Genetic and pharmacological manipulations of glyoxalase 1 mediate ethanol withdrawal seizure susceptibility in mice

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Funding support: This work was supported by NIH-NIAAA grant R01 AA026281. AMB-L was supported by NIH-NIAAA grants F32 AA025515 and K99 AA07835.

Abstract

Central nervous system (CNS) hyperexcitability is a clinically significant feature of acute ethanol withdrawal. There is evidence for a genetic contribution to withdrawal severity, but specific genetic risk factors have not be identified. The gene glyoxalase 1 (*Glo1*) has been previously implicated in ethanol consumption in mice, and GLO1 inhibition can attenuate drinking in mice and rats. Here, we investigated whether genetic and pharmacological manipulations of GLO1 activity can also mediate ethanol withdrawal seizure severity in mice. Mice from two transgenic lines overexpressing *Glo1* on different genetic backgrounds (C57BL/6J [B6] and FVB/NJ [FVB]) were tested for handling induced convulsions (HICs) as a measure of CNS hyperexcitability during acute ethanol withdrawal. During withdrawal from an acute injection of 4 g/kg alcohol, both B6 and FVB mice overexpressing *Glo1* showed increases in HICs from their wild type littermates. We also administered daily ethanol injections (2 g/kg + 9 mg/kg 4-methylpyrazole) to wild type B6 mice for 10 days and tested them for HICs on the 10th day, following treatment with either vehicle or a GLO1 inhibitor (S-bromobenzylglutathione cyclopentyl diester [pBBG]). Treatment with pBBG reduced seizure activity, although this effect was only statistically significant following two 10-day cycles of ethanol exposure and withdrawal. Taken together, these results provide converging genetic and pharmacological evidence that GLO1 can mediate ethanol withdrawal seizure susceptibility. We discuss the likely role of GLO1's substrate, methylglyoxal, acting as an agonist at $GABA_A$ receptors.

Introduction

Alcohol (ethanol) withdrawal is a key feature of alcohol dependence and includes physiological and affective symptoms such as tremors, nausea, vomiting, irritability, insomnia, and anxiety. Withdrawal symptoms are included in the diagnostic criteria for an alcohol use disorder (DSM V), and withdrawal may be related to relapse risk (e.g. Becker 2008; Breese et al. 2011; Heilig et al. 2010). In severe cases, acute withdrawal can lead to serious consequences such as delirium, seizures, or even death (Jesse et al. 2017; McKeon et al. 2008; Saitz 1998). The determinants of ethanol withdrawal severity are not fully known, but there are likely multiple contributing factors including drinking history, past withdrawal experience, co-occurring use of other drugs, structural brain lesions, and genetics (Jesse et al. 2017; Schmidt & Sander 2000). Understanding the biological factors that confer risk for severe ethanol withdrawal may therefore lead to improved prevention and treatment options.

There is considerable evidence from both human and model organism studies for genetic contributions to ethanol withdrawal severity (Kendler et al. 2012; Kosobud & Crabbe 1986; Metten & Crabbe 2005; Fehr et al. 2004; Scott et al. 2017). In humans, gene polymorphisms associated with many neurotransmitter systems have been explored in relation to ethanol withdrawal (for review see Schmidt and Sander 2000). Several of these candidate gene and association studies have identified relationships between specific genetic variants and ethanol withdrawal severity (e.g. Grzywacz et al. 2012; Sander et al. 1997; Dutta et al. 2016; Huang et al. 2014; Smith et al. 2018). However, large-scale human genetics studies have predominately focused on alcohol consumption and alcohol use disorder diagnosis, rather than withdrawal severity. Animal models are a useful complementary tool for studying novel genetic contributions to ethanol related traits, since animal models can isolate specific aspects of ethanol withdrawal such as seizure susceptibility. Heritability of susceptibility to ethanol withdrawal seizures has been well-established in mouse models: inbred mouse strains and recombinant inbred panels show a high degree of phenotypic variation for this trait (Metten & Crabbe 2005, 1994; Metten et al. 2011; Crabbe 1998), and selective breeding has produced withdrawal seizure prone and resistant lines of mice (Crabbe et al. 1983; Kosobud & Crabbe 1986).

Here, we use a mouse model of ethanol withdrawal to investigate the role of glyoxalase 1 (GLO1) in withdrawal seizure severity. GLO1 is a ubiquitously expressed enzyme that metabolizes the glycolytic byproduct methylglyoxal (MG). We have shown previously that MG

can act as a partial agonist at $GABA_A$ receptors and that inhibition of GLO1 leads to increased levels of MG (Distler et al. 2012). Genetic and pharmacological manipulations of the GLO1 system, as well as direct administration of MG, have been associated with changes in anxiety- and depression-like behaviors, ethanol consumption, and seizure threshold. Specifically decreased anxiety-like behavior (Distler et al.2012, McMurray et al. 2016), decreased depression-like behavior (McMurray et al.2018, Perez et al. 2020), decreased ethanol consumption (McMurray et al. 2017, de Guglielmo et al. 2018), and decreased susceptibility to pilocarpine- and picrotoxin-induce seizures (Distler et al. 2013) have all been seen following GLO1 inhibitor treatment. Some of these effects are also produced by genetic knockdown of *Glo1* or direct MG administration. In contrast, *Glo1* overexpression produces opposite effects (i.e. increased anxiety-like behavior and ethanol consumption) (Distler et al. 2012; McMurray et al. 2017, 2018).

Significant changes in GABAergic signaling following chronic ethanol use are believed to underlie in part the central nervous system hyperexcitability seen in ethanol withdrawal (e.g. Kumar et al. 2009; Rogawski 2005; Sachdeva et al. 2015), and we hypothesize that GLO1's effects on MG levels and subsequent GABAergic activation could mediate some of these changes. In the present studies, we examined whether overexpression of *Glo1* could potentiate ethanol withdrawal seizure severity, and whether pharmacological manipulation of GLO1 activity by treatment with a GLO1 inhibitor could attenuate ethanol withdrawal seizure severity in mice.

Methods

Animals and husbandry

All *Glo1* transgenic mice used in Experiments 1 and 2 were bred in house. Generation of the *Glo1* transgenic mice by insertion of a BAC transgene has been previously described (Distler et al., 2012). The *Glo1* transgenic lines are maintained by breeding male mice heterozygous for the *Glo1* overexpression to wild type female mice (B6 or FVB depending on the line). Resulting heterozygous and wild type littermates were used as experimental animals. For Experiment 3, wild type B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). For all experiments, mice were housed 2-5 per cage on wood chip bedding and food (Envigo 8604,

Indianapolis, IN) and water were provided *ad libitum*. Mice were maintained on a 12hr/12hr light/dark cycle with lights on at 06:00. Behavioral testing was conducted during the light phase. All procedures were approved by the local Institutional Animal Care and Use Committees and were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Handling induced convulsions

Handling induced convulsions (HICs) are used to assess susceptibility to ethanol withdrawal seizures and serve as a measure of CNS hyperexcitability. We used the 7-point scale developed by Crabbe and colleagues (e.g. Crabbe et al. 1991) based on the original procedure by Goldstein and Pal (1971). Briefly, mice are lifted by the tail and observed for convulsion signs. If lifting alone does not produce a convulsion, the mouse is gently spun in a 360-degree arc. Scores range from 0 (no convulsion) to 7 (lethal seizure). Baseline HIC assessments are made prior to ethanol administration. In Experiments 1 and 3, HICs were assessed hourly from 2-10 hours after the final ethanol injection. In Experiment 2, HICs were assessed only during peak withdrawal (6-8 hr after the final ethanol injection). In all experiments, a final HIC measurement was taken at 24 hr into withdrawal. Experimenters were blinded to genotype and treatment for all HIC assessments, and the same experimenter performed all HIC assessments within a given experiment. A withdrawal severity score was calculated by finding the area under the curve (AUC) of the HIC time course (Metten et al. 2018). Peak HIC score was the highest HIC score recorded for each animal throughout the time course.

Drugs

The GLO1 inhibitor S-bromobenzylglutathione cyclopentyl diester (pBBG) was synthesized in the laboratory of Alexander Arnold at the University of Wisconsin Milwaukee, as previously described (McMurray et al., 2017). The dose of 25 mg/kg was chosen because it was believed to be an intermediate dose in the behaviorally-active range; 12.5 mg/kg and 50 mg/kg doses have been shown to reduce ethanol drinking and anxiety-like behavior, respectively in previous studies (McMurray et al. 2017, Distler et al. 2012), and 25 mg/kg can attenuate withdrawal-associated escalation of ethanol drinking in dependent rats (de Guglielmo et al. 2018). pBBG was dissolved in vehicle (8% DMSO/18% tween80/saline) and administered i.p. (injection volume 0.01 ml/g). Ethanol (Deacon Laboratories Inc., King of Prussia, PA) was

mixed in saline (20% v/v) and administered i.p. at a dose of 4 g/kg in Experiments 1 and 2. In Experiment 3, the alcohol dehydrogenase inhibitor 4-methylpyrazole (9 mg/kg; Sigma -Aldrich, St. Louis, MO) was dissolved in 20% ethanol solution, such that animals received only a single injection for both ethanol and pyrazole administration.

Experiment 1: Acute ethanol withdrawal in Glo1 transgenic mice on a B6 background

Male and female mice from the B6 background *Glo1* transgenic line were used in this experiment (n=4/sex/genotype). All animals were between 59-153 days at the start of testing. Mice were weighed, assessed for baseline HIC score, and then injected i.p. with 4 g/kg ethanol and returned to the home cage. Starting two hours after the ethanol injection, mice were assessed hourly for HICs with the final HIC assessment made at 10 hr after injection. The following morning, 24 hr after the previous injection, the final HIC assessment was made.

Experiment 2: Acute ethanol withdrawal in Glo1 transgenic mice on an FVB background

Because B6 background mice in Experiment 1 showed limited ethanol withdrawal seizure susceptibility, we sought to extend our studies to include FVB *Glo1* transgenic mice because FVB mice have higher HIC scores during ethanol withdrawal than B6 (Metten et al. 2011). Peak withdrawal scores were seen from 6-8 hr after injection in Experiment 1, so in Experiment 2 we limited HIC assessments to this peak withdrawal window. Only females were used for Experiment 2 due to animal availability (n=9-12/genotype); all mice were between 241-287 days of age at testing. As in Experiment 1, mice were weighed, given a baseline HIC assessment, and then injected with 4 g/kg ethanol and returned to their home cages. HICs were assess at 6, 7, and 8 hr after injection and again the next morning at 24 hr after injection.

Experiment 3: GLO1 inhibitor effects on chronic ethanol withdrawal

To assess whether pharmacological manipulations of GLO1 activity could also influence ethanol withdrawal, we tested whether the GLO1 inhibitor pBBG could attenuate withdrawal seizure severity following chronic ethanol exposure. Importantly, GLO1 inhibition should produce effects that are opposite to those produced by *Glo1* overexpression. Male B6 mice were used for this experiment (n=11-13/treatment group); all animals were 75-77 days of age at the start of testing. Male B6 animals were chosen because the majority of our studies examining GLO1 inhibitor effects on behavior and effective dose ranges have used male B6 mice. Because B6 animals show limited withdrawal seizure susceptibility during acute withdrawal, we used a

chronic ethanol dosing model to induce dependence. Specifically, we used a 10-day ethanol injection paradigm adapted from Perez and De Biasi (2015) that has been shown previously to produce somatic and affective signs of withdrawal in male B6 mice. Mice received a daily injection of 2 g/kg ethanol with 9 mg/kg 4-methylpyrazole for 10 days.

Baseline HICs were assessed before the first injection was given. On Day 10, HIC time course was assessed starting 2 hr after the final injection and again hourly until 10 hr post-injection. At 3.5 hr after the final ethanol injection, mice received an i.p. injection of either 25 mg/kg pBBG or vehicle. This pretreatment time was chosen to give sufficient time for GLO1 inhibition to lead to increased MG levels during peak withdrawal. A final HIC assessment was made the next day at 24 hr after the final ethanol injection.

To determine whether the effectiveness of pBBG changed with increased ethanol exposure, and due to low levels of withdrawal seizure susceptibility seen following the initial ethanol exposure, we repeated the same procedure for a second cycle of ethanol exposure and withdrawal. Following the first 10-day ethanol withdrawal cycle, mice were allowed to remain undisturbed for 6 weeks in their home cages before we repeated the 10-day procedure described above. All mice received the same drug treatment and the same experimental procedures were used in both ethanol withdrawal cycles, including a re-baselining HIC assessment before the start of the second ethanol exposure period.

Statistical analyses

All statistical analyses were performed using SPSS (version 27; IBM Corp, Armonk, NY). HIC time course data were analyzed by mixed-model analysis of variance (ANOVA) with a repeated measure of time point and between-subjects factors of genotype, treatment group, and/or sex, as relevant for the given experiment. A Huynh-Feldt correction for violations of sphericity was used for repeated measures analyses. HIC AUC and peak HIC score data were analyzed by one- or two-way ANOVA. Significance was set at $\alpha=0.05$ for all tests.

Results

Experiment 1: Acute ethanol withdrawal in Glo1 transgenic mice on a B6 background

Withdrawal HIC time course (**Fig. 1a**) was analyzed using a mixed-model ANOVA with a repeated measure of time and between-subjects factors of genotype and sex. There were

statistical trends toward main effects of time ($F_{5.83, 70}$=1.95, p=0.086) and genotype ($F_{1,12}$=4.2, p=0.063), and a main effect of sex ($F_{1,14}$=4.2, p=0.022). There were no significant interactions between any factors (p>0.1 for all).

To determine differences in overall withdrawal severity, we analyzed area under the HIC curve. HIC AUC was significantly greater in transgenic mice compared to wild type animals, indicating *Glo1* overexpression increased cumulative seizure activity ($F_{1,12}$=6.28, p=0.028; **Fig. 1b**). Peak HIC score also showed trends toward main effects of genotype and sex, with higher scores in the transgenic mice than wild type mice and in males than females ($F_{1,12}$=3.43, p=0.089). There were no significant genotype x sex interactions for either HIC AUC or peak HIC score (p>0.1 for all).

Experiment 2: Acute ethanol withdrawal in Glo1 transgenic mice on an FVB background

Withdrawal HIC time course (**Fig. 2a**) was analyzed using a mixed-model ANOVA with a repeated measure of time and a between-subjects factor of genotype. There were main effects of time ($F_{3.88,73.68}$=37.02, p<0.001) and genotype ($F_{1,19}$=6.32, p=0.021), but no significant time x genotype interaction. Analysis of HIC AUC (**Fig. 2b**) and peak HIC score (**Fig. 2c**) also showed that transgenic mice had significantly greater withdrawal severity ($F_{1,19}$=4.94, p=0.039) and higher peak HIC scores ($F_{1,19}$=5.08, p=0.036) than wild type mice.

Experiment 3: GLO1 inhibitor effects on chronic ethanol withdrawal

Figure 3 shows withdrawal HIC measures after one or two cycles of chronic ethanol treatment. Analysis with mixed-model ANOVA of HIC time course found a main effect of time following a single ethanol cycle (Fig 3a: $F_{5.89, 129.57}$=7.61, p<0.001), but no main effect of inhibitor treatment and no treatment x time interaction (p>0.1 for both). After two cycles of ethanol exposure, there was a significant treatment x time interaction (Fig 3b: $F_{5.39,118.49}$=3.04, p=0.011) and significant main effects of both treatment and time ($F_{1,22}$=12.26=0.002 and $F_{5.39, 118.49}$=14.54, p<0.001). Follow-up comparisons found that inhibitor-treated mice had significantly lower HIC scores than vehicle-treated mice at 5, 6, and 8 hr after the final ethanol injection (p≤0.05) and a trend toward lower HIC scores at 7 hr (p<0.07). Analysis of area under the HIC curve showed that following one cycle of ethanol, there was a statistical trend toward lower HIC AUC in the inhibitor-treated group compared to vehicle (Fig 3c: $F_{1,22}$=3.37, p=0.08). Similarly, there was no significant effect of treatment group on peak HIC score after one cycle of ethanol

(Fig 3d). In contrast, after two cycles of ethanol exposure, there were significant effects of treatment group on both HIC AUC (Fig 3e) and peak HIC score (Fig 3f) with GLO1 inhibition significantly reducing both measures ($F_{1,22}$=11.29, p=0.003 and $F_{1,22}$=7.51, p=0.012).

Discussion

In these experiments, we showed that overexpression of the gene *Glo1* on two different genetic backgrounds can produce increased ethanol withdrawal severity as assessed by HICs. Furthermore, treatment of wild type animals with a GLO1 inhibitor had the opposite effect, namely attenuating ethanol withdrawal seizure severity in mice undergoing a chronic ethanol exposure procedure. Taken together, these results provide evidence that GLO1 activity modulates CNS hyperexcitability during ethanol withdrawal in mice.

GLO1 activity has not been previously examined as a potential mediator of ethanol withdrawal severity. Chronic ethanol produces significant neuroadaptations, particularly in the GABA and glutamate systems, which underlie some of the symptoms of ethanol withdrawal (e.g. Olsen & Spigelman 2012; Kumar et al. 2009; Lovinger & Roberto 2013; Becker & Mulholland 2014). GLO1's role in ethanol withdrawal may therefore be due to its effects on MG levels and subsequent activity at $GABA_A$ receptors. We have shown previously that MG treatment reduces both the severity and duration of pharmacologically induced seizures, as well as EEG measures of seizure activity (Distler et al. 2013). It is possible that ethanol withdrawal may result in lower levels of MG, either through upregulation of GLO1or due to changes in glycolytic activity, and this reduction in MG could contribute to the blunted GABAergic signaling. In this case, the brains of ethanol-naïve *Glo1* overexpressing transgenic mice may parallel some of the neuroadaptations seen in dependent animals, which could explain some of their behavioral phenotypes such as increased ethanol consumption and increased anxiety-like behavior (McMurray et al. 2018; Distler et al. 2012). Similarly, treatment with a GLO1 inhibitor in dependent wild type mice could attenuate ethanol withdrawal severity by bringing MG levels closer to baseline conditions. These possibilities are speculative, and direct measurement of MG levels and *Glo1* expression during withdrawal will be an important step for determining whether and how the glyoxalase system is altered by ethanol treatment. GLO1 effects on negative affective changes during protracted abstinence should also be examined, since many of these changes (e.g. increased anxiety- and depression-like behavior) have been seen with genetic and

pharmacological manipulation of GLO1 activity in non-dependent mice (McMurray et al. 2019; Distler et al. 2012).

The finding in Experiment 3 that pBBG treatment only produced a trend toward a significant effect following one cycle of ethanol exposure but led to robust decreases in HIC severity after two cycles, suggests that GLO1 inhibition may be more effective in dependent animals than in non-dependent animals. This is consistent with previous findings where pBBG was more effective at reducing ethanol drinking in dependent rats compared to non-dependent rats (de Guglielmo et al. 2018). In the present study there was not a significant ethanol cycle x treatment group interaction (data not shown), so we cannot state conclusively what may be driving the increased efficacy seen with two ethanol withdrawal cycles. There is considerable evidence that ethanol withdrawal severity increases with repeated withdrawal experience ("kindling" effect) (e.g. McCown & Breese 1990; Booth & Blow 1993; Becker & Hale 1993). It could be that withdrawal severity increased with the repeated ethanol cycles, thereby eliminating any floor effect and making it easier to see a statistically significant reduction in seizure activity following pBBG treatment. Alternatively, it is possible that pBBG is more effective in animals with a greater ethanol history due to the subsequent neuroadaptations, or a combination of these factors.

There are some limitations of the present studies that should be noted. First, potential sex differences were only directly examined in Experiment 1, and the number of animals per sex per genotype likely provided limited power for identifying interactions with sex. However, we did see significant and consistent effects of GLO1 manipulations in both male and female animals in Experiments 2 and 3, even though the sexes were not directly compared in these experiments. Mice in Experiment 2 were also older on average than mice in the other two experiments, and potential age effects cannot be ruled out with the current experimental design. However, the similar effects of *Glo1* overexpression seen in Experiments 1 and 2 used transgenic mice on two different genetic backgrounds, the parallel effect of a GLO1 inhibitor in wild type animals, and the consistency in findings in both acute and chronic withdrawal models suggest that the role of GLO1 in ethanol withdrawal severity generalizes across multiple populations and conditions. Finally, the overall level of withdrawal severity seen in Experiment 3 was low, likely due to the use of B6 mice, and future studies in this genotype might benefit from a more extensive ethanol

exposure method to induce greater dependence (e.g. chronic-intermittent ethanol vapor exposure).

In conclusion, we provided converging genetic and pharmacological evidence that GLO1 activity mediates ethanol withdrawal seizure susceptibility. In mice, overexpression of *Glo1* contributes to increased withdrawal seizure severity, and inhibition of GLO1 attenuates withdrawal seizure severity. GLO1 inhibition could therefore potentially be a useful strategy for treatment of neural hyperexcitability during acute withdrawal. Further studies will be needed to determine how the glyoxalase system is altered by chronic ethanol exposure, and whether GLO1 inhibitors may have utility for treating negative affective changes seen during protracted abstinence.

Author Contributions

All authors contributed to design of these studies. AMB-L and AL were responsible for data collection and analysis. AMB-L drafted the manuscript. All authors provided critical input on the manuscript and approved the final version for submission.

Conflicts of Interest

The authors declare no conflicts of interest.

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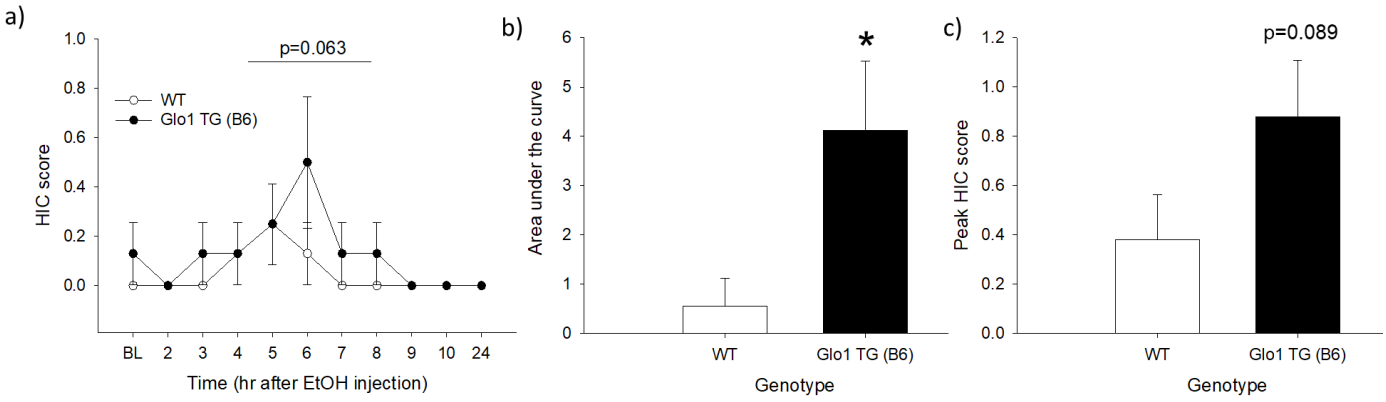

Figure 1. Acute ethanol withdrawal as assessed by handling-induced convulsions (HICs) in wild type (WT) and *Glo1* overexpressing (TG) mice on a B6 genetic background. Panel **a** shows time course of withdrawal, with baseline HIC measurements made immediately before ethanol injection. There was a statistical trend toward a main effect of genotype, with *Glo1* overexpressing mice trending toward higher HIC scores across the time course. *Glo1* overexpressing mice also showed significantly greater cumulative seizure susceptibility (area under the curve [AUC] panel **b**), and a trend toward higher peak HIC scores (panel **c**). * indicates $p<0.05$ N=8/genotype.

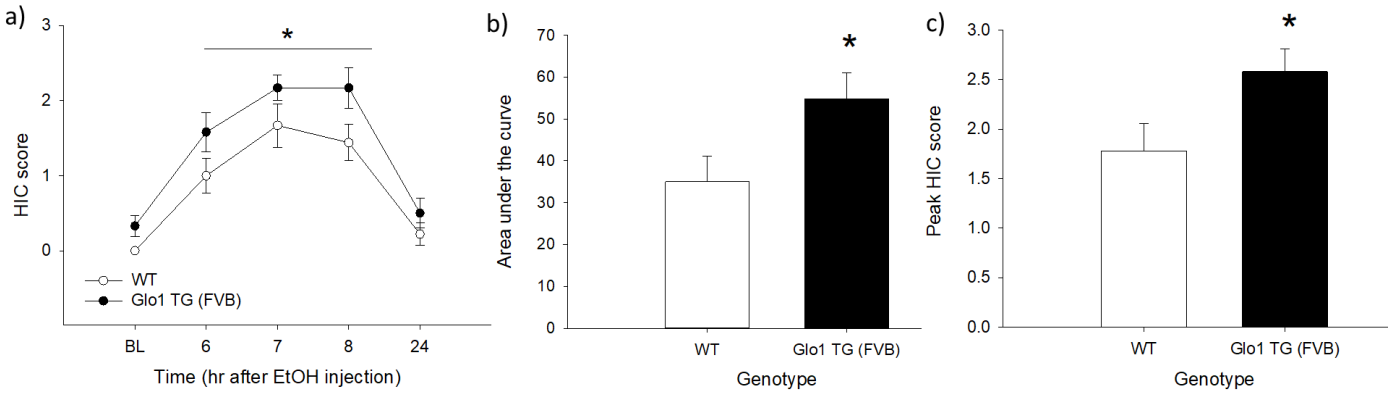

Figure 2. Acute ethanol withdrawal as assessed by handling-induced convulsions (HICs) in wild type (WT) and *Glo1* overexpressing (TG) mice on an FVB genetic background. Panel **a** shows time course of withdrawal, with baseline HIC measurements made immediately before ethanol injection. There was a main effect of genotype, with *Glo1* overexpressing mice having higher HIC scores across the time course that WT mice. *Glo1* overexpressing mice also showed greater cumulative seizure susceptibility (area under the curve, panel **b**), and higher peak HIC scores than WT littermates (panel **c**). * indicates $p<0.05$ N=9-12/genotype.

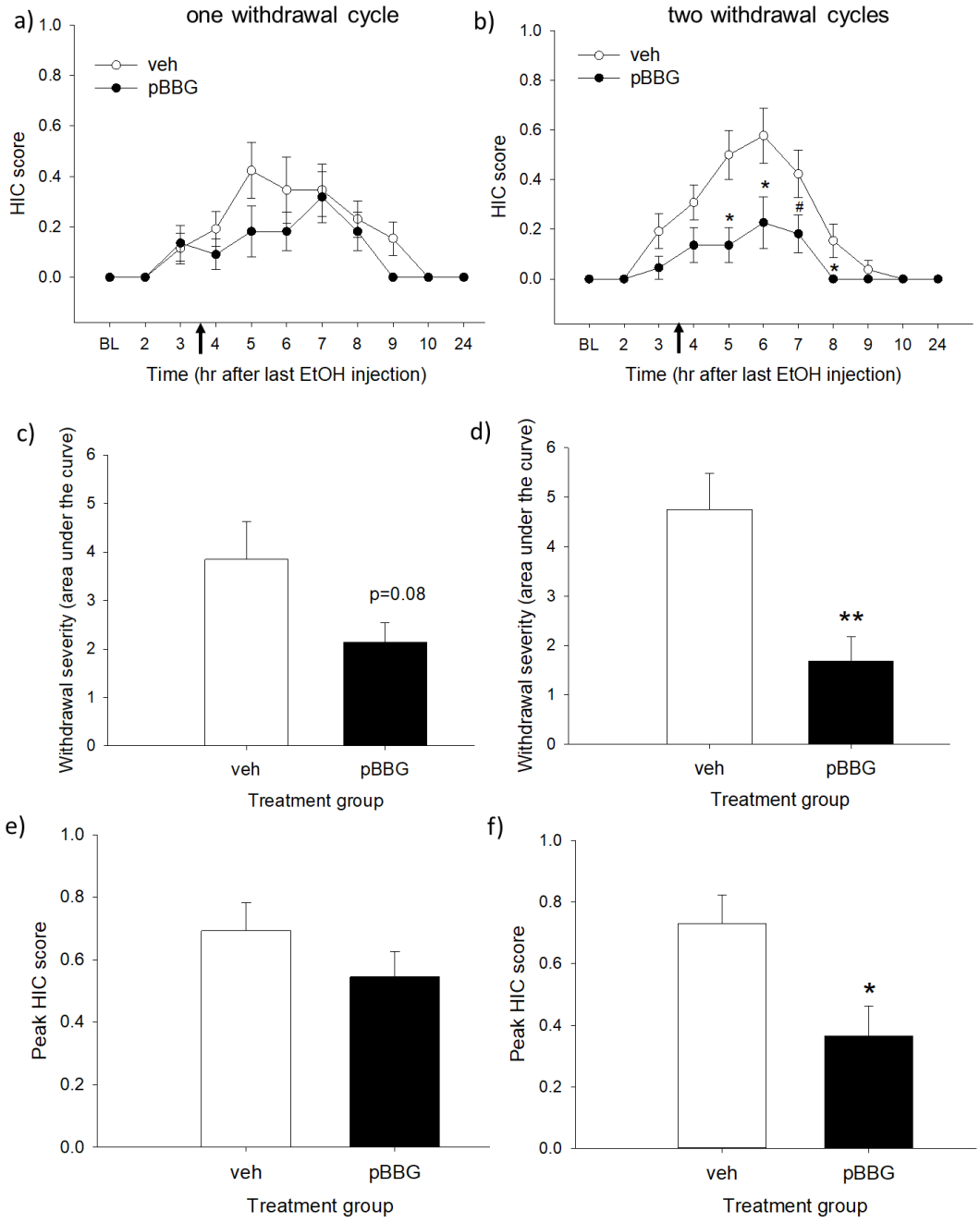

Figure 3. HIC time courses (a & b), area under the HIC curve (c & d) and peak HIC scores (e & f) after one (left column) and two (right column) withdrawal cycles. Baseline HIC measurements were made before the start of each 10-day ethanol exposure period. Injections of GLO1 inhibitor (pBBG, 25 mg/kg) or vehicle were given at 3.5 hr after the final ethanol injection (indicated by arrows). ** indicates statistically significant difference from vehicle-treated mice ($p<0.01$). * indicates $p<0.05$. # indicates trend toward statistically significant difference ($p<0.07$). N=11-13/treatment group.