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ARTICLE

Modulation of Gpr39, a G-protein coupled receptor associated with alcohol use in non-human primates, curbs ethanol intake in mice

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Alcohol use disorder (AUD) is a chronic condition with devastating health and socioeconomic effects. Still, pharmacotherapies to treat AUD are scarce. In a prior study aimed at identifying novel AUD therapeutic targets, we investigated the DNA methylome of the nucleus accumbens core (NAcc) of rhesus macaques after chronic alcohol use. The G-protein coupled receptor 39 (*GPR39*) gene was hypermethylated and its expression downregulated in heavy alcohol drinking macaques. *GPR39* encodes a Zn^{2+}-binding metabotropic receptor known to modulate excitatory and inhibitory neurotransmission, the balance of which is altered in AUD. These prior findings suggest that a GPR39 agonist would reduce alcohol intake. Using a drinking-in-the-dark two bottle choice (DID-2BC) model, we showed that an acute 7.5 mg/kg dose of the GPR39 agonist, TC-G 1008, reduced ethanol intake in mice without affecting total fluid intake, locomotor activity or saccharin preference. Furthermore, repeated doses of the agonist prevented ethanol escalation in an intermittent access 2BC paradigm (IA-2BC). This effect was reversible, as ethanol escalation followed agonist "wash out". As observed during the DID-2BC study, a subsequent acute agonist challenge during the IA-2BC procedure reduced ethanol intake by ~47%. Finally, Gpr39 activation was associated with changes in *Gpr39* and *Bdnf* expression, and in glutamate release in the NAcc. Together, our findings suggest that GPR39 is a promising target for the development of prevention and treatment therapies for AUD.

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INTRODUCTION

Alcohol use disorder (AUD) afflicted ~6.2% (15 million) adults in the United States in 2015 [1]. Yet despite the long-lasting health and economic burdens, current AUD treatment options are limited to a 30-day rehabilitation program and three FDA approved drugs (naltrexone, acamprosate, and disulfiram). These treatments have limited success, as two-thirds of individuals with AUD relapse within months of treatment cessation [2]. Thus, the critical challenge remains to identify effective pharmacological treatments that curb alcohol abuse.

We published a genome-wide DNA methylation (GW-DNAm) analysis to identify differentially methylated regions (DMRs) associated with chronic alcohol use in the nucleus accumbens core (NAcc) of non-human primates [3]. The identified DMRs mapped to genes involved in synaptic function, potentially mediating the neuroadaptations associated with sustained alcohol abuse. Here, we test the strength of our GW-DNAm approach to identify novel therapeutic targets by altering the protein activity of one of the differentially methylated genes and testing its impact on alcohol consumption. The G-protein coupled receptor 39 (*GPR39*) gene was hypermethylated and downregulated in heavy alcohol drinking macaques, which consumed >3 g/kg/day over 12 months of access. As selective GPR39 agonists are commercially available, we tested the hypothesis that increased GPR39 activity would reduce alcohol intake.

GPR39 encodes a Zn^{2+}-binding receptor that is a member of the ghrelin receptor family A [4–6]. The widely expressed *GPR39* gene has been linked to a range of physiological functions, including glucose homeostasis, gastric emptying, and inflammation [7–13]. In the central nervous system, the endogenous ligand of GPR39, Zn^{2+}, modulates N-methyl-D-aspartate (NMDA), γ-aminobutyric acid type A ($GABA_A$), and glycine receptors. Furthermore, Zn^{2+}/GPR39 activity regulates glutamate and GABA signaling in the hippocampus, amygdala, and dorsal cochlear nucleus [14, 15], thereby modulating excitatory and inhibitory responses [16–21]. Consistent with its role in maintaining neurotransmitter homeostasis, GPR39 has been linked to psychiatric disorders. GPR39 is downregulated in the frontal cortex and hippocampus of Zn^{2+}-deficient rodents and suicide victims [13], but upregulated after chronic antidepressant treatment [22]. Furthermore, anxiety- and depressive-like behaviors were reported in *Gpr39* knockout mice [23], and an antidepressant response was observed following a Gpr39 agonist treatment in wild-type mice [23, 24]. In both of these studies, lack of Gpr39 was accompanied by decreased Creb and Bdnf expression, while Gpr39 agonist increased Bdnf expression [23, 24]; these two proteins are widely associated with psychiatric disorders, including alcohol

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abuse [25]. While the mechanism(s) linking GPR39 and neurotransmitter homeostasis remains unclear, its association with psychiatric disorders, in combination with our alcohol-associated differential DNAm findings, warrants the study of its role in regulating alcohol intake.

Here we report the first study examining the potential use of a Gpr39 agonist for the reduction of alcohol intake in a preclinical rodent model of AUD. TC-G 1008 is a potent and selective GPR39 agonist, with EC_{50} values of 0.4 and 0.8 nM for rat and human receptors, respectively [26]. TC-G 1008 reduced anxiety- and depression-like behaviors in mice [24] and showed marked activity in the absence of Zn^{2+} [27]. Our study demonstrates that administration of 7.5 mg/kg TC-G 1008 prevented alcohol escalation and reduced established drinking in mice after acute administration, potentially through a shift towards neuronal excitation in the NAcc.

MATERIALS AND METHODS

Mice

Ten-week-old male C57BL/6J mice (Jackson Laboratory, Sacramento, CA, USA) were individually housed in custom lickometer chambers under a reversed 12-h light/dark cycle (lights on at 21.30) and acclimated to a temperature- and humidity-controlled room for 1 week before experiment onset. Food and water were available ad libitum. Mice (26.27 ± 0.24 g) were weighed daily before each drinking session. There were no changes in weight over time or between treatment groups (Figure S1). All animal procedures were conducted according to the National Institutes of Health Animal Care and Use Committee Guidelines and were approved by the ONPRC Institutional Animal Care and Use Committee.

Drinking paradigms

Two bottle choice (2BC). Modified drinking-in-the-dark (DID) and intermittent access (IA) procedures involved 2BC. Following acclimation, mice were presented with 2 water bottles for two consecutive days before alcohol sessions started to counterbalance groups based on baseline fluid intake. During 2BC sessions, mice had access to a 10% v/v ethanol bottle (Supplementary Methods) and a water bottle. Drinking patterns were acquired by lickometer circuits and recorded via MED-PC IV software (MED Associates, Inc., St. Albans, VT USA) [28]. Lick events were analyzed by custom R scripts (www.r-project.org), and a 'bout' was defined as ≥20 licks with <5-min pause between consecutive licks.

For DID, 10 ml graduated tubes were filled with 10% v/v ethanol or water, and fluid intake was determined by volume displacement to the nearest 0.05 ml. For IA, 50 ml bottles were used and fluid intakes were determined by weight displacement after the session. For ethanol 'off' days, two 50 ml water bottles were provided. Two control chambers (no resident mouse) were monitored to account for changes in volume due to evaporation or housing rack movement.

Dose-response using DID-2BC. Based on prior work [29, 30], DID-2BC was selected for assessing TC-G 1008 dose-response effects on alcohol intake. Mice ($n = 11$) began DID-2BC 2 h into the dark cycle (11.30) for a duration of 4-h/day, 7 days/week. At the end of each drinking session, the volume of fluid tubes was recorded and tubes were replaced with two water bottles. Mice reached stable alcohol drinking levels over a 2-week period (Fig. 1a) before starting a TC-G 1008 dose-response analysis using a within-subject design and 4 drug doses that were selected based on earlier in vivo mouse studies [24]: 2, 5, 10, and 15 mg/kg. However, since we noted that mice significantly decreased water consumption following the 10 mg/kg dose, the planned 15 mg/kg dose was substituted with 7.5 mg/kg (Fig. 1a). Mice received vehicle or TC-G 1008 10 min prior to the DID-2BC sessions. To ensure recovery of baseline alcohol intake, at least two vehicle pretreatment sessions interspersed TC-G 1008 doses (Fig. 1a). Details on drug preparation can be found in Supplementary Methods.

Alcohol escalation using IA-2BC. A cohort of naive mice ($n = 32$) underwent a modified IA-2BC procedure [31, 32] to determine the effect of TC-G 1008 on alcohol use escalation. Twenty-two mice had access to 10% v/v ethanol and water bottles while the remaining 10 mice had access to two water bottles (water-vehicle group). All mice had 2BC access for 22-h, every other day, for a total of 4 weeks (Fig. 3a). Each session started 2-h into the dark cycle (11.30). Baseline water intake (ml/kg) and side preference were used to counterbalance mice across groups ($n = 10$, 11, 11, water-vehicle, ethanol-vehicle, and ethanol-agonist; respectively). For the first 6 IA-2BC sessions, water-/ethanol-vehicle and ethanol-agonist mice received vehicle or 7.5 mg/kg TC-G 1008, respectively. For sessions 6–13, water and both ethanol groups received vehicle injections to determine the impact of discontinued TC-G 1008 treatment in the ethanol-agonist group. An acute drug challenge was given to the ethanol-agonist group on session 14. During a final IA-2BC session mice again received either vehicle or agonist, and were euthanized 3-h post injection. Blood was immediately collected to measure blood ethanol concentration (BEC) via an ambient headspace sampling gas chromatography method [33].

Statistical analysis. Repeated measures ANOVA with simple contrasts and Bonferroni *post hoc* tests were used to analyze TC-G 1008 dose effects on ethanol and water intake, ethanol preference ratio (EPR) and total fluid intake (TFI). Two-way mixed ANOVA was used to compare TC-G 1008 effects on ethanol intake, EPR, water intake and TFI in the IA-2BC procedure. If there was an interaction between the experimental groups (vehicle vs agonist), session and the dependent variables (ethanol intake, EPR, water intake, and TFI), we followed up with a one-way ANOVA with Bonferroni *post hoc* testing. Linear mixed models determined the effect of mediator variables (bout frequency, bout size, inter-bout interval, bout duration, and lick rate) on ethanol drinking associated with 7.5 mg/kg versus baseline/vehicle. Two-way ANOVA tested data from the locomotor study. The effect of the agonist on saccharin intake, preference and TFI was investigated using two-way mixed ANOVA. Two-way repeated measures ANOVA was used to analyze the hourly lick-values. A one-way ANOVA with Bonferroni *post hoc* testing was used to compare differential gene expression across groups. Finally, we used a one-way ANOVA with Sidak *post hoc* testing to compare the effects of the agonist on spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs, respectively) across the different experimental groups. To compare the effects of the agonist on sEPSCs and sIPSCs within the same cells we used a paired *t*-test. In general, if the assumption on homogeneity of variance was violated, we applied the nonparametric Kruskal-Wallis test (i.e. *Bdnf*) or Welch-ANOVA (i.e. percent change of sIPSCs). In addition, if the sphericity was not met, as assessed by Maulchly's test, we applied a Greenhouse-Geisser correction. Mean ± SEMs are presented and $p < 0.05$ was considered significant.

RESULTS

Acute administration of TC-G 1008 reduces ethanol intake and preference

Under a DID-2BC paradigm, mice escalated ethanol intake until reaching a plateau (day 1: 1.2 g/kg vs day 12: 6.0 g/kg, Fig. 1b) that was maintained for 6 consecutive days. While acute administration of lower TC-G 1008 doses (2 and 5 mg/kg) exhibited no effect on ethanol intake, EPR or TFI (Fig. 1b–f), the 7.5 and 10 mg/kg doses significantly reduced ethanol

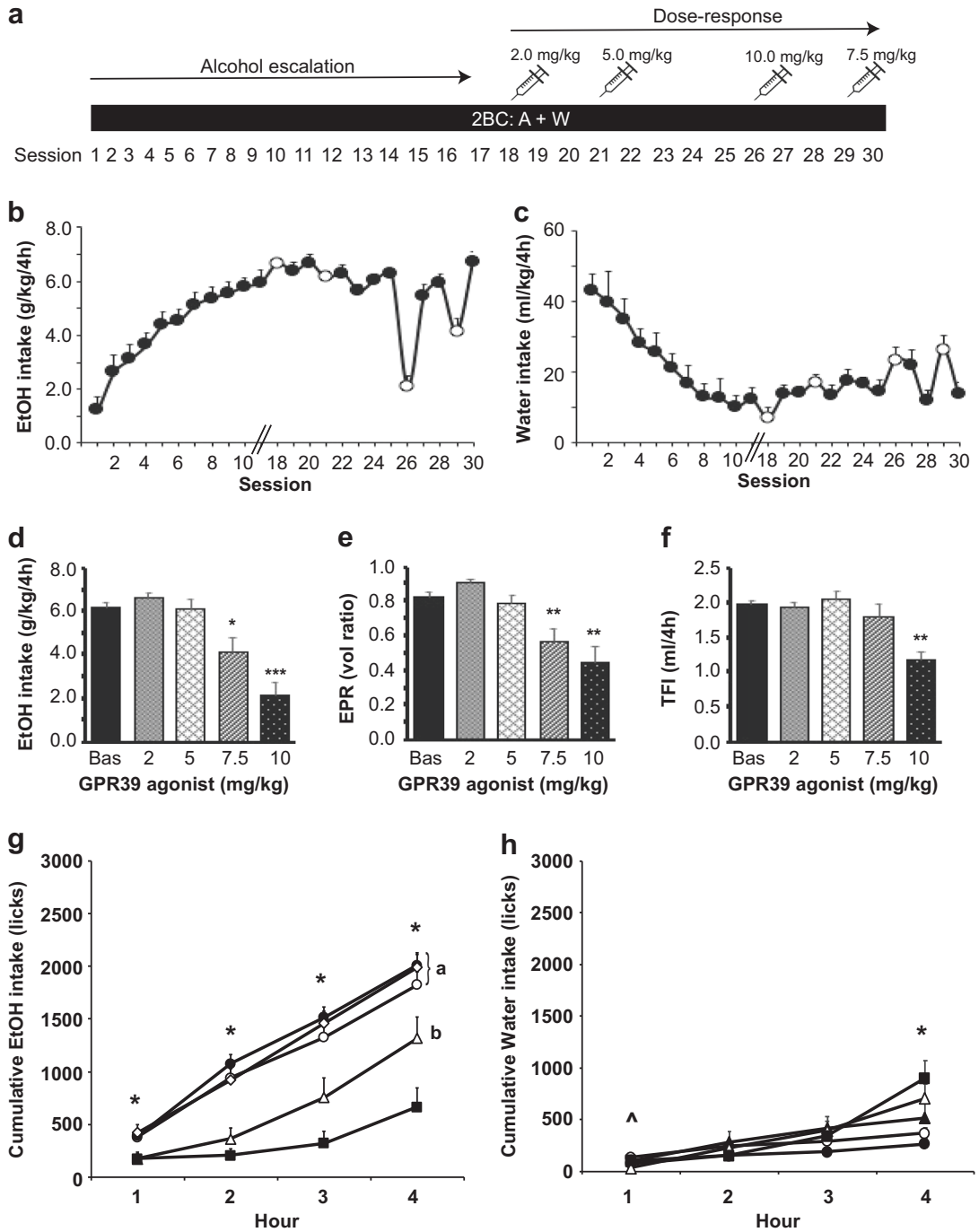


Fig. 1 Effects of different doses of the GPR39 agonist, TC-G 1008, on ethanol intake in a Drinking-in-the-Dark 2 bottle choice (DID-2BC) paradigm. **a** Schematic representation of the DID-2BC and drug administration timeline. After reaching an alcohol intake plateau, mice received 4 doses of the agonist (2, 5, 7.5, and 10 mg/kg), allowing recovery time in between doses. "A" is for alcohol and "W" is for water. The syringe represents the day of vehicle or TC-G 1008 administration. **b** Average daily ethanol intake (g/kg/4 h) and **c** water intake (ml/kg/4 h). White circles represent the drug-administration sessions. **d** Alcohol intake (g/kg/4 h), **e** preference (EPR, volume ratio) and **f** total fluid intake (TFI, ml/kg/4 h) were measured at the end of the 4-h drinking sessions. Data are represented as mean ± SEM, $n = 11$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, repeated measures ANOVA with Bonferroni post hoc test (baseline against each dose). Each dose is represented by a different pattern. **g**, **h** Cumulative ethanol (g/kg) intake and TFI (ml/kg) at each hour of the 4-h session. Data are represented as mean ± SEM, $n = 11$. Between group at each hour: *$p < 0.05$ vehicle vs 7.5 and 10 mg/kg, ^$p < 0.05$ vehicle vs 10.0 mg/kg; Within subjects per each agonist dose: [a]$p < 0.05$ all hours vs all hours, [b]$p < 0.05$ h 1 vs 3–4, [c]$p < 0.05$ h 1 vs 2, [d]$p < 0.05$ h 2 vs 3–4, [e]$p < 0.05$ h 1 vs 4, [f]$p < 0.05$ h 2 vs 3–4, [g]$p < 0.05$ h 3 vs 4. Vehicle (white circle), 2 mg/kg (black circle), 5 mg/kg (white diamond), 7.5 mg/kg (white triangle), 10 mg/kg (black square)

consumption by 33% and 67%, respectively ($F_{1.99,19.88} = 17.40$, $p = 4.40e{-5}$; Bonferroni: $p_{7.5\ mg/kg} = 0.04$; $p_{10\ mg/kg} = 4.56e{-4}$; Fig. 1b, d). Neither 7.5 nor 10 mg/kg TC-G 1008 altered alcohol intake 24-h after drug administration (Fig. 1b). Relative to baseline, the 7.5 mg/kg dose reduced EPR ($F_{1.86,18.56} = 11.35$, $p = 1e{-3}$; Bonferroni: $p_{7.5\ mg/kg} = 8e{-3}$; Fig. 1e) by both decreasing alcohol consumption (Fig. 1d) and significantly increasing water intake (baseline: 13.14 ± 7.06 ml/kg; 7.5 mg/kg:

26.35 ± 13.01 ml/kg; $F_{4,40} = 6.75$, $p = 2.99e{-4}$; Bonferroni: $p_{7.5\ mg/kg} = 4e{-3}$), which culminated in an unaltered TFI ($F_{2.07,20.68} = 10.49$, $p = 1e{-3}$; Bonferroni: $p_{7.5\ mg/kg} = 0.98$; Fig. 1f). Similarly, 10 mg/kg TC-G 1008 reduced EPR ($p_{10\ mg/kg} = 8e{-3}$; Fig. 1e). However, the increase in water intake was modest (23.36 ± 13.11 g/kg; $p_{10\ mg/kg} = 0.16$), resulting in a significant reduction in TFI ($p_{10\ mg/kg} = 4e{-3}$; Fig. 1f) as compared to baseline. Moreover, this modest increase in water intake persisted 24 h following drug administration (Fig. 1c) before reaching baseline levels 48 h post drug administration. Overall, these results identified 7.5 mg/kg TC-G 1008 as an optimal dose for subsequent study. Drinking pattern analyses of this particular dose revealed that the reduction in ethanol intake was attributable to decreases in both bout frequency and average bout size ($p = 2.14e{-9}$, $p = 2.32e{-8}$; respectively, Tables S1, S2).

To delineate the temporal effects of TC-G 1008 treatment on fluid intakes, we evaluated hourly ethanol and water lick distributions throughout the DID-2BC sessions. Overall, 7.5 mg/kg TC-G 1008 significantly suppressed cumulative ethanol licks during the first 2 h compared to baseline (Table S3a, 3b). This effect persisted through hour 4, although it was not significant ($p_{h\ 3} = 0.06$, $p_{h\ 4} = 0.06$, Fig. 1g, Table S3b). Importantly, cumulative water licks were significantly lower during hour 1, but higher during hour 4 compared to baseline (Table S3b), suggesting that the lower TFI intake with TC-G 1008 (7.5 mg/kg) observed during the first 3 h (Fig. 1h) was driven by reduced ethanol and water intake during hour 1, but without alterations in water intake during hours 2 and 3. At hour 4, the increase in water licks (Fig. 1g, Table S3a–c) and recovery of ethanol licks resulted in a similar cumulative TFI as compared to baseline (Fig. 1h, Table S3a–c).

TC-G 1008 (7.5 mg/kg) did not alter LMA (Supplementary Methods), as there was no distance or velocity × group interaction ($F_{4.88,78.08} = 0.99$, $p = 0.42$, partial $\eta^2 = 0.06$, $\varepsilon = 0.44$, $F_{5.14,87.34} = 0.79$, $p = 0.57$, partial $\eta^2 = 0.04$, $\varepsilon = 0.47$, respectively, Fig. 2b, c). Further, TC-G 1008 did not affect saccharin intake (Supplementary Methods), as there was no session × group interaction (saccharin intake: $F_{1.36,24.38} = 2.71$, $p = 0.10$, partial $\eta^2 = 0.13$, $\varepsilon = 0.68$; saccharine preference: $F_{1.25,22.56} = 2.44$, $p = 0.13$, partial $\eta^2 = 0.12$, $\varepsilon = 0.63$; TFI: $F_{1.37,24.56} = 1.28$, $p = 0.28$, partial $\eta^2 = 0.07$, $\varepsilon = 0.68$, Fig. 2d–f), simple main effect of treatment group on the day of the agonist treatment ($p_{Sac.intake} = 0.17$, $p_{Sac.preference} = 0.12$, $p_{TFI} = 0.20$, Fig. 2d–f), or within-subject effect between baseline and session of agonist treatment ($p_{Sac.intake} = 0.50$, $p_{Sac.preference} = 0.51$, $p_{TFI} = 0.60$, Fig. 2d–f) on saccharin intake, preference or TFI. Therefore, reductions in ethanol intake cannot be explained by agonist-induced changes in general reward sensitivity, and further suggest that TC-G 1008 effects exhibit specificity for alcohol preference.

TC-G 1008 prevents ethanol escalation

After demonstrating that 7.5 mg/kg TC-G 1008 acutely reduces established ethanol drinking in a DID-2BC procedure, we tested its ability to prevent ethanol escalation with repeated administration (Fig. 3a) in alcohol naive mice. There was an interaction between session and group on ethanol intake, EPR and TFI ($F_{2.12,42.46} = 3.93$, $p = 0.03$; $F_{2.28,45.55} = 6.50$, $p = 2e{-}03$; $F_{3,30} = 7.01$, $p = 4.04e{-}04$; respectively). Main effects analyses showed that during the first IA-2BC session, mice receiving TC-G 1008 had a significant reduction in ethanol intake (ethanol-vehicle: 8.20 ± 1.60 g/kg; ethanol-agonist: 3.21 ± 1.60 g/kg; $F_{1,20} = 4.86$, $p = 0.04$), and no effect on TFI (ethanol-vehicle: 169.56 ± 10.49 ml/kg; ethanol-agonist: 155.83 ± 10.49 ml/kg, $F_{1,20} = 0.86$, $p = 0.37$). EPR was also lower in the ethanol-agonist group as compared to the ethanol-vehicle group, although the difference was not significant (0.21 ± 0.09 vs 0.48 ± 0.09; respectively, $F_{1,20} = 3.98$, $p = 0.06$). The agonist's effects were even more pronounced in sessions 2–6 when ethanol-vehicle mice demonstrated an anticipated escalation in ethanol intake; whereas, ethanol-agonist mice continued to exhibit significantly blunted ethanol intakes (session 6, $F_{1,20} = 37.67$, $p = 5e{-}6$) and EPR (session 6, $F_{1,20} = 0.96$, $p = 2e{-}6$). The dramatic reduction in ethanol intake by the ethanol-agonist group was accompanied by a significant increase in both water intake (session 6, ethanol-vehicle: 26.02 ± 15.60 ml/kg; ethanol-agonist: 171.74 ± 15.60 ml/kg; $F_{1,20} = 43.62$, $p = 2e{-}6$) and TFI (session 6, $F_{1,20} = 6.07$, $p = 0.02$, Fig. 3g). The reduction in ethanol intake was driven by significant decreases in ethanol bout frequency ($F_{1,20} = 35.76$, $p = 8e{-}6$, Fig. 3h), mean bout size ($F_{1,20} = 14.9$, $p = 2e{-}3$, Fig. 3i) first bout size ($F_{1,15} = 5.53$, $p = 0.03$, Fig. 3j), mean lick rate ($F_{1,20} = 8.92$, $p = 9e{-}3$) and increases in inter-bout interval (IBI, $F_{1,20} = 2.28$, $p = 0.01$) (Table S4a).

To test whether the behavioral effects of the agonist were reversible, TC-G 1008 treatment was discontinued in the ethanol-agonist group during sessions 7–13 (Fig. 3a). Overall, ethanol intakes and EPR partially recovered in the ethanol-agonist group as compared to session 6 ($F_{1,10} = 12.75$, $p = 0.05$, $F_{1,10} = 12.06$, $p = 0.03$; respectively, Fig. 3e, f). Although these levels were lower than those observed in the ethanol-vehicle group ($F_{1,20} = 10.80$, $p = 4e{-}3$, $F_{1,20} = 9.63$, $p = 6e{-}3$), when excluding 4 of the 11 ethanol-agonist animals that showed no reversibility in ethanol intake (Figure S2) in the presence or absence of the agonist, ethanol intake and preference at session 13 were similar to those from ethanol-vehicle subjects ($F_{1,16} = 3.84$, $p = 0.07$, $F_{1,16} = 3.05$, $p = 0.10$, respectively). The recovery of ethanol intake and preference in the whole ethanol-agonist group ($n = 11$) was accompanied by a shift in drinking patterns that included increases in bout frequency, mean and first bout sizes, and lick rate as well as a decrease in IBI ($p > 0.05$; Fig. 3h–j, Table S4a).

In order to assess TC-G 1008 effects after alcohol escalation, and to replicate earlier findings from the dose-response analysis (DID-2BC experiment), an acute challenge with the 7.5 mg/kg was administered to the ethanol-agonist group on session 14. Ethanol intake and preference were significantly reduced as compared to session 13 ($F_{1,10} = 12.75$, $p = 0.01$, $F_{1,10} = 12.06$, $p = 0.01$; respectively, Fig. 3e, f). When considering only the 7 subjects that exhibited ethanol escalation after the drug was "washed out", ethanol intake and preference were reduced by 47 and 60%, respectively ($F_{1,16} = 21.89$, $p = 2.50e{-}4$, $F_{1,16} = 77.55$, $p = 1.56e{-}7$; respectively, Figure S2). These results confirm the ability of TC-G 1008 to acutely reduce established ethanol intake levels in the IA-2BC paradigm. Attenuated ethanol intake in the ethanol-agonist group ($n = 11$) during session 14 was accompanied by an increase in latency to 1st ethanol bout (Table S4a).

TC-G 1008 treatment caused a significant increase in water intake (ethanol-vehicle: 26.02 ± 15.60 ml/kg, ethanol-agonist: 171.74 ± 15.60 ml/kg, $F_{1,20} = 43.62$, $p = 2e{-}6$) and TFI ($F_{1,20} = 6.07$, $p = 0.02$, Fig. 3g) on session 6 as compared to the vehicle group. A similar water intake increase was observed after acute TC-G 1008 administration (session 14: ethanol-vehicle: 10.03 ± 10.02 ml/kg, ethanol-agonist: 151.07 ± 10.02 ml/kg, $F_{1,20} = 99.08$, $p = 3.42e{-}9$) as compared to session 13 ($F_{1,10} = 28.42$, $p = 0.045$); however, no change in TFI between sessions 13 and 14 was observed ($F_{1,10} = 687.60$, $p = 0.47$), suggesting the reduction in ethanol intake was compensated for by water intake after acute dosing.

The hourly drinking patterns of sessions 6, 13, and 14 (Fig. 3k, l) were further examined because of their potential relevance to the temporal TC-G 1008 effects on drinking escalation and maintenance of excessive intake levels. Ethanol-vehicle animals consumed the vast majority of alcohol during the dark cycle (hours 0–10, Fig. 3k, l), whereas during repeated TC-G 1008 treatment (session 6) subjects maintained very low levels of ethanol licking throughout the 22 h session, a finding consistent with the significant reductions in bout frequency and bout size (Fig. 3h–j, Table S4a). When TC-G

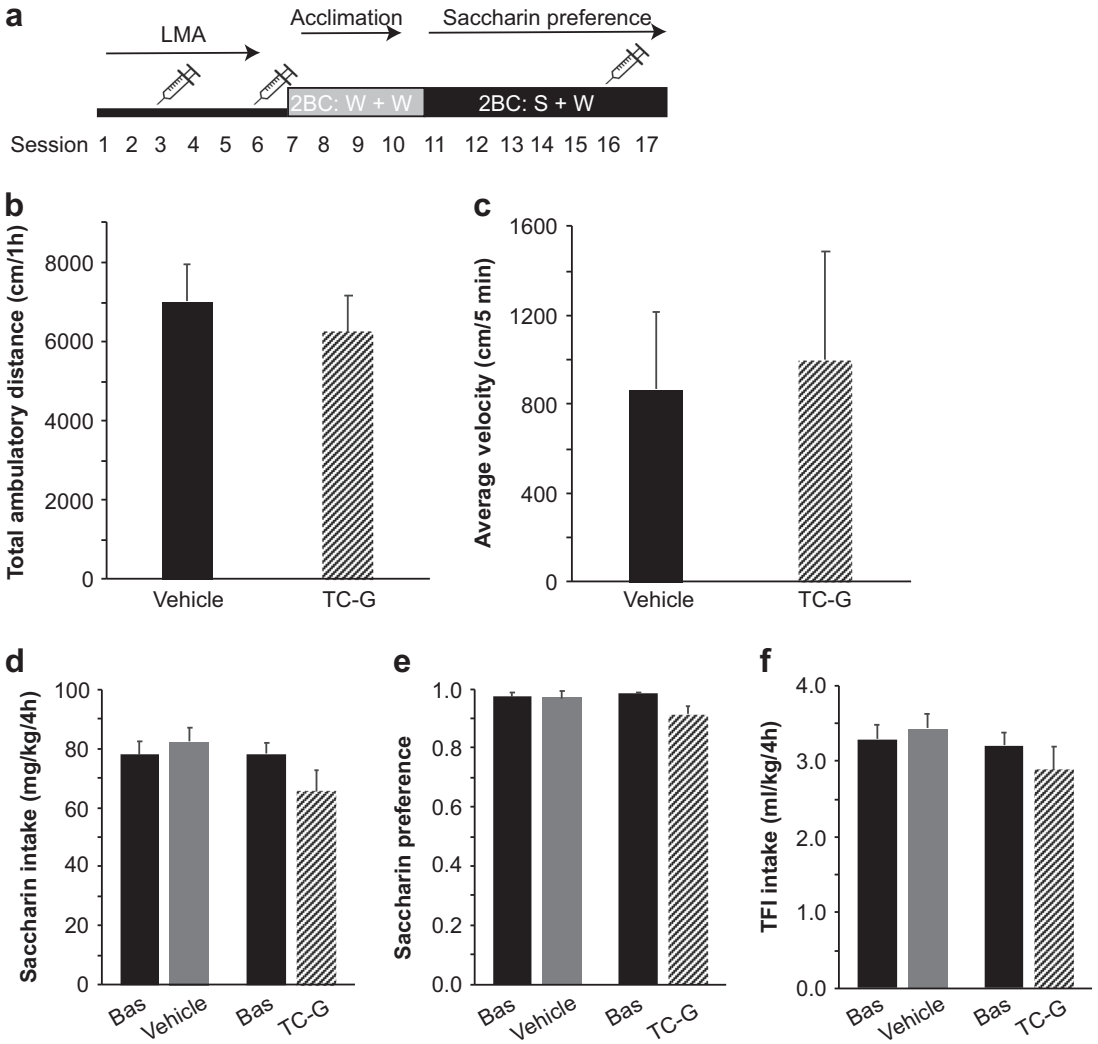


Fig. 2 Effects of the 7.5 mg/kg TC-G 1008 agonist dose on locomotor activity (LMA) and saccharin intake in a DID-2BC paradigm. **a** Schematic representation of the LMA, saccharin studies and drug administration (dose of 7.5 mg/kg). "W" is water and "S" is saccharin. The syringe represents the day of vehicle or TC-G 1008 administration. LMA is reported as **b** total ambulatory distance (cm/1 h) and **c** average velocity (cm/5 min) during the 1 h duration of the LMA study. **d** Saccharin intake (mg/kg/4 h), **e** preference (volume ratio) and **f** total fluid intake (TFI, ml/kg/4 h) prior, during and 24 h after 7.5 mg/kg administration. Data are represented as mean ± SEM. In **d**–**f**, Bas (black bars) refers to the corresponding measurement at baseline (average of the two days prior to vehicle or TC-G 1008 administration). Saccharin intake, preference or TFI is represented in dark gray or in a dotted pattern in the vehicle- or TC-G1008-receiving group, respectively

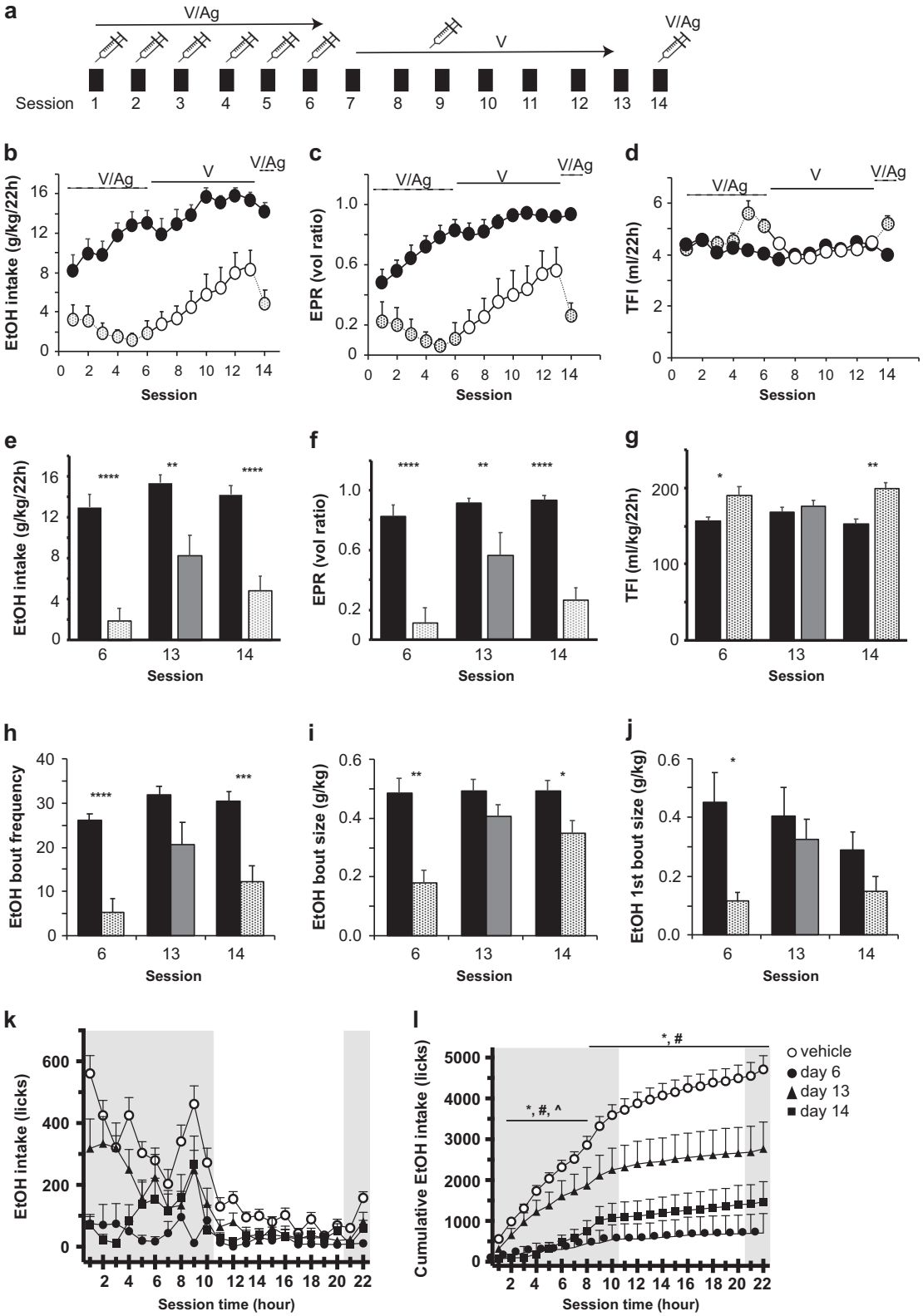
1008 treatment was discontinued, ethanol-agonist mice adopted similar drinking patterns (i.e., elevated bout frequency and bout sizes), as observed in ethanol-vehicle animals in session 13 (Fig. 3h–k, Table S4a). Consequently, the cumulative licks were similar to the ethanol-vehicle group and were significantly different from session 6 (Fig. 3l, Table S4b). When an acute TC-G 1008 dose was administered (session 14), the drinking during the dark-phase was largely suppressed, with the greatest disruption occurring during the first 3–5 h post drug administration (Fig. 3k, Table S4b). Taken in conjunction with the temporal influence of TC-G 1008 noted above (Fig. 1g), the therapeutic effects of TC-G 1008 in reducing alcohol drinking is most pronounced during the first 3 h, but can extend to ~5 h. We examined the most sensitive 3-h time period for agonist effects on BECs (session 15) and found that levels were significantly lower in the ethanol-agonist group as compared to ethanol-vehicle group (3 ± 3 and 82 ± 16 mg%, respectively, $p = 4.52e{-}4$). Furthermore, BEC and ethanol intake levels were positively correlated ($r^2 = 0.73$, $p = 2.62e{-}7$).

Gpr39 and *Bdnf* expression are increased after TC-G 1008 administration

Agonist treatment resulted in a significant increase in *Gpr39* expression as compared to water-vehicle mice (Fig. 4a; $F_{2,27} = 5.34$, $p = 0.01$). A trend in the same direction was observed between ethanol-agonist and ethanol-vehicle groups (Fig. 4a; $p = 0.08$). Similarly, *Bdnf* was significantly overexpressed in ethanol-agonist as compared to both water- and ethanol-vehicle ($H_2 = -9.88$, $p = 0.02$ and $H_2 = -8.58$, $p = 0.046$; respectively, Fig. 4b). The expression of *Kcc2* was not different between groups. See Supplementary Methods for details on gene expression analysis.

TC-G 1008 biases towards enhanced excitatory transmission

Spontaneous excitatory postsynaptic currents (sEPSC) and spontaneous inhibitory postsynaptic currents (sIPSC) were recorded from MSNs located within the NAcc (Fig. 4d) in ex vivo slices before (baseline), during bath application of TC-G 1008 at different concentrations (0.1 nM–1 μM, Supplementary Methods; Fig. 4e),



and following wash out of the drug (data not shown). The mean ± SEM frequency (Fig. 4f), amplitude, rise time, decay time and area are presented in Table S5. Baseline frequency of sEPSC and sIPSCs did not differ across treatment group (sEPSC frequency $F_{3,40} = 1.49$, $p = 0.23$; sIPSC frequency $F_{3,40} = 1.51$, $p = 0.23$; Fig. 4f). However, sEPSC frequency was significantly different across TC-G 1008 doses (sEPSCs: $F_{4,48} = 5.44$, $p = 6e{-}4$; sIPSCs: $F_{4,48} = 1.29$, $p = 0.28$), with 100 nM showing significantly higher sEPSC frequency as compared to the other doses ($p_{0.1\ vs\ 100\ nM} = 0.03$, $p_{1\ vs\ 100\ nM} = 0.04$, $p_{100\ nM\ vs\ 1\ \mu M} = 2e{-}4$; Fig. 4f). The percent change of sEPSC and sIPSC frequency across the different agonist doses was not different ($F_{3,48} = 1.50$, $p = 0.23$ and $F_{3,48} = 2.44$,

Fig. 3 Effects of the GPR39 agonist, TC-G 1008, on ethanol escalation. **a** Timeline of the Intermittent Access, 2BC, paradigm (IA-2BC). "V" is for vehicle and "Ag" is for agonist. The syringe represents the day of vehicle and/or TC-G 1008 administration. **b** Daily ethanol intake (g/kg/22 h), **c** ethanol preference ratio (EPR, volume ratio) and **d** total fluid intake (TFI, ml/kg/22 h) of ethanol-vehicle group (black circles) and ethanol-agonist group (patterned and white circles). The patterned circle represents the drug-administration sessions, and the white circles the sessions with no agonist administered. **e** Ethanol intake (g/kg/22 h), **f** EPR and **g** TFI (ml/kg/22 h) were measured at the end of three 22 h drinking sessions: 6, 13, and 14. While one group always received a vehicle injection at each one of these sessions, the other group received TC-G 1008 during sessions 6 and 14 (dotted bars), but vehicle at session 13 (dark gray). **h** Ethanol bout frequency, **i** mean ethanol bout size (g/kg) and **j** mean first bout size (g/kg) measured at the end of three 22 h drinking sessions: 6, 13, and 14 are shown. Data are represented as mean ± SEM, $n = 11$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, repeated measures ANOVA with Bonferroni *post hoc* test. **k** Number of ethanol licks and **l** cumulative ethanol licks at each hour of the 22 h session. For **k**, see Table S6 for detailed significance data. For **l**, the statistical significance is as follows: *$p < 0.05$ vehicle vs TC-G 1008 at session 6 and 14, #$p < 0.05$ session 6 vs session 13, ^$p < 0.05$ session 13 vs session 14, two-way repeated measures ANOVA with Bonferroni *post hoc* test. The legend for **k** and **l** is shown on the top right corner of panel **l**. Briefly, vehicle (white circle), day 6 (black circle), day 13 (black triangle), day 14 (black square)

$p = 0.27$; respectively). However, when we measured the percent change in sEPSC and sIPSC frequency and amplitude before and after each dose of the agonist, our data revealed that the most consistent effect of the agonist was on sEPSC frequency. Lower concentrations of TC-G 1008 (<1 nM) had a trend toward an increase in the percent change of sEPSC frequency from baseline that was not significant (Fig. 4g $p_{0.1\ nM} = 0.06$; $p_{1\ nM} = 0.07$). However, concentrations above 1 nM led to a significant increase in sEPSC frequency over baseline (Fig. 4g; $p_{100\ nM} = 0.048$; $p_{1\ \mu M} = 0.048$). Regarding sIPSCs, TC-G 1008 exhibited mixed effects on frequency in that there was a 79.69 ± 6.78 % decrease under baseline only with the application of 1 nM TC-G 1008 (Fig. 4g; $p_{0.1\ nM} = 0.26$; $p_{1\ nM} = 0.01$; $p_{100\ nM} = 0.90$; $p_{1\ \mu M} = 0.34$). The significant decrease in percent change in sIPSC frequency following 1 nM TC-G 1008 and increase in sEPSCs frequency at concentrations above 100 nM resulted in a significantly greater excitatory/inhibitory ratio of frequency over that of baseline (Fig. 4h; $p_{0.1\ nM} = 0.09$; $p_{1\ nM} = 0.02$; $p_{100\ nM} = 0.01$; $p_{1\ \mu M} = 3e\text{-}3$). At the highest dose tested (1 μM), wash out of the drug for 20 min brought sEPSC and sIPSC frequency back to baseline levels (sEPSC: 97.40 ± 11.61 %; sIPSC: 93.32 ± 5.50 %), confirming that the observed effects on sEPSC frequency were due to bath application of the drug. Together this suggests a bias towards excitatory neurotransmission in the presence of TC-G 1008 as evidenced by an excitatory/inhibitory ratio >1 that increases above that of baseline.

DISCUSSION

Our results illustrate the power of integrating epigenetic, transcriptomic, circuitry, pharmacological, and behavioral approaches to identify novel targets for the treatment of hazardous alcohol use. By selecting a candidate gene identified in our prior GW-DNAm analysis of the non-human primate NAcc (*GPR39*) and using the selective agonist TC-G 1008, we demonstrated that manipulation of a DNAm-identified target significantly altered alcohol intake in murine models of binge and escalating drinking. We further showed that TC-G 1008 effects were not attributable to a global suppression of fluid intake or locomotor activity, and were specific to alcohol.

Repeated daily TC-G 1008 administration during the IA-2BC escalation phase markedly attenuated ethanol intake over 22 h access periods. When agonist treatment was discontinued, a delayed escalation of alcohol consumption ensued, indicating that the treatment effects were largely reversible after 7 ethanol sessions. We noted, however, that 4 of the 11 TC-G 1008 mice did not increase alcohol intake post treatment. While this individual variability in escalation rate was similar to that observed in ethanol-vehicle subjects (data not shown), it is possible that additional sessions were needed for some drug-treated animals to obtain control intake levels [34–36]. Additionally, given that BECs were essentially undetectable 3 h into an IA-2BC session following acute TC-G 1008 challenge, it is possible that some ethanol-agonist mice were unable to associate the positive reinforcing, intoxicating and discriminative stimulus effects of alcohol with its consumption, thereby precluding the ability of these abuse-related attributes to maintain drug seeking behavior [37]. Additional investigation will be required to distinguish whether Gpr39 receptor activity modulates sensitivity to the rewarding versus the aversive properties of ethanol.

While analyzing quantity and frequency of alcohol intake as a measure of hazardous drinking patterns [38] we found that acute treatment with 7.5 mg/kg TC-G 1008 significantly reduced ethanol consumption by consistently suppressing bout frequency (and bout quantity/size to a lesser extent) in both DID-2BC and IA-2BC procedures. Based on temporal lick patterns, TC-G 1008 exhibited the greatest efficacy throughout the first 3–5 h following injection, observations that are congruent with the reported 7h half-life of TC-G 1008 (10 mg/kg; i.p.) in mice [26]. Taken in conjunction with the ability of TC-G 1008 to prevent an escalating frequency of drinking bouts that typically accompanies consecutive alcohol-access sessions in mice [35], TC-G 1008 may show pharmacotherapeutic promise when administered during active drinking or abstinence when a relapse-associated escalation in drinking is possible [39].

In agreement with a prior study reporting increased Gpr39 after TC-G 1008 administration [24], we similarly observed *Gpr39* overexpression with TC-G 1008 treatment. Although the underpinnings of *Gpr39* transcriptional regulation in response to agonist are currently unknown, others have suggested that a Gpr39 desensitization mechanism in response to prolonged Zn^{2+} treatment is important for the regulation of Zn^{2+}/Gpr39 signaling [40]. Similarly, prolonged Gpr39 activation by TC-G 1008 would lead to receptor internalization and degradation, requiring transcription/translational upregulation to produce de novo Gpr39 receptor. Further studies are needed to associate these changes in gene expression with the activity of the functional protein.

Activated Gpr39 upregulates Gαq, $G\alpha_{12/13}$ and Gαs pathways [5], leading to transcription mediated by increased inositol turnover and activation of cAMP- and serum-response elements [4, 41]. Prior studies showed reduced hippocampal Creb and Bdnf levels in *Gpr39*-KO mice [23], while systemic TC-G 1008 significantly increased Bdnf in the hippocampus of wild-type mice [24]. In agreement with these results, we report a significant increase in *Bdnf* expression in the NAcc of ethanol-agonist mice. Bdnf plays an important role in mediating synaptic transmission and plasticity [42], and is extensively studied for its role in regulating alcohol intake [43]. Accordingly, acute and moderate ethanol intake elevates striatal Bdnf levels, which activates downstream signaling cascades that ultimately limit further ethanol intake. However, after chronic heavy drinking, this protective pathway becomes unresponsive, and striatal Bdnf levels are unaltered [43]. In agreement, our results showed no differences in *Bdnf* levels between water-vehicle and chronic ethanol-drinkers (ethanol-vehicle). However, acute TC-G 1008 administration may reestablish the Bdnf-dependent protective system, and by increasing *Bdnf* levels, reduce ethanol intake.

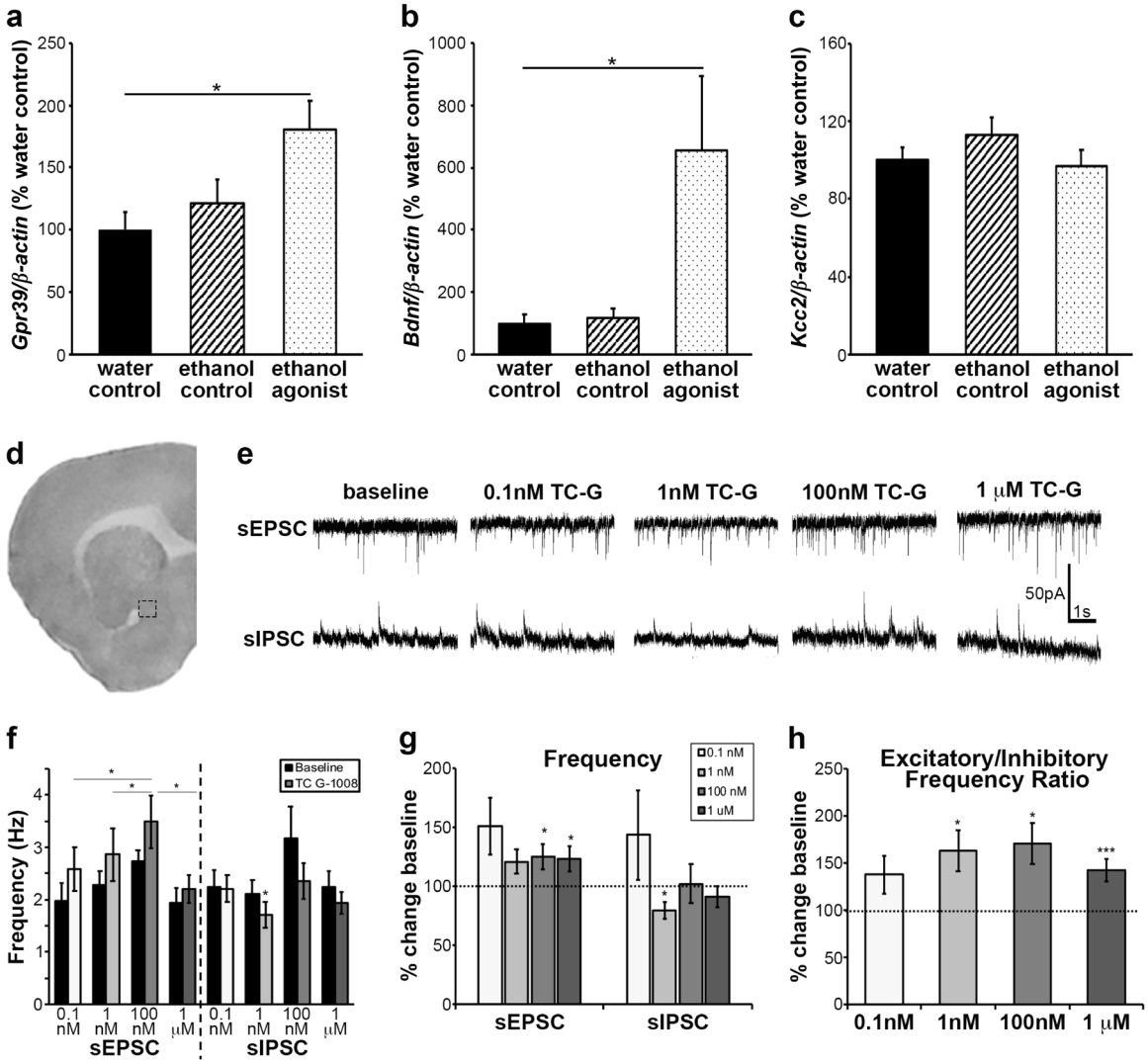


Fig. 4 Effects of the GPR39 agonist, TC-G 1008, on the nucleus accumbens core (NAcc). Relative gene expression of **a** *Gpr39*, **b** *Bdnf* and **c** *Kcc2* measured in the NAcc of mice that consumed water and were treated with vehicle (water-vehicle), consumed ethanol and were treated with vehicle (ethanol-vehicle), or consumed ethanol and were treated with 7.5 mg/kg TC-G 1008 (ethanol-agonist). **d** An image of a coronal section containing the NAcc. The box denotes the location in which medium spiny neurons (MSNs) were targeted for electrophysiological recordings. **e** Whole-cell patch clamp recording of MSNs ($n = 14–20$ cells) located within the NAcc. Spontaneous excitatory postsynaptic currents (sEPSC) and spontaneous inhibitory postsynaptic currents (sIPSC) were recorded before (baseline) and during bath application of TC-G 1008 (0.1 nM, 1 nM, 100 nM, or 1 μM). **f** Average frequency of sEPSC and sIPSC during baseline (black bars) and bath application of TC-G 1008 (shades of gray bars). Data analyzed by paired *t*-test. **g**, **h** Percent change of bath application of TC-G 1008 (0.1–1 μM) from baseline (dotted line) in sEPSC and sIPSC characteristic of frequency **g** and the excitatory/inhibitory ratio of frequency **h**. Data are represented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

Gpr39 and Bdnf regulate multiple neurotransmitter systems in different brain regions, including glutamate through the endocannabinoid system [44], GABA mediated by the K+/Cl− cotransporter 2 (Kcc2) and NMDA activity [45–48]. Our ex vivo analysis of glutamatergic and GABAergic synapses in alcohol-naive NAcc slices showed that TC-G 1008-induced alterations in sEPSC frequency ultimately led to a bias towards excitation, suggesting an increase in glutamate release associated with Gpr39 activation. This effect, if also observed in the presence of alcohol, could be mediated by the increase in *Bdnf* expression in ethanol-agonist mice, as it has been reported that Bdnf enhances glutamate release and increases the frequency of sESPCs in hippocampal neurons [49–51]. While further studies are needed to understand the relationship between Gpr39, Bdnf and glutamate release in the presence of alcohol, our results suggest a role for Gpr39 in maintaining excitatory/inhibitory homeostasis in the NAcc.

Extensive evidence shows that ethanol, generally, potentiates GABA and glycine receptors, while it inhibits glutamate receptors suggesting a bias towards inhibition [52–55]. In terms of neurotransmitter release, while ethanol enhances presynaptic GABA release, its effects on glutamate release are controversial [52, 56, 57]. These variable results could be attributable to the dose of alcohol consumed, with glutamate release inversely related to ethanol concentrations in the striatum [58–60]. It has been suggested that the increase in accumbal glutamate with alcohol intake [61, 62] may be a compensatory mechanism to adapt to the chronic inhibitory effects of ethanol [63]. Based on this evidence, we propose a model in which TC-G 1008, by increasing extracellular glutamate, may compensate for the decrease in postsynaptic glutamate activity and increased GABA that has been reported with ethanol exposure. In combination, with the trend towards an increased activity of the postsynaptic

glutamatergic receptors, TC-G 1008 may "normalize" the excitatory/inhibitory ratio, reducing ethanol intake.

While the effects of TC-G 1008 in the NAcc lead to a consistent effect on sEPSC frequency, we hypothesize that only an effect of 100 nM TC-G 1008 is seen on postsynaptic characteristics of $GABA_A$ receptors (Table S6) due to the promiscuous activities of the GPR39 endogenous ligand, Zn^{2+}. Zinc is known to activate different pathways, including the endocannabinoid system, calcium release, KCC2 activity [14, 15, 64], which could all be contributing to this effect on glutamate and GABA signaling. Future experiments will be needed to explore the role of GPR39 on microcircuitry within the NAcc and the interplay between ethanol, Zn^{2+} and GPR39 receptor.

Since *GPR39* is widely expressed [65, 66], we cannot exclude the possibility that reduced alcohol use is the result of the combined effect of TC-G 1008 on multiple brain and body regions. In addition to the described role of Gpr39 in the NAcc, Gpr39 alterations in brain areas influencing depression and anxiety-like behaviors [12, 13, 22, 23] could contribute to alcohol intake. Furthermore, a recent study showed that two other GPR39 agonists induced somatostatin release and inhibited ghrelin release in gastric mucosal cells [67]. Further evidence has shown that ghrelin inhibits water intake [68] by modulating angiotensin II [69], and ghrelin also stimulates alcohol intake [70–72]. Thus, if TC-G 1008 reduced ghrelin levels, it may contribute to reduced alcohol intake. In addition, Zn^{2+} and Gpr39 in colonocytic cells enhance tight junctional complexes and epithelial barrier function [11, 73]. A loss of tight junction function during Zn^{2+}/Gpr39 dysfunction may contribute to alterations of the gut barrier function, also referred to as "leaky gut", reported in alcohol-exposed rodents and alcohol-dependent humans [74]. Finally, Gpr39 activity is highly dependent on Zn^{2+} levels, which are dramatically reduced in the brain of alcoholics [75, 76]. The decrease in Zn^{2+} (15–25%) was found to be consistent across the brain regions analyzed (9–14 different brain regions), although the frontal cortex, thalamus, amygdala, and hippocampus were more dramatically affected (>20% reduced zinc levels) [75, 76]. While short-term ethanol exposure seems to be insufficient to alter Zn^{2+} levels in the brain of rodents [74], the combination of chronic ethanol use, together with associated hepatic failure (the liver is actively involved with the metabolism of ingested Zn^{2+}), and dietary deficiencies, are posited to alter cerebral Zn^{2+} homeostasis and drive a reduction in brain Zn^{2+} content in human alcoholics. Interestingly, low Zn^{2+} levels are not only associated with alcoholism; preclinical and clinical data report low Zn^{2+} levels in the serum of subjects suffering from depression and anxiety disorders (see [41]). Further studies indicate that Zn^{2+} supplementation may have antidepressant effects (see [41]) and that chronic antidepressant treatment upregulates GPR39 [22]. Extensive evidence supports a causal relationship between AUD and depression (see [77]), that together with a role of a Gpr39 agonist in ameliorating depression-like behaviors [24] and reducing alcohol intake (present study), suggest a common molecular pathway and potential novel therapeutic target for the treatment of comorbid alcohol abuse and depression disorders. Future studies are needed to clarify the interplay of multiple brain regions and/or peripheral systems in the role of GPR39 agonist(s) in suppressing ethanol intake as reported in this study.

In conclusion, this study demonstrates that GW-DNAm approaches can successfully identify novel candidate genes that are promising targets for new AUD therapies. Moreover, this study reports successful cross-species validation, identifying a candidate gene in a non-human primate alcohol use model, then leveraging rodent alcohol models to evaluate potential treatment strategies. In total, these studies identify Gpr39 as a promising, novel candidate for the treatment of hazardous alcohol use. Future studies are needed to clarify the precise molecular and cellular mechanisms linking Gpr39 activity to alterations in synaptic plasticity that ultimately lead to alcohol use.

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ADDITIONAL INFORMATION

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