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TITLE PAGE

Targeting Glycine Reuptake in Alcohol Seeking and Relapse

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RUNNING TITLE

GlyT1 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 behaviour. 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 behavioural models, the alcohol deprivation effect (ADE) 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 anti-craving and anti-relapse drugs.

ABBREVIATIONS

ADE, alcohol deprivation effect; ANOVA, Analysis of variance; CS, conditioned stimulus; CSA, cocaine self-administration; FR, fixed ratio; GlyR, glycine receptor; GlyT1, glycine transporter 1; NMDAR, N-methyl-D-aspartate receptors; S, stimulus.

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INTRODUCTION

Drugs currently approved for the indication alcohol use disorder are of limited effectiveness, and there is still a need for new treatment options. 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 alcohol directly affects several neurotransmitter systems that control the activity of the dopaminergic reward/reinforcement system, including glycine receptors (GlyRs) and N-methyl-D-aspartate receptors (NMDARs), where glycine functions as a co-agonist (Molander and Söderpalm, 2005; Vengeliene et al., 2008; Spanagel, 2009; Jonsson et al., 2014; Clarke et al., 2015; Söderpalm et al., 2017).

One key target to modulate glycinergic activity is the 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 Org25935 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 Org25935 were not confirmed in a recent clinical study. A multicenter, randomized, double-blind, placebo-controlled clinical trial of Org25935 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, Org25935 was tested in two preclinical studies, both of which used voluntary alcohol consuming rats under either baseline or relapse-like conditions (Molander et al., 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

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

behaviour could be modified by different GlyT1 blockers. For this purpose we performed a dose-response study of three GlyT1 blockers: SSR504734 (Depoortère et al., 2005), A-1246399 and RO4993850 (Kohli et al., 2016). To study the effect of the GlyT1 blockade, two behavioural 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 behaviour becomes compulsive (Vengeliene et al., 2013; 2014), similar to a relapse situation in alcoholic patients. Preclinical studies have shown that sub-acute treatment with GlyT1 blocker Org25935 or with NMDAR antagonists, attenuated the expression of ADE (Hölter et al., 2000; 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 behaviour (Bäckström and Hyytiä, 2004; Bachteler et al., 2005; Spanagel, 2009; Holmes et al., 2013). The most effective way to reduce drug-seeking behaviour though this receptor seems to be by targeting its glycine binding site (Bäckström and Hyytiä, 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 behaviour.

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.

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

METHODS

Animals

Ninety-five two-month-old male Wistar rats (from our own breeding colony at the CIMH, Mannheim, Germany) were used for the ADE experiments and 33 two-month-old male Wistar rats (Harlan Laboratories, Venray, Netherlands) were used for ethanol cue-induced reinstatement experiments. All animals were housed individually in standard rat cages (Ehret, Emmendingen, Germany) under a 12 h artificial light-dark cycle (lights on at 7:00 a.m.). Standard laboratory rat food (Ssniff, 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, Germany) and were carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs

Alcohol drinking solutions were prepared from 96% ethanol (Sigma-Aldrich, Taufkirchen, Germany) and then diluted with tap water. SSR504734 (generously provided by AbbVie, Ludwigshafen, Germany) was dissolved in water for injections (aqua ad iniectabilia, Braun, Melsungen AG, Germany). A-1246399 (generously provided by AbbVie, Ludwigshafen, Germany) was dissolved in polyethylene glycol 400 (PEG 400, Sigma-Aldrich Co., St. Louis, MO, USA) and then diluted with injection water to a final PEG 400 concentration of 20%. SSR504734 and A-1246399 were freshly prepared and injected intraperitoneally (i.p.) 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 (p.o.) in a volume of 5 ml/kg.

Long-term voluntary alcohol consumption with repeated deprivation phases

After two 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 (ml per kg of body weight per day; ml/kg/day) and alcohol consumption (g of pure alcohol per kg of body weight per day; g/kg/day) was calculated. The first two-week deprivation period was introduced after eight weeks of continuous alcohol availability. After the deprivation period, rats were given access to alcohol again and 4-6 more deprivation periods were introduced in a

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

random manner. The long-term voluntary alcohol drinking procedure including all deprivation phases lasted for approximately one year.

Relapse-like drinking (pharmacological studies I)

In order 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 one 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 two weeks. Thereafter, the first eight groups of animals were subjected to a total of 5 i.p. injections (starting at 7 p.m. with 12 h intervals) of either vehicle or SSR504734 (1 mg/kg, 3 mg/kg and 10 mg/kg) or A-1246399 (3 mg/kg, 10 mg/kg and 30 mg/kg). The other four groups received 3 p.o. administrations (once daily at 9 a.m.) of either vehicle or RO4993850 (3 mg/kg, 10 mg/kg and 30 mg/kg). The doses of SSR504734 were selected according to the previous published research (Depoortère 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 CSF 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 of body weight/day) and water intake (ml/kg of body weight/day) were measured daily for a subsequent week.

Home-cage locomotor activity measurements by the E-motion system

In order 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 by 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

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

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 four 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 ~30µl 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-min 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 (non-reinforcement). Discriminative stimuli predicting ethanol or water availability were presented during each ethanol or water self-administration session (one 30-min session/day). An orange flavour extract served as the contextual stimulus (S+) for ethanol, whereas water availability was signalled by a lemon grass extract (S-). These olfactory stimuli were generated by depositing 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-s blinking-light conditioned stimulus (CS+), whereas a 5-s constant-light stimulus (CS-) was presented with water delivery. The 5-s 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.

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

During the first two 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-min extinction sessions for five 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 three 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+ (blinking-light) in the S+ condition or the CS- (constant-light) 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 two 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, while the other two groups of animals were injected with the respective vehicle. Drug administration was performed 30 min 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.

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

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, we tested 15 two-month-old male Sprague-Dawley rats (Charles River, Germany) in the cocaine cue-induced reinstatement model.

Catheter implantation

Rats were anesthetised with 4% isoflurane and maintained anesthetised 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, Düsseldorf, Germany) was passed under the skin and protruded in the mid-scapular region. Rats were given 9-14 days recovery before cocaine self-administration (CSA) sessions began. Cocaine-HCl (Sigma-Aldrich, Germany) was dissolved in sterile saline. Catheters were flushed daily with a heparinized solution (100 I.U./ml) containing 1 mg/ml of enrofloxacin (Baytril®).

Self-administration Apparatus

Cue-induced reinstatement of cocaine seeking was carried out in operant chambers (Imetronic, 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 µl (0.26 mg) of cocaine over a period of 2 sec. 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-h daily sessions until stable responding was established. During the first three sessions animals were trained under a fixed

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

ratio 3 (FR3) schedule of reinforcement. FR5 schedule was used for the reminder of conditioning phase. Cocaine availability was signalled 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 s the infusion pump was activated. The white cue light and beep-sound was maintained for 4 s in total. Cocaine infusion was followed by a 40 s 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 cocaine or the presentation of response-contingent cues. Extinction lasted for seventeen 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 which 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 s 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, while 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 min before the reinstatement test.

Statistical analysis

Data obtained from ADE measurements (total alcohol intake, water intake) and locomotor activity was analysed using a two-way ANOVA with repeated measures [factors were:

JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

treatment group and day]. Locomotor activity data was analysed using recordings of 12-hour post-injection 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 analysed 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 $p < 0.05$.

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

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 over-all increase in alcohol intake after a deprivation phase compared to 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 – SSR504734, A-1246399 and RO4993850 – treatments significantly reduced alcohol intake during post-abstinence days compared to that of vehicle treated animals [factor treatment group × 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 post-abstinence week (data on drinking during subsequent post-abstinence 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 post-abstinence day compared to 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 to that of vehicle treated animals [factor treatment group × 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. 1B, 1D, 1F).

Analysis of locomotor activity data revealed that there was a general reduction in home-cage activity in all animal groups, which was likely caused by alcohol intoxication during the first post-abstinence 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

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

treatment group and treatment group × 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 × 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 × 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 (~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 behaviour

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 5 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 to 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 S+/CS+ reinstatement test was seen in 10 mg/kg of SSR504734 treated rats [factor treatment group × 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 behaviour. In A-1246399 treated rats, responding on the active lever during the reinstatement test did not differ from

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

that seen during the extinction sessions [factor treatment group × 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 to 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 towards the lever (active vs. inactive), indicating that it may have been caused by a general arousal [factor session × lever interaction: $p=0.29$ and $p=0.12$ for SSR504734 and A-1246399 treatment groups, respectively]. Both treatments significantly reduced responding on the water-associated lever [factor treatment group × 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 3C, 3D and Fig. 4C, 3D).

The fact that water responding was affected as well by GlyT1 blockade is suggestive of a more general involvement of glycinergic system in stimulus-reward association. To test if GlyT1 blockade selectively affects only alcohol-related behaviours we further tested two of the drugs in the cocaine cue-induced reinstatement model.

SSR504734 and A-1246399 reduced cue-induced reinstatement of cocaine-seeking behaviour

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 20 extinction sessions to 42±4 of the previously cocaine-reinforced nose-pokes, and to 30±6 of the non-reinforced nose-pokes.

Three-way ANOVA with repeated measures revealed that the number of nose-pokes increased during the cocaine cue-induced reinstatement test compared to 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

JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

the extinction phase [factor treatment group × session interaction: $F(1,26)=12.1$, $p < 0.01$] (Fig 5A). Similarly, active nose-pokes during the reinstatement test were abolished by A-1246399 treatment [factor treatment group × session interaction: $F(1,22)=16.2$, $p < 0.001$] (Fig. 6A). Inactive nose-pokes were not reduced by either SSR504734 or A-1246399 treatment compared to the vehicle treated group (Fig. 5B, 6B).

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

DISCUSSION

Our study demonstrated that three different GlyT1 blockers reduced or even abolished relapse-like alcohol consumption in male rats during the first post-abstinence 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. In order to compensate for decreased ethanol consumption, water intake increased in drug-treated rats compared to vehicle-treated rats, demonstrating the selectivity of tested compounds towards 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 behaviour 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. Non-reinforced 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 behaviour.

In our earlier study we showed that repeated treatment with GlyT1 blocker Org25935 reduced relapse-like drinking in chronically drinking rats, which may have resulted from a reversal of alcohol-induced alterations of glycinergic and glutamatergic signalling. Hence, multiple changes were found in gene expression profiles associated with glycinergic and glutamatergic signalling. 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 behavioural effects of Org25935 were likely 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, 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 water cue-induced responding. This indicates a more general effect of GlyT1 blockade on stimulus-reward learning, and indeed, cue-induced reinstatement of cocaine-seeking behaviour was reduced as

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

well by SSR504734 and A-1246399. SSR504734 treatment has also been shown to reduce nicotine-seeking behaviour (Cervo et al., 2013). In contrast, 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-generalisation may have occurred in these animals. This could have interfered with the treatment effect on animal behaviour 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 of NMDARs in mediating the cue-induced drug-seeking response have been outlined in numerous studies on other drugs of abuse, primarily cocaine (Bäckström and Hyytiä, 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 behaviour, independently if animals are 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 behaviour. However, neither competitive NMDAR antagonists nor NMDAR channel blockers were found effective in either alcohol or cocaine cue-induced reinstatement (Bespalov et al., 2000; Bachteler et al., 2005; Bäckström and Hyytiä, 2004; 2006). Blocking the co-agonist glycine binding site of the NMDAR seems to be the only effective way of targeting this receptor in order to reduce cue-induced drug-seeking behaviour (Bäckström and Hyytiä, 2004; 2006). Elevated extracellular glycine levels were shown to potentiate NMDAR function but also cause internalisation of both AMPA and NMDA receptors and induce long-term depression of excitatory post-synaptic currents (Nong et al., 2003; Chen et al., 2011; Alberati et al., 2012), which in turn may have been also responsible for impaired animal performance during cue-induced reinstatement tests. Accumulation of extracellular Gly 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 behaviours has also been demonstrated using GlyR subunit mutant mice (Blednov et al., 2015).

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

All 3 tested drugs had a very low side effect profile. Some hypolocomotion was observed during drug testing in the ADE model; however, a general CNS depressant effect can be excluded as inactive lever responding in the reinstatement test was not affected. Drug treatment-induced hypolocomotion during ADE testing was likely related to activation of inhibitory GlyR. This effect was absent in case of SSR504734 and short-lasting in 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 behaviour 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, Bespalov and Spanagel.
Conducted experiments: Vengeliene, Roßmanith and Takahashi.
Contributed new compounds: Alberati, Behl and Bespalov.
Performed data analysis: Vengeliene.
Wrote or contributed to the writing of the manuscript: Vengeliene and Spanagel.

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

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FOOTNOTES

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 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

LEGENDS FOR FIGURES

Figure 1. Intake of total ethanol, calculated in g of pure alcohol per kg of body weight per day (A, C, E), and water, calculated in ml per kg of body weight per day (B, D, F), in (A, B) vehicle, 1 mg/kg of SSR504734, 3 mg/kg of SSR504734 and 10 mg/kg of SSR504734 (n=8 per treatment condition), (C, 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, 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 three weeks. The last week measurements of ethanol intake is given as baseline drinking – BL. Animals received a total of five, once every 12 h, i.p. injections of SSR504734 and A-1246399 and three, once daily, oral administrations of RO4993850 (arrows). The ethanol bottles were reintroduced after the second drug administration. Data are presented as means ± S.E.M. * indicates significant differences from the vehicle control group, $p < 0.05$.

Figure 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 post-injection 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 "^". The arrow indicates re-exposure of animals to alcohol solutions (Ethanol). Data are presented as means ± S.E.M. * indicates significant differences from the vehicle control group, $p < 0.05$.

Figure 3. The effect of vehicle and 10 mg/kg of SSR504734 (n=8 per treatment condition) on ethanol (A, B) and water (C, D) cue-induced reinstatement. Data are shown as the average number of lever presses on the active (A, C) and inactive (B, 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 ± S.E.M. * indicates significant differences to the extinction lever responses; + indicates significant differences from the vehicle control group, $p < 0.05$.

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

Figure 4. The effect of vehicle and 30 mg/kg of A-1246399 (n=8-9 per treatment condition) on ethanol (A, B) and water (C, D) cue-induced reinstatement. Data are shown as the average number of lever presses on the active (A, C) and inactive (B, 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 ± S.E.M. * indicates significant differences to the extinction lever responses; + indicates significant differences from the vehicle control group, $p < 0.05$.

Figure 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 ± S.E.M. * indicates significant differences to the extinction nose-pokes; + indicates significant differences from the vehicle control group, $p < 0.05$.

Figure 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 ± S.E.M. * indicates significant differences to the extinction nose-pokes; + indicates significant differences from the vehicle control group, $p < 0.05$.

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

FIGURES

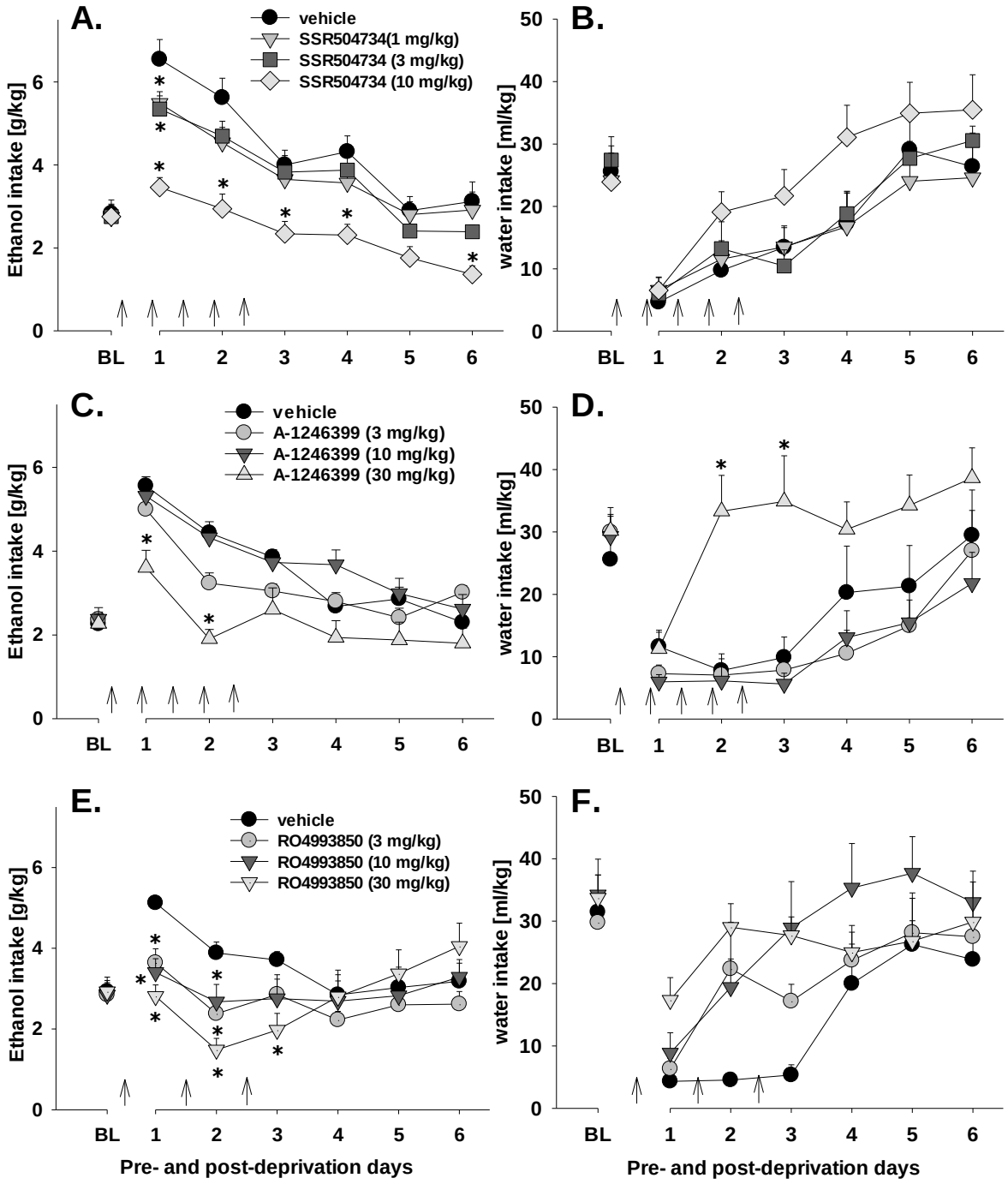

Figure 1

JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

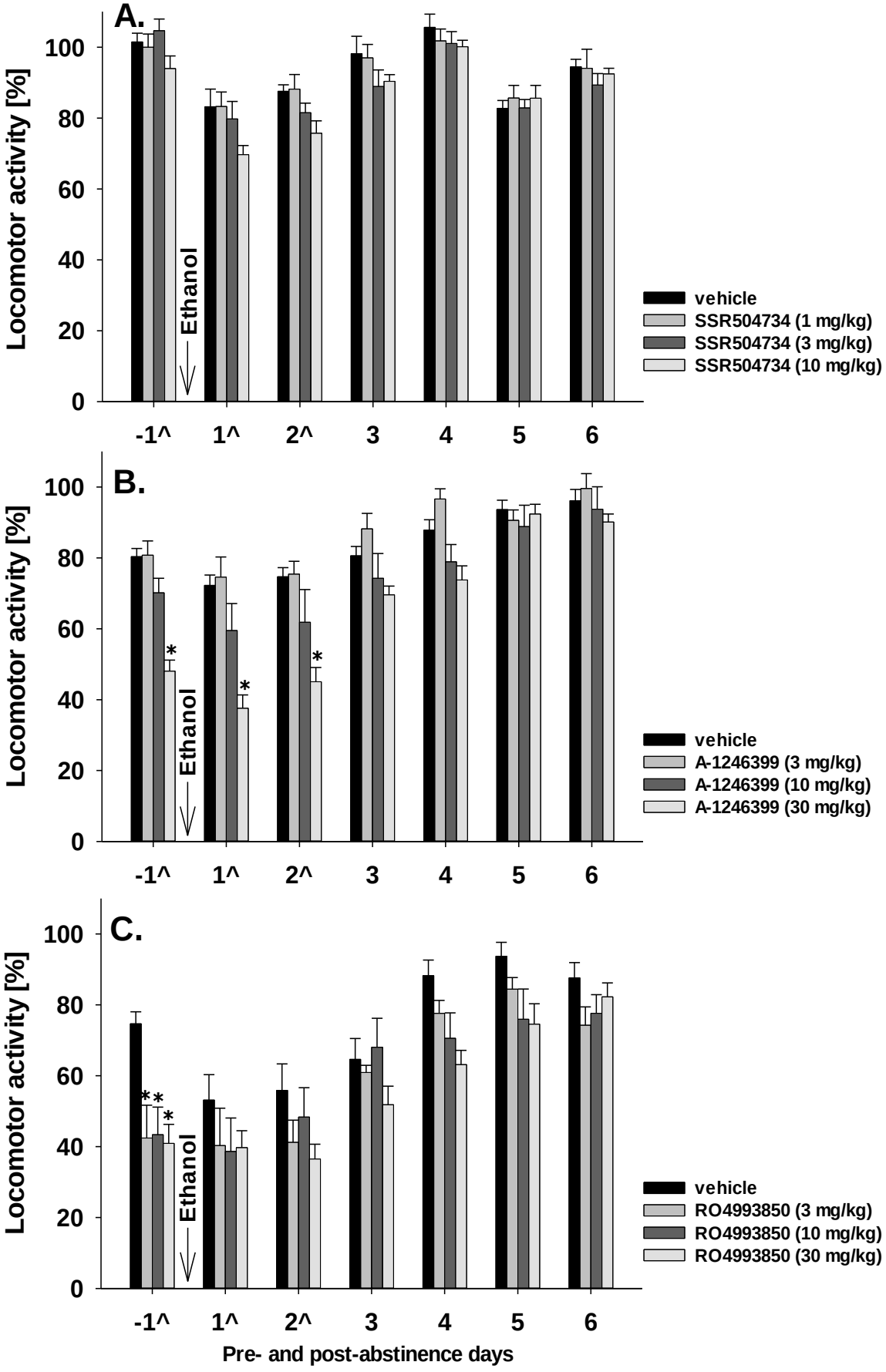

Figure 2

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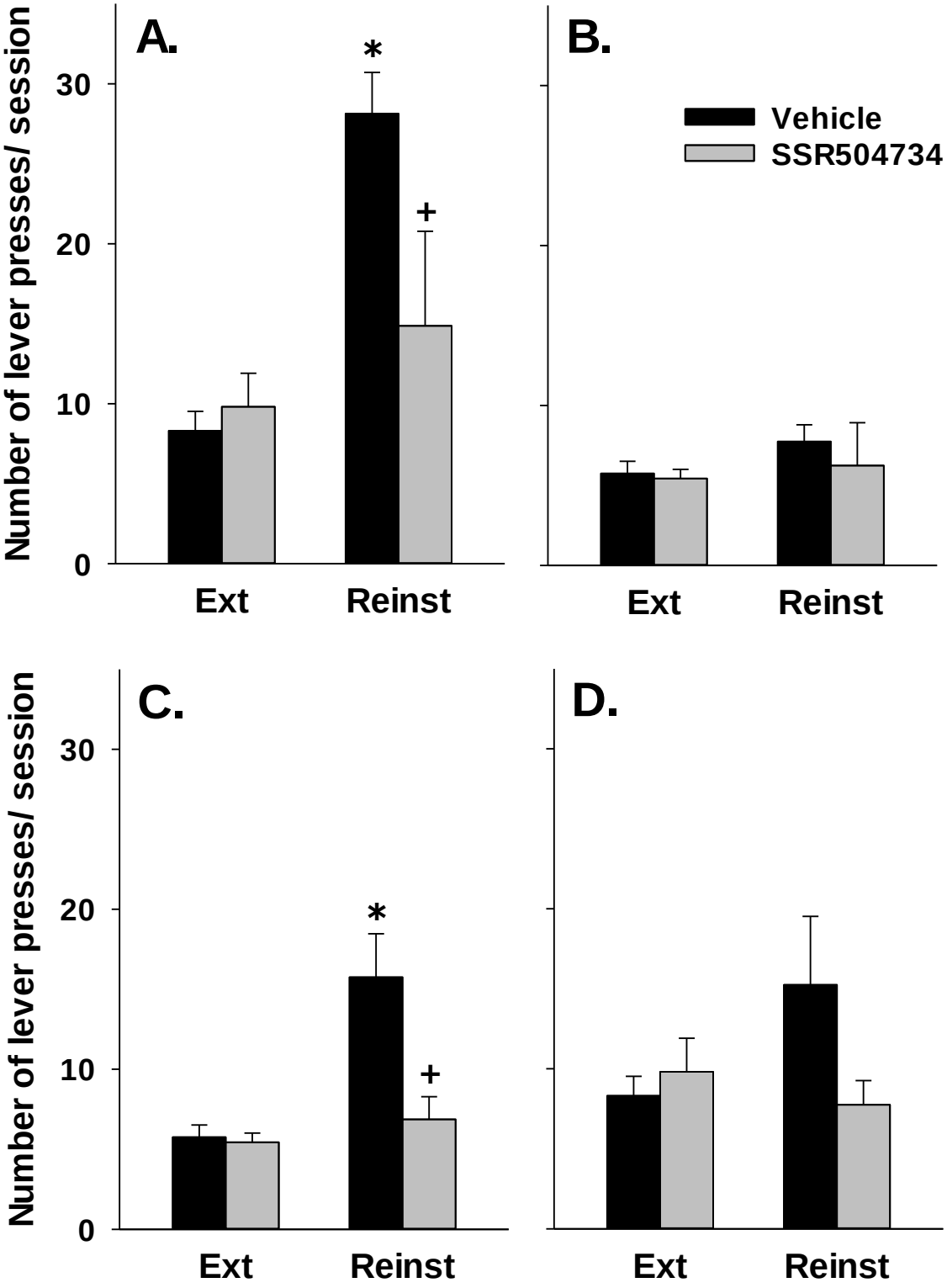

Figure 3

 JPET Fast Forward. Published on January 24, 2018 as DOI: 10.1124/jpet.117.244822

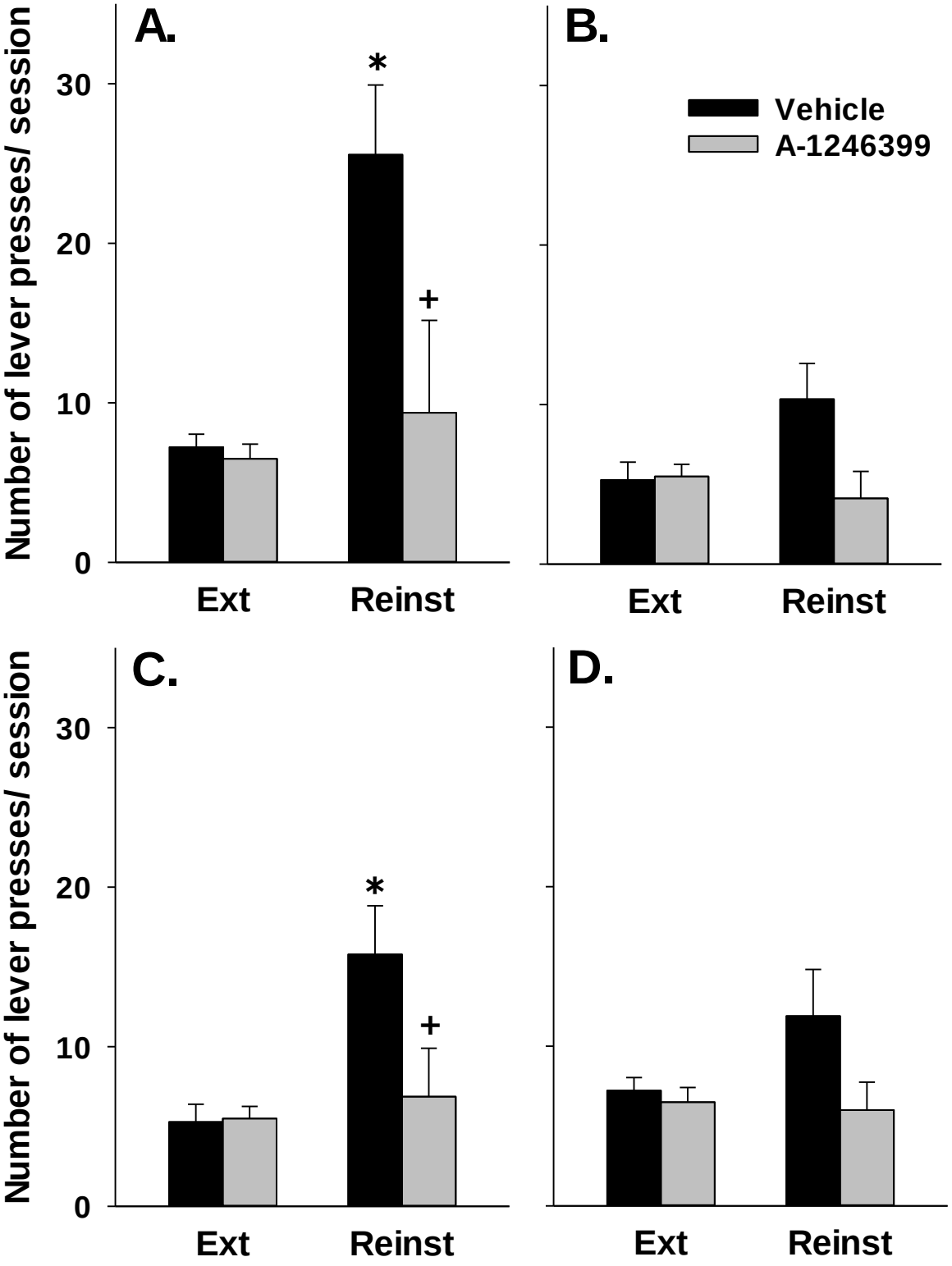

Figure 4

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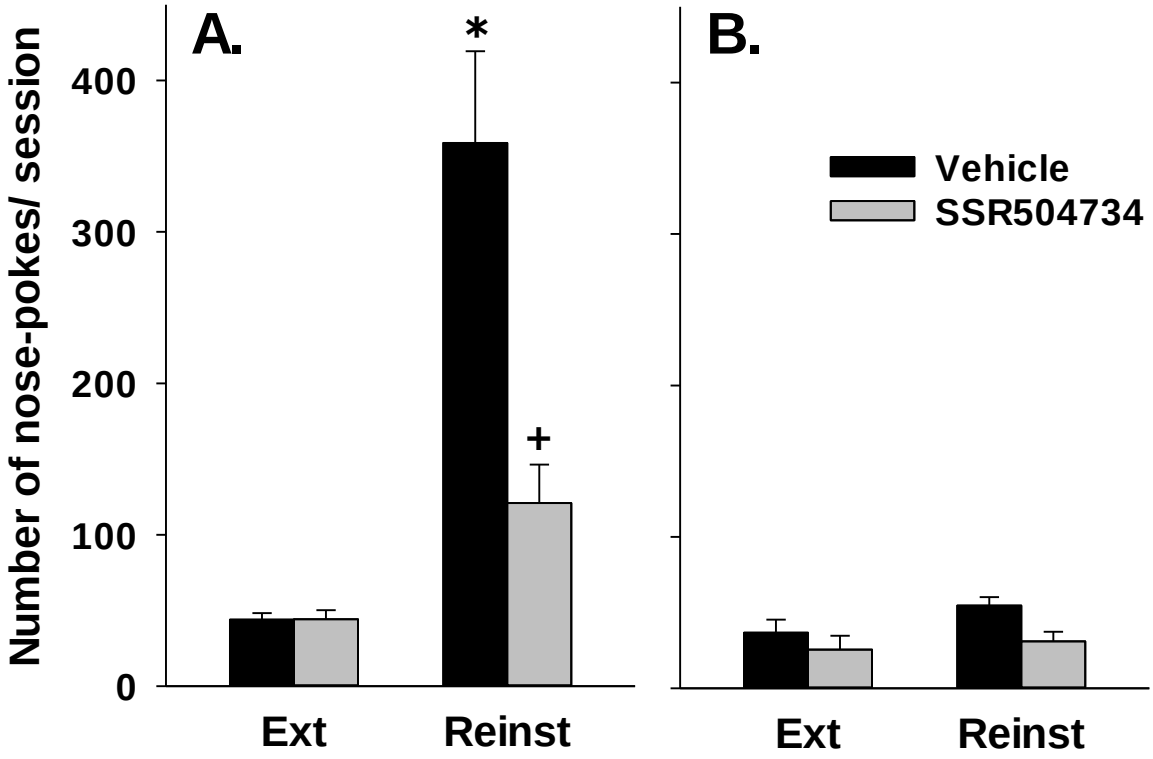

Figure 5

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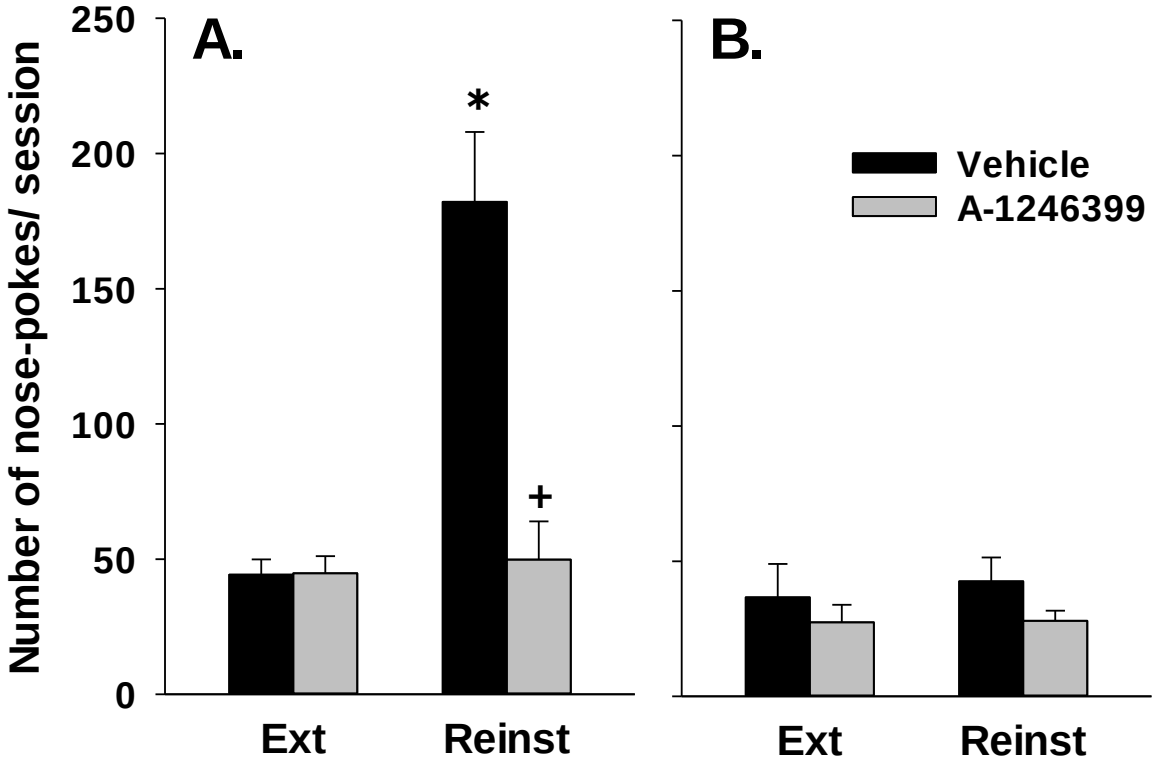

Figure 6