprivation period of 3 weeks. The last week's measurements of ethanol intake is given as baseline drinking (BL). Animals received a total of five, once every 12 hours, i.p. injections of SSR504734 and A-1246399 and three, once daily, oral administrations of RO4993850 (vertical arrows). The ethanol bottles were reintroduced after the second drug administration. Data are presented as means $\pm$ S.E.M. *Significant differences from the vehicle control group, $P < 0.05$.

S+/CS+ reinstatement test was seen in 10 mg/kg of SSR504734-treated rats [factor treatment group $\times$ session interaction: $F(1,28) = 4.6$, $P < 0.05$] (Fig. 3A). Further post hoc analysis demonstrated that responding on the active lever in the SSR504734-treated group during the S+/CS+ reinstatement test did not differ from the last extinction sessions. Inactive lever responses were not significantly different between vehicle- and SSR504734-treated rat groups (Fig. 3B). Administration of 30 mg/kg of A-1246399 had similar effect on cue-induced reinstatement of ethanol-seeking behavior. In A-1246399-treated rats, responding on the active lever during the reinstatement test did not differ

from that seen during the extinction sessions [factor treatment group $\times$ session interaction: $F(1,30) = 9.0$, $P < 0.01$] (Fig. 4A). Responding on the inactive lever was not affected significantly (Fig. 4B).

Interestingly, animals also increased number of responding during the S-/CS- test compared with the last extinction sessions [factor session: $F(1,28) = 7.0$, $P < 0.05$ and $F(1,30) = 11.2$, $P < 0.01$ for SSR504734 and A-1246399 treatment groups, respectively]. This increase was not selective toward the lever (active vs. inactive), indicating that it may have been caused by a general arousal of the animal [factor session $\times$ lever interaction: $P = 0.29$ and $P = 0.12$ for SSR504734 and

Fig. 2. Locomotor activity in (A) vehicle, 1 mg/kg of SSR504734, 3 mg/kg of SSR504734 and 10 mg/kg of SSR504734 ($n = 8$ per treatment condition), (B) vehicle, 3 mg/kg of A-1246399, 10 mg/kg of A-1246399, and 30 mg/kg of A-1246399 ($n = 7-8$ per treatment condition), and (C) vehicle, 3 mg/kg of RO4993850, 10 mg/kg of RO4993850, and 30 mg/kg of RO4993850 ($n = 8$ per treatment condition)-treated animal groups. Locomotor activity is shown as 12-hour postinjection intervals of the animals' active phase. The percentage of each rat's locomotor activity during and after treatment days was calculated with respect to basal activity prior to treatment. Injection days are marked as " $\wedge$ ". The arrow indicates re-exposure of animals to ethanol solutions. Data are presented as means $\pm$ S.E.M. *Significant differences from the vehicle control group, $P < 0.05$.

A-1246399 treatment groups, respectively]. Both treatments significantly reduced responding on the water-associated lever [factor treatment group $\times$ session interaction: $F(1,28) = 8.2$, $P < 0.01$ and $F(1,30) = 8.9$, $P < 0.01$ for SSR504734 and A-1246399 treatment groups, respectively]; reduced responding on the inactive lever did not reach statistical significance (Fig. 3, C and D; Fig. 4, C and D).

SSR504734 and A-1246399 Reduced Cue-Induced Reinstatement of Cocaine-Seeking Behavior. Rats reached 660 ± 184 cocaine-associated nose-pokes and 51 ± 12 inactive nose-pokes by the end of the conditioning phase. Number of nose-pokes progressively decreased over 17 extinction sessions to 42 ± 4 of the previously cocaine-reinforced nose-pokes, and to 30 ± 6 of the nonreinforced nose-pokes.

Three-way ANOVA with repeated measures revealed that the number of nose-pokes increased during the cocaine cue-induced reinstatement test compared with that during the last extinction sessions [factor session: $F(1,26) = 33.3$, $P < 0.0001$ and $F(1,22) = 19.0$, $P < 0.001$ for SSR504734 and A-1246399 treatment groups, respectively]. This was mostly driven by increased responding in the active nose-poke hole in the vehicle-treated rat group. Administration of 10 mg/kg of SSR504734 abolished cocaine seeking. The number of active nose-pokes during the reinstatement testing in SSR504734-treated animals did not differ from the extinction phase [factor treatment group $\times$ session interaction: $F(1,26) = 12.1$, $P < 0.01$] (Fig. 5A). Likewise, active nose-pokes during the reinstatement test were abolished by A-1246399 treatment [factor treatment group $\times$ session interaction: $F(1,22) = 16.2$, $P < 0.001$]

Fig. 3. The effect of vehicle and 10 mg/kg of SSR504734 ($n = 8$ per treatment condition) on ethanol (A and B) and water (C and D) cue-induced reinstatement. Data are shown as the average number of lever presses on the active (A and C) and inactive (B and D) levers during the last extinction sessions and as the number of responses after the presentation of stimuli previously paired with either ethanol or water. Data are presented as means $\pm$ S.E.M. *Significant differences to the extinction lever responses; +Significant differences from the vehicle control group, $P < 0.05$.

(Fig. 6A). Inactive nose-pokes were not reduced by either SSR504734 or A-1246399 treatment compared with the vehicle treated group (Fig. 5B; Fig. 6B).

Discussion

Our study demonstrated that three different GlyT1 blockers reduced or even abolished relapse-like alcohol consumption in male rats during the first postabstinence days in a four-bottle free-choice setting. Repeated administration of SSR504734 or RO4993850 abolished relapse-like drinking, and significant reduction was measured after treatment with A-1246399. To compensate for decreased ethanol consumption, water intake increased in drug-treated rats compared with vehicle-treated rats, demonstrating the selectivity of tested compounds toward alcohol consumption. The blockade of GlyT1 caused some hypolocomotion in case of A-1246399 and RO4993850 treatments but no other side effects could be observed. Cue-induced reinstatement of alcohol-seeking behavior was abolished by administration of the GlyT1 blockers SSR504734 and A-1246399. Both compounds also reduced cue-induced

reinstatement of water-seeking and cocaine-seeking, suggesting a more general involvement of glycinergic system in stimulus-reward association. Nonreinforced responding was not significantly affected during all three reinstatement tests, ruling out the possibility that the reduced responding during the reinstatement tests was caused by an unselective effect of treatment on lever-pressing behavior.

In our earlier study we showed that repeated treatment with GlyT1 blocker Org-25935 reduced relapse-like drinking in chronically drinking rats, which may have resulted from a reversal of alcohol-induced alterations of glycinergic and glutamatergic signaling. Hence, multiple changes were found in gene expression profiles associated with glycinergic and glutamatergic signaling. Administration of Glyt1 blocker reversed the altered expression levels of these genes to those of alcohol-naïve rats. As both GlyRs and NMDARs are primary targets of alcohol, it was suggested that behavioral effects of Org-25935 were probably mediated via either of these receptors (Vengeliene et al., 2010). In the present study we used three GlyT1 blockers with unrelated basic chemical structures provided by three different suppliers. In all three experiments,

Fig. 4. The effect of vehicle and 30 mg/kg of A-1246399 ($n = 8$ to 9 per treatment condition) on ethanol (A and B) and water (C and D) cue-induced reinstatement. Data are shown as the average number of lever presses on the active (A and C) and inactive (B and D) levers during the last extinction sessions and as the number of responses after the presentation of stimuli previously paired with either ethanol or water. Data are presented as means $\pm$ S.E.M. *indicates significant differences to the extinction lever responses; +indicates significant differences from the vehicle control group, $P < 0.05$.

the treatment caused a significant reduction of ethanol consumption during a relapse-like situation, thus confirming that the glycinergic system is indeed involved in relapse-like alcohol drinking.

In our study, blockade of GlyT1 reduced not only alcohol but also cue-induced water responding. This may indicate a more general effect of GlyT1 blockade on stimulus-reward learning, and indeed, cue-induced reinstatement of cocaine-seeking behavior was reduced as well by SSR504734 and A-1246399. SSR504734 treatment has also been shown to reduce nicotine-seeking behavior (Cervo et al., 2013). However, the study by Cervo et al. (2013) demonstrated that cue-induced reinstatement of sucrose-seeking was not affected by SSR504734 administration, suggesting that GlyT1 blockade is selective toward drug-associated stimuli. In our study, ethanol and water-cue conditioning was done in the same animal; therefore, some level of cue-generalization may have occurred in these animals. This could have interfered with the treatment effect on animal behavior during the water reinstatement test. Again, we do not know about the contribution of GlyRs and/or NMDARs to the effect of GlyT1 blockers. However, a critical role for NMDARs in mediating the cue-induced drug-seeking response has been outlined in numerous studies on other

drugs of abuse, primarily cocaine (Bäckström and Hyttiä, 2006, 2007; Feltenstein and See, 2007; Engblom et al., 2008; Mameli et al., 2009), which suggests a contribution of NMDARs to the effects of GlyT1 blockade. Glutamate release is known to be elevated during cue-induced reinstatement of drug-seeking behavior, in animals trained to self-administer alcohol (Gass et al., 2011), cocaine (McFarland et al., 2003), or heroin (LaLumiere and Kalivas, 2008). Therefore, it is assumed that a reduction of glutamatergic activity during the conditioned cue exposure could be expected to lower the drug-seeking behavior. However, neither competitive NMDAR antagonists nor NMDAR channel blockers were found effective in either alcohol or cocaine cue-induced reinstatement (Bespalov et al., 2000; Bäckström and Hyttiä, 2004, 2006; Bachteler et al., 2005). Blocking the coagonist glycine binding site of the NMDAR seems to be the only effective way of targeting this receptor to reduce cue-induced drug-seeking behavior (Bäckström and Hyttiä, 2004, 2006). Elevated extracellular glycine levels were shown to potentiate NMDAR function but also cause internalization of both AMPA and NMDA receptors and induce long-term depression of excitatory postsynaptic currents (Nong et al., 2003; Chen et al., 2011; Alberati et al., 2012), which in turn may have been also

Fig. 5. The effect of vehicle and 10 mg/kg of SSR504734 ($n = 7-8$ per treatment condition) on cocaine cue-induced reinstatement. Data are shown as the average number of active (A) and inactive (B) nose-pokes during the last extinction sessions and as the number of pokes after the presentation of stimuli previously paired with cocaine. Data are presented as means $\pm$ S.E.M. *Significant differences to the extinction nose-pokes; +Significant differences from the vehicle control group, $P < 0.05$.

Fig. 6. The effect of vehicle and 30 mg/kg of A-1246399 ($n = 6-7$ per treatment condition) on cocaine cue-induced reinstatement. Data are shown as the average number of active (A) and inactive (B) nose-pokes during the last extinction sessions and as the number of pokes after the presentation of stimuli previously paired with cocaine. Data are presented as means $\pm$ S.E.M. *Significant differences to the extinction nose-pokes; +Significant differences from the vehicle control group, $P < 0.05$.

responsible for impaired animal performance during cue-induced reinstatement tests. Accumulation of extracellular glycine leads to tonic activation of GlyRs, which is a part of the homeostatic mechanism to overcome neuronal hyperexcitability (Zhang et al., 2008). To date, glycine release has not been measured during the cue-induced reinstatement testing; however, GlyRs have been shown to modulate the activity of the mesocorticolimbic dopaminergic system (Jonsson et al., 2014; Clarke et al., 2015), which plays a key role in drug-seeking responses (Ito et al., 2000). A significant role of GlyRs in alcohol-related behaviors has also been demonstrated using GlyR subunit mutant mice (Blednov et al., 2015).

All three tested drugs had a very low side-effect profile. Some hypolocomotion was observed during drug testing in the ADE model; however, a general central nervous system depressant effect can be excluded as inactive-lever responding in the reinstatement test was not affected. Drug treatment-induced hypolocomotion during ADE testing was probably related to activation of inhibitory GlyR. This effect was absent in the case of SSR504734 and short-lasting in the case of RO4993850. Loss of body weight caused by drug treatment was very small or absent. These findings suggest that the risk of severe side effects caused by GlyT1 blockade is very low. In agreement, administration of GlyT1 blockers in healthy volunteers and alcoholic patients demonstrated that these compounds are generally well tolerated (de Bejczy et al., 2014; Hofmann et al., 2016).

In conclusion, our results demonstrated that GlyT1 blockade impaired alcohol-seeking and relapse-like behavior in rats. This treatment also affected cocaine cue-induced responding. Earlier studies reported that cue-induced reinstatement of nicotine seeking was reduced by GlyT1 blockade as well (Cervo et al., 2013). The mechanism for such general effect of GlyT1 blockade on drug cue-induced responding is not clear at the moment. We propose GlyT1 as a novel pharmacological target for relapse prevention. However, further studies are needed to better understand the mechanism of action of GlyT1 blockers.

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Authorship Contributions

Participated in research design: Vengeliene, Alberati, Behl, Bernalov, Spanagel.

Conducted experiments: Vengeliene, Roßmanith, Takahashi.

Contributed new compounds: Alberati, Behl, Bernalov.

Performed data analysis: Vengeliene.

Wrote or contributed to the writing of the manuscript: Vengeliene, Spanagel.

References

- Alberati D, Moreau JL, Lengyel J, Hauser N, Mory R, Borroni E, Pinard E, Knoflach F, Schlotterbeck G, Hainzl D, et al. (2012) Glycine reuptake inhibitor RG1678: a pharmacologic characterization of an investigational agent for the treatment of schizophrenia. *Neuropharmacology* **62**:1152–1161.
- Bachteler D, Economidou D, Danysz W, Ciccocioppo R, and Spanagel R (2005) The effects of acamprosate and neramexane on cue-induced reinstatement of ethanol-seeking behavior in rat. *Neuropsychopharmacology* **30**:1104–1110.
- Bäckström P and Hyttä P (2004) Ionotropic glutamate receptor antagonists modulate cue-induced reinstatement of ethanol-seeking behavior. *Alcohol Clin Exp Res* **28**:558–565.
- Bäckström P and Hyttä P (2006) Ionotropic and metabotropic glutamate receptor antagonism attenuates cue-induced cocaine seeking. *Neuropsychopharmacology* **31**:778–786.
- Bäckström P and Hyttä P (2007) Involvement of AMPA/kainate, NMDA, and mGlu5 receptors in the nucleus accumbens core in cue-induced reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl)* **192**:571–580.
- Bernalov AY, Zvartau EE, Balster RL, and Beardsley PM (2000) Effects of N-methyl-D-aspartate receptor antagonists on reinstatement of cocaine-seeking behavior by priming injections of cocaine or exposures to cocaine-associated cues in rats. *Behav Pharmacol* **11**:37–44.
- Blednov YA, Benavidez JM, Black M, Leiter CR, Osterndorff-Kahanek E, and Harris RA (2015) Glycine receptors containing $\alpha 2$ or $\alpha 3$ subunits regulate specific ethanol-mediated behaviors. *J Pharmacol Exp Ther* **353**:181–191.
- Bossert JM, Marchant NJ, Calu DJ, and Shaham Y (2013) The reinstatement model of drug relapse: recent neurobiological findings, emerging research topics, and translational research. *Psychopharmacology (Berl)* **229**:453–476.
- Cervo L, Di Clemente A, Orrù A, Moro F, Cassina C, Pich EM, Corsi M, Gozzi A, and Bifone A (2013) Inhibition of glycine transporter-1 reduces cue-induced nicotine-seeking, but does not promote extinction of conditioned nicotine cue responding in the rat. *Addict Biol* **18**:800–811.
- Chen L, Muhlhauser M, and Yang CR (2003) Glycine transporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons in vitro and in vivo. *J Neurophysiol* **89**:691–703.
- Chen RQ, Wang SH, Yao W, Wang JJ, Ji F, Yan JZ, Ren SQ, Chen Z, Liu SY, and Lu W (2011) Role of glycine receptors in glycine-induced LTD in hippocampal CA1 pyramidal neurons. *Neuropsychopharmacology* **36**:1948–1958.
- Clarke RB, Söderpalm B, Lotfi A, Ericson M, and Ademark L (2015) Involvement of inhibitory receptors in modulating dopamine signaling and synaptic activity

- following acute ethanol exposure in striatal subregions. *Alcohol Clin Exp Res* **39**: 2364–2374.
- de Bejczy A, Nations KR, Szegedi A, Schoemaker J, Ruwe F, and Söderpalm B (2014) Efficacy and safety of the glycine transporter-1 inhibitor org 25935 for the prevention of relapse in alcohol-dependent patients: a randomized, double-blind, placebo-controlled trial. *Alcohol Clin Exp Res* **38**:2427–2435.
- Depoortere R, Dargazanli G, Estenne-Bouhtou G, Coste A, Lanneau C, Desvignes C, Poncelet M, Heaulme M, Santucci V, Decobert M, et al. (2005) Neurochemical, electrophysiological and pharmacological profiles of the selective inhibitor of the glycine transporter-1 SSR504734, a potential new type of antipsychotic. *Neuropsychopharmacology* **30**:1963–1985.
- de Wit H and Stewart J (1981) Reinstatement of cocaine-reinforced responding in the rat. *Psychopharmacology (Berl)* **75**:134–143.
- Engblom D, Bilbao A, Sanchis-Segura C, Dahan L, Perreau-Lenz S, Ballard B, Parkitna JR, Luján R, Halbout B, Mamelí M, et al. (2008) Glutamate receptors on dopamine neurons control the persistence of cocaine seeking. *Neuron* **59**: 497–508.
- Feltenstein MW and See RE (2007) NMDA receptor blockade in the basolateral amygdala disrupts consolidation of stimulus-reward memory and extinction learning during reinstatement of cocaine-seeking in an animal model of relapse. *Neurobiol Learn Mem* **88**:435–444.
- Gass JT, Sinclair CM, Cleva RM, Widholm JJ, and Olive MF (2011) Alcohol-seeking behavior is associated with increased glutamate transmission in basolateral amygdala and nucleus accumbens as measured by glutamate-oxidase-coated biosensors. *Addict Biol* **16**:215–228.
- Gomez J, Hülsmann S, Ohno K, Eulenburg V, Szöke K, Richter D, and Betz H (2003) Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. *Neuron* **40**:785–796.
- Hofmann C, Pizzagalli F, Boetsch C, Alberati D, Ereshefsky L, Jhee S, Patat A, Boutouyrie-Dumont B, and Martin-Facklam M (2016) Effects of the glycine reuptake inhibitors bitopertin and RG7118 on glycine in cerebrospinal fluid: results of two proofs of mechanism studies in healthy volunteers. *Psychopharmacology (Berl)* **233**:2429–2439.
- Holmes A, Spanagel R, and Krystal JH (2013) Glutamatergic targets for new alcohol medications. *Psychopharmacology (Berl)* **229**:539–554.
- Ito R, Dalley JW, Howes SR, Robbins TW, and Everitt BJ (2000) Dissociation in conditioned dopamine release in the nucleus accumbens core and shell in response to cocaine cues and during cocaine-seeking behavior in rats. *J Neurosci* **20**: 7489–7495.
- Jonsson S, Adermark L, Ericson M, and Söderpalm B (2014) The involvement of accumbal glycine receptors in the dopamine-elevating effects of addictive drugs. *Neuropharmacology* **82**:69–75.
- Kohli S, Alberati D, Ballard TM, Steward LJ, King MV, and Fone KCF (2016) The GlyT1 inhibitor RO4993850 alters social behaviour and ultrasonic vocalisation calls in a neonatal-phencyclidine isolation-reared rat model for schizophrenia. *Eur Neuropsychopharmacol* **26**:S494–S495.
- LaLumiere RT and Kalivas PW (2008) Glutamate release in the nucleus accumbens core is necessary for heroin seeking. *J Neurosci* **28**:3170–3177.
- Lidö HH, Jonsson S, Hyttiä P, Ericson M, and Söderpalm B (2017) Further characterization of the GlyT-1 inhibitor Org-25935: anti-alcohol, neurobehavioral, and gene expression effects. *J Neural Transm (Vienna)* **124**:607–619.
- Lidö HH, Marston H, Ericson M, and Söderpalm B (2012) The glycine reuptake inhibitor Org24598 and acamprosate reduce ethanol intake in the rat; tolerance development to acamprosate but not to Org24598. *Addict Biol* **17**: 897–907.
- Lidö HH, Stomberg R, Fagerberg A, Ericson M, and Söderpalm B (2009) The glycine reuptake inhibitor org 25935 interacts with basal and ethanol-induced dopamine release in rat nucleus accumbens. *Alcohol Clin Exp Res* **33**:1151–1157.
- Mamelí M, Halbout B, Creton C, Engblom D, Parkitna JR, Spanagel R, and Lüscher C (2009) Cocaine-evoked synaptic plasticity: persistence in the VTA triggers adaptations in the NAc. *Nat Neurosci* **12**:1036–1041.
- McFarland K, Lapish CC, and Kalivas PW (2003) Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior. *J Neurosci* **23**:3531–3537.
- Molander A, Lidö HH, Löf E, Ericson M, and Söderpalm B (2007) The glycine reuptake inhibitor Org 25935 decreases ethanol intake and preference in male wistar rats. *Alcohol Alcohol* **42**:11–18.
- Molander A, Löf E, Stomberg R, Ericson M, and Söderpalm B (2005) Involvement of accumbal glycine receptors in the regulation of voluntary ethanol intake in the rat. *Alcohol Clin Exp Res* **29**:38–45.
- Molander A and Söderpalm B (2005) Glycine receptors regulate dopamine release in the rat nucleus accumbens. *Alcohol Clin Exp Res* **29**:17–26.
- Nong Y, Huang YQ, Ju W, Kalia LV, Ahmadian G, Wang YT, and Salter MW (2003) Glycine binding primes NMDA receptor internalization. *Nature* **422**:302–307.
- Sanchis-Segura C and Spanagel R (2006) Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. *Addict Biol* **11**:2–38.
- Söderpalm B, Lidö HH, and Ericson M (2017) The glycine receptor-A functionally important primary brain target of ethanol. *Alcohol Clin Exp Res* **41**:1816–1830.
- Spanagel R (2009) Alcoholism: a systems approach from molecular physiology to addictive behavior. *Physiol Rev* **89**:649–705.
- Spanagel R, Durstewitz D, Hansson A, Heinz A, Kiefer F, Köhr G, Matthäus F, Nöthen MM, Noori HR, Obermayer K, et al.; Addiction GWAS Resource Group (2013) A systems medicine research approach for studying alcohol addiction. *Addict Biol* **18**:883–896.
- Vengeliene V, Bachteler D, Danysz W, and Spanagel R (2005) The role of the NMDA receptor in alcohol relapse: a pharmacological mapping study using the alcohol deprivation effect. *Neuropharmacology* **48**:822–829.
- Vengeliene V, Bilbao A, Molander A, and Spanagel R (2008) Neuropharmacology of alcohol addiction. *Br J Pharmacol* **154**:299–315.
- Vengeliene V, Bilbao A, and Spanagel R (2014) The alcohol deprivation effect model for studying relapse behavior: a comparison between rats and mice. *Alcohol* **48**:313–320.
- Vengeliene V, Leonardi-Essmann F, Sommer WH, Marston HM, and Spanagel R (2010) Glycine transporter-1 blockade leads to persistently reduced relapse-like alcohol drinking in rats. *Biol Psychiatry* **68**:704–711.
- Vengeliene V, Noori HR, and Spanagel R (2013) The use of a novel drinkometer system for assessing pharmacological treatment effects on ethanol consumption in rats. *Alcohol Clin Exp Res* **37** (Suppl 1):E322–E328.
- Zhang LH, Gong N, Fei D, Xu L, and Xu TL (2008) Glycine uptake regulates hippocampal network activity via glycine receptor-mediated tonic inhibition. *Neuropsychopharmacology* **33**:701–711.

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