Dysregulated Glycine Signaling Contributes to Increased Impulsivity during Protracted Alcohol Abstinence

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Persons with alcoholism who are abstinent exhibit persistent impairments in the capacity for response inhibition, and this form of impulsivity is significantly associated with heightened relapse risk. Brain-imaging studies implicate aberrant prefrontal cortical function in this behavioral pathology, although the underlying mechanisms are not understood. Here we present evidence that deficient activation of glycine and serine release in the ventral medial prefrontal cortex (vmPFC) contributes to increased motor impulsivity during protracted abstinence from long-term alcohol exposure. Levels of 12 neurotransmitters were monitored in the rat vmPFC during the performance of a challenging variant of the five-choice serial reaction time task (5-CSRTT) in which alcohol-exposed rats exhibit excessive premature responding. Following long-term ethanol exposure, rats showed blunted task-related recruitment of vmPFC glycine and serine release, and the loss of an inverse relationship between levels of these neurotransmitters and premature responding normally evident in alcohol-naïve subjects. Intra-vmPFC administration of the glycine transport inhibitor ALX5407 prevented excessive premature responding by alcohol-exposed rats, and this was reliant on NMDA glycine site availability. Alcohol-exposed rats and controls did not differ in their premature responding and glycine and serine levels in vmPFC during the performance of the standard 5-CSRTT. Collectively, these findings provide novel insight into cortical neurochemical mechanisms contributing to increased impulsivity following long-term alcohol exposure and highlight the NMDA receptor coagonist site as a potential therapeutic target for increased impulsivity that may contribute to relapse risk.

Key words: alcohol dependence; five-choice serial reaction time task; glycine; motor impulsivity

Significance Statement

Persons with alcoholism demonstrate increased motor impulsivity during abstinence; however, the neuronal mechanisms underlying these behavioral effects remain unknown. Here, we took advantage of an animal model that shows deficiencies in inhibitory control following prolonged alcohol exposure to investigate the neurotransmitters that are potentially responsible for dysregulated motor impulsivity following long-term alcohol exposure. We found that increased motor impulsivity is associated with reduced recruitment of glycine and serine neurotransmitters in the ventromedial prefrontal cortex (vmPFC) cortex in rats following long-term alcohol exposure. Administration of glycine transport inhibitor ALX5407 in the vmPFC alleviated deficits in impulse control.

Introduction

Alcoholism is associated with significant disruptions in executive function that contribute to loss of control and relapse (Jentsch

and Taylor, 1999; Duka et al., 2011a; Goldstein and Volkow, 2011). Increased impulsivity contributes to binge drinking and long-term use (Weafer et al., 2014) and is associated with heightened relapse risk (Bowden-Jones et al., 2005; MacKillop and Kahler, 2009; Loree et al., 2015). Persons with alcoholism who have undergone detoxification exhibit increased impulsivity in tasks that index the capacity for response inhibition (Duka et al., 2003; Kamarajan et al., 2005; Rubio et al., 2008; Lawrence et al.,

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2009; Salgado et al., 2009), an effect that is most prevalent following several weeks of abstinence (Stavro et al., 2013) when relapse risk is high (Moos and Moos, 2006).

The restraint of improper responses relies on the function of the ventral medial prefrontal cortex (vmPFC; Iversen and Mishkin, 1970; Swick et al., 2011). In persons with alcoholism, these regions exhibit reduced volumes (Fein et al., 2002; Duka et al., 2011b) and aberrant activation during response inhibition tasks (Li et al., 2009). While compensatory activation of other brain regions allows persons with alcoholism to perform simple tasks at levels similar to those of persons without alcoholism (Chanraud et al., 2013), performance of more challenging tasks is impaired (Noël et al., 2001; Chanraud et al., 2013). Despite knowledge of regional disruptions in cortical function associated with long-term alcohol use (Sullivan and Pfefferbaum, 2005), the neurochemical underpinnings of dysregulated impulse control have not been elucidated.

Animal models provide an essential tool for elucidating the mechanisms underlying impaired impulse control associated with long-term alcohol exposure. The five-choice serial reaction time task (5-CSRTT) is particularly well suited for translating animal studies to the human condition, as this task has uncovered increased motor impulsivity (premature responding) in rodents undergoing protracted ethanol (EtOH) abstinence (Walker et al., 2011; Irimia et al., 2015) and in detoxified alcohol-dependent and binge-drinking humans (Sanchez-Roige et al., 2014; Voon et al., 2014). Following a month of abstinence, EtOH-dependent rodents adequately perform the standard version of the 5-CSRTT but display increased motor impulsivity under conditions of enhanced cognitive load during the challenge task (Walker et al., 2011; Irimia et al., 2014; Irimia et al., 2015). Likewise, alcohol-dependent humans display delayed emergence of impulsivity during abstinence (Stavro et al., 2013) that is most evident in cognitively challenging tasks (Moselhy et al., 2001; Noël et al., 2001; Jung et al., 2014).

The neural mechanisms mediating the constraint of waiting impulsivity in the 5-CSRTT are not fully understood. Lesions of the vmPFC increase motor impulsivity as evaluated by the standard 5-CSRTT (Chudasama et al., 2003), and NMDA receptor antagonism in this region results in excessive premature responding (Murphy et al., 2012), suggesting an important role for the vmPFC and NMDA signaling in the constraint of motor impulsivity in this task. Less is known about the specific neurotransmitter signals that are important for proper response inhibition during more cognitively challenging variants of the 5-CSRTT or following long-term EtOH exposure and abstinence.

The present study investigates neurochemical mechanisms in the vmPFC that underlie increased impulsivity during protracted abstinence from long-term EtOH exposure. We used *in vivo* microdialysis and mass spectrometry to quantify changes in 12 neurotransmitters evoked in the vmPFC during performance of a more challenging version of the 5-CSRTT task (Song et al., 2012; Buczynski et al., 2016). In rodents, repeated exposure to EtOH results in structural damage (Crews and Boettiger, 2009; Crews and Nixon, 2009), upregulation of NMDA-mediated activity (Kroener et al., 2012), and functional impairment (Holmes et al., 2012) in the vmPFC. Thus, we hypothesized that neurotransmitters that signal through the NMDA receptor will be reduced in the vmPFC of EtOH-dependent rats, but not of controls. Our analytical approach allowed us for the first time to measure extracellular levels of multiple neurotransmitters previously implicated in impulse control as well as several novel candidates.

Materials and Methods

Subjects. Male Wistar rats (71 rats; weight, 250 g; Charles River Laboratories) were housed two per cage on a 12 h circadian cycle (lights off at 10:00 AM). With the exception of 7 d of postsurgical recovery and EtOH vapor exposure, rats were maintained at 90% of free feeding weight to enable 5-CSRTT performance. All procedures strictly adhered to the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and were approved by The Scripps Research Institute Institutional Animal Care and Use Committee.

Five-choice serial reaction time task: standard and challenge sessions. All 5-CSRTT training and testing was performed as previously described (Irimia et al., 2015). Standard sessions were composed of 100 trials with a 2 s visual stimulus duration, a 2 s limited hold, and a 5 s intertrial interval (ITI). Performance was indexed as follows: (1) response accuracy [$100 \times \text{correct}/(\text{correct} + \text{incorrect})$] responses, an index of attentional capacity; (2) premature responses (failure to withhold responding during the ITI, an index of impulsive action); (3) perseverative responses; (4) omissions (index of ability to complete the task or motivation); (5) latency to correct response (index of information processing speed); and (6) feeder latency (index of motivation). Challenge sessions had the same parameters as the standard task, with the exception of the ITI, which was varied pseudorandomly among 5, 7, 9, and 11 s; and the stimulus duration, which was 1 s. All rats received standard 5-CSRTT training. Twenty-seven rats were tested under the challenge conditions, and 12 rats were tested under standard conditions during *in vivo* microdialysis sampling. Figure 1C depicts the experimental time line.

Long-term intermittent EtOH exposure. Rats were housed two per cage in inhalation chambers and exposed to EtOH vapor (EtOH group) or air (CON) 12 h/d for 4 weeks. Blood alcohol levels (BALs) were determined one time per week (Analox Instrument). This procedure has been shown to induce dependence (Schulteis et al., 1995; George et al., 2014) and to result in significant increases in motor impulsivity during abstinence (Irimia et al., 2014, 2015).

In vivo microdialysis. Rats were stereotactically implanted with a unilateral vmPFC microdialysis guide cannula [PlasticsOne; anteroposterior (AP), +3.0 mm from bregma; mediolateral (ML), ± 0.75 mm from bregma; dorsoventral (DV), -4.2 mm from dura; probe extension, 1 mm]. Once stable 5-CSRTT performance was re-established, neurochemical collection was performed by *in vivo* microdialysis as previously described (Caillé et al., 2007). Briefly, probes were implanted in the guide cannula and were subsequently perfused with artificial CSF overnight at $0.1 \mu\text{l}/\text{min}$. The following day, the flow rate was increased to $0.6 \mu\text{l}/\text{min}$, and samples were collected at 5 min intervals before and during the 5-CSRTT sessions. The six baseline samples before the beginning of the test session correspond to -30 to 0 min time points in Figure 1 (and see Figs. 3, 5). After the last baseline sample was collected, the animal was placed in the 5-CSRTT box, and the session started. The first sample corresponding to the first 5 min of the task was collected at the 5 min time mark. Dialysate levels of glycine, serine, aspartate, glutamate, GABA, dopamine, norepinephrine, serotonin, acetylcholine, taurine, histamine, and glutamine were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Song et al., 2012; Buczynski et al., 2016). The analytical method used cannot distinguish between D-serine and L-serine. Following the session, brains were snap frozen and analyzed for probe placement.

Drug treatment by site-specific infusion. Rats were stereotactically implanted with a bilateral vmPFC guide cannula for intracranial infusion (AP, +3.0 mm from bregma; ML, ± 0.75 mm from bregma; DV, -1.9 mm from dura; injector extension, 2.5 mm). Once stable 5-CSRTT performance was re-established, rats were site-specifically treated with vmPFC infusions of vehicle (20% DMSO, 20% Emulphor, 60% Saline; $0.6 \mu\text{l}$, $n = 16$ rats), the glycine transport inhibitor ALX5407 (2 ng/site, Sigma-Aldrich; $n = 16$ rats; Atkinson et al., 2001) and/or the glycine site NMDA receptor antagonist L-701,324 (2 ng/site, Santa Cruz Biotechnology; $n = 5$ rats; Vengeliene et al., 2005). Intra-vmPFC infusions ($0.6 \mu\text{l}$ over 2 min, injectors removed 2 min after infusion) were administered 90 min before behavioral testing. Following the session, brains were snap frozen and analyzed for cannula placement.

Figure 1. Experimental setup. **A**, The standard 5-CSRTT procedure is depicted. During the session, rats must withhold responding (left) then elicit a response upon presentation of a light stimulus in one of five apertures (middle). Nose poke in the correct aperture results in food reward. Premature responses (e.g., during the intertrial interval) are not rewarded and provide an index of motor impulsivity (right). In the challenge task, the wait time is varied randomly between 5, 7, 9, and 11 s. **B**, vmPFC microdialysates collected before and during 5-CSRTT performance were analyzed by LC-MS/MS for levels of 12 neurotransmitters. **C**, The time line of experiments is depicted. Performance of the challenge task changes the neurotransmitter profile in the vmPFC in CON rats. **D–J**, Task performance resulted in significant elevations in vmPFC dialysate dopamine (**D**), norepinephrine (**E**), glycine (**F**), serine (**G**), GABA (**H**), histamine (**I**), and taurine (**J**) in CON rats. Neurotransmitter temporal profiles show the percentage change from baseline during the 5-CSRTT session. Samples collected during the 5-CSRTT session are shown on a light gray background.

Statistical analyses. All data were analyzed using the PASW package (SPSS, version 18.0). Before chronic intermittent ethanol (CIE) exposure, the behavioral performance of the two groups (CON and pre-EtOH) was evaluated using a one-way ANOVA comparison for all behavioral indices calculated as the average of the last 5 d. The effect of CIE on standard task performance following long-term EtOH exposure was evaluated using a repeated-measures (RM) ANOVA with time (abstinence weeks 2, 3, and 4) as the within-subjects factor and group (CON, EtOH) as between-subjects factors. Behavioral performance during the challenge task following CIE exposure and abstinence was evaluated with a one-way ANOVA with group (CON, EtOH) as a factor for all behavioral indices. To test whether premature responses change during the course of the challenge task, we binned responses in six 5 min bins and conducted a two-way ANOVA with time (six bins) as a within-subjects factor and group (CON, EtOH) as a between-subjects factor. Group differences in baseline neurotransmitter levels were examined by one-way ANOVA with group (CON, EtOH) as the between-subjects factor. Neurotransmitter levels were expressed as nanomolar concentrations averaged over the six pre-session baseline samples. Analysis of overall task-related changes in vmPFC neurotransmitter levels was made using area under the curve (AUC) measures, which were obtained by subtracting the average baseline concentration from individual sample concentrations.

Figure 2. Challenge 5-CSRTT disrupts performance in EtOH-exposed rats. A 5-CSRTT challenge task was used to evaluate the behavior of drug-naïve (CON, $n = 14$) and CIE rats (EtOH, $n = 13$). **A–E**, Rats with a history of CIE exposure made more (**A**) premature responses than CON rats, but did not differ in terms of accuracy (**B**), omissions (**C**), perseverative responses (**D**), or total trials (**E**). **F**, Time course of premature responses during the session.

tration levels and summing the resultant values obtained during task performance. Changes in neurotransmitter levels over time as CON rats engaged in the challenge task were evaluated by one-way ANOVA with time (12 total 5 min bins; 6 bins of baseline and 6 bins of challenge task samples) as a within-subjects factor. The effect of EtOH history on 5-CSRTT behavior and neurotransmitter levels during the challenge task was examined using RM ANOVA with time (six 5 min bins) as the within-subjects factor and group (CON, EtOH) as the between-subjects factor. We used a one-step false discovery rate (FDR) analysis (Benjamini and Hochberg, 1995; Pike, 2011) to correct *p* values obtained from ANOVAs of individual neurotransmitters for multiple comparisons. The FDR adjustment was applied after grouping the neurotransmitters into three groups based on our original hypothesis (NMDA agonists, monoamines, and other neurotransmitters). Reported *p* values in text represent corrected values. Associations between dialysate neurotransmitter levels and premature responses were evaluated by Pearson's correlation followed by one-sided significance tests. The effects of intra-vmPFC drug infusions on challenge 5-CSRTT performance were examined by three-way RM ANOVA with group and treatment as between-subjects factors and ITI level as the within-subjects factor. The Greenhouse–Geisser correction was used for all RM ANOVAs when sphericity assumptions were violated, and *F* values in the text represent the corrected values. Significant interactions were followed by simple effects ANOVA and Student's *t* test. Data are presented as the mean $\pm$ SEM. In all cases, the criterion for significance was as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Lack of significance was noted as “NS” (nonsignificant).

Results

Long-term ethanol exposure does not affect standard task performance

To evaluate the effect of long-term EtOH exposure on motor impulsivity, rats (CON, *n* = 14; EtOH, *n* = 13) were trained to perform a standard 5-CSRTT that required them to wait for a fixed amount of time (5 s) until they could respond in the appropriate aperture for a reward (Fig. 1). Behavioral indices of standard task performance before EtOH exposure did not differ between the two groups (CON vs pre-EtOH: $F_{(1,26)} < 3.2$, NS; *p* > 0.08, NS for all parameters). The EtOH group sustained average BALs of 296 ± 18 mg% during long-term EtOH vapor exposure, yet, consistent with prior observations (Walker et al., 2011; Irimia et al., 2014), no significant group differences in standard performance were evident during a 3 week post-EtOH period (CON vs EtOH: $F_{(1,25)} < 3.9$, NS; *p* > 0.06, NS for all parameters).

Baseline neurotransmitter levels before the challenge five-choice session

To evaluate the effect of long-term EtOH exposure on cognitive performance, CON and EtOH rats were tested on a challenging version of the 5-CSRTT 4 weeks into abstinence. Neurotransmit-

Figure 3. Challenge 5-CSRTT differentially recruits neurotransmitters in the vmPFC of CON and EtOH rats. **A–C**, An unbiased LC-MS/MS analysis of the levels of 12 neurotransmitters in the vmPFC during the performance of the challenge 5-CSRTT (**A**) found blunted release of glycine (**B**) and serine (**C**) in EtOH rats (*n* = 13) compared with CON rats (*n* = 14). **D, E**, EtOH rats showed a trend toward increased dopamine release (*p* = 0.051; **D**) and a reduced initial burst of norepinephrine compared with CON rats (*p* = 0.051; **E**). Neurotransmitter temporal profiles show the percentage change from baseline during the 5-CSRTT session. Baseline samples were collected from *t* = 0–30 min. Samples collected during the 5-CSRTT session are shown on a light gray background. AUC values for individual neurotransmitters were calculated by summing the relative change from baseline in each of the six samples collected during the session. **p* < 0.05 and ***p* < 0.01.

ter release in the vmPFC before and during the performance of the challenge task was measured using *in vivo* microdialysis. Baseline samples collected before the session showed no group differences in neurotransmitter content ($F_{(1,26)} < 3.9$, *p* > 0.06 for all neurotransmitters, NS).

Performance of the challenge task changes the neurotransmitter profile in the vmPFC in control rats

In CON rats, neurotransmitter levels in the vmPFC significantly increased during the performance of the challenge task compared with pre-session baseline levels (CON, dopamine: time, $F_{(11,143)} = 3.6$, *p* = 0.028; norepinephrine: $F_{(11,143)} = 3.0$, *p* = 0.03; glycine: $F_{(11,143)} = 9.2$, *p* = 0.000036; serine: $F_{(11,143)} = 5.9$, *p* = 0.005; GABA: $F_{(11,143)} = 3.3$, *p* = 0.02; histamine: $F_{(11,143)} = 9.5$, *p* = 0.0009; taurine: $F_{(11,143)} = 3.3$, *p* = 0.0009; Fig. 1D–J).

EtOH rats are more impulsive during the performance of the challenge task

In comparison with CON rats, EtOH rats exhibited significantly higher levels of premature responding during the challenge session (Fig. 2*A*; $F_{(1,26)} = 5.1$, $p = 0.03$). No group differences in response accuracy (Fig. 2*B*) or other performance indices ($F_{(1,26)} < 2.9$, NS; $p > 0.09$, NS for all other behavioral indices; Fig. 2*C–E*) were evident, which is consistent with our prior observations (Irimia et al., 2014, 2015). Although EtOH rats made overall more premature responses during the 5-CSRTT probe session compared with CON rats, there was no significant change over time in the number of premature responses elicited (Fig. 2*F*; premature responses: time: $F_{(5,125)} = 1.4$, $p = 0.22$, NS; time $\times$ group interaction, $F_{(5,125)} = 1.5$, $p = 0.18$, NS).

Long-term ethanol exposure reduces release of NMDA coagonists glycine and serine during the challenge task

To evaluate potential alcohol-induced disruptions in signaling that drive impulsivity, we compared neurotransmitter release between CON and EtOH rats during the challenge task performance in the vmPFC (Fig. 3*A–E*). Rats previously exposed to EtOH exhibited blunted vmPFC release of the NMDA coagonists glycine and serine (CON vs EtOH: glycine: $F_{(1,25)} = 8.6$, $p = 0.02$; serine: $F_{(1,25)} = 6.8$, $p = 0.03$; Fig. 3*B,C*). There was a trend toward increased vmPFC dopamine levels and reduced norepinephrine levels in EtOH rats compared with CON rats that, although significant in individual ANOVAs, was no longer significant after adjusting p values to account for repeated comparisons (CON vs EtOH: dopamine, $F_{(1,25)} = 5.1$, $p = 0.051$; norepinephrine, $F_{(1,25)} = 5.0$, $p = 0.051$; Fig. 3*D,E*).

To further investigate the relationship between vmPFC neurochemical responses and motor impulsivity, we constructed a regression model to probe the relationship between dialysate neurotransmitter content and premature responses. The independent variables were each of the neurotransmitter concentration in each of the 5 min samples for each animal, and the dependent variable was the number of premature responses in the time bin. This model accounted for 48% of the variance in premature responses by CON rats ($r = 0.691$, $R^2 = 0.478$, $F_{(12,83)} = 5.4$, $p < 0.001$) and 47% of the variance in premature responses by EtOH rats ($r = 0.686$, $R^2 = 0.470$, $F_{(12,77)} = 4.8$, $p < 0.001$). These models explained more of the behavioral variance than previous models restricted to monoamines (Dalley et al., 2002), validating the utility of more comprehensive neurotransmitter evaluations. Probing the influence of individual neurotransmitters selected based on ANOVAs (Fig. 3) revealed significant negative correlations between glycine and serine levels and premature responses during the challenge sessions in CON rats (glycine: $r = -0.204$, $p < 0.05$; serine: $r = -0.182$, $p < 0.05$) but not in EtOH rats (glycine: $r = -0.056$, NS; serine: $r = 0.142$, NS). In contrast, dopamine was positively correlated with premature responses in EtOH rats, but not CON rats, during the challenge sessions (EtOH: $r = 0.200$, $p < 0.05$; CON: $r = -0.042$, NS). Norepinephrine levels were not correlated with premature responses in either group. Collectively, these results suggest that deficient recruitment of vmPFC glycine and other NMDA coagonists may underlie enhanced impulsivity observed during protracted EtOH abstinence.

Treatment with a glycine transport inhibitor reduces EtOH-specific impulsivity through the NMDA receptor

To test the hypothesis that deficient glycine coactivation of the NMDA receptor contributes to impulsivity during protracted

Figure 4. Intra-vmPFC infusion of the glycine transport inhibitor ALX5407 reduces excessive premature responding by EtOH rats in the challenge task. *A, C*, Following intra-vmPFC VEH infusion EtOH rats ($n = 6$) exhibited significantly more premature responses than CON rats ($n = 10$; *A, C*). Intra-vmPFC ALX5407 (2 ng/site) significantly reduced premature responding by EtOH rats ($n = 8$; *A*) without altering premature responding by CON rats ($n = 8$; *C*). *B, D*, There was no significant effect of intra-vmPFC ALX5407 on response accuracy in either EtOH (*B*) or CON (*D*) rats. *E*, ALX5407-induced reductions in premature responding by EtOH rats were reversed by coadministration of the NMDA receptor glycine site B antagonist L-701,324 (2 ng/site, each; ALX, $n = 7$; ALX + L-701, $n = 5$; VEH, $n = 6$). *F*, Coadministration of ALX5407 and L-701,324 did not alter the response accuracy by EtOH rats. $^{\#}p < 0.05$.

EtOH abstinence (VEH-treated EtOH vs CON: $F_{(1,14)} = 5.4$, $p = 0.032$; Fig. 4*A,C*), we evaluated the effects of intra-vmPFC infusion of the glycine transport inhibitor ALX5407 (Atkinson et al., 2001) on challenge task performance. The administration of ALX5407 significantly reduced premature responding by EtOH rats (Fig. 4*A*; EtOH, VEH vs ALX5407: $F_{(1,12)} = 7.4$, $p = 0.018$) to levels observed in vehicle-treated CON rats (group $\times$ treatment: $F_{(1,28)} = 4.4$, $p = 0.044$; Fig. 4*C*). Intra-vmPFC ALX5407 had no effect on premature responding by CON rats (Fig. 4*C*; CON rats: $F_{(1,16)} = 1.0$, NS) and did not alter response accuracy (Fig. 4*B,D*) or other performance indices in either group. The attenuation of premature responding by EtOH rats following intra-vmPFC ALX5407 was fully blocked by coadministration of the selective glycine-site NMDA receptor antagonist L-701,324 (Vengeliene et al., 2005; Fig. 4*E*; effect of pretreatment: $F_{(2,15)} = 3.8$, $p = 0.045$; ALX vs VEH: $F_{(1,11)} = 4.9$, $p = 0.047$; ALX vs ALX + L701: $F_{(1,10)} = 11.6$, $p = 0.006$; ALX + L701 vs VEH: $F_{(1,9)} < 1$, NS), and combined ALX5407 and L-701,324 administration did not significantly alter response accuracy (Fig. 4*F*) or other indices of challenge task performance. Together, these results demonstrate a functional link between a deficient NMDA coagonist signaling and increased impulsivity during protracted EtOH abstinence.

CON and EtOH rats perform a standard 5-CSRTT at similar levels

A separate group of rats (CON, $n = 6$; EtOH, $n = 6$) acquired the standard 5-CSRTT. Before undergoing long-term EtOH vapor exposure, CON and pre-EtOH rats had similar behavioral performance on the standard 5-CSRTT (Con vs pre-EtOH rats: $F_{(1,11)} > 2.8$, $p > 0.12$, NS for all parameters). As expected from the experiment described above and previous results (Irimia et al., 2014, 2015), following EtOH exposure CON and EtOH rats performed at similar levels on the standard task throughout 4 weeks of abstinence ($F_{(1,10)} > 2.5$, $p > 0.142$, NS; for all param-

Figure 5. EtOH rats demonstrate increased aspartate levels during the performance of the standard 5-CSRTT. **A, B.** *In vivo* microdialysis sampling and LC-MS/MS analysis of samples collected while CON ($n = 6$) and EtOH ($n = 6$) rats were performing a standard 5-CSRTT session (**A**) showed that EtOH rats had significantly higher levels of aspartate compared with CON rats (**B**). Neurotransmitter temporal profiles show the percentage change from baseline during the 5-CSRTT session. Samples collected during the 5-CSRTT session are shown on a light gray background.

eters with the exception of latency to correct response: $F_{(1,10)} = 9.5$, $p = 0.012$). At the end of 4 weeks of abstinence, microdialysis samples were collected from the vmPFC for 30 min of baseline values and then for 30 min as the rats solved a standard 5-CSRTT.

Baseline neurotransmitter levels before a standard five-choice session

Microdialysis samples collected 30 min before the start of the standard 5-CSRTT showed no difference in baseline levels of neurotransmitters between the two groups (CON vs EtOH: $F_{(1,10)} < 1.6$, $p > 0.2$, NS). These results mirror the lack of baseline group differences observed in rats before the start of the challenge task.

Long-term ethanol exposure increases release of aspartate during the standard task

During the standard 5-CSRTT session in which *in vivo* microdialysis samples were collected from the vmPFC, rats in the CON and EtOH group performed at similar levels, showing no significant group differences in any behavioral index ($F_{(1,10)} < 2.1$, $p > 0.176$). CON and EtOH rats had similar neurotransmitter profiles in vmPFC throughout the session (Fig. 5A; $F < 6.5$, $p > 0.14$, NS for 11 neurotransmitters) with the exception of aspartate, which was significantly elevated in the EtOH group (Fig. 5B; $F_{(1,10)} = 9.4$, $p = 0.047$).

Discussion

Rats with long-term ethanol exposure demonstrate improper response inhibition in the 5-CSRTT challenge task (Walker et al., 2011; Irimia et al., 2015). The present study replicates these behavioral observations and reveals that increased motor impulsivity in the EtOH group is associated with diminished recruitment of vmPFC glycine and serine. EtOH-exposed rats do not demonstrate a correlation between levels of glycine and serine and the premature responding that is evident in CON rats. Intra-vmPFC administration of the glycine transport inhibitor ALX5407 ameliorates excessive premature responding by EtOH-exposed rats through a mechanism reliant on the availability of the glycine site on the NMDA receptor. These results indicate that deficient signaling at the NMDA receptor 1 (NR1) coagonist site contributes to increased motor impulsivity during protracted EtOH abstinence.

The relevance of the present observations for the clinical population relies on the strength of the models used to obtain the mechanistic insight. Here, the increase in motor impulsivity in alcoholics was modeled by an increase in premature responses in

rats with CIE vapor exposure. Most clinical data on the tasks of response inhibition require inhibition of specific stimuli (Noël, 2012; Stavro et al., 2013), while in the challenge 5-CSRTTs rats made more responses before the presentation of the Go stimulus. However, a recent study (Voon et al., 2014) showed that persons with alcoholism are also deficient in their ability to inhibit prepotent motor responses while solving the 5-CSRTT. CIE exposure through vapor inhalation (Rogers et al., 1979; O'Dell et al., 2004; Gilpin and Koob, 2008) is a valuable model of alcohol dependence as, in rats, prolonged exposure leads to several signs of dependence, such as increased EtOH consumption (Gilpin and Koob, 2008), the presence of withdrawal when the drug is not available (Schulteis et al., 1995), increased likelihood of relapse (Liu and Weiss, 2002), and continued consumption of EtOH despite negative consequences (Vendruscolo et al., 2012).

Glycine has not previously been implicated in modulating impulse control. The importance of NMDA receptor signaling for cognitive processing has long been recognized (Tang et al., 1999), and, due to the role of glycine as an obligatory coagonist for the NMDA receptor, glycine signaling has been evaluated as a therapeutic target to improve cognitive function in pathologic conditions such as schizophrenia (Harvey and Yee, 2013; Young et al., 2015). NMDA receptors also modulate response inhibition (Murphy et al., 2012; Finlay et al., 2015). The present evidence supports and extends these observations by implicating vmPFC glycine signaling at the NMDA coagonist site in the constraint of motor impulsivity. As a coagonist, glycine may improve response inhibition by reversing hypofunctioning states of the NMDA receptor (Coyle, 2006). Serine is also an agonist at the NMDA receptor coagonist site (Araque et al., 2014), and the present finding of blunted serine recruitment during challenge task performance by EtOH-exposed rats suggests a combined influence of deficient glycine and serine signaling in the excessive premature responding elicited by these rats. However, we note that using our analytical procedure, we could not distinguish between D-serine and L-serine. EtOH-naïve rats demonstrate impaired impulse control following NMDA antagonism in vmPFC (Murphy et al., 2012), which is blocked by GABA_A antagonism (Murphy et al., 2012), or following lesions of vmPFC (Chudasama et al., 2003). Thus, impulse control may depend on the balance of excitation and inhibition in the vmPFC circuit (Lisman, 2012; Murphy et al., 2012). Although in the current experiments there were no group differences in GABA levels during the challenge task (Fig. 3), performance of the challenge probe resulted in significantly increased

GABA levels compared with baseline (Fig. 1*H*). A lack of compensatory activation of NMDA receptors by glycine and serine in the EtOH group could result in increased inhibition of pyramidal cells in vmPFC. The ability of the glycine transporter inhibitor ALX-5407 to improve impulse control in the EtOH group provides further evidence that glycine and serine signaling at the NMDA coagonist site modulated motor impulsivity, although a limitation of the current study is the lack of microdialysis measurements following ALX-5407 infusion, confirming an increase in glycine levels (Perry et al., 2008). Together, the present findings suggest that bolstering NMDA receptor coagonist site occupancy ameliorates post-EtOH increases in motor impulsivity at this protracted abstinence time point.

Diminished expression of NMDA receptor subunits and glycine signaling at the strychnine-sensitive glycine receptors may contribute to excessive premature responding by EtOH-exposed rats. Indeed, reduced NR1 expression in the mPFC emerges during abstinence from long-term EtOH exposure (Holmes et al., 2012; Kroener et al., 2012). In the nucleus accumbens, EtOH perfusion increases dopamine levels, and the effect is blocked by the antagonism of glycine receptors (Jonsson et al., 2014), raising the possibility that, in the current study, reduced glycine levels in the EtOH group may contribute to the trend in increased dopamine and impaired impulse control observed during the challenge task.

Higher levels of premature responses in the EtOH group during the challenge 5-CSRTT session could result from impaired learning in this group. Rats experience the variable ITI for the first time during the challenge 5-CSRTT session, and NMDA receptor function has previously been shown to be important for instrumental learning (Sakimura et al., 1995; Baldwin et al., 2002). However, the lack of an improvement in premature responses with time in CON rats performing the challenge task (Fig. 2*F*) does not support an effect of learning in the current experiments. Because previous work (Walker et al., 2011) has shown that CON rats can learn to restrain premature responses following repeated challenge tests, it is possible that group differences in response inhibition would be exacerbated if CON and EtOH rats were given repeated challenge tests closely spaced in time.

Existing evidence demonstrates the therapeutic potential of targeting glycine for alcoholism. Glycine transport inhibition or D-cycloserine administration (a glycine-B site agonist) reduces the positive effects of EtOH, enhances the extinction of ethanol-related cues, and reduces the hyperglutamatergic state associated with alcoholism (Trevisan et al., 2008; Vengeliene et al., 2008, 2010; Spanagel, 2009; Lidö et al., 2012; Holmes et al., 2013; Seif et al., 2015). The present findings suggest that these therapeutic approaches may additionally improve impulse control under conditions of enhanced cognitive load, a capacity that is impaired in individuals with problematic alcohol use (Moselhy et al., 2001; Noël et al., 2001; Jung et al., 2014) and may contribute to heightened risk of relapse (Bowden-Jones et al., 2005; MacKillop and Kahler, 2009; Loree et al., 2015).

The present analytical approach allowed the novel unbiased evaluation of parallel neurotransmitter changes needed to characterize complex cognitive functions that engage multiple signaling systems. For example, we found that performance in the 5-CSRTT challenge session by naive rats led to the cortical release of GABA, taurine, and histamine (Fig. 1*H–J*). Infusion of GABA_A agonists in the vmPFC increases premature responses during 5-CSRTT baseline sessions (Murphy et al., 2012), suggesting that behavioral inhibition can be modulated through GABA receptors

in the vmPFC cortex. Taurine levels correlate with behavioral inhibition as evaluated by the Stroop test in schizophrenic patients (Shirayama et al., 2010). Increased histamine release supports a proposed role for this neurotransmitter in promoting wakefulness (Passani et al., 2014). Together, these findings highlight the overall neurochemical diversity underlying complex behaviors, such as impulsivity, and the importance of evaluating cortical dysfunction using a comprehensive analytical approach.

Neuroimaging studies suggest that in persons with alcoholism the recruitment of compensatory cortical mechanisms serves to sustain control levels of cognitive task performance (Li et al., 2009; Chanraud et al., 2013; Jung et al., 2014). In the present experiments, EtOH-exposed and CON rats exhibited comparable performance of the standard 5-CSRTT, although EtOH-exposed rats displayed significantly greater recruitment of vmPFC aspartate during this task. Aspartate is a weak NMDA receptor coagonist (Patneau and Mayer, 1990), and robust elevations in vmPFC aspartate levels may serve to sustain NMDA receptor signaling in EtOH-exposed rats during standard 5-CSRTT performance. However, this group difference in aspartate recruitment is lost during the challenge task, which, together with deficient recruitment of glycine and serine, may contribute to impaired motor impulsivity.

In summary, the present data provide evidence that increased motor impulsivity during protracted EtOH abstinence is associated with deficient recruitment of glycine and serine release in the vmPFC, and that bolstering NMDA receptor coagonist site activation through intra-vmPFC administration of a glycine uptake inhibitor ameliorates EtOH-related increases in premature responding. These results shed new light on the possible therapeutic benefits of glycine transport inhibitors for alcohol use disorders and alcoholism. Furthermore, our unbiased LC-MS/MS approach allowed us to provide a comprehensive picture of the changes in neurotransmitter levels associated with the performance of the challenge 5-CSRTT.

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