Published in final edited form as:

Alcohol Clin Exp Res. 2016 August ; 40(8): 1617–1626. doi:10.1111/acer.13136.

Alcohol suppresses tonic GABA_A receptor currents in cerebellar granule cells in the prairie vole: a neural signature of high alcohol consuming genotypes

JS Kaplan, Ph.D.^{a,b,d}, C. Mohr, Ph.D.^{a,c}, C.M. Hostetler, Ph.D.^{a,b}, A.E. Ryabinin, Ph.D.^a, D.A. Finn, Ph.D.^{a,b}, and D.J. Rossi, Ph.D.^{a,c}

^aOregon Health & Science University, Department of Behavioral Neuroscience, Portland, OR

^bVA Portland Health Care System, Portland, OR

^cWashington State University, Department of Integrative Physiology and Neuroscience, Pullman, WA

Abstract

Background—Evidence indicates that the cerebellum plays a role in genetic predilection to excessive alcohol (ethanol, EtOH) consumption in rodents and humans, but the molecular mechanisms mediating such predilection are not understood. We recently determined that EtOH has opposite actions (enhancement or suppression) on tonic GABA_A receptor (GABA_AR) currents in cerebellar granule cells (GCs) in low and high-EtOH consuming rodents, respectively, and proposed that variation in GC tonic GABA_AR current responses to EtOH contributes to genetic variation in EtOH consumption phenotype.

Methods—Voltage-clamp recordings of GCs in acutely prepared slices of cerebellum were used to evaluate the effect of EtOH on GC tonic GABA_AR currents in another high EtOH consuming rodent, prairie voles (PVs).

Results—EtOH (52mM) suppressed the magnitude of the tonic GABA_AR current in 57% of cells, had no effect in 38% of cells, and enhanced the tonic GABA_AR current in 5% of cells. This result is similar to GCs from high EtOH consuming C57BL/6J (B6) mice, but it differs from the enhancement of tonic GABA_AR currents by EtOH in low EtOH consuming DBA/2J (D2) mice and Sprague Dawley (SD) rats. EtOH suppression of tonic GABA_AR currents was not affected by the sodium channel blocker, tetrodotoxin (TTX, 500nM), and was independent of the frequency of phasic GABA_AR-mediated currents, suggesting that suppression is mediated by post synaptic actions on GABA_ARs, rather than a reduction of GABA release. Finally, immunohistochemical analysis of neuronal nitric oxide synthase (nNOS, which can mediate EtOH enhancement of GABA release) demonstrated that nNOS expression in the GC layer of PV cerebellum was similar to the levels seen in B6 mice, both being significantly reduced relative to D2 mice and SD rats.

Corresponding author contact information: Dr. David Rossi, Department of Integrative Physiology and Neuroscience, 1815 Ferdinand's Lane, Washington State University, Pullman, WA 99164-7620, rossid@vetmed.wsu.edu, Phone: 509-335-7671.

^dCurrent location: University of Washington, Department of Pharmacology, Seattle, WA

Conflict of Interest: The authors declare that they have no conflicts of interest.

Conclusions—Combined, these data highlight the GC GABA_AR response to EtOH in another species, the high EtOH consuming PV, which correlates with EtOH consumption phenotype and further implicates the GC GABA_AR system as a contributing mechanism to high EtOH consumption.

Keywords

Alcohol Use Disorder; Genetic Predisposition; GABA; Cerebellum; Nitric Oxide

Introduction

Alcohol use disorder (AUD) is a leading cause of preventable death and illness that contributes to substantial emotional, social, and economic costs (Harwood, 2000). Adoption and twin studies indicate that genetic factors contribute 50–60% of risk for developing an AUD (Prescott and Kendler, 1999; Hill, 2010). Among them, variation in cerebellar structure, processing and cerebellar dependent behavioral sensitivity to ethanol (EtOH) are heritably associated with susceptibility to AUDs (Schuckit, 1985; Schuckit and Smith, 1996; Hill et al., 2007; Herting et al., 2011; Cservenka, 2016). The behavioral mechanisms through which the cerebellum mediates risk for developing AUD are unclear, but may stem from a combination of EtOH's action on motor (Schuckit, 1985; Hancher et al., 2005; Dar, 2015) and non-motor cerebellar functions (Miquel et al., 2009; Strick et al., 2009; Schmahmann, 2010; Stoodley, 2012). Regardless of the specific behavioral mechanisms, cerebellar related genetic risk manifests in studies of the low level of response (LLR) to EtOH phenotype, which is defined by requiring a higher dose of EtOH to attain a given effect, and is associated with risk for developing an AUD (Schuckit, 2009). The cerebellar LLR phenotype is common in young men and women with a family history of AUDs (i.e. high genetic risk) (Schuckit, 1985; Eng et al., 2005) and predicts development of AUD (Schuckit and Smith, 1996), while in rodents, it is most common in strains with high EtOH consumption phenotypes (Gallagher et al., 1996). Thus, cerebellar aspects of the LLR to EtOH phenotype are heritable and associated with increased risk for developing an AUD in humans and high EtOH consumption in rodents. However, the cellular mechanisms that underlie cerebellar contributions to the LLR behavioral phenotype are not well understood.

Cerebellar granule cells (GCs) are key targets through which EtOH differentially affects cerebellar processing in high and low-EtOH consuming phenotypes (Kaplan et al., 2013; Mohr et al., 2013). GCs are the main relay of afferent information through the cerebellar cortex to Purkinje cells (PCs), the primary integrator and sole output of the cerebellar cortex. Accordingly, GCs are critical targets of pharmacological modulation of cerebellar processing (Hamann et al., 2002; Duguid et al., 2012). GC activity is regulated by two forms of GABA_A receptor (GABA_AR)-mediated inhibition: traditional phasic GABA_AR-mediated inhibitory postsynaptic currents (IPSCs), as well as a tonic form of GABA_AR current (Brickley et al., 1996; Wall and Usowicz, 1997) mediated by extrasynaptic GABA_ARs containing the α_6 and δ subunits (Brickley et al., 2001; Hamann et al., 2002; Stell et al., 2003; Meera et al., 2011). The tonic form of GABA_AR inhibition mediates 75% of the entire inhibitory charge of the GC, making it a powerful regulator of signal transmission through the cerebellar cortex (Hamann et al., 2002). Both GC spontaneous IPSCs (sIPSCs) and tonic

GABA_AR currents are sensitive to low, socially relevant concentrations of EtOH (9–30mM), and the magnitude and polarity of modulation varies in parallel with EtOH consumption phenotype. Specifically, while it is well established that EtOH increases the frequency of GC sIPSCs and the magnitude of the GC tonic GABA_AR current in low EtOH consuming Sprague Dawley (SD) rats and DBA/2J (D2) mice (Carta et al., 2004; Hancher et al., 2005; Kaplan et al., 2013), we recently determined that in high EtOH consuming C57BL/6J (B6) mice, EtOH has little impact on GC sIPSCs, and actually suppresses GC tonic GABA_AR currents (Kaplan et al., 2013). EtOH has intermediate impact on GC sIPSCs and tonic GABA_AR currents in rodents and non-human primates that exhibit intermediate EtOH consumption phenotypes (Kaplan et al., 2013; Mohr et al., 2013). Importantly, with respect to cerebellar contributions to the LLR and EtOH consumption, B6 mice are significantly less sensitive to EtOH-induced disruption of rotarod performance when compared to D2 mice (Gallagher et al., 1996). Therefore, genetic regulation of EtOH's effect on tonic GABA_AR currents in cerebellar GCs may contribute to EtOH-related behavioral phenotypes that promote risk for developing an AUD.

In an effort to understand the molecular underpinnings of genetic variation in GC GABA_AR current sensitivity to EtOH, we determined that the net effect of EtOH on the magnitude and polarity of the tonic GABA_AR current is dictated by two genetically regulated mechanisms: 1) the degree of neuronal nitric oxide synthase (nNOS) expression in the GC layer determines the magnitude by which EtOH enhances presynaptic vesicular GABA release to increase tonic GABA_AR currents in GCs, whereas 2) low GC protein kinase C (PKC) activity enables EtOH to postsynaptically inhibit the extrasynaptic $\alpha_6\delta$ -containing GABA_ARs that mediate the tonic GABA_AR current (Kaplan et al., 2013; Mohr et al., 2013). Thus, low EtOH consuming SD rats have high levels of expression of nNOS and high GC PKC activity, whereas the high EtOH consuming, behaviorally insensitive B6 mice have low nNOS expression and low GC PKC activity that, together, promote EtOH-induced suppression of tonic GABA_AR inhibition of GCs. Interestingly, D2 mice have high levels of nNOS but low levels of GC PKC activity, giving them a mixed molecular phenotype. However, at the cellular level, increased vesicular GABA release mediated by EtOH inhibition of nNOS overpowers direct suppression of postsynaptic GABA_ARs (enabled by low PKC activity) resulting in a net enhancement of tonic GABA_AR currents, the proposed cellular signature of high behavioral sensitivity to EtOH and low EtOH consumption.

Based on the above, we hypothesize that EtOH-induced suppression of GC tonic GABA_AR current is a common trait across high-EtOH consuming genotypes that is mediated by low nNOS expression and low GC PKC activity levels, with low nNOS expression being the more critical factor. The purpose of the present study was to test this hypothesis in another species known for high EtOH consumption, the prairie vole (PV) (Anacker et al., 2011; Hostetler et al., 2012).

Methods

Subjects

Male and female PVs (68 – 97 days old) were used in all experiments. Since PVs reach sexual maturity at 30–45 days old, and have an average lifespan of 1–3 years in the wild and

captivity respectively (Kurta, 1995), the animals used in this study are fully mature adults. The animals were supplied from a breeding colony at the Veterinary Medical Unit of the VA Portland Health Care System. For the immunohistochemistry studies, we used age-matched male and female B6 and D2 mice (Jackson Laboratory-West), and SD rats (Charles River Laboratories). 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 at the VA Portland Health Care System and Washington State University.

Preparation of brain slices

Cerebellar slices were prepared acutely on each day of experimentation (Brady et al., 2010; Mohr et al., 2010; Kaplan et al., 2013). Male and female PVs were anaesthetized with isoflurane and killed by decapitation. The whole brain was rapidly isolated and immersed in ice cold (0–2°C) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 26 NaHCO₃, 1 NaH₂PO₄, 2.5 KCl, 2.5 CaCl₂, 2 MgCl₂, 10 D-glucose, and bubbled with 95% O₂/5% CO₂ (pH 7.4). The cerebellum was dissected out of the brain and mounted, parallel to the sagittal plane, in a slicing chamber filled with ice cold (0–2°C) ACSF. Parasagittal slices (225µm) were made with a vibrating tissue slicer (Vibratome). Slices were incubated in warmed ACSF (33±1°C) for one hour after dissection and then held at 22–23°C until used. Kynurenic acid (1 mM) was included in the dissection, incubation and holding solution, but was omitted from the experimental solutions.

Electrophysiology

Slices were placed in a submersion chamber on an upright microscope, viewed with an Olympus 60 $\times$ (0.9 numerical aperture) water immersion objective with DIC and infrared optics, and perfused with ACSF at a rate of ~ 7 ml/min. Drugs were dissolved in ACSF and applied by bath perfusion. Patch pipettes were constructed from thick-walled borosilicate glass capillaries and filled with an internal solution containing (in mM): CsCl 130, NaCl 4, CaCl₂ 0.5, HEPES 10, EGTA 5, MgATP 4, Na₂GTP 0.5, QX-314 5, pH adjusted to 7.2 with CsOH. Electrode resistance was 4 to 10 M Ω . Cells were rejected if access resistance was greater than 15 M Ω .

Analysis of GABA_AR currents

Membrane currents were acquired at 20 kHz, filtered at 10 kHz, and analyzed with pClamp software (Molecular Devices). For analysis and display of sIPSCs, data were filtered at 2 kHz. sIPSCs were defined as current deflections that have an amplitude (measured from the mean current) greater than the peak-to-peak amplitude of the current noise, with a decay time constant >3-fold slower than the rise time. Tonic current magnitude was assessed by fitting the Gaussian distribution of all data points not skewed by synaptic events from a point 3pA to the left of the peak value to the rightmost (smallest) value of the histogram distribution. Drug-induced changes in tonic GABA_AR current magnitude and sIPSC frequency were calculated by comparing the amplitude/frequency in the drug versus the mean amplitude/frequency of the currents before and after drug application.

Immunohistochemistry

Brain slices were prepared from age-matched PVs, B6 and D2 mice, and SD rats in an identical manner as for electrophysiology experiments. Slices were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 17–24 hours. Slices were then washed and incubated for 40 minutes in blocking solution (PBS, 0.5% Triton X-100, and bovine serum albumin (0.5mg/ml)). Next, they were incubated for 24 hours with primary antibody in PBS and Triton. Slices were washed 3 times (10 minutes each) in PBS, then incubated for 45 minutes with an Alexa-conjugated secondary antibody. Slices were mounted in ProLong® Gold Antifade and imaged with confocal microscopy.

Confocal microscopy

Images were acquired with a Leica SP8-X point scanning confocal microscope using accompanying software for acquisition, processing and subsequent analysis. A laser line falling within 20nm of the peak absorbance was used for Hoechst 33342, a white line laser was used for each of the other fluorophores, with appropriate excitation, dichroic, and emission filters. One objective was used for all experiments: a 20×, 0.7 N.A. Plan Apochromat air objective. Pinhole diameter and slice step thickness were optimized for the objective used. With our protocol, antibody fluorescence is detectable up to 100µm into the slice, but begins to diminish in intensity starting at ~50µm into the slice, due to a combination of poorer antibody penetration, poorer laser penetration, and increasing laser and fluorescence scatter. Additionally, the first 5µm of the slice tends to be affected by slicing damage. Hence, for quantification of GC encirclement by nNOS (see Kaplan et al., 2013), a single image plane at 10µm from the surface of the slice was analyzed as follows. Individual GCs were identified by nuclear stain Hoechst 33342, and the nNOS signal was amplified until ~5% of the pixels were saturated. Subsequently, an experimenter, blind to the experimental condition, analyzed individual GC nuclei for percent encirclement by detectable nNOS signal (as in Kaplan et al., 2013). Values were obtained and averaged from at least 3 animals of each species, at least 2 slices from each animal, and at least 5 distinct regions of each slice.

Statistics

All data are expressed as the mean $\pm$ the standard error of the mean. One-way ANOVA was used to detect significant main effects, and post hoc comparisons were made using Bonferroni corrected *t*-tests. All other statistical tests are specified in the results. In all cases, the threshold for significance was $P < 0.05$.

Reagents

All reagents were from Sigma Chemicals except GABA_Azine and kynurenic acid (Abcam). Primary antibodies were (host/supplier/dilution): nNOS (rabbit/Cayman chemicals 160870/1:200), GAD65 (mouse/Millipore MAB 351/1:200), GAD67 (mouse/Millipore MAP 5406/1:200). Secondary antibodies were conjugated with Alexafluors of various excitation maxima (Invitrogen), with specificity against immunoglobulins of the hosts of the primary antibodies, diluted 1:500. We previously demonstrated the specificity of the nNOS

antibody by lack of staining of cerebellar slices obtained from mice in which the nNOS gene was deleted (Kaplan et al., 2013).

Results

PV GCs exhibit phasic and tonic GABA_AR-mediated currents

PVs are a socially monogamous and high EtOH-consuming species (Anacker et al., 2011). This has led to robust behavioral characterization of these animals, but little is known about properties of signal transmission in this species and whether electrophysiologically-detected similarities across previously investigated genotypes extend to PVs. To determine if PV cerebellar GCs express similar phasic and tonic GABA_AR-mediated forms of inhibition to those previously described in mice, rats, and non-human primates (Kaplan et al., 2013; Mohr et al., 2013), we made whole-cell voltage-clamp ($V_h = -60\text{mV}$, with E_{Cl^-} set to $\sim 0\text{mV}$, making GABA_AR-mediated currents inward, downward deflections in the holding current) recordings of GCs in sagittal slices of cerebellum. PV GCs express both phasic sIPSCs and a tonic current that are mediated by GABA_ARs, as evidenced by their block by the broad spectrum GABA_AR antagonist, GABAzine ($10\mu\text{M}$; Fig. 1A,B&E; mean tonic current amplitude = $12.26 \pm 1.89\text{pA}$, mean sIPSC frequency = $0.125 \pm 0.02\text{ Hz}$, see insets and Table 1). In all other species studied, the GC tonic GABA_AR current is mediated by $\alpha 6$ and δ -subunit containing extrasynaptic GABA_ARs (Brickley et al., 2001; Hamann et al., 2002; Stell et al., 2003; Meera et al., 2011; Kaplan et al., 2013; Mohr et al., 2013), but this has not been established in the PV. We used pharmacology to determine if the PV GC tonic GABA_AR current is mediated by $\alpha 6, \delta$ -subunit containing GABA_ARs (Fig. 1C–E). Similar to reports from other species, the tonic current amplitude was reduced by the $\alpha 6$ subunit-selective antagonist, furosemide ($100\mu\text{M}$, at which concentration it is selective for GABA_ARs containing the $\alpha 6$ subunit (Korpi et al., 1995; Hamann et al., 2002); Fig 1C,E; furosemide-induced current block = $4.80 \pm 1.12\text{pA}$), and enhanced by the δ -subunit containing GABA_AR agonist, THIP (500nM ; at which concentration it is selective for δ -subunit containing GABA_ARs (Meera et al., 2011); Fig 1D,E; THIP-induced current = $14.03 \pm 1.67\text{pA}$). Together, these data confirm that PVs express both GABA_AR-mediated sIPSCs as well as a tonic current that is mediated by $\alpha 6$ and δ -subunit containing GABA_ARs.

EtOH suppresses tonic GABA_AR inhibition of PV GCs

In contrast to the well characterized EtOH enhancement of GC tonic GABA_AR current in low EtOH consuming SD rats and D2 mice, in cerebellar slices from high EtOH-consuming B6 mice, 52mM EtOH mainly suppresses the GC tonic GABA_AR current (Kaplan et al., 2013). We reasoned that if such suppression plays a meaningful role in promoting or enabling high EtOH consumption, then EtOH should suppress GC tonic GABA_AR current in other high EtOH consuming species, including the PV. In support of our hypothesis, in voltage-clamped PV GCs, bath application of 52mM EtOH predominately suppressed or had no effect on the tonic GABA_AR current (Fig 2A&B; suppression: 12/21 cells recorded; no effect: 8/21 cells; enhancement: 1/21 cells; recorded from 5 animals). These group proportions were conserved across individual PVs, suggesting reasonable consistency across subregions of cerebellar cortex and across individuals. Specifically, in 21 recordings from 5 different animals, the proportion of cells showing suppression, no response or potentiation

was respectively (3/6, 2/4, 4/5, 2/2, 1/4), (2/6, 2/4, 1/5, 0/2, 3/4), and (1/6, 0/4, 0/5, 0/2, 0/4). Importantly, EtOH failed to induce any current in the presence of the GABA_AR antagonist, GABAzine, thereby confirming that the EtOH-induced outward current is mediated by suppression of the tonic GABA_AR current (Fig 2C; representative of $n = 10$ from 4 animals; see Fig. 3C for mean value). We next calculated the mean response across all the GCs we recorded from in order to gain insight into the effect of EtOH on the population of GCs, which in turn, would likely impact the efficacy of transmission from mossy fibers (main afferent input) through GCs to PCs (the sole output of the cerebellar cortex). On average, EtOH induced a significant outward current (mean EtOH-induced outward current for all cells tested = -1.16 ± 0.34 pA; $t[20] = 3.39$, $P = 0.003$, one-sample t -test) which represents a suppression of the total tonic GABA_AR current by $14.12 \pm 2.63\%$ in all responding cells (calculated by dividing the magnitude of the EtOH-induced current by the magnitude of the GABAzine-induced current in each cell in which both drugs were tested; Fig. 2C&D).

In order to determine how EtOH suppression of GC tonic GABA_AR current in PV GCs compares to EtOH actions in other genotypes, we reanalyzed data from our previously published studies of SD rats and B6 & D2 mice (Kaplan et al., 2013) to facilitate direct cross-species comparisons. In particular, to account for differences across species in the basal magnitude of tonic GABA_AR currents, we normalized the magnitude of the EtOH-induced current to the magnitude of the total tonic GABA_AR current (i.e. the magnitude of the current blocked by GABAzine) in each cell for each species, enabling us to compare the percent enhancement or suppression of the tonic GABA_AR current by 52mM EtOH in each species (Fig. 2D). A one-way ANOVA revealed a significant main effect of genotype on EtOH's impact on GC tonic GABA_AR current as a percent of the total GABA_AR current ($F(3,63) = 23.61$, $P < 0.001$). Bonferroni post-hoc comparisons confirmed that EtOH enhancement of tonic GABA_AR currents in low-drinking SD rats ($35.28 \pm 6.08\%$, $n = 19$) and D2 mice ($10.21 \pm 5.41\%$, $n = 15$) significantly differed from EtOH suppression of tonic GABA_AR currents in high-consuming B6 mice ($-10.67 \pm 3.43\%$, $n = 15$) and PVs ($-14.12 \pm 2.63\%$, $n = 13$). The clear parallel across species between GC response to EtOH and consumption phenotype highlights genetic differences in GC sensitivity to EtOH as a potential contributor to behavioral phenotypes that affect EtOH intake in animals.

EtOH acts via a postsynaptic mechanism to inhibit the tonic GABA_AR current

We previously determined that in B6 mice, EtOH suppresses the tonic GABA_AR current via postsynaptic inhibition of extrasynaptic GABA_ARs (Kaplan et al., 2013). To determine if the suppression of PV GC tonic GABA_AR currents (observed in 57% of PV GCs) is also via postsynaptic actions, as opposed to being due to reduced Golgi cell vesicular release of GABA, we compared the impact of EtOH on sIPSC frequency to the impact on the tonic GABA_AR current (Fig. 3A). Consistent with postsynaptic EtOH action, EtOH did not reduce sIPSC frequency in cells where EtOH suppressed the tonic GABA_AR current (% change in sIPSC frequency = $7.92 \pm 14.50\%$; Fig. 3A), and EtOH enhanced sIPSC frequency in cells where EtOH had no effect on the tonic GABA_AR current (% change = 25.99 ± 10.28 ; Fig 3A). In further support of postsynaptic EtOH action, the sodium channel antagonist, tetrodotoxin (TTX; 500nM; Fig 3B), which blocks action potential-dependent vesicular GABA release from Golgi cells, failed to block EtOH suppression of tonic GABA_AR

currents (Fig. 3B&C). These results are consistent with our previous findings in B6 mice showing that in cells in which EtOH suppresses the tonic GABA_AR current (57%), it is via postsynaptic inhibition of extrasynaptic GABA_ARs without significant change in GABA release, and in some cells (38%) where there is an increase in vesicular GABA release (as indicated by increased sIPSC frequency), the direct postsynaptic suppression is counteracted by the increase in extracellular GABA, resulting in no net effect of EtOH on the tonic GABA_AR current.

Lack of enhancement of PV GC tonic GABA_AR current is mirrored by low expression of nNOS

Earlier reports demonstrated that EtOH enhanced GABAergic transmission to GCs by increasing Golgi cell activity (Carta et al., 2004). More recent work determined that the increased Golgi cell activity is generated by EtOH inhibition of nNOS which excites Golgi cells (Kaplan et al., 2013), possibly via consequent inhibition of K⁺ channels and/or the Na⁺/K⁺-ATPase (Valenzuela and Jotty, 2015). Accordingly, nNOS expression is low in the GC layer of B6 mice (which do not exhibit EtOH enhancement of the GC tonic GABA_AR current), and high in the GC layer of D2 mice and SD rats (which do exhibit EtOH enhancement of GC tonic GABA_AR currents, via increased vesicular release of GABA) (Kaplan et al., 2013). We therefore hypothesized that the lack of EtOH enhancement of PV GC tonic GABA_AR currents also resulted from reduced expression of nNOS in the GC layer of PV cerebellum. We addressed this hypothesis by measuring nNOS immunoreactivity (IR) using confocal microscopy in the GC layer of age-matched PVs, B6 and D2 mice, and SD rats (Fig. 4). Notably, the nNOS IR pattern differed across rodents as a function of their EtOH-consumption phenotype and the net effect of EtOH on GC tonic GABA_AR currents ($F(3,88) = 62.10$, $P < 0.001$, one-way ANOVA; Fig 4). nNOS IR, quantified as the mean percent GC encirclement (see Methods and Kaplan et al., 2013), was similarly low in the high-EtOH consuming PVs (mean % encirclement = $32.53 \pm 1.67\%$) and B6 mice (mean % encirclement = $36.32 \pm 2.39\%$) relative to high nNOS expression in the cerebellum of low EtOH-consuming D2 mice (mean % encirclement = $58.70 \pm 1.35\%$) and SD rats (mean % encirclement = $56.34 \pm 1.28\%$; all $P < 0.001$, pairwise comparisons by Student's *t* tests). Similar to previous descriptions of B6 mouse and non-human primates (Kaplan et al., 2013, Mohr et al., 2013), the majority of PV GCs were not fully surrounded by nNOS, and many were entirely void of nNOS. Thus, the reduced nNOS IR in the GC layer of the PV is consistent with the predominant absence of EtOH-induced enhancement of GC tonic GABA_AR currents in PV GCs, both of which appear to be restricted to low EtOH consuming genotypes.

Discussion

Based on our prior results that variation in GC tonic GABA_AR current responses to EtOH correlates with genetic variation in EtOH consumption phenotype (Kaplan et al., 2013; Mohr et al., 2013), we further explored this association by examining cerebellar GCs from another high EtOH consuming rodent genotype, the socially monogamous PV (Anacker et al., 2011; Hostetler et al., 2012). Our specific prediction was that EtOH should suppress the tonic GABA_AR current in PV GCs to a similar degree as in the high EtOH consuming B6 mouse.

Importantly, this is the first report of *ex vivo* whole-cell patch-clamp recordings in the PV cerebellum. We found that similar to other rodent and non-human primate genotypes, PV GCs exhibit both GABA_AR-mediated sIPSCs and tonic GABA_AR-mediated currents (Fig. 1 and Table 1). Similarly to high EtOH consuming B6 mice, EtOH primarily either suppressed or had no impact on the magnitude of PV GC tonic GABA_AR currents (Fig. 2). Such suppression/lack of effect is mediated by a balance of direct suppression on PV GC postsynaptic GABA_ARs and a relatively low level of EtOH-induced increased GABA release (Fig. 3). The relative lack of increased GABA release is associated with reduced expression of nNOS in the PV GC layer (Fig. 4), which we have previously shown mediates EtOH-induced increased vesicular release of GABA (Kaplan et al., 2013). Collectively, the data are compatible with the hypothesis that genetically determined low expression of cerebellar nNOS diminishes the ability of EtOH to enhance GABA_AR inhibition of GCs, thereby contributing to the behavioral LLR to EtOH that is associated with genetic predilection for elevated EtOH consumption and abuse.

Prairie Vole cerebellar granule cell responses to EtOH are similar to those of the B6 mouse

In agreement with our *a priori* prediction mentioned above, bath application of EtOH (52mM) suppressed the tonic GABA_AR current in more than half of the PV GCs recorded via postsynaptic inhibition of extrasynaptic GABA_ARs (Fig. 2), as was observed in B6 mice (Kaplan et al., 2013). Consistent with postsynaptic EtOH action, suppression of the GC tonic GABA_AR current was not blocked by TTX nor decreased GC sIPSCs, demonstrating that it was independent of changes in action-potential-dependent vesicular GABA release from Golgi cells. Although EtOH suppressed the tonic GABA_AR current in the majority of cells recorded (57%), EtOH had no effect on the tonic GABA_AR current in 37% of the recorded GCs (Fig. 2B), which is similar to what we reported for B6 mice (Kaplan et al., 2013). This variability in EtOH suppression of tonic inhibition of GCs has been observed in cultured neurons (Yamashita et al., 2006) and in slice recordings (Kaplan et al., 2013), and likely reflects a combination of factors. First, there could be across-cell variability in the activity or expression of GC PKC, the activity of which suppresses the ability of EtOH to inhibit extrasynaptic GABA_ARs (Kaplan et al., 2013). Additionally, in some cells, direct suppression of postsynaptic GABA_ARs may have been masked by a concomitant counteracting increase in vesicular GABA release. Indeed, in cells in which EtOH did not affect the tonic GABA_AR current, there was on average, a larger EtOH-induced increase in sIPSC frequency than in cells that showed suppression of the tonic GABA_AR current (Fig. 3A). Although the current study does not further elucidate the mechanism of EtOH-induced suppression of tonic GABA_AR currents, numerous previous studies strongly implicate PKC activity as a key regulator of EtOH modulation of GABA_ARs that mediate tonic GABA_AR currents (Harris et al., 1995; Choi et al., 2008; Lesscher et al., 2009; Ron and Messing, 2011; Werner et al., 2011; Kaplan et al., 2013; Bohnsack et al., 2016).

Response to EtOH varies by consumption phenotype

Until recently it had been generally accepted that the main action of EtOH on GC GABA_AR currents was an increase in the frequency of GABA_AR mediated sIPSCs and an associated increase in the magnitude of the tonic GABA_AR current, both primarily mediated by

increased vesicular release of GABA from Golgi cells onto postsynaptic GCs (Carta et al., 2004; Hancher et al., 2005). However, this view was based largely on evidence collected from a low-EtOH consuming SD rat model. In contrast to this view, in PV GCs, 52mM EtOH had a more varied and smaller impact on sIPSC frequency (Fig. 3A), and on average, reduced the PV GC tonic GABA_AR current by ~14% (Fig. 2D). This cellular phenotype is in line with our previous observations from a variety of mammalian genotypes: the magnitude and polarity of the EtOH effect on the tonic GABA_AR current in cerebellar GCs parallels their EtOH consumption phenotype, ranging from strong enhancement in animals predisposed to low EtOH consumption to suppression in animals predisposed to high EtOH consumption (Kaplan et al., 2013; Mohr et al., 2013).

Cerebellar nNOS expression is a key determinant of GC GABA_AR current response to EtOH

Directed by evidence that blocking nNOS increased GABAergic transmission to GCs (Wall, 2003), and that EtOH inhibited NO production by nNOS (Persson and Gustafsson, 1992; Fataccioli et al., 1997; Al-Rejaie and Dar, 2006), we previously determined that EtOH increased Golgi cell activity, and consequently increased vesicular GABA release, by blocking nNOS production of NO in SD rats (Kaplan et al., 2013). Accordingly, the lack of EtOH-enhancement of GABAergic inhibition of PV GCs was paralleled by low nNOS expression (Fig. 4). The low nNOS expression in the PV GC layer was similar to low nNOS expression in age-matched B6 mice, both being significantly less than the high nNOS expression in age-matched D2 mice and SD rats (Fig. 4).

Could cerebellar nNOS expression provide the molecular link between cerebellar LLR and elevated EtOH consumption? While our data indicate that low cerebellar nNOS expression level is correlated with high EtOH consumption phenotype, behaviorally, EtOH impairment of rotarod performance, a cerebellar-dependent task, is mediated by EtOH suppression of NO production (Al-Rejaie and Dar, 2006; Dar, 2015). Thus, since animals with high EtOH consumption phenotypes have low nNOS expression levels, they may lack a main mechanism mediating EtOH disruption of rotarod performance. Indeed, B6 mice which have low cerebellar nNOS expression levels (Fig. 4) are also one of the most resistant rodents to EtOH impairment of rotarod performance (Gallagher et al., 1996).

Limitations of this study

Although our studies of several rodent genotypes have revealed a striking correlation between the polarity of the response of GC tonic GABA_AR currents to EtOH and sensitivity to EtOH-induced motor impairment, both of which are inversely related to the typical amount of EtOH consumed by the rodents (Fig. 2D) (Kaplan et al., 2013), evidence for causal relationships is lacking. Extensive studies by Dar and colleagues have shown that various pharmacological manipulations of cerebellar cortical processing can reduce motor impairment caused by systemic EtOH, but it is currently unknown if such interactions involve GC tonic GABA_AR currents (Dar, 2015). In the future, it would be useful to determine if pharmacologically counteracting EtOH actions on GC tonic GABA_AR currents affects EtOH consumption or motor impairment. Furthermore, although an inverse relationship between EtOH-induced motor impairment and excessive EtOH consumption in

rodents is well established for some genotypes (Malila, 1978; Gallaher et al., 1996; Bell et al., 2001), there are also rodent models that show the opposite relationship (Stinchcomb et al., 1989; Shram et al., 2004; Fritz et al., 2012). In this regard, to the extent that EtOH-induced cerebellar-dependent motor impairment is a deterrent to EtOH consumption, genetic differences in the initial sensitivity as well as in tolerance to motor impairment may be important (Gallaher et al., 1996; Fritz et al., 2012; Matson et al., 2014). Finally, the role of cerebellum in motor impairment and EtOH consumption is only a component of the complex brain reward and motor systems (Koob, 2014), making it unlikely that variation in GC tonic GABA_AR current response to EtOH will be perfectly linked to any EtOH-related phenotype.

Although it is difficult to make direct comparisons of absolute current magnitudes across species studied in different labs, different projects, and different aged animals, a perusal of the published mean values suggests that all aspects of GABA_AR inhibition (tonic current amplitude, sIPSC frequency and amplitude; Table 1) are smaller in PV GCs than in a variety of other rodents and non-human primates, which are themselves fairly similar (Brickley et al., 2001; Hamann et al., 2002; Meera et al., 2011; Kaplan et al., 2013; Mohr et al., 2013). Accordingly, we compared EtOH impacts across species by calculating the percent inhibition or potentiation of the tonic GABA_AR current for each cell (Fig. 2D). However, without knowing whether glutamatergic excitation or cell autonomous excitability scales in parallel with reduced tonic currents in PV GCs, it remains possible that despite similar percent block in PV and B6 mouse GCs, the overall impact of smaller EtOH-induced outward currents will be less in PV GCs. Clearly, future studies are needed to examine other aspects of PV GC excitability. However, regardless of the magnitude of the impact, there is a clear relationship between polarity of EtOH impact on tonic GABA_AR current and EtOH consumption phenotype (Fig. 2D).

The smaller magnitude basal tonic current in PV GCs raises another perplexing question: if EtOH-block of nNOS is the mechanism mediating increased Golgi cell firing and consequent increased GC sIPSC frequency and increased tonic GABA_AR current magnitude, then why don't genotypes with low nNOS expression have higher basal sIPSC frequencies and tonic current magnitudes? Instead, PV and B6 mouse GCs respectively have smaller and similar sIPSC frequencies and tonic current magnitudes compared to other genotypes with high nNOS expression (Kaplan et al., 2013). Although a rigorous answer to this question is beyond the scope of this manuscript, we propose that proper GC function is dependent on having optimal levels of tonic inhibition, and in the case of B6 mice this is accomplished by maintaining similar magnitude tonic GABA_AR currents despite reduced nNOS activity, via an as yet unknown mechanism. In the case of PV GCs, we predict that other aspects of GC excitability are shifted in parallel with reduced tonic currents to enable optimal function under basal conditions. In fact, genetic deletion of the $\alpha 6$ subunit of the GABA_AR, which abolishes tonic GABA_AR currents, does not affect overall GC excitability due to a compensatory upregulation of a tonic K⁺ conductance, highlighting the importance of a basal excitability set point (Brickley et al., 2001). Regardless of the complex mechanisms maintaining basal GC properties, the relative lack of nNOS clearly reduces the capacity of EtOH to modulate the system via the nNOS pathway, and thus is a likely contributing molecular determinant of EtOH-related behaviors.

Conclusions

Our findings that EtOH predominately suppresses GC tonic GABA_AR current in the PV cerebellum are consistent with our general hypothesis that the polarity and magnitude of EtOH's effect on GC tonic GABA_AR currents varies as a function of EtOH-consumption phenotype, ranging from enhancement in low-EtOH consuming genotypes, to suppression in high-EtOH consuming genotypes. The consistency of this relationship across genotypes and species suggests that there is some overlap in the genes that influence ethanol consumption and granule cell GABA_AR responses to ethanol. Future studies should investigate how EtOH's effects on cerebellar GC tonic GABA_AR currents influence signal transmission through the cerebellar cortex, and in turn, behavioral intoxication and EtOH consumption.

Acknowledgments

Support: This work was supported by NIH RO1 grants AA012439 (DAF and DJR, MPIs), AA019793 (AER), Washington State University institutional support funds (DJR), and space and resources from the Department of Veterans Affairs (DAF). JSK was supported by F31 AA022267.

References

- Al-Rejaie S, Dar MS. Antagonism of ethanol ataxia by intracerebellar nicotine: possible modulation by mouse cerebellar nitric oxide and cGMP. *Brain Res Bull.* 2006; 69:187–196. [PubMed: 16533669]
- Anacker AM, Loftis JM, Kaur S, Ryabinin AE. Prairie voles as a novel model of socially facilitated excessive drinking. *Addict Biol.* 2011; 16:92–107. [PubMed: 20579002]
- Bell RL, Stewart RB, Woods JE, Lumeng L, Li TK, Murphy JM, McBride WJ. Responsivity and development of tolerance to the motor impairing effects of moderate doses of ethanol in alcohol-preferring (P) and -nonpreferring (NP) rat lines. *Alcohol Clin Exp Res.* 2001; 25:644–650. [PubMed: 11371712]
- Bohnsack JP, Carlson SL, Morrow AL. Differential regulation of synaptic and extrasynaptic alpha4 GABA(A) receptor populations by protein kinase A and protein kinase C in cultured cortical neurons. *Neuropharmacology.* 2016
- Brady JD, Mohr C, Rossi DJ. Vesicular GABA release delays the onset of the Purkinje cell terminal depolarization without affecting tissue swelling in cerebellar slices during simulated ischemia. *Neuroscience.* 2010
- Brickley SG, Cull-Candy SG, Farrant M. Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABAA receptors. *J Physiol.* 1996; 497(Pt 3):753–759. [PubMed: 9003560]
- Brickley SG, Revilla V, Cull-Candy SG, Wisden W, Farrant M. Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance. *Nature.* 2001; 409:88–92. [PubMed: 11343119]
- Carta M, Mameli M, Valenzuela CF. Alcohol enhances GABAergic transmission to cerebellar granule cells via an increase in Golgi cell excitability. *J Neurosci.* 2004; 24:3746–3751. [PubMed: 15084654]
- Choi DS, Wei W, Deitchman JK, Kharazia VN, Lesscher HM, McMahon T, Wang D, Qi ZH, Sieghart W, Zhang C, Shokat KM, Mody I, Messing RO. Protein kinase Cdelta regulates ethanol intoxication and enhancement of GABA-stimulated tonic current. *J Neurosci.* 2008; 28:11890–11899. [PubMed: 19005054]
- Cservenka A. Neurobiological phenotypes associated with a family history of alcoholism. *Drug Alcohol Depend.* 2016; 158:8–21. [PubMed: 26559000]
- Dar MS. Ethanol-Induced Cerebellar Ataxia: Cellular and Molecular Mechanisms. *Cerebellum.* 2015; 14:447–465. [PubMed: 25578036]

- Duguid I, Branco T, London M, Chadderton P, Hausser M. Tonic inhibition enhances fidelity of sensory information transmission in the cerebellar cortex. *J Neurosci.* 2012; 32:11132–11143. [PubMed: 22875944]
- Eng MY, Schuckit MA, Smith TL. The level of response to alcohol in daughters of alcoholics and controls. *Drug Alcohol Depend.* 2005; 79:83–93. [PubMed: 15943947]
- Fataccioli V, Gentil M, Nordmann R, Rouach H. Inactivation of cerebellar nitric oxide synthase by ethanol in vitro. *Alcohol Alcohol.* 1997; 32:683–691. [PubMed: 9463722]
- Fritz BM, Grahame NJ, Boehm SL. Selection for high alcohol preference drinking in mice results in heightened sensitivity and rapid development of acute functional tolerance to alcohol's ataxic effects. *Genes Brain Behav.* 2012
- Gallagher EJ, Jones GE, Belknap JK, Crabbe JC. Identification of genetic markers for initial sensitivity and rapid tolerance to ethanol-induced ataxia using quantitative trait locus analysis in BXD recombinant inbred mice. *J Pharmacol Exp Ther.* 1996; 277:604–612. [PubMed: 8627537]
- Hamann M, Rossi DJ, Attwell D. Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. *Neuron.* 2002; 33:625–633. [PubMed: 11856535]
- Hancher HJ, Dodson PD, Olsen RW, Otis TS, Wallner M. Alcohol-induced motor impairment caused by increased extrasynaptic GABA(A) receptor activity. *Nat Neurosci.* 2005; 8:339–345. [PubMed: 15696164]
- Harris RA, McQuilkin SJ, Paylor R, Abeliovich A, Tonegawa S, Wehner JM. Mutant mice lacking the gamma isoform of protein kinase C show decreased behavioral actions of ethanol and altered function of gamma-aminobutyrate type A receptors. *Proc Natl Acad Sci U S A.* 1995; 92:3658–3662. [PubMed: 7731960]
- Harwood, H. Updating Estimates of the Economic Costs of Alcohol Abuse in the United States: Estimates, Update Methods, and Data. National Institute on Alcohol Abuse and Alcoholism; 2000. <http://pubs.niaaa.nih.gov/publications/economic-2000/>
- Herting MM, Fair D, Nagel BJ. Altered fronto-cerebellar connectivity in alcohol-naïve youth with a family history of alcoholism. *Neuroimage.* 2011; 54:2582–2589. [PubMed: 20970506]
- Hill SY. Neural plasticity, human genetics, and risk for alcohol dependence. *Int Rev Neurobiol.* 2010; 91:53–94. [PubMed: 20813240]
- Hill SY, Muddasani S, Prasad K, Nutche J, Steinhauer SR, Scanlon J, McDermott M, Keshavan M. Cerebellar volume in offspring from multiplex alcohol dependence families. *Biol Psychiatry.* 2007; 61:41–47. [PubMed: 16533498]
- Hostetler CM, Anacker AM, Loftis JM, Ryabinin AE. Social housing and alcohol drinking in male-female pairs of prairie voles (*Microtus ochrogaster*). *Psychopharmacology (Berl).* 2012; 224:121–132. [PubMed: 22903359]
- Kaplan JS, Mohr C, Rossi DJ. Opposite actions of alcohol on tonic GABA(A) receptor currents mediated by nNOS and PKC activity. *Nat Neurosci.* 2013; 16:1783–1793. [PubMed: 24162656]
- Koob GF. Neurocircuitry of alcohol addiction: synthesis from animal models. *Handb Clin Neurol.* 2014; 125:33–54. [PubMed: 25307567]
- Korpi ER, Kuner T, Seeburg PH, Luddens H. Selective antagonist for the cerebellar granule cell-specific gamma-aminobutyric acid type A receptor. *Mol Pharmacol.* 1995; 47:283–289. [PubMed: 7870036]
- Kurta, A. Mammals of the Great Lakes Region. Ann Arbor, Michigan: The University of Michigan Press; 1995.
- Lesscher HM, Wallace MJ, Zeng L, Wang V, Deitchman JK, McMahon T, Messing RO, Newton PM. Amygdala protein kinase C epsilon controls alcohol consumption. *Genes Brain Behav.* 2009; 8:493–499. [PubMed: 19243450]
- Malila A. Intoxicating effects of three aliphatic alcohols and barbitol on two rat strains genetically selected for their ethanol intake. *Pharmacol Biochem Behav.* 1978; 8:197–201. [PubMed: 652828]
- Matson LM, Kasten CR, Boehm SL, Grahame NJ. Selectively bred crossed high-alcohol-preferring mice drink to intoxication and develop functional tolerance, but not locomotor sensitization during free-choice ethanol access. *Alcohol Clin Exp Res.* 2014; 38:267–274. [PubMed: 23909817]
- Meera P, Wallner M, Otis TS. Molecular basis for the high THIP/gaboxadol sensitivity of extrasynaptic GABAA receptors. *J Neurophysiol.* 2011

- Miquel M, Toledo R, Garcia LI, Coria-Avila GA, Manzo J. Why should we keep the cerebellum in mind when thinking about addiction? *Curr Drug Abuse Rev.* 2009; 2:26–40. [PubMed: 19630735]
- Mohr C, Brady JD, Rossi DJ. Young age and low temperature, but not female gender delay ATP loss and glutamate release, and protect Purkinje cells during simulated ischemia in cerebellar slices. *Neuropharmacology.* 2010; 58:392–403. [PubMed: 19825379]
- Mohr C, Kolotushkina O, Kaplan JS, Welsh J, Daunais JB, Grant KA, Rossi DJ. Primate cerebellar granule cells exhibit a tonic GABAAR conductance that is not affected by alcohol: a possible cellular substrate of the low level of response phenotype. *Front Neural Circuits.* 2013; 7:189. [PubMed: 24324408]
- Persson MG, Gustafsson LE. Ethanol can inhibit nitric oxide production. *Eur J Pharmacol.* 1992; 224:99–100. [PubMed: 1451748]
- Prescott CA, Kendler KS. Genetic and environmental contributions to alcohol abuse and dependence in a population-based sample of male twins. *Am J Psychiatry.* 1999; 156:34–40. [PubMed: 9892295]
- Ron D, Messing RO. Signaling Pathways Mediating Alcohol Effects. *Curr Top Behav Neurosci.* 2011
- Schmahmann JD. The role of the cerebellum in cognition and emotion: personal reflections since 1982 on the dysmetria of thought hypothesis, and its historical evolution from theory to therapy. *Neuropsychol Rev.* 2010; 20:236–260. [PubMed: 20821056]
- Schuckit MA. Ethanol-induced changes in body sway in men at high alcoholism risk. *Arch Gen Psychiatry.* 1985; 42:375–379. [PubMed: 3977555]
- Schuckit MA. An overview of genetic influences in alcoholism. *J Subst Abuse Treat.* 2009; 36:S5–14. [PubMed: 19062348]
- Schuckit MA, Smith TL. An 8-year follow-up of 450 sons of alcoholic and control subjects. *Arch Gen Psychiatry.* 1996; 53:202–210. [PubMed: 8611056]
- Shram MJ, Bahroos M, Beleskey JI, Tampakeras M, Le AD, Tomkins DM. Motor impairing effects of ethanol and diazepam in rats selectively bred for high and low ethanol consumption in a limited-access paradigm. *Alcohol Clin Exp Res.* 2004; 28:1814–1821. [PubMed: 15608597]
- Stell BM, Brickley SG, Tang CY, Farrant M, Mody I. Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by delta subunit-containing GABAA receptors. *Proc Natl Acad Sci U S A.* 2003; 100:14439–14444. [PubMed: 14623958]
- Stinchcomb A, Bowers BJ, Wehner JM. The effects of ethanol and Ro 15–4513 on elevated plus-maze and rotarod performance in long-sleep and short-sleep mice. *Alcohol.* 1989; 6:369–376. [PubMed: 2510766]
- Stoodley CJ. The cerebellum and cognition: evidence from functional imaging studies. *Cerebellum.* 2012; 11:352–365. [PubMed: 21373864]
- Strick PL, Dum RP, Fiez JA. Cerebellum and nonmotor function. *Annu Rev Neurosci.* 2009; 32:413–434. [PubMed: 19555291]
- Valenzuela CF, Jotty K. Mini-Review: Effects of Ethanol on GABAA Receptor-Mediated Neurotransmission in the Cerebellar Cortex--Recent Advances. *Cerebellum.* 2015; 14:438–446. [PubMed: 25575727]
- Wall MJ. Endogenous nitric oxide modulates GABAergic transmission to granule cells in adult rat cerebellum. *Eur J Neurosci.* 2003; 18:869–878. [PubMed: 12925012]
- Wall MJ, Usowicz MM. Development of action potential-dependent and independent spontaneous GABAA receptor-mediated currents in granule cells of postnatal rat cerebellum. *Eur J Neurosci.* 1997; 9:533–548. [PubMed: 9104595]
- Werner DF, Kumar S, Criswell HE, Suryanarayanan A, Fetzer JA, Comerford CE, Morrow AL. PKCgamma is required for ethanol-induced increases in GABA(A) receptor alpha4 subunit expression in cultured cerebral cortical neurons. *J Neurochem.* 2011; 116:554–563. [PubMed: 21155805]
- Yamashita M, Marszalec W, Yeh JZ, Narahashi T. Effects of ethanol on tonic GABA currents in cerebellar granule cells and mammalian cells recombinantly expressing GABA(A) receptors. *J Pharmacol Exp Ther.* 2006; 319:431–438. [PubMed: 16844844]

Figure 1.

PV GCs exhibit a tonic current mediated by $\alpha 6, \delta$ -subunit containing GABA_ARs. **A&B.** Example voltage-clamp recording ($V_h = -60$ mV, $E_{Cl^-} = \sim 0$ mV) of a PV GC showing block of sIPSCs and tonic current by the GABA_AR antagonist GABA_zine (10 μ M). The gray arrows (A) and dashed lines (B) point to an expanded time scale of the recording from different time points of the main traces (sIPSC marked by * in A). **C.** Example trace showing block of tonic current by GABA_AR antagonist furosemide (100 μ M, at which concentration it is specific for GABA_ARs containing the $\alpha 6$ subunit). **D.** Example trace showing enhancement of tonic current by GABA_AR agonist THIP (500 nM, at which concentration it is selective for GABA_ARs containing the δ subunit). **E.** Plot of mean current blocked by furosemide (Furo, $n=9$) and GABA_zine (GBZ, $n=23$), and enhanced by THIP ($n=10$).

Figure 2.

EtOH suppresses or has no effect on PV GC tonic GABA_AR currents. **A.** Representative voltage-clamp traces of PV GCs showing that EtOH (52mM) can suppress (left) or does not affect (right) GC tonic GABA_AR current. **B.** Plot of percentage of cells (from 21 recordings) showing potentiation, suppression or no change induced by EtOH (52mM). **C.** Representative trace showing that EtOH suppression of the tonic GABA_AR current is abolished by the GABA_AR antagonist, GABAzine (10μM). **D.** Plot of percent modulation (enhancement up and suppression down) of the tonic GABA_AR current by EtOH (52mM) in GCs from various genotypes listed from left to right based on relative EtOH consumption in typical two bottle choice paradigms: Sprague-Dawley rat (SDR), DBA/2J (D2) mouse, C57BL/6J (B6) mouse, and Prairie Vole (PV). Percent modulation of tonic GABA_AR current was derived by dividing the magnitude of the current induced by EtOH by the current induced by GABAzine for individual cells within a species. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Values for SDR, D2, and B6 GCs were derived by reanalyzing (as described above) raw values from studies published in Kaplan et al. (2013).

Figure 3.

EtOH suppression of PV GC tonic GABA_AR current is due to direct action on postsynaptic GABA_ARs. **A.** Plot of mean EtOH-induced change in sIPSC frequency in GCs that exhibited EtOH-induced suppression or no change of the tonic GABA_AR current. **B.**

Representative recording showing that EtOH suppression of the tonic GABA_AR current is not prevented by blocking action potential dependent release of GABA with TTX (500nM).

C. Plot of mean EtOH-induced change in tonic GABA_AR current under control conditions, in TTX (500nM), or GABAzine (10μM). # = 0.053, **P* < 0.05, ***P* < 0.01, by one-sample *t* test.

Figure 4.

PV and B6 mouse cerebellums have less granule cell layer expression of nNOS than SD rat and D2 mouse cerebellums. **A.** Confocally acquired images of immunohistochemistry for nNOS (red), GAD-65/67 (green) and nuclear stain Hoechst (blue) in the granule cell layer of Sprague-Dawley rat (SDR), DBA/2J mouse (D2), C57BL/6J mouse (B6) and prairie vole (PV) cerebellum. **B.** Plot of mean percent encirclement of GC soma/nuclei for age matched SDRs, D2 mice, B6 mice, and PVs. *** indicates SDRs and D2s both have significantly greater nNOS encirclement than B6s and PVs, $P < 0.001$.

Properties of Prairie Vole cerebellar granule cell GABA_AR-mediated sIPSCs (frequency, amplitude & 10–90% decay time) and tonic current (magnitude), under various pharmacological conditions.

Table 1

	Control		Ethanol		TTX		TTX + Ethanol	
	<i>M</i>	<i>SEM</i>	<i>M</i>	<i>SEM</i>	<i>M</i>	<i>SEM</i>	<i>M</i>	<i>SEM</i>
sIPSC Frequency (Hz)	0.13	0.02	0.15	0.03	0.13	0.03	0.15	0.03
sIPSC Amplitude (pA)	14.97	1.15	15.64	1.44	15.04	2.32	14.28	2.3
sIPSC Decay Time (ms)	5.49	0.43	5.22	0.47	4.01	0.45	4.13	0.75
Tonic GABA _A R current magnitude (pA)	12.26	1.88	11.1	0.34	8.37	1.18	6.45	0.78

Note, because this is the first report of recording from such cells, the values given are meant to provide absolute values under various conditions (basal, EtOH, TTX, and TTX + EtOH) for comparison to other species. Thus, values are means ± S.E.M. from all recordings made of each condition, and do not reflect within cell changes. Accordingly, in many cases, cell to cell variability may obscure actual significant changes induced by a given condition within a given cell. For within cell comparisons of percent change induced by a given drug, see values in the main text.