Increased Extracellular Glutamate In the Nucleus Accumbens Promotes Excessive Ethanol Drinking in Ethanol Dependent Mice

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Using a well-established model of ethanol dependence and relapse, this study examined adaptations in glutamatergic transmission in the nucleus accumbens (NAc) and their role in regulating voluntary ethanol drinking. Mice were first trained to drink ethanol in a free-choice, limited access (2 h/day) paradigm. One group (EtOH mice) received repeated weekly cycles of chronic intermittent ethanol (CIE) exposure with intervening weeks of test drinking sessions, whereas the remaining mice (CTL mice) were similarly treated but did not receive CIE treatment. Over repeated cycles of CIE exposure, EtOH mice exhibited significant escalation in drinking (up to ~3.5 g/kg), whereas drinking remained relatively stable at baseline levels (2–2.5 g/kg) in CTL mice. Using *in vivo* microdialysis procedures, extracellular glutamate (GLU_{EX}) levels in the NAc were increased approximately twofold in EtOH mice compared with CTL mice, and this difference was observed 7 days after final CIE exposure, indicating that this hyperglutamatergic state persisted beyond acute withdrawal. This finding prompted additional studies examining the effects of pharmacologically manipulating GLU_{EX} in the NAc on ethanol drinking in the CIE model. The non-selective glutamate reuptake antagonist, *threo*- β -benzyloxyaspartate (TBOA), was bilaterally microinjected into the NAc and found to dose-dependently increase drinking in nondependent (CTL) mice to levels attained by dependent (EtOH) mice. TBOA also further increased drinking in EtOH mice. In contrast, reducing glutamatergic transmission in the NAc via bilateral injections of the metabotropic glutamate receptor-2/3 agonist LY379268 reduced drinking in dependent (EtOH) mice to nondependent (CTL) levels, whereas having a more modest effect in decreasing ethanol consumption in CTL mice. Taken together, these data support an important role of glutamatergic transmission in the NAc in regulating ethanol drinking. Additionally, these results indicate that ethanol dependence produces adaptations that favor elevated glutamate activity in the NAc which, in turn, promote excessive levels of ethanol consumption associated with dependence.

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INTRODUCTION

Alcoholism is a chronic relapsing disease. Prolonged excessive alcohol (ethanol) consumption can lead to the development of dependence, which is characterized by a host of neuroadaptive changes in the brain reward and stress systems (Hansson *et al*, 2008; Koob and Le Moal, 2008; Spanagel, 2009). These changes are thought to underlie expression of withdrawal symptoms when drinking is terminated, increase vulnerability to relapse, and facilitate

a shift from regulated drinking to less controlled and more excessive ethanol consumption (Becker, 2008; Heilig *et al*, 2010; Vengeliene *et al*, 2009).

The interaction of ethanol with ionotropic and metabotropic glutamate receptor systems in the brain is known to have a significant role in mediating a variety of acute pharmacological effects of ethanol, and neuroadaptive changes in glutamate activity following chronic ethanol exposure contribute to tolerance and dependence (Gass and Olive, 2008). Although a role for glutamate activity in mediating ethanol drinking, particularly in the context of dependence, is not fully understood, evidence from preclinical and clinical investigations suggests that glutamate transmission may significantly contribute to motivational processes that regulate ethanol consumption. For example, NMDA glutamate receptor antagonism has been reported to mimic the subjective stimulus (intoxicating)

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effects of ethanol in humans (Krystal *et al*, 2003), monkeys (Vivian *et al*, 2002), and rodents (Hodge *et al*, 2006), and animal studies have shown that metabotropic glutamate receptor-5 (mGluR5) activity in the nucleus accumbens (NAc) has a key role in mediating this effect (Besheer *et al*, 2009). The NAc is a critical brain structure involved in regulating motivated behaviors, and it is an important site for ethanol–glutamate interactions. Microdialysis studies in mice and rats indicate that systemic administration of ethanol alters glutamate levels in NAc, with the effect dependent on the genotype and ethanol dose (Kapasova and Szumlinski, 2008; Melendez *et al*, 2005). Additionally, blockade of metabotropic glutamate receptors in the NAc attenuates ethanol reinforcement and/or drinking (Cozzoli *et al*, 2012; Gass and Olive, 2009), suggesting that glutamate signaling in this structure has a role in the maintenance of ethanol consumption.

Adaptive upregulation of glutamate activity following chronic ethanol treatment is well-documented in animal studies (Gass and Olive, 2008). Microdialysis studies in rats have revealed elevated extracellular levels of glutamate following chronic ethanol treatment in several brain regions including dorsal striatum, NAc, and hippocampus (Dahchour and De Witte, 2003; Dahchour *et al*, 2000; Rossetti and Carboni, 1995). Similarly, in rat studies involving magnetic resonance spectroscopy (MRS) analyses, increased glutamate activity has been noted in the prefrontal cortex (Hermann *et al*, 2012) and basal ganglia (Gu *et al*, 2013; Zahr *et al*, 2009). This aligns with recent MRS studies in human alcoholics that show elevated glutamate activity in anterior cingulate and the NAc (Bauer *et al*, 2013; Hermann *et al*, 2012). However, it is important to note that in all these studies, glutamate activity was examined during the acute withdrawal period. Whether the noted increase in glutamate activity following chronic ethanol exposure persists beyond acute withdrawal and what impact this effect may have in relation to drinking is not known.

We have developed a mouse model of ethanol dependence and relapse drinking that involves repeated cycles of chronic ethanol exposure and withdrawal periods and produces significant escalation of voluntary alcohol drinking in dependent compared with nondependent mice (Becker, 2008; Becker and Lopez, 2004; Griffin *et al*, 2009b; Lopez and Becker, 2005). Given that chronic ethanol induces significant alterations in glutamate activity in several brain regions, and adaptive changes in glutamate transmission have been postulated to have a significant role in the addiction process (Kalivas, 2009), our mouse model of dependence and relapse drinking would appear ideal for examining the relationship between dependence-related adaptations in glutamate activity and increased alcohol consumption. Thus, the present series of studies were conducted to investigate the role of glutamatergic transmission in the NAc in mediating/promoting escalation of drinking associated with ethanol dependence. It was hypothesized that disruption of accumbal glutamate homeostasis induced by dependence significantly contributes to increased propensity to engage in excessive alcohol drinking behavior in dependent compared to non-dependent subjects.

MATERIALS AND METHODS

Subjects

Male C57BL/6J mice (10 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME), individually housed, and maintained in an AAALAC accredited animal facility under a 12 h light cycle (lights on 0200 hours). Mice had free access to food and water at all times. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and were consistent with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). Mice were acclimated to the vivarium for 4 weeks before the experiments.

General Study Design

Mice were given access to ethanol using a 2-bottle choice, limited access procedure (described below). After establishing stable (baseline) ethanol intake, mice were separated into chronic ethanol exposure/withdrawal (EtOH) and control (CTL) groups. The EtOH group received chronic intermittent exposure to ethanol vapor in inhalation chambers (16 h/day for 4 days), whereas CTL mice were similarly handled, but placed in control chambers (see below). Limited access drinking sessions were suspended during inhalation exposure. After inhalation treatment, mice entered a 72 h abstinence period and then were tested for voluntary ethanol intake for 5 consecutive days using limited access conditions as before. This pattern of weekly chronic intermittent ethanol (CIE) (or air) exposure alternating with 5-day limited access drinking sessions was repeated for four (microdialysis experiments) or five cycles (microinjection experiments). Microdialysis procedures were conducted on the fifth day of Test Cycle 4. Dialysate samples were collected for 90 min to determine baseline levels of extracellular glutamate (GLU_{EX}). Mice were then administered either 1 or 2 g/kg ethanol by gavage and dialysate samples were collected for 135 min. Collection of dialysate samples both before and following ethanol gavage were conducted ~18–20 h after the previous day's limited access drinking session. In separate studies, microinjection procedures were conducted on the third or fourth day of Test Cycles 3–5. In these experiments, mice were provided the opportunity to drink ethanol in the 2-bottle choice limited access situation following microinjection of pharmacological agents (see below for more details).

Limited Access Drinking Procedures

Mice were trained to drink ethanol in the home cage under a limited access, 2-bottle choice situation as previously described (Becker and Lopez, 2004; Griffin *et al*, 2009a; Griffin *et al*, 2009b; Lopez and Becker, 2005). A modified sucrose fading procedure was used, with 15% (v/v) ethanol as the final solution along with tap water as the alternative fluid. The 2-h drinking sessions started at 1330 hours Monday through Friday. The amount consumed was recorded daily (± 0.1 ml) and body weights were recorded

weekly. The position of ethanol and water bottles were alternated randomly to avoid side preferences.

CIE Exposure Procedures

CIE (or air) vapor exposure was delivered in Plexiglas inhalation chambers as previously described (Becker and Lopez, 2004; Griffin *et al*, 2009b). Chamber ethanol concentrations were monitored daily and air flow was adjusted to maintain ethanol concentrations within a range that yielded stable blood ethanol levels throughout exposure (175–225 mg/dl). Mice were placed in inhalation chambers at 1600 hours and removed 16-h later at 0800 hours. Before each 16-h exposure during CIE treatment, EtOH mice were administered ethanol (1.6 g/kg; 8% w/v) and the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg) to maintain a stable level of intoxication during each cycle of ethanol vapor exposure (Griffin *et al*, 2009a). CTL mice were handled similarly, but received injections of saline and pyrazole before being placed in control chambers. All injections were given intraperitoneally in a volume of 20 ml/kg body weight.

Stereotaxic Surgery

Mice were anesthetized using isoflurane mixed with medical-grade air (4% induction, 2% maintenance), given a subcutaneous injection of 5 mg/kg carprofen (Pfizer), and placed in a Kopf stereotaxic instrument with digital display (Model 942). For microdialysis experiments, unilateral guide cannulae (CMA/7; CMA Microdialysis, Sweden) were positioned above the NAc (relative to Bregma: AP +1.7, ML $\pm$ 0.8, and DV –3.7). For microinjection studies, bilateral guide cannulae (Plastics One; Roanoke, VA) were positioned above the NAc (relative to Bregma: AP +1.7, ML $\pm$ 0.75, and DV –3.9). In both cases, guides were secured to the skull using a light-cured resin system and the guide obdurators remained in place until use (Griffin *et al*, 2009b). Mice were allowed a minimum of 10 days to recover from surgery before starting an experiment.

Microdialysis Procedures

As previously described (Griffin *et al*, 2009b; Griffin *et al*, 2007), microdialysis probes (CMA/7) were implanted the day before sample collection and aCSF was perfused through the probe during the overnight period at 0.2 μ l/min (probes extended 1 mm beyond the guide cannulae). The next day, the flow was increased to 1 μ l/min and allowed to equilibrate for 2 h before beginning the serial dialysate collection in 15 min intervals. An aliquot (10 μ l) of each sample was immediately frozen on dry ice and stored at –80 °C until analysis.

Glutamate Determination

Glutamate was quantified in dialysate samples using isocratic high-performance liquid chromatography with fluorescence detection. Samples were stored in the cooling tray (8 °C) of a Shimadzu SIL-10AF autosampler and derivatized in a 3 min reaction with the addition of 30 μ l of a solution containing O-phthaldehyde (10 mg%; dissolved

first in 1 ml 100% methanol and added to 29 ml of OPA Diluent (Pickering Laboratories)) mixed with 100% beta-mercaptoethanol in a 1:2000 ratio. Sample separation was accomplished using an ESA HR-80 $\times$ 3.2 column with 120 micron pore size (ESA; Chelmsford, MA). The mobile phase was 0.1 M Sodium Phosphate Dibasic (pH 6.75) mixed with 28% methanol and continuously pumped at 0.5 ml/min using a Shimadzu LC-20AD Prominence pump. A Shimadzu RF-10XL Fluorescence Detector was set with emission wavelength at 348 nm and an excitation wavelength at 450 nm. Glutamate concentrations were determined against external standards using peak heights determined by LabSolutions Software (Shimadzu, LC solution Version 1.22 SP1). The limit of detection for this assay is $\sim$ 25 nM and the limit of quantification is $\sim$ 135 nM.

Microinjection Procedures

Microinjections were administered on Wednesday or Thursday, 30 min before the test drinking sessions. Mice were removed from their home cage and gently restrained to insert the bilateral injectors, which extended 0.5 mm beyond the end of the guides. DL-Threo- β -benzyloxyaspartate (TBOA) and LY379268 were diluted in phosphate-buffered saline (vehicle) and delivered in a volume of 0.25 μ l over 2 min with an additional 2 min for diffusion using Harvard Apparatus Pump 11 (Harvard Apparatus, Holliston, MA). Doses of the drugs (including vehicle) were administered in a quasi-randomized design, with animals receiving 1, 2, or 3 injections (no animal received more than three microinjections and each injection was separated by 2 weeks). Thus, the factor Dose was treated as a between-subjects variable in analysis of these data.

Dual Dialysis/Microinjection Guide Implants and Procedures

The implant was constructed by positioning a single microinjector guide (Plastics One) at approximately an 8° angle next to a CMA/7 dialysis guide so that when the injection cannula was inserted the tip was within 50 microns of the middle of the active membrane. This was accomplished by using fine grit sandpaper to shave the walls of both guides at slight angles. The proximity of the guide tips was adjusted by additional sanding if necessary and distances were checked under a $\times$ 10 microscope with a micron reticle. The two guides along with a piece of wire used to tether the mouse to the fluid swivel were secured together using epoxy glue. Microdialysis procedures were similar to that described above. After collecting baseline samples, the mouse was removed from the cage and gently restrained. The microdialysis probe remained in place while the injector was inserted. Vehicle (phosphate-buffered saline), TBOA, and LY379268 were injected as described above and, after completing the injection procedure, the injector was removed and collection resumed.

Histology

Microdialysis probe and microinjector placements were verified at the end of each study and only mice with correct placements were included in the final analysis. Briefly, at the

end of the study, mice were killed by anesthetic overdose and transcardiac perfusion, and the brains were extracted and preserved in 10% formalin. The brains were sectioned on a cryostat and stained with cresyl violet. For microdialysis, mice were excluded from analysis if the probe was determined to be <70% in the NAc. Microinjector placements were determined by measuring 0.5 mm beyond the most ventral point of the guide cannula track. The reference atlas was (Paxinos and Franklin, 2001).

Drugs

DL-TBOA and LY379268 were purchased from Tocris Bioscience (Bristol, UK). A 10 × phosphate-buffered saline stock was purchased from Fisher and diluted 1:10 for use as a vehicle for the microinjection studies. TBOA and LY37928 were frozen as 2.5 and 10 mM stocks, respectively, in 1 × PBS and diluted as needed for microinjection.

Statistical Analysis

Analyses were conducted with SPSS (Version 19) using Student's *t*-test or two-way analysis of variance (ANOVA) with repeated measures, as appropriate. Significant interactions were followed up using Bonferroni corrected pair-wise comparisons. For all analyses, significance was set at $P < 0.05$.

RESULTS

Effects of CIE Exposure on Ethanol Drinking

Repeated cycles of CIE exposure produced significant escalation of voluntary ethanol consumption over successive test periods compared with relatively stable levels of intake displayed by nondependent controls throughout the study (Figure 1). A two-way repeated measures ANOVA supported this impression by revealing a significant

Figure 1 Escalation of ethanol drinking in dependent (chronic intermittent ethanol, CIE-exposed) mice compared with relatively stable ethanol intake in nondependent controls. The data shown are weekly averages (means $\pm$ SEM) for ethanol consumption (g/kg) during Baseline and Test cycles for EtOH and CTL groups. * $P < 0.05$ significantly different from CTL group; ^ $P < 0.05$ significantly different from respective baseline level.

Group $\times$ Test Cycle interaction ($F(4,132) = 4.14$, $P < 0.01$). *Post hoc* analyses indicated that Baseline ethanol intake was similar for EtOH mice (2.0 ± 0.1 g/kg) and CTL mice (2.1 ± 0.1 g/kg) and, whereas consumption only slightly increased above baseline levels for CTL mice, ethanol consumption significantly escalated in CIE-exposed (EtOH) mice above their baseline level of intake as well as amount consumed by the CTL group (intake reaching 3.3 ± 0.2 g/kg in EtOH mice compared with 2.5 ± 0.2 g/kg in CTL mice during Test Cycle 4) (p -values < 0.05).

GLU_{EX} Concentrations in the NAc of Ethanol-Dependent and Nondependent Mice

Conventional microdialysis procedures were used to examine GLU_{EX} in the NAc at the end of Test 4 (1 week after completing the final cycle of CIE (or air) exposure and at least 18 h after the last limited access drinking session). Glutamate concentrations (μ M) recovered in NAc dialysate samples were serially collected during a 1.5 h (six samples) baseline period and then for an additional 2.25 h (nine samples) following intragastric intubation of either 1 or 2 g/kg ethanol. As can be seen, baseline GLU_{EX} dialysate concentrations were consistently greater (approximately twofold) in EtOH mice compared with CTL mice (Figure 2a). A three-way repeated measures ANOVA of these baseline data revealed a significant main effect of Group ($F(1,31) = 7.79$, $P < 0.005$). The effects of Dose and Time were not significant, and these factors did not interact with the main effect of Group ($F_s < 1.0$). Baseline data were analyzed further by collapsing over Dose and Time factors. This analysis also indicated that GLU_{EX} dialysate concentrations were significantly greater in EtOH compared with CTL mice ($t(33) = 2.83$, $P < 0.005$) (Figure 2b).

Analysis of GLU_{EX} levels in dialysate samples collected after mice were challenged by gavage of 1 or 2 g/kg ethanol using a two-way repeated measures ANOVA revealed a significant main effect of Group ($F(1,31) = 3.29$, $P < 0.0001$). Similar to the analysis of baseline samples, Dose and Time factors were not significant and these factors did not significantly influence the Group effect of EtOH mice displaying higher GLU_{EX} levels than CTL mice (all $F_s < 2.4$, p -values > 0.1) (Figure 2a).

To further evaluate the potential effects of administering 1 or 2 g/kg ethanol on GLU_{EX} levels in the NAc, each subject's data were transformed to a percentage of its average baseline value and the data were analyzed separately for the two ethanol doses. For mice treated with 1 g/kg ethanol, a two-way repeated measures ANOVA revealed no significant effects of Group, Time, or the Group $\times$ Time interaction ($F_s < 1.4$, p -values > 0.1) (Figure 2c). Analysis of samples collected from separate groups of mice gavaged with 2 g/kg ethanol indicated a significant main effect of Time ($F(9,171) = 2.03$, $P < 0.05$), consistent with the modest increase in GLU_{EX} levels exhibited in both EtOH and CTL mice that returned to baseline levels within 1 h. However, a main effect of Group and the Group $\times$ Time interaction were not significant ($F_s < 1.0$) (Figure 2d). Thus, even when accounting for baseline differences in GLU_{EX} levels between EtOH and CTL groups, neither 1 nor 2 g/kg doses of ethanol administered by gavage significantly altered GLU_{EX} concentrations in the NAc.

Figure 2 Extracellular glutamate concentrations in dialysate samples from NAc of ethanol dependent (EtOH) and nondependent (CTL) mice collected at baseline and after administration of 1 or 2 g/kg ethanol gavage. (a) Extracellular glutamate (GLU_{EX}; μM) levels were approximately twofold higher in EtOH ($n = 19$) compared with CTL ($n = 16$) mice. Analysis indicated that EtOH mice displayed significantly higher baseline of GLU_{EX} levels than CTL mice, but there was no effect of ethanol gavage in either group or either dose. * $P < 0.05$ significantly differs from CTL values. (b) Significantly higher baseline GLU_{EX} levels in EtOH mice compared with CTL mice was confirmed by expressing data collapsed across Dose and Time factors. * $P < 0.05$ significantly differs from CTL group. (c) Administering 1 g/kg ethanol by gavage did not alter GLU_{EX} levels in either EtOH or CTL groups of mice; data are expressed as percent of average baseline values for EtOH and CTL groups. (d) Administration of 2 g/kg ethanol by gavage did not significantly alter GLU_{EX} levels in either EtOH or CTL groups; data are expressed as percent of average baseline values for EtOH and CTL groups.

Microinjection of the Non-Selective Glutamate Uptake Inhibitor DL-TBOA into the NAc of Ethanol-Dependent and Nondependent Mice

Given the observed elevation in baseline levels of accumbal extracellular glutamate in EtOH mice compared with CTL mice, it is possible that higher glutamatergic tone in the NAc may have a role in promoting/driving escalation of drinking displayed in dependent mice. To test this hypothesis, the non-selective glutamate transporter inhibitor TBOA was injected into the NAc to examine whether pharmacologically increasing glutamate activity in this brain region increases ethanol consumption in the CIE relapse drinking model. As expected, CIE exposure produced increased voluntary ethanol drinking in ethanol-dependent (EtOH) mice compared with nondependent control (CTL) mice over two test cycles of drinking. This was supported by a significant Group $\times$ Test Cycle interaction in the two-way repeated measures ANOVA ($F(2,134) = 13.30$, $P < 0.001$). *Post hoc* analyses indicated that EtOH mice consumed significantly more ethanol during Test 1 and Test 2 compared with both their baseline level of intake and the amount consumed by CTL mice (p -values < 0.05). Baseline intake was similar for EtOH and CTL groups (Figure 3a). Intra-NAc injections of TBOA 30 min before limited access (2 h) drinking sessions during Test Cycles 3–5 increased ethanol consumption in a dose-related manner in EtOH and CTL groups (Figure 3b). The two-way ANOVA revealed main effects of Group ($F(1,83) = 4.86$, $P < 0.05$) and Dose ($F(2,83) = 14.43$,

$P < 0.0001$), but the Group $\times$ Dose interaction did not achieve statistical significance. Thus, blockade of glutamate transporters in NAc increased voluntary ethanol drinking in nondependent mice to levels exhibited by vehicle-treated dependent mice, and this manipulation also further elevated drinking in dependent mice.

Microinjection of the mGluR2/3 Agonist LY379268 into the NAc of Ethanol-Dependent and Nondependent Mice

This study examined whether reducing presynaptic glutamate release in the NAc via microinjection of the mGluR2/3 agonist LY379268 would reduce ethanol drinking in EtOH and CTL groups. Analysis of drinking behavior during Baseline, Test 1, and Test 2 (before microinjections) using a two-way repeated measures ANOVA indicated a significant Group $\times$ Test Cycle interaction ($F(2,116) = 17.26$, $P < 0.0001$). *Post hoc* analyses found that ethanol drinking was greater in EtOH mice during Test 1 and Test 2 relative to their baseline intake and intake in CTL mice (p -values < 0.05). CTL mice also showed a relatively small, yet significant increase in drinking during Test 2 compared with their baseline level of consumption ($P < 0.05$) (Figure 4a). Analysis of the effects of intra-NAc injection of LY379268 on drinking during Test Cycles 3–5 using a two-way ANOVA revealed a significant main effect of Dose ($F(4,66) = 9.68$, $P < 0.001$) and a significant Group $\times$ Dose interaction ($F(4,66) = 3.40$, $P < 0.01$). *Post hoc* comparisons indicated that the three highest doses of the mGluR2/3

Figure 3 Effects of pharmacologically increasing extracellular glutamate (GLU_{EX}) levels in NAc via microinjection of the non-selective glutamate transporter blocker *threo*- β -benzyloxyaspartate (TBOA) on voluntary ethanol drinking in dependent and nondependent mice. (a) Ethanol consumption (g/kg) significantly increased over successive chronic intermittent ethanol (CIE) exposure cycles in EtOH mice ($n=30$) compared with CTL mice ($n=39$) before intra-NAc TBOA treatment. * $P<0.05$ significantly different from CTL group; $^{\wedge}P<0.05$ significantly different from respective baseline level. (b) Bilateral microinjection of TBOA 30 min before limited access drinking sessions increased voluntary ethanol consumption in a dose-related manner in both CTL and EtOH groups. * $P<0.05$ significantly differs from CTL group; $^{\wedge}P<0.05$ significantly differs from vehicle condition. Values are means $\pm$ SEM.

agonist significantly reduced voluntary ethanol intake relative to vehicle in EtOH mice ($P<0.05$). In contrast, although there was an apparent dose-dependent trend for reduced ethanol intake in CTL mice, this effect did not achieve statistical significance (Figure 4b). These data suggest that ethanol-dependent mice are more sensitive than nondependent mice to the effects of intra-NAc LY379268 administration on ethanol drinking.

Effects of Microinjecting TBOA and LY379268 on GLU_{EX} in the NAc

To confirm that intra-NAc injection of TBOA and LY379268 produced the expected increase and decrease in GLU_{EX}

Figure 4 Effects of pharmacologically reducing extracellular glutamate (GLU_{EX}) levels in NAc via microinjection of the metabotropic glutamate receptor-2/3 (mGluR2/3) agonist LY379268 on voluntary ethanol drinking in dependent and nondependent mice. (a) Ethanol consumption (g/kg) significantly increased over successive chronic intermittent ethanol (CIE) exposure cycles in EtOH mice ($n=23$) compared to CTL mice ($n=37$) prior to intra-NAc LY379268 treatment. * $P<0.05$ significantly different from CTL group; $^{\wedge}P<0.05$ significantly different from respective baseline level. (b) Bilateral microinjection of LY379268 given 30 min before limited access drinking sessions decreased voluntary ethanol consumption in a dose-related manner in EtOH mice while producing a modest (non-significant) reduction in ethanol intake in CTL mice. * $P<0.05$ significantly differs from CTL group; $^{\wedge}P<0.05$ significantly differs from respective vehicle condition. Values are means $\pm$ SEM.

levels, respectively, the microinjector was positioned adjacent to the microdialysis probe (see Materials and methods). Results indicated that compared with vehicle injection, TBOA (500 μM) increased extracellular glutamate for about 30 min, whereas LY379268 (5 nmol) robustly reduced GLU_{EX} levels over the entire 90 min collection period (Table 1). This was supported by a two-way repeated measures ANOVA, with the average value from three samples collected before injection serving as the baseline point (Time = 0 min). The analysis indicated significant main effects of Drug ($F(2,13) = 3.55$, $P = 0.027$) and Time ($F(12,78) = 3.199$, $P = 0.008$) as well as a significant Drug $\times$ Time interaction ($F(12,78) = 2.590$, $P = 0.006$). *Post hoc* pair-wise comparisons indicated that TBOA increased GLU_{EX} ($\sim 100\%$) above baseline levels at 15 min (91%) and 30 min (25%), with values returning to baseline during the remainder of the collection period. In contrast, LY379268 significantly reduced GLU_{EX} below baseline levels starting at 30 min (71%), with the reduction sustained (76–81%) over the 90 min collection period. GLU_{EX} levels

registered after vehicle administration did not differ from baseline values throughout the collection period.

Histological Verification of Probes and Injector Tips

Figures 5a and b show representative brain sections stained with cresyl violet indicating placements of microdialysis probes and microinjector tips, respectively. The gray shaded areas in the atlas plates show areas targeted by the dialysis and microinjection experiments (Figure 5c). For the dialysis experiment, mice were excluded from the analysis if the microdialysis probe was more than 70% outside the shaded area. For these experiments, the distribution of placement of dialysis probes within subregions of the NAc is as follows: NAc core (34%), NAc shell (40%), and NAc core-shell transition region (25%). Data analyses did not indicate significance differences in profile of results among these regions. In the microinjector experiments, mice were excluded if one or both tips were outside the shaded area.

DISCUSSION

Consistent with our previous reports, our mouse model of ethanol dependence and relapse drinking involving repeated cycles of CIE exposure produced significant escalation of voluntary drinking in dependent compared with nondependent mice (Becker and Lopez, 2004; Griffin *et al*, 2009b; Lopez and Becker, 2005). That is, once stable intake was established under limited access conditions (2 h/day), free-choice ethanol consumption progressively increased over testing cycles in CIE-exposed mice, whereas intake remained relatively unchanged in air-exposed controls. This level of increased ethanol consumption attained in the present series of experiments has been previously shown to result in significant elevation (two- to threefold increase) in blood ethanol levels and brain ethanol concentrations (Griffin *et al*, 2009b). These results (increased alcohol consumption) are congruent with those reported by others using similar procedures in mice (Finn *et al*, 2007; Dhaher *et al*, 2008) and rats (Gehlert *et al*, 2007; Roberts *et al*, 1996; Roberts *et al*, 2000; Sommer *et al*, 2008).

Results from this study also indicated that extracellular concentrations of glutamate (GLU_{EX}) in the NAc were increased approximately twofold in dependent compared with nondependent mice. In general, this finding is consistent with other studies showing increased basal

GLU_{EX} in the NAc (Ding *et al*, 2013; Kapasova and Szumlinski, 2008; Melendez *et al*, 2005) as well as other brain regions such as hippocampus (Chefer *et al*, 2011; Dahchour and De Witte, 1999; Moghaddam and Bolinao, 1994) and the amygdala (Roberto *et al*, 2004). However, results from the present study are unique in two ways. First, although some studies have demonstrated increased basal GLU_{EX} levels in the NAc following repeated injections of ethanol (Kapasova and Szumlinski, 2008; Melendez *et al*, 2005), this study examined this effect in a dependence model where elevated glutamate levels were observed only in dependent mice that exhibited escalation of voluntary drinking. Second, although some reports have indicated elevated accumbal GLU_{EX} levels following chronic ethanol (vapor inhalation) exposure, these measurements were collected during acute withdrawal (within 24 h of exposure) (Dahchour and De Witte, 2003; Dahchour *et al*, 2000).

Figure 5 Microdialysis probe and microinjector tip placements in the NAc. Cresyl violet stained tissue sections showing typical placements for microdialysis probes (a) and the bilateral microinjector guides and tips (b). (c) Shaded areas depicted on plates from the atlas of Paxinos and Franklin (2001) indicating the areas where microdialysis probes and microinjection tips were located. Data were not included in analyses if more than 70% of the dialysis probe or one or both microinjector tips were outside the boundaries of the shaded area.

Table 1 GLU_{EX} Response to Microinjection of TBOA (500 μ M) or LY379268 (5 nmol) in the NAc

Drug	Time Post Injection (min)						
	0	15	30	45	60	75	90
VEH (<i>n</i> = 6)	1.24 $\pm$ 0.40	1.37 $\pm$ 0.27	1.42 $\pm$ 0.40	1.33 $\pm$ 0.34	1.27 $\pm$ 0.34	1.31 $\pm$ 0.35	1.36 $\pm$ 0.43
TBOA (<i>n</i> = 6)	1.29 $\pm$ 0.15	2.46 $\pm$ 0.41 ^{a,b}	1.61 $\pm$ 0.20 ^a	1.47 $\pm$ 0.17	1.48 $\pm$ 0.21	1.40 $\pm$ 0.13	1.29 $\pm$ 0.14
LY379268 (<i>n</i> = 3)	0.89 $\pm$ 0.10	0.96 $\pm$ 0.32	0.26 $\pm$ 0.11 ^a	0.19 $\pm$ 0.04 ^{a,b}	0.19 $\pm$ 0.03 ^a	0.17 $\pm$ 0.04 ^{a,b}	0.21 $\pm$ 0.04 ^a

Abbreviations: GLU_{EX}, extracellular glutamate; TBOA, *threo*- β -benzyloxyaspartate.

Values are μ M and expressed as mean $\pm$ SEM.

^aDifferent from baseline ($P < 0.05$).

^bDifferent from VEH at the same time point ($P < 0.05$).

Results from the present study demonstrate increased GLU_{EX} levels in NAc associated with the CIE exposure model that extend well beyond acute withdrawal (at least 7 days following exposure), suggesting a persistent alteration in glutamatergic activity. Taken together, results from this study suggest a relationship between increased glutamatergic tone in the NAc and the propensity for increased drinking exhibited by ethanol-dependent mice.

Although repeated cycles of CIE exposure produced significant elevation in accumbal GLU_{EX} levels 7 days following the final CIE exposure, subsequent oral challenge with ethanol (1 and 2 g/kg) did not significantly alter GLU_{EX} levels in either dependent or nondependent mice. Although there appeared to be a trend to increase GLU_{EX} at the higher dose (2 g/kg), the lack of a significant response to the ethanol challenge was observed even when differences between the groups in baseline GLU_{EX} levels were taken into account. This was somewhat surprising given that other microdialysis studies in mice and rats have indicated systemic administration of low ethanol doses (0.5–1 g/kg) increase accumbal GLU_{EX} levels (Lominac *et al*, 2006; Moghaddam and Bolinao, 1994; Selim and Bradberry, 1996; Smith *et al*, 2004; Szumlinski *et al*, 2008), whereas higher doses (2 to 3 g/kg) either decrease GLU_{EX} or produce no change (Lominac *et al*, 2006; Piepponen *et al*, 2002; Smith *et al*, 2004; Yan *et al*, 1998). All of these earlier studies administered ethanol via the intraperitoneal route during dialysis, whereas ethanol was administered by gavage in this study. Thus, it is possible that pharmacokinetic differences produced by different routes of administration might contribute to differences in the accumbal GLU_{EX} response to acute ethanol challenge. Another possibility is that glutamatergic transmission in some subregions of the NAc may be more responsive to ethanol challenge than others. For example, studies with probe placements largely, but not exclusively, within the NAc shell suggest this subregion responds to ethanol challenge in a dose-dependent manner (Lominac *et al*, 2006). Analysis of the present data did not reveal differences in GLU_{EX} levels across NAc subregions following ethanol gavage. Nevertheless, additional studies will be needed to more fully examine these possibilities. It also will be of interest to determine whether volitional (self-administered) ethanol induces similar or unique changes in accumbal GLU_{EX} levels.

Given the apparent sustained elevation in accumbal baseline GLU_{EX} levels following CIE exposure, the possibility that this hyperglutamatergic tone in the NAc may be linked with escalated drinking in ethanol-dependent mice prompted additional studies that examined the effects of pharmacologically manipulating extracellular glutamate levels in the NAc on voluntary ethanol consumption. The first approach used DL-TBOA, a non-selective antagonist at the major glutamate reuptake proteins residing in the glial and neuronal membranes, with little affinity for ionotropic and metabotropic glutamate receptors (Shimamoto *et al*, 1998; Shimamoto *et al*, 2000). Consistent with this mechanism, TBOA microinjected next to dialysis probes in the NAc was shown to significantly increase GLU_{EX} levels. Further, bilateral TBOA microinjection into the NAc significantly increased ethanol drinking in a dose-related fashion. It is not clear why the TBOA-induced increase in GLU_{EX} levels was evident for about 30 min, returning to

baseline levels thereafter. Extracellular glutamate levels are tightly controlled by a number of mechanisms (Danbolt, 2001), and it is likely that such mechanisms were operable in restoring homeostatic glutamate levels. Despite the relatively short-lived increase in GLU_{EX} levels, TBOA produced a clear dose-related increase in amount of ethanol consumed over the 2-h drinking session. Additional studies that track the temporal pattern of ethanol intake following intra-NAc TBOA administration will be required to ascertain whether this pharmacologically induced increase in GLU_{EX} levels (even for only 30 min) is sufficient to drive increased drinking over a longer period of time.

It is noteworthy that a similar finding has been reported for intra-NAc TBOA effects on ethanol drinking in mice using a modified 24-h drinking paradigm (Kapasova and Szumlinski, 2008). In the present study, the highest dose of TBOA increased ethanol drinking in nondependent mice to levels similar to that measured in ethanol-dependent mice. Thus, despite exhibiting relatively stable levels of intake over several weeks, nondependent mice were induced to drink greater amounts of ethanol that ultimately reached the higher levels of intake displayed by dependent mice when GLU_{EX} levels were elevated in NAc via TBOA injection. Escalated drinking in dependent mice was also further increased by intra-NAc TBOA administration. Thus, these results suggest that increasing glutamatergic activity in the NAc is associated with increased propensity to consume ethanol.

Considering these results, it was predicted that reducing glutamatergic transmission in the NAc would reduce ethanol drinking in the CIE exposure dependence model. Activation of mGluR2 and mGluR3 receptors reduces glutamate release (Cartmell and Schoepp, 2000; Schoepp, 2001), and this effect was verified in the present study when the mGluR2/3 agonist LY37928 was injected next to a dialysis probe implanted in the NAc and found to significantly decrease GLU_{EX} levels. Intra-NAc LY379268 administration significantly reduced voluntary ethanol consumption in dependent and nondependent mice in a dose-related manner. This effect was most robust in dependent mice where LY379268 produced a maximal 78% decrease in ethanol consumption and, importantly, reduced ethanol drinking to levels similar to nondependent mice. Although LY379268 also reduced ethanol intake in nondependent mice, this effect was more modest and did not achieve statistical significance (maximal reduction relative to vehicle = 44%). These findings are consistent with other reports showing that systemic administration of LY379268 attenuated ethanol reinforcement, stress-induced ethanol seeking, and conditioned reinstatement, with the most robust effects seen in animals with a history of ethanol dependence (Kufahl *et al*, 2011; Sidhpura *et al*, 2010). Because mGluR2/3 receptors are widely expressed in forebrain regions including the NAc (Ohishi *et al*, 1998; Tamaru *et al*, 2001), the present findings extend previous studies to specifically include a role for mGluR2/3 receptors in the NAc in regulating voluntary ethanol consumption.

Taken together, results from the present microdialysis and microinjection experiments indicate adaptations in ethanol-dependent mice that contribute to enhanced glutamate transmission in the NAc. However, the mechanism underlying these adaptations and the source of elevated

glutamatergic activity is not known. Extracellular glutamate levels are tightly regulated by numerous neuronal and glial release and uptake functions. One possibility is that repeated cycles of CIE exposure produce a persistent hyperexcitable state in the NAc driven by increased activity in glutamatergic afferents arising from the prefrontal cortex and limbic (hippocampus and amygdala) nuclei (Britt *et al*, 2012). In the NAc, mGluR2 and mGluR3 receptors are predominantly located in the neurons, pre- and post-synaptically (Ohishi *et al*, 1998; Tamaru *et al*, 2001). These group II metabotropic glutamate receptors are known to function together to reduce glutamate release (Cartmell and Schoepp, 2000; Schoepp, 2001) and dampen neuronal excitability (Lovinger and McCool, 1995). Our finding that LY379268 was more effective in reducing ethanol drinking in dependent mice suggests greater inherent glutamatergic transmission in the NAc of these mice, consistent with the higher GLU_{EX} concentrations in the NAc.

Another possibility is that elevated accumbal GLU_{EX} concentrations result from CIE-induced alterations in expression and/or function of excitatory amino-acid transporters (EAATs). EAAT1 (GLAST) and EAAT2 (GTL-1) are predominantly expressed in glia and these sodium-dependent glutamate transporters are critically involved in maintaining low extracellular levels of GLU_{EX} in the central nervous system (Amara and Fontana, 2002; Danbolt, 2001; Tzingounis and Wadiche, 2007). Elevated GLU_{EX} levels in the NAc following a subchronic ethanol exposure regimen were shown to be associated with the downregulation of sodium-dependent glutamate uptake, but no change in EAAT1 or EAAT2 expression in rats (Melendez *et al*, 2005). Similarly, reduced glutamate clearance was reported to accompany increased accumbal GLU_{EX} concentrations in C57BL/6J mice after repeated ethanol injection treatment (Kapasova and Szumlinski, 2008). In a recent study in alcohol-preferring (P) rats, several weeks of free-choice ethanol consumption produced elevated GLU_{EX} levels in NAc that was associated with reduced glutamate clearance and decreased expression of EAAT1 but not EAAT2 (Ding *et al*, 2013). Thus, altered expression and/or function of EAATs expressed in glia (EAAT1 and EAAT2) and possibly in neurons (EAAT3/EAAC1) following chronic ethanol exposure may contribute to the resultant elevated glutamate activity in NAc. To date, we have not observed differences in EAAT1 or EAAT2 expression in NAc in our CIE exposure model (unpublished findings), but on-going studies are examining the possibility of a functional change in uptake capacity that could contribute to increased accumbal GLU_{EX} concentrations. In addition, it is possible that increased glutamate release from glial cells via the cystine–glutamate exchanger may contribute to elevated glutamatergic tone in the NAc and increased propensity to drink, as altered function of this sodium-independent transporter has been implicated in drug addiction (Kalivas, 2009).

Finally, results from the present study indicating dependence-related enhanced glutamate tone in the NAc that is associated with increased drinking may have implications regarding treatment strategies for alcoholism. As noted above, clinical MRS studies have reported increased glutamate activity in anterior cingulate and NAc in abstinent human alcoholics compared with age- and sex-matched healthy controls (Bauer *et al*, 2013; Hermann *et al*,

2012). In another MRS study, acamprosate was found to reduce glutamate levels in anterior cingulate several weeks after abstinence (Umhau *et al*, 2010). Acamprosate is an FDA-approved medication for alcohol dependence that is thought to alter central glutamate neurotransmission, although its mechanism of action is not fully understood and reports on its clinical efficacy in treating alcohol dependence have been mixed (Berger *et al*, 2013; Mann *et al*, 2012; Mason and Leher, 2012; Mann *et al* 2012; Yahn *et al*, 2013). Likewise, studies with other therapeutics such as antiepileptics (eg, topiramate, gabapentin, pregabalin, and levetiracetam) that indirectly alter central glutamate activity have produced mixed results in terms of reducing alcohol craving/drinking (De Sousa, 2010; Guglielmo *et al*, 2012; Johnson and Ait-Daoud, 2010). Clearly, the relationship between dependence, drinking, and glutamate transmission warrants further investigation, especially in the context of identifying new and better pharmacotherapeutics for alcoholism. Indeed, the CIE model of dependence and relapse drinking used in the present study may be particularly valuable for evaluating the efficacy of various pharmacological agents in reducing dependence-related escalated drinking and elevated glutamatergic activity.

In summary, in a model of dependence and relapse drinking, repeated cycles of CIE exposure produced escalation of drinking along with elevated extracellular levels of glutamate in the NAc in dependent compared with nondependent mice. This twofold increase in accumbal GLU_{EX} concentrations persisted well beyond acute withdrawal. Further, pharmacologically manipulating glutamatergic tone in the NAc altered ethanol consumption. Increasing GLU_{EX} with the non-selective transporter blocker, TBOA, increased drinking in nondependent mice to levels attained by dependent animals, whereas decreasing GLU_{EX} using the mGluR2/3 agonist, LY379268, significantly reduced drinking in dependent mice to moderate levels exhibited by nondependent mice. Collectively, these results indicate that CIE exposure produces adaptations that favor the elevation of glutamate activity in the NAc, and this hyperglutamatergic state drives and/or promotes increased drinking characteristic of alcohol dependence.

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