Brief Communications

Pharmacologically Counteracting a Phenotypic Difference in Cerebellar $GABA_A$ Receptor Response to Alcohol Prevents Excessive Alcohol Consumption in a High Alcohol-Consuming Rodent Genotype

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Cerebellar granule cell $GABA_A$ receptor responses to alcohol vary as a function of alcohol consumption phenotype, representing a potential neural mechanism for genetic predilection for alcohol abuse (Kaplan et al., 2013; Mohr et al., 2013). However, there are numerous molecular targets of alcohol in the cerebellum, and it is not known how they interact to affect cerebellar processing during consumption of socially relevant amounts of alcohol. Importantly, direct evidence for a causative role of the cerebellum in alcohol consumption phenotype is lacking. Here we determined that concentrations of alcohol that would be achieved in the blood after consumption of 1–2 standard units (9 mM) suppresses transmission through the cerebellar cortex in low, but not high, alcohol consuming rodent genotypes (DBA/2J and C57BL/6J mice, respectively). This genotype-selective suppression is mediated exclusively by enhancement of granule cell $GABA_A$ receptor currents, which only occurs in DBA/2J mice. Simulating the DBA/2J cellular phenotype in C57BL/6J mice by infusing the $GABA_A$ receptor agonist, 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol hydrochloride, into cerebellar lobules IV–VI, *in vivo*, significantly reduced their alcohol consumption and blood alcohol concentrations achieved. 4,5,6,7-Tetrahydroisoxazolo-[5,4-c]pyridine-3-ol hydrochloride infusions also significantly decreased sucrose consumption, but they did not affect consumption of water or general locomotion. Thus, genetic differences in cerebellar response to alcohol contributes to alcohol consumption phenotype, and targeting the cerebellar $GABA_A$ receptor system may be a clinically viable therapeutic strategy for reducing excessive alcohol consumption.

Key words: addiction; alcohol; cerebellum; GABA; THIP

Significance Statement

Alcohol abuse is a leading cause of preventable death and illness; and although alcohol use disorders are 50%–60% genetically determined, the cellular and molecular mechanisms of such genetic influences are largely unknown. Here we demonstrate that genetic differences in cerebellar granule cell $GABA_A$ receptor responses to recreational concentrations of alcohol are the primary determinant of alcohol's impact on cerebellar processing and that pharmacologically modifying such responses alters alcohol consumption. These data highlight the cerebellum as an important neuroanatomical region in alcohol consumption phenotype and as a target for pharmacological treatment of alcohol use disorders. The results also add to the growing list of cognitive/emotional roles of the cerebellum in psychiatric disease and drug abuse.

Introduction

Alcohol (ethanol) abuse is a leading cause of preventable death and disability (Lim et al., 2012), and accumulating evidence links the cerebellum to genetic risk for developing an alcohol use disorder (AUD) in humans (Schuckit, 1985; Hill et al., 2007; Hill, 2010; Herting et al., 2011) and excessive ethanol consumption in rodents (Gallaher et al., 1996; Yoneyama et al., 2008). We recently determined that physiologically relevant concentrations of etha-

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nol (9–52 mM) differentially affect granule cell (GC) tonic $GABA_A$ receptor ($GABA_A$R) currents, as a function of ethanol consumption phenotype (Kaplan et al., 2013; Mohr et al., 2013). Specifically, ethanol enhances tonic $GABA_A$R-mediated inhibition in low ethanol-consuming rodent genotypes, including DBA/2J (D2) mice, but suppresses it in high ethanol-consuming C57BL/6J (B6) mice (Kaplan et al., 2013). Ethanol has an intermediate effect on GC tonic $GABA_A$R currents in moderate ethanol-consuming rodents and nonhuman primates (Kaplan et al., 2013; Mohr et al., 2013). Collectively, there is a striking genetic correlation between the polarity/magnitude of ethanol's impact on GC $GABA_A$R currents and ethanol consumption phenotype. However, ethanol has numerous potential targets in the cerebellum, and it is not known whether ethanol's differential effect on GCs plays a primary role in modulating signal processing through the cerebellum or whether altering cerebellar processing influences ethanol consumption. Here, we demonstrate that low concentrations of ethanol (9 mM, a concentration achieved in the blood after consumption of 1–2 standard units of ethanol) act exclusively at GC $GABA_A$R currents to suppress transmission through the cerebellar cortex in D2 but not B6 mice, and that pharmacologically activating cerebellar $GABA_A$Rs significantly reduces ethanol consumption in B6 mice.

Materials and Methods

Ethical approval. All procedures conform to the regulations detailed in the National Institutes of Health *Guide for the care and use of laboratory animals* and were approved by the Institutional Animal Care and Use Committees of Oregon Health & Science University, Portland Veterans Administration Health Care System, and Washington State University.

Preparation of brain slices. Cerebellar slices were prepared acutely each day of experimentation (Kaplan et al., 2013). Male B6 and D2 mice (24–35 d old; 2–6 animals/cage) were maintained on a standard 12 h light-dark cycle. Animals were anesthetized with isoflurane and killed by decapitation. Whole brain was isolated and immersed in aCSF (0°C–2°C) containing the following (in mM): 124 NaCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 2.5 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, and bubbled with 95%O_2/5% CO_2, pH 7.4. The cerebellum was dissected out of the brain and mounted, parallel to the sagittal plane, in a slicing chamber filled with aCSF (0°C–2°C). Parasagittal slices (225 μm) were made with a vibrating tissue slicer (Vibratome). Slices were incubated in warmed aCSF (33 ± 1°C) for 1 h after dissection, and then held at 22°C–23°C until used. Kynurenic acid (1 mM) was included in the dissection, incubation, and holding solution but was omitted from the experimental solutions. In a subset of brain slice experiments (n = 30 cells) in which we wanted to determine whether key observations in brain slices from young animals also occurred in slices from animals that were the same age as used for *in vivo* behavioral experiments (9–12 weeks), we used a modified dissection/slicing solution to improve the health of slices, as follows (in mM): 26 $NaHCO_3$, 1.25 $NaPO_4$, 2 KCl, 5 $MgCl_2$, 2 Na-pyruvate, 0.5 $CaCl_2$, 10 D-glucose, 220 sucrose, 1 kynurenate, and bubbled with 95% O_2/5% CO_2, pH 7.4.

Electrophysiology. Slices were placed in a submersion chamber on an upright microscope and viewed with an Olympus 60× (0.9 NA) water-immersion objective with differential interference contrast and infrared optics. Slices were perfused with aCSF (31°C-34°C) at a rate of ~7 ml/min. Drugs were dissolved in aCSF and applied by bath perfusion. Visually identified Purkinje cells (PCs) were either voltage-clamped or current-clamped with patch pipettes constructed from thick-walled borosilicate glass capillaries and filled with internal solutions optimized for current- or voltage-clamp recording, as described below. For testing the effect of 9 mM ethanol on PC spontaneous IPSC (sIPSC) frequency in the presence of the ionotropic glutamate receptor antagonist, kynurenic acid (2 mM), the internal solution contained the following (in mM): 130 CsCl, 4 NaCl, 0.5 $CaCl_2$, 10 HEPES, 5 EGTA, 4 MgATP, 0.5 Na_2GTP, 5 QX-314, pH adjusted to 7.2–7.3 with CsOH. In separate voltage-clamp experiments, in which glutamate receptors were not blocked (but PC sIPSCs were nonpharmacologically isolated by recording with V_h = 10 mV, the empirically determined reversal potential for spontaneous EPCSs [sEPSCs]), the internal solution contained the following (in mM): 130 Cs-gluconate, 4 NaCl, 10 HEPES, 5 BAPTA, 4 ATP Mg^{2+} salt, 0.5 GTP Na^+ salt, 5 QX-314 Cl, 10 TEA, and 100 nM paxilline. The internal solution for all current-clamp experiments was (in mM) as follows: 132.3 K-gluconate, 7.7 KCl, 4 NaCl, 0.5 $CaCl_2$, 10 HEPES, 5 EGTA free acid, 4 ATP Mg^{2+} salt, 0.5 GTP Na^+ salt, pH buffered to 7.2–7.3 with KOH. Electrode resistance was 1.5–3 MΩ. Cells were rejected if access resistance was >10 MΩ or if it changed by >15%. Only one cell was recorded from a given slice. For current-clamp recordings, constant current was injected to set the PC membrane potential between −60 and −65 mV. There were no strain differences in holding current (B6: −243.04 ± 18.14 pA; D2: −254.13 ± 9.90 pA, p = 0.61). In cases where the slice was exposed to more than one drug or different doses of the same drug, order of drug application was randomized across slices, and stable baselines were obtained between drug applications for a minimum of 4 min.

Animals for behavioral experiments. Male B6 mice (~9 weeks of age, from The Jackson Laboratory) were individually housed, maintained on a 12 h reverse light-dark cycle (lights off 0800) for the drinking studies, and those experiments were conducted during the dark cycle. For the locomotor activity study, mice were acclimated to a 12 h light-dark cycle (lights on 0600) for 3 weeks, and the study was conducted during the light cycle.

Cannula implantation surgery. Mice were maintained under isoflurane anesthesia and implanted with a unilateral guide cannula (26Ga, stainless steel, 12.0 mm long) aimed at lobes IV/V/VI of the cerebellar cortex (anteroposterior: −7.2, mediolateral: ±0.1, dorsoventral: −0.8 mm [−1.8 mm with injectors]; Experiments 1–4) or lobes II/III of the cerebellar cortex (anteroposterior: −5.6 mm, mediolateral: ±0.36 mm, dorsoventral: −1.5 mm [−2.5 mm with injectors]; Experiment 5). Mice were administered a single intraperitoneal injection of ketoprofen (3 mg/kg; Abbott Laboratories) for postoperative analgesia and were given a minimum of 1 week to recover before testing.

Ethanol and sucrose consumption protocol. Experiments were performed in the dark phase of the light-dark cycle to measure high levels of consumption (Ramaker et al., 2012, 2015). Beginning 3 h into the dark cycle, mice had 2 h access to a 25 ml bottle containing a 10% v/v ethanol solution or 2% w/v sucrose solution, and a 25 ml bottle containing tap water; bottle positions were counterbalanced between the left and right side across subjects. At the conclusion of the 2 h period, water and ethanol or sucrose consumption were measured to the nearest 0.2 ml, and bottles were replaced with two 25 ml bottles containing tap water. Before drug infusions, mice were given sham infusions where the injector was lowered through the cannula, but no fluid was administered. Subsequent drug infusions were given 5 min before the ethanol or sucrose access session in 200 nl of solution delivered over 1 min, with an additional 30 s allowed for diffusion. Infusions were spaced a minimum of 3 d apart to ensure that ethanol or sucrose intake had returned to baseline for at least 2 d before subsequent infusions.

Ethanol consumption: Experiment 1. Mice were given ethanol access 5 d/week (Monday-Friday) for 15 d before surgery. Following surgery, their drinking behavior stabilized (defined as <10% variation for 3 d) by the seventh day of ethanol access. Mice were administered an infusion of vehicle (aCSF), followed 3 d later by an infusion of 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol hydrochloride (THIP; 500 ng). For analysis of blood ethanol content on infusion days (vehicle and THIP), orbital blood samples were collected immediately following the 2 h ethanol access period. Upon completion of the THIP infusion trial, after ethanol intake returned to baseline levels, mice were only given access to water during the 2 h drinking session. After 5 d, their water

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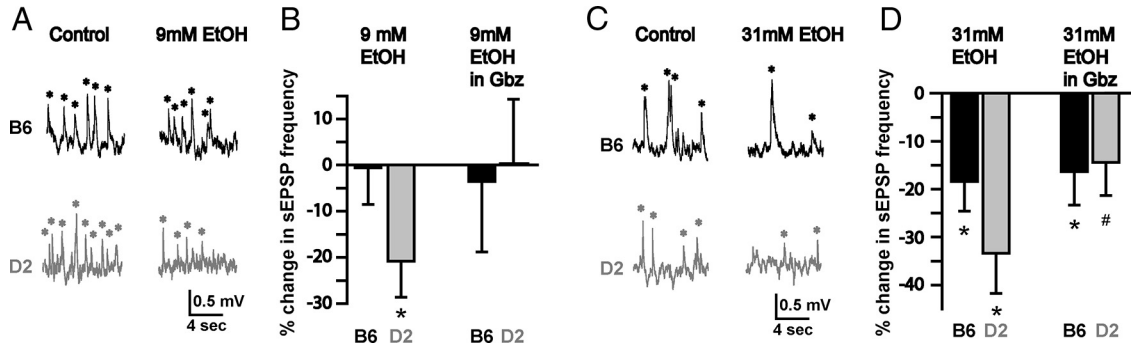

Figure 1. 9 mM ethanol (EtOH) reduces spontaneous excitatory input to PCs in D2 but not B6 mice by enhancing $GABA_AR$ inhibition. ***A***, Current-clamp recording of PC sEPSPs in control and 9 mM EtOH in B6 mice (black) and D2 mice (gray). ***B***, Mean percentage change in PC sEPSP frequency by 9 mM EtOH under control conditions and in GABAzine (Gbz) in B6 (black) and D2 (gray) mice. ***C***, PC sEPSPs in control and 31 mM EtOH in B6 mice (black) and D2 mice (gray). ***D***, Mean percentage change in PC sEPSP frequency by 31 mM EtOH under control conditions and in Gbz in B6 (black) and D2 (gray) mice. *Occurrence of an sIPSP (raw data). *$p < 0.05$, significant difference from baseline (bar charts). #$p = 0.08$, significant difference at trend level (one-sample *t* tests).

intake had stabilized and they were again administered vehicle and then, 3 d later, THIP (500 ng).

Ethanol consumption: Experiment 2. Mice underwent surgery before receiving ethanol access, and ethanol intake was measured 7 d/week. Following stabilization of ethanol consumption levels, mice received infusions of drug in the following order: vehicle, 500 ng THIP, 50 ng THIP, 100 ng THIP, 250 ng THIP, and vehicle. Pre- and post-THIP vehicle infusions were used to determine whether mechanical or psychological stress induced by multiple infusions altered ethanol intake. Ethanol intake following the second vehicle infusion did not differ from ethanol intake after the first vehicle infusion ($p > 0.05$, $n = 12$), confirming that the THIP-induced decrease in ethanol intake was not a general consequence of repeated brain infusions over the course of the experiment.

Sucrose consumption: Experiment 3. Mice underwent surgery before receiving 2 h sucrose access, which was measured 5 d/week. Following stabilization of sucrose intake (8 drinking days), mice received a sham infusion and then an infusion of 500 ng THIP 5 d later (including the nondrinking weekend days). After confirming that sucrose consumption returned to baseline on the following day, the mice were given a vehicle infusion 2 d later. Upon completion of the drug and vehicle infusions, mice were given water only to drink for 3 weeks and acclimated to a regular light-dark cycle before testing locomotor activity.

Locomotor activity: Experiment 4. The locomotor effects of 500 ng THIP versus vehicle were tested in automated locomotor activity chambers (40 × 40 × 30 cm; AccuScan Instruments), as described by Ramaker et al. (2015). The chambers contained 8 pairs of photocell beams positioned 2 cm above the floor. Beam breaks were collected by VersaDat software (AccuScan Instruments) and used as the index of horizontal activity. Mice were tested during the light phase (between 0900–1300) each day to allow for the detection of either drug-induced increases or decreases in activity during 2 h sessions. Mice were acclimated to the activity chambers on day 1. On day 2 (baseline), mice received a sham infusion immediately before placement in the activity chambers. On day 3, separate groups of mice ($n = 7$/group) were infused with either vehicle or 500 ng THIP and immediately placed in the activity chambers.

Ethanol consumption: Experiment 5. For this study, mice were surgically implanted with a unilateral guide cannula aimed at lobes II/III of the cerebellar cortex before the measurement of 2 h ethanol intake (5 d/week). Following stabilization of ethanol intake (8 drinking days), mice received a sham infusion and then an infusion of 500 ng THIP 5 d later (including the nondrinking weekend days). After confirming that ethanol consumption returned to baseline on the following day, the mice were given a vehicle infusion 2 d later.

Assessment of blood ethanol concentrations (BECs). Immediately following the 2 h ethanol access sessions on vehicle and THIP infusion days in Experiment 1, blood samples (20 μl) were collected from the orbital sinus in all subjects and analyzed using headspace gas chromatography, as described previously (Finn et al., 2007). Concentrations of samples were interpolated from a standard curve with six pairs of external standards with known ethanol concentrations (from 0.1 to 3.0 mg/ml).

Histological determination of infusion sites. At the conclusion of each behavioral study, mice were killed and infused with 20 mg/ml methylene blue dye in aCSF. Intact whole brains were removed and flash frozen in isopentane and stored at −80°C. Brains were sliced (35 μm sections) using a Leica cryostat, mounted on glass slides, and photographed with an IM50 imaging system (Leica Microsystems). Data from mice with confirmed placements were used in the analyses.

Statistics. For assessment of electrophysiology experiments, all data are expressed as mean ± SEM. Between-strain analyses were conducted using Student's *t* tests. In the cases where a mixed-measures ANOVA or repeated-measures ANOVA was used, mouse genotype was treated as a between-subjects measure and drug condition was a within-subjects measure. *Post hoc* comparisons were made using Student's *t* tests. All other statistical comparisons were made with unpaired or one-sample *t* tests, or a Mann–Whitney Rank Sum Test (as indicated). For analysis of behavioral data, repeated-measures ANOVA and *post hoc t* tests or planned comparisons assessed the effect of THIP on ethanol intake, water intake, and BEC. Correlations between BEC and ethanol intake were assessed using a Pearson correlation. For locomotor studies, THIP was analyzed as a between-subjects factor, and time (20 min bins) was analyzed as a repeated measure. In all cases, statistical comparisons were two-tailed, and the significance threshold was set at $p < 0.05$. No statistical methods were used to predetermine sample sizes, but sample sizes for electrophysiology experiments are similar to those reported in the field (Hanchar et al., 2005; Kaplan et al., 2013), and sample sizes for behavioral experiments are similar to those recently reported following bilateral microinjection of THIP into the nucleus accumbens shell in B6 mice (Ramaker et al., 2015).

Reagents. For behavioral experiments, ethanol (200 proof; Pharmco Products) was diluted in tap water to yield a 10% (v/v) solution for consumption, sucrose (Sigma-Aldrich) was diluted in tap water to yield a 2% (w/v) solution for consumption, and THIP (Tocris Bioscience) was dissolved in aCSF comprised of the following: 124 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2P0_4, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 2.5 mM KCl, for brain infusions. For electrophysiology experiments, all reagents were from Sigma Chemicals, except for GABAzine and kynurenic acid (Abcam).

Results

Because PCs are the sole output of the cerebellar cortex (Cerminara et al., 2015), any cerebellar-specific actions of ethanol that affect behavior (including differences across genotypes) should manifest in PCs, either via their afferent inputs or intrinsic properties. Accordingly, we made current-clamp recordings from PCs in cerebellar slices from B6 and D2 mice, and assessed the effect of ethanol on PC membrane potential (V_m) and spontaneous synaptic potentials (Fig. 1). PCs from both B6 and D2 mice had similar baseline V_m values (mean = −61.6 ± 0.5 and −61.2 ± 0.5 mV, respectively, $n = 16$ and $n = 14$, $p = 0.57$, unpaired *t* test)

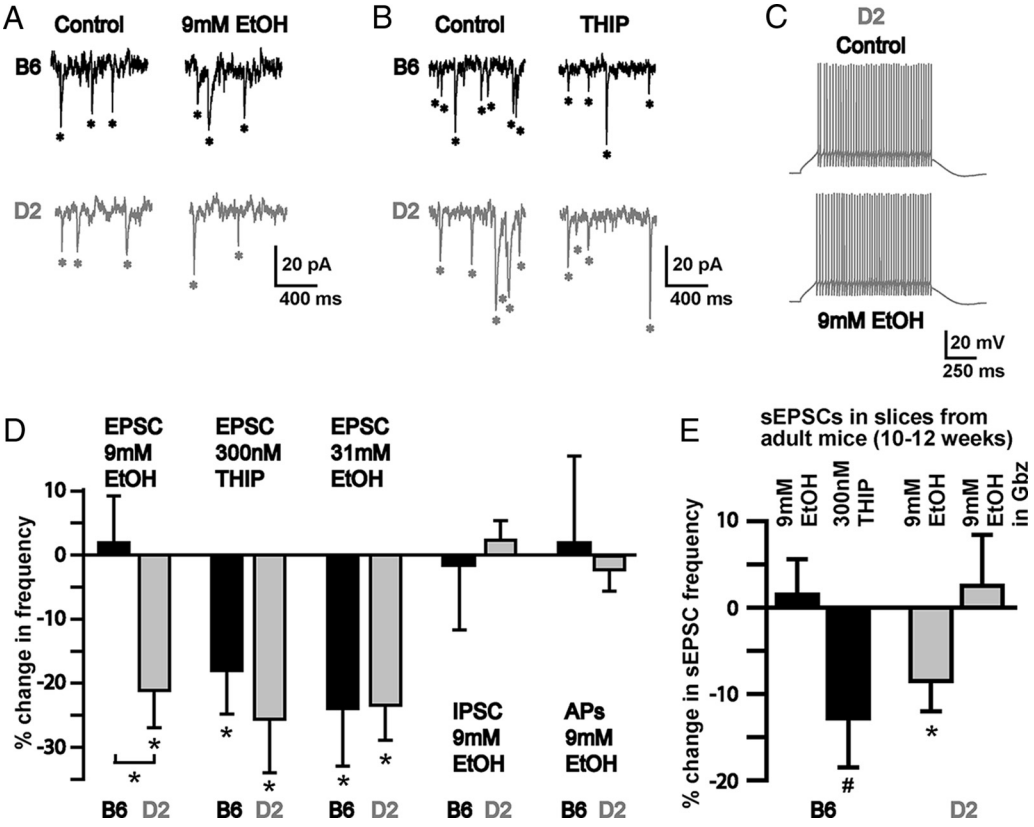

Figure 2. 9 mM ethanol (EtOH) decreases PC sEPSC frequency but not sIPSC frequency or native PC conductance properties. ***A***, ***B***, Voltage-clamp recording ($V_h = -60$, $E_{Cl^-} = -60$ mV) of PC sEPSCs in control and 9 mM EtOH (***A***) or 300 nM THIP (***B***) in B6 (black) and D2 (gray) mice. ***C***, Current-clamp recording of PC action potential response to current injection (150 pA, 1 s) under control conditions (top) and in the presence of 9 mM EtOH (bottom) from a D2 mouse. ***D***, Mean percentage change in sEPSC, sIPSC (in kynurenate, 2 mM, $V_h = -60$ mV, $E_{Cl^-} = 0$ mV), and action potential frequency in 9 or 31 mM EtOH, and in THIP (300 nM) in B6 mice (black) and D2 mice (gray). ***E***, Mean percentage change in sEPSC frequency in 9 mM (alone or in GABAzine, Gbz; 10 μM) or in THIP (300 nM) in adult (10–12 weeks) B6 mice (black) and D2 mice (gray). *Occurrence of sEPSCs (raw data). $^*p < 0.05$, drug effect versus control (one-sample *t* test; bar charts); and comparisons between genotypes (paired *t* test; indicated by bars). $^\#p = 0.06$, significant difference at trend level.

and exhibited frequent sEPSPs (mean frequency = 0.27 ± 0.03 and 0.53 ± 0.05 Hz). However, 9 mM ethanol (a blood concentration at which only D2 mice display significant cerebellar ataxia) (Gallaher et al., 1996) and only B6 mice achieve during voluntary consumption of ethanol (Yoneyama et al., 2008) selectively reduced sEPSP frequency in D2 PCs (−20.85 ± 7.79%, $n = 16$ cells; $t_{(15)} = 2.68$, $p = 0.017$, one-sample *t* test) but had no effect in B6 PCs (−0.56 ± 7.96%, $n = 12$, $p = 0.95$; Fig. 1*A*,*B*). The $GABA_AR$ antagonist, GABAzine (10 μM) blocked ethanol-induced attenuation of sEPSP frequency in D2 mice (D2: 0.35 ± 13.02%, $n = 12$ cells; B6: −3.52 ± 15.28%, $n = 12$; Fig. 1*B*), suggesting that ethanol selectively reduces glutamatergic input to D2 PCs by enhancing GC $GABA_AR$ inhibition. Importantly, this genotype and receptor type selectivity was lost at 31 mM ethanol (a concentration that causes ataxia in both D2 and B6 mice) (Gallaher et al., 1996; Rhodes et al., 2007), and one only rarely achieved during voluntary consumption of ethanol in B6 mice (Rhodes et al., 2007; Yoneyama et al., 2008; Ramaker et al., 2012). Indeed, 31 mM EtOH suppressed sEPSP frequency in PCs from both mouse genotypes (D2: −33.34 ± 8.40%, $n = 7$, $p = 0.007$, one-sample *t* test; B6: −18.38 ± 6.18, $n = 9$, $p = 0.018$; Fig. 1*C*,*D*), and the suppression was not fully blocked by GABAzine (10 μM, D2: −14.43 ± 6.93%, $t_{(6)} = 2.12$, $p = 0.078$, B6: −16.37 ± 6.95%, $t_{(8)} = 0.21$, $p = 0.84$, by paired *t* tests; Figure 1*D*).

To confirm that the observed ethanol-induced genotype-selective changes in sEPSPs are due to specific actions on excitatory inputs from GCs rather than changes in inhibitory inputs to PCs from their molecular layer afferent basket and stellate cells, we made voltage-clamp recordings of PCs, and held PCs at the reversal potential for $GABA_AR$ currents ($V_h = -60$ mV, with E_{Cl^-} set to −60 mV) to nonpharmacologically isolate PC glutamate-mediated sEPSCs (Fig. 2). Under such conditions, PCs in both mouse genotypes exhibited similarly frequent sEPSCs (mean baseline frequency, D2 = 1.62 ± 0.18 Hz and B6 = 1.66 ± 0.19, $p = 0.87$, paired *t* test), and as predicted, 9 mM ethanol selectively reduced the frequency of sEPSCs in D2 PCs without affecting B6 PC sEPSCs (D2: −21.13 ± 5.79%, $n = 6$ cells, $p = 0.015$, one-sample *t* test; B6: 1.94 ± 7.31%, $n = 6$, $p = 0.80$; Fig. 2*A*,*D*). Importantly, the lack of effect of 9 mM ethanol on B6 PC sEPSCs is not due to innate differences in the ability of extrasynaptic $GABA_AR$-mediated GC tonic inhibitory currents to modify GC excitation of PCs because the extrasynaptic, $GABA_AR$ δ-subunit specific agonist THIP (300 nM) (Meera et al., 2011) reduced sEPSC frequency to the same degree in both mouse genotypes (D2: −25.62 ± 8.33%, $n = 6$ cells; B6: −18.00 ± 6.83%, $n = 7$; main effect of THIP, $F_{(1,11)} = 5.18$, $p = 0.044$, but no main effect of mouse genotype, $p = 0.23$, by mixed-measures ANOVA; Fig. 2*B*,*D*). Similar to the effects of ethanol on sEPSPs, the genotype specificity of ethanol action on sEPSCs was lost at higher concentrations of ethanol (31 mM), which reduced sEPSC frequency similarly in D2 and B6 mice (D2: −23.41 ± 5.50%, $n = 8$ cells, B6: −23.97 ± 8.96%, $n = 8$; main effect of 31 mM ethanol, $F_{(1,14)} = 9.77$, $p = 0.007$, but no main effect of mouse genotype,

$p = 0.71$, by mixed-measures ANOVA; Fig. 2*D*). To definitively rule out actions of ethanol on inhibitory afferent input to PCs, we made voltage-clamp recordings from PCs ($V_h = -60$ mV, with E_{Cl^-} set to 0 mV) and pharmacologically isolated $GABA_AR$-mediated sIPSCs by applying the broad-spectrum glutamate receptor antagonist, kynurenate (2 mM). Under such conditions, PCs from both genotypes exhibited similar baseline frequencies of sIPSCs (mean frequency, D2 = 4.51 ± 2.02 Hz and B6 = 3.00 ± 0.67, $p = 0.95$, Mann–Whitney Rank Sum Test), and they were not affected by 9 mM ethanol in either genotype (D2: 2.32 ± 3.06%, $n = 6$, $p = 0.49$, one-sample *t* test; B6: −1.62 ± 10.05%, $n = 6$, $p = 0.88$; Fig. 2*D*).

Having systematically tested the ethanol sensitivity of all of the synaptic inputs to PCs, we next tested whether 9 mM ethanol had any impact on PC excitability. In current-clamp recordings of PC action potential responses to current injection, application of 9 mM ethanol did not affect PC action potential firing frequency (Fig. 2*C*,*D*), which, combined with a lack of effect on PC membrane potential or inhibitory synaptic inputs (see above), confirms that genotypic differences in the actions of 9 mM ethanol on PCs are mediated exclusively by differential actions on GC $GABA_AR$ currents via their control of GC glutamatergic inputs to PCs (Figs. 1*A*,*B*, 2*A*,*D*).

Because the health of acutely prepared brain slices is typically reduced when slices are obtained from older animals (which could artifactually affect neural network processing), we typically use brain slices from animals that are anatomically and molecularly fully mature (as in the studies above) (Liscovitch and Chechik, 2013; Leto et al., 2015). Nonetheless, such animals are still adolescent, and it is well established that adolescents have different ethanol-related phenotypes from fully mature adults (Alfonso-Loeches and Guerri, 2011; Leeman et al., 2012; Bell et al., 2014). Accordingly, we replicated key experiments in cerebellar slices from rodents that were the same age as those for subsequent behavioral experiments. Importantly, all experiments that were replicated in cerebellar slices from fully adult mice showed similar outcomes as in slices from younger animals, including the result that 9 mM EtOH suppresses excitatory transmission onto D2 mouse PCs (8.45 ± 3.55%, $n = 17$, $p = 0.03$; Fig. 2*E*) in a $GABA_AR$-dependent fashion (−2.53 ± 5.87% in GABAzine, $n = 4$, $p = 0.70$) but not B6 mouse PCs (1.45 ± 4.11%, $n = 11$, $p = 0.73$; Fig. 2*E*), and THIP replicates this suppression when applied to slices from B6 mice (−12.84 ± 5.67%; $n = 8$, $p = 0.06$; Fig. 2*E*).

Given that genotypic differences in ethanol consumption and sensitivity to ataxia manifest at a concentration of ethanol (i.e., 9 mM) (Gallaher et al., 1996; Yoneyama et al., 2008) that differentially affects transmission through the cerebellar cortex (Figs. 1, 2), we hypothesized that, if such cellular response differences contributed to behavioral differences, then pharmacologically mimicking a cerebellar response to ethanol that occurs in low ethanol-consuming genotypes (i.e., enhancement of $GABA_AR$ currents) in a high ethanol-consuming genotype *in vivo* should reduce their ethanol consumption. To test this hypothesis, we took advantage of past and present findings that THIP enhances the GC tonic $GABA_AR$ current (Meera et al., 2011; Kaplan et al., 2013) and induces a consequent suppression of transmission through the cerebellar cortex in B6 mice very similar to the effect of ethanol in D2 mice (Fig. 2*B*,*D*). The initial studies examined the effect of unilateral THIP infusion into lobes IV/V/VI of the cerebellar cortex (lobes known to influence dopamine release in the forebrain) (Dempsey and Richardson, 1987; Mittleman et al., 2008; Rogers et al., 2011, 2013) (Fig. 3*A*,*B*) on 2 h ethanol intake in B6 mice (Ramaker et al., 2012). THIP infusion (500 ng) significantly reduced ethanol consumption and the highly correlated BEC achieved ($t_{(16)} = 2.47$, $p = 0.025$, $n = 17$, paired *t* test; Fig. 3*C*,*D*). As a control for potential influences of THIP infusions on fluid consumption, we gave mice access to only water during the 2 h period when they normally received ethanol access. After water intake stabilized, we found that THIP (500 ng) had no effect on water intake compared with vehicle infusion (vehicle: 0.65 ± 0.06 ml; THIP: 0.67 ± 0.08 ml, $t_{(16)} = 0.27$, $p = 0.80$, paired *t* test; Fig. 3*C*). A separate dose–response assessment of THIP microinjection (50–500 ng) on ethanol consumption indicated that the threshold concentration for reducing ethanol consumption was between 100 and 250 ng (vehicle: 2.28 ± 0.14 g/kg; 100 ng THIP: ↓ 15% $p = 0.08$; 250 ng THIP: ↓ 23%; 500 ng THIP: ↓ 26%, both $p < 0.05$, planned pairwise comparisons using paired *t* tests; Fig. 3*E*). On the day following THIP infusion trials, ethanol consumption returned to baseline values (data not shown; $p > 0.05$ compared with baseline).

To gain insight into whether THIP-induced suppression of ethanol consumption is a function of reward-related processing, we next evaluated the impact of THIP infusions on sucrose consumption. Similar to the effect on ethanol consumption, local infusion of THIP (500 ng) into lobes IV–VI also decreased sucrose consumption (vehicle: 1.52 ± 0.10 g/kg; 500 ng THIP ↓ 40% to 0.91 ± 0.16 g/kg, $F_{(1,13)} = 13.97$, $p = 0.002$, $n = 14$, repeated-measures ANOVA; Fig. 3*F*), indicating that suppression of GC excitability may broadly impact ingestion of rewarding substances. Similar to ethanol, sucrose consumption returned to baseline levels on the day following THIP infusion (data not shown, $p > 0.05$ compared with baseline). THIP (500 ng) injections did not affect gross locomotor activity, having no significant impact on horizontal locomotion compared with vehicle-infused subjects (as assessed by beam breaks, during a 2 h test session: vehicle = 21,704 ± 4576, THIP = 15,226 ± 3770, $n = 7$/group, $p > 0.05$; Fig. 3*G*; or when broken into 20 min segments Fig. 3*H*, repeated-measures ANOVA, main effect of time $F_{(5,60)} = 6.36$, $p < 0.001$, no main effect of drug $p = 0.296$, and no significant interaction $p = 0.571$). Finally, to test for specificity of the observed effects to lobes IV–VI, we conducted additional THIP infusions into lobe III. Similar to the lobe IV–VI infusions, infusion of THIP (500 ng) into lobe III significantly reduced 2 h ethanol intake (vehicle: 2.13 ± 0.22 g/kg; 500 ng THIP ↓ 49% to 1.09 ± 0.31 g/kg, $F_{(1,7)} = 6.58$, $p = 0.037$, $n = 8$, repeated-measures ANOVA; Fig. 3*I*). Importantly, our previous studies determined that intracerebroventricular infusion of 500 ng THIP does not affect 2 h ethanol consumption (Ramaker et al., 2015), demonstrating the cerebellar specificity of our current study. Collectively, the results suggest that suppression of GC excitability decreases limited-access ethanol and sucrose intake.

Discussion

Our data suggest that the GC tonic $GABA_AR$ current is the primary molecular target mediating cerebellar-dependent behavioral responses to concentrations of ethanol (9 mM) achieved during recreational, nonabusive consumption in humans (1–2 standard units). Furthermore, because relevant responses (enhancement of GC tonic $GABA_AR$ currents) (Kaplan et al., 2013) and consequent suppression of transmission through the cerebellar cortex (Figs. 1, 2) are only observed in low ethanol-consuming rodent genotypes, our findings highlight GC tonic $GABA_AR$ current resistance to enhancement by ethanol as a potential cellular contributor to the low level of response behavioral phenotype that is genetically associated with high ethanol consumption in rodents and predilection to develop AUD in humans (Schuckit,

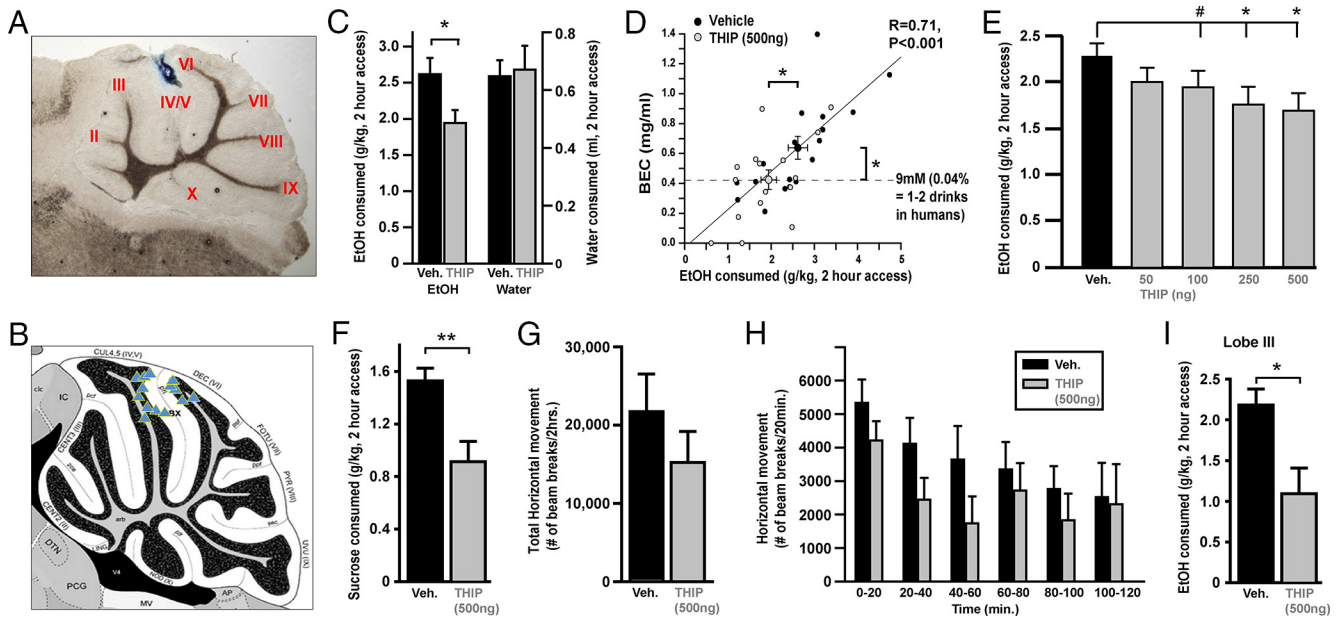

Figure 3. THIP reduces ethanol (EtOH) intake and BEC achieved. ***A***, Representative cerebellar section showing cannula placement in lobe IV–VI, indicated by the presence of postexperimentally injected methylene blue (blue dye). ***B***, Reference image of the mouse cerebellum (0.225 lateral) taken from the Allen Brain Atlas. Blue triangles represent injection sites from representative experiments. ***C***, Bar chart showing mean EtOH (left set of bars) or water (right set of bars) consumed after vehicle (black) and THIP (500 ng; gray) infusion into cerebellar cortical lobes IV–VI. ***D***, Scatterplot showing that EtOH consumption during the 2 h access period (g/kg) was significantly positively correlated with BEC levels achieved, either after vehicle injection (black; $r = 0.75$; $p < 0.001$, $n = 17$), in the presence of THIP (500 ng; gray, $r = 0.54$; $p = 0.02$, $n = 17$), or when combined ($r = 0.71$; $p < 0.001$, $n = 34$). Mean values with error bars are overlaid showing that THIP (500 ng; gray symbol) significantly reduces EtOH consumed and BEC achieved versus vehicle (black symbol). Dashed gray horizontal line indicates 9 mM EtOH (for reference with *in vitro* experiments). ***E***, Bar chart showing mean EtOH consumed (g/kg) after microinjection of vehicle (aCSF; black) and four THIP doses (ng; gray) into cerebellar cortical lobes IV–VI. ***F***, Bar chart showing mean sucrose consumed (g/kg during 2 h test session) after microinjection of vehicle (aCSF; black) and THIP (500 ng; gray) into cerebellar cortical lobes IV–VI. ***G***, ***H***, Bar charts showing mean horizontal movement (assessed as the number of beam breaks during a 2 h test session, ***G***; or broken into 20 min bins, ***H***) after vehicle (black) or THIP (500 ng; gray) infusion into cerebellar cortical lobes IV–VI. ***I***, Bar chart showing mean EtOH consumed (g/kg during 2 h test session) after vehicle (black) or THIP (500 ng; gray) infusion into cerebellar cortical lobe III. $^*p < 0.05$ (paired *t* tests or repeated-measures ANOVA). $^{**}p < 0.01$ (paired *t* tests or repeated-measures ANOVA). $^{\#}p < 0.08$ (paired *t* tests or repeated-measures ANOVA).

1985; Gallaher et al., 1996). This contention is supported by our finding that pharmacologically inducing a high response cellular phenotype (by directly activating $GABA_AR$ currents with THIP in a range of cerebellar lobules), in a rodent genotype that is insensitive to ethanol impairment (B6 mice), reduces their ethanol consumption from excessive (14 mM, equivalent to 3–4 units consumed by a typical adult male in 2 h) to recreational blood levels (9 mM, equivalent to 1–2 units; Fig. 3*C–E*,*I*). The parallel impact on ethanol and sucrose consumption (Fig. 3*F*) is compatible with suppression of GC excitability altering the processing of rewarding substances or neural processes underlying caloric ingestion, or both (Berthoud et al., 2011). Furthermore, the lack of THIP effect on water consumption (Fig. 3*C*) or locomotor activity (Fig. 3*G*,*H*) in the present studies, combined with the return to baseline consumption levels on the day subsequent to THIP infusion (data not shown), argues against suppression of GC excitability as being incapacitating or aversive per se (McMahon and Wellman, 1998). However, further experimentation is needed to substantiate these conclusions.

Regardless of the specific underlying psychological mechanisms, based on these findings, we propose that ethanol-induced suppression of signal propagation through the cerebellar cortex is a deterrent to continued ethanol consumption, and thus that genotypes in which 9 mM ethanol suppresses cerebellar signaling (D2 mice) are less likely to consume beyond that level. Further, because the cerebellum in high ethanol-consuming genotypes (B6 mice) does not respond to 9 mM ethanol (Figs. 1, 2), it follows that there is no such cerebellar deterrent, thereby enabling continued consumption. Beyond the implications for genetic predilection to AUD, our study is the first to demonstrate that cerebellar-specific manipulations can substantially curtail high ethanol consumption without gross motor impairment.

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