THE CEREBELLAR $GABA_A$ RECEPTOR CONTRIBUTION TO ALCOHOL INTAKE AND INTOXICATION

By

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Abbreviations

ADHD	Attention-deficit/hyperactivity disorder
aCSF	Artificial cerebral spinal fluid
AP	Action potential
AUD	Alcohol use disorder
B6	C57BL/6J mouse
BEC	Blood ethanol concentration
D2	DBA/2J mouse
DCN	Deep cerebellar nuclei
DM	Differentiator model
EPSP	Excitatory postsynaptic potential
eEPSP	Evoked EPSP
EtOH	Ethanol
FHP	Family history positive
FHN	Family history negative
fMRI	Functional magnetic resonance imaging
$GABA_AR$	$GABA_A$ receptor
IPSC	Inhibitory postsynaptic current
mIPSC	Miniature IPSC
sIPSC	spontaneous IPSC
LLR	Low level of response
LTD	Long-term depression
mGluR	Metabotropic glutamate receptor
MRI	Magnetic resonance imaging
NHP	Non-human primate
NOS	Nitric oxide synthase

nNOS	Neuronal nitric oxide synthase
PC	Purkinje cell
PET	Positron emission tomography
PV	Prairie vole
PKC	Protein kinase C
SDR	Sprague-Dawley rat
TTX	Tetrodotoxin
WSP1	Withdrawal Seizure-Prone replicate line 1
WSR1	Withdrawal Seizure-Resistant replicate line 1

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General abstract

Alcohol use disorders (AUDs) are a leading cause of preventable death and illness that contribute significant social and economic costs to society. Genetic factors contribute substantially to AUD development but identifying and characterizing their phenotypic expression at the cellular level has been incomplete. One of the greatest challenges in treating problem alcohol (ethanol; EtOH) use is our poor understanding of the diversity of factors that promote sensitivity to EtOH reward and intoxication.

The cerebellum has long been ignored as a brain region that could influence EtOH intake. However, recent functional, anatomical, and behavioral studies have established a cerebellar link to cognitive function, elements of reward processing, and risk for developing AUDs in humans and enhanced EtOH consumption in rodents. Low sensitivity to EtOH-induced cerebellar ataxia is a genetically-regulated factor associated with increased EtOH consumption and elevated risk for developing AUDs, but the cellular mechanisms that promote variation in sensitivity to EtOH impairment are unknown. In this dissertation I sought to identify the cellular mechanisms that underlie sensitivity to EtOH-induced cerebellar impairment in rodents with opposite sensitivity to EtOH-induced cerebellar ataxia and EtOH consumption phenotypes.

Previous reports have identified that EtOH enhancement of cerebellar $GABA_A$ receptor ($GABA_AR$) signaling induces ataxia, and $GABA_AR$ inhibition of granule cells (GCs) is a particularly sensitive target for modulation by EtOH. I therefore hypothesized that sensitivity to EtOH enhancement of GC $GABA_AR$ inhibition is a cellular substrate linking genetic risk for high EtOH consumption to a low sensitivity to EtOH-induced cerebellar impairment. Using patch-clamp slice electrophysiology, immunohistochemistry, and behavioral techniques, I confirmed this hypothesis by revealing that two genetically-regulated factors, GC layer neuronal nitric oxide synthase (nNOS) expression and GC protein kinase C (PKC) determine sensitivity to EtOH-induced enhancement of GC $GABA_AR$ inhibition and modulate glutamatergic input to

Purkinje cells, the sole output of the cerebellar cortex. Lastly, I demonstrate that EtOH's action on GC tonic $GABA_AR$ inhibition contributes to EtOH intake.

In Chapter 2, I tested whether EtOH differentially affected GC $GABA_ARs$ in rats and mice with opposite sensitivities to EtOH-induced cerebellar ataxia and consumption phenotypes. I specifically predicted that EtOH would strongly enhance $GABA_AR$ inhibition in Sprague-Dawley rats (SDRs) and DBA/2J (D2) mice with high sensitivity to EtOH-induced cerebellar impairment and low EtOH consumption phenotypes, and induce little enhancement in behaviorally insensitive, high EtOH consuming, C57BL/6J (B6) mice. Instead, I found that EtOH's effect on GC tonic $GABA_AR$ inhibition ranged across a spectrum from strong enhancement in behaviorally sensitive, low EtOH consuming, SDRs and D2 mice to suppression in behaviorally insensitive, high EtOH consuming B6 mice. The net effect of EtOH on GC tonic $GABA_AR$ inhibition was determined by the relative expression of two genetically regulated factors: nNOS expression in the GC layer determined the magnitude of enhancement in GABAergic transmission to GCs by EtOH, and low PKC activity enabled EtOH to directly inhibit the $GABA_ARs$ that mediate the tonic inhibitory current. Thus, Chapter 2 revealed that low nNOS expression and low GC PKC activity are genetic factors, common in high EtOH consuming rodents, which may perhaps underlie cerebellar-mediated genetic AUD risk in humans.

Based on the findings from Chapter 2, Chapter 3 tested the predictive validity of the hypothesis that EtOH-induced suppression of GC tonic $GABA_AR$ inhibition is a neural substrate common in high EtOH consuming genotypes. I tested and confirmed this hypothesis in the high-EtOH consuming prairie vole (PV) genotype. Consistent with the results from Chapter 2, EtOH suppression of tonic $GABA_AR$ inhibition in the majority of GCs was associated with relatively low nNOS expression in comparison to age-matched low EtOH consuming rat and mice genotypes. Together with the genotypes investigated in Chapter 2, as well as our lab's previously collected data from non-human primate GCs, I confirmed in seven different genotypes that the effect of EtOH on GC tonic $GABA_AR$ inhibition varied as a function of EtOH-consumption phenotype,

ranging from strong enhancement in low EtOH consuming genotypes to suppression in high EtOH consuming genotypes. These results suggest an important contribution of EtOH's actions on GC tonic $GABA_AR$ inhibition in behavioral phenotypes associated with elevated EtOH intake in rodents and AUD risk in humans.

Despite EtOH's differential effects on GC tonic $GABA_AR$ inhibition, it was unknown if EtOH differentially regulated excitatory transmission through the cerebellar cortex. Chapter 4 tested the hypothesis that EtOH-induced suppression of GC tonic $GABA_AR$ inhibition would be less disruptive of spontaneous and mossy fiber-evoked transmission to PCs. This hypothesis was tested in B6 and D2 mice that have high- and low-EtOH consumption phenotypes, respectively. In support of my hypothesis, a low 9mM EtOH concentration reduced spontaneous glutamatergic input to PCs in D2s, but not B6s, through effects on GC $GABA_AR$ inhibition. However, 9mM EtOH paradoxically caused slight enhancement of mossy fiber-evoked glutamatergic input to PCs in D2 mice and a slight reduction in B6 mice. GABAergic molecular layer interneurons were found to be relatively insensitive to low-moderate EtOH concentrations, further supporting the importance of EtOH's opposite actions on GC tonic $GABA_AR$ inhibition in the diversity of cerebellar-mediated EtOH-related behavioral phenotypes.

Chapters 2-4 suggested that EtOH suppression of GC tonic $GABA_AR$ inhibition is common in high-EtOH consuming genotypes and differentially regulates glutamatergic input to PCs. So in Chapter 5, I tested the hypothesis that EtOH-induced suppression of GC tonic $GABA_AR$ inhibition contributes to EtOH intake. To test this, B6 mice were stereotaxically implanted with unliteral cannula into lobes IV/V/VI of the cerebellar cortex and given limited access to 10% EtOH in a two-bottle choice drinking paradigm. GC tonic $GABA_AR$ inhibition was then selectively enhanced by microinjection of the selective δ subunit-containing $GABA_AR$ agonist, THIP, which significantly reduced EtOH intake compared to vehicle infusion in two separate experimental passes. THIP reduced EtOH intake without impairing the ability to

consume fluids. These results are the first to show an isolated cerebellar contribution to EtOH intake by revealing that enhancing GC tonic $GABA_AR$ inhibition suppresses EtOH consumption.

Together, the findings presented in this dissertation provide important insight into the cellular mechanism underlying the link between heritable cerebellar-related behavioral phenotypes and EtOH consumption. GC tonic $GABA_AR$ inhibition may be a promising target for directed pharmacological intervention for treatment and prevention of AUDs. Future research should be directed towards gaining a more thorough understanding of the cerebellum in reward processing and EtOH intake.

Chapter 1: General Introduction

1.1. Overview

The cerebellum is a distinct subregion of the vertebrate brain, and its cellular organization has been evolutionarily conserved across species. However, phylogenetic expansion of cerebellar volume closely parallels neocortical expansion across primate phylogeny, suggesting an important relationship between cerebellar and neocortical function (Barton & Harvey, 2000). Intriguingly, the lobes of the cerebellum that are known to communicate with the prefrontal cortex are proportionally largest in higher-order primates, and largest in humans. This volume expansion parallels a high degree of interconnectivity between the cerebellum with the neocortex, and the cerebellum with reward circuitry, pointing to a cerebellar role beyond its traditional role in motor control. Indeed, accumulating behavioral studies clearly support a cerebellar contribution to learning, attention, language, and reward processing. These studies implicate a powerful cerebellar influence on the performance and execution of tasks traditionally thought to be solely allocated to other structures that likely stem from the anatomical and functional connectivity between the cerebellum and a host of brain regions. As a consequence of this broad cerebellar involvement across cognitive and motor tasks, abnormal cerebellar function has been associated with neuropsychiatric disorders and risk for developing problem drug use.

The following dissertation proposes a cerebellar role in alcohol (ethanol; EtOH)-related behaviors beyond motor discoordination, including genetic propensity for elevated EtOH consumption and abuse. I will present both cellular and behavioral evidence in support of a cerebellar influence on behaviors associated with risk for development of alcohol use disorders (AUDs) and provide evidence that manipulation of cerebellar processing can directly affect EtOH consumption. The data and conclusions submitted here should promote collaboration and integration of the seemingly independent cerebellar and addiction fields so that we can develop

effective individualized addiction treatment methods against the spectrum of behavioral and genetic risk factors that have been characterized for this destructive disease.

1.2. Alcohol Use Disorder is a pervasive global problem

EtOH consumption is ubiquitous throughout the world. In the United States, over 88% of Americans over the age of 18 have consumed EtOH (SAMHSA, 2014). However, binge drinking (defined as 5 or more drinks in one occasion in men, and 4 or more drinks in women; Bouchery et al., 2011) is extremely common, making EtOH consumption a leading cause of preventable death and disability. Globally, EtOH is the fifth highest contributor to death and disability, but it is first among people 15-49 years old (Lim et al., 2012). Approximately 25% of Americans 18 years and over reported binge drinking within the last month (SAMHSA, 2014), resulting in an annual economic burden of approximately $170 billion annually due to lost productivity, health care costs, and property damage (Bouchery et al., 2011). However, the high prevalence of binge EtOH use has a burden that stretches well beyond economics.

Repeated binges can result in the development of an AUD, which encompasses both dependence and repeated abuse that does not reach the level of dependence. Over 17 million Americans are estimated to have an AUD (Grant et al., 2004). Heavy EtOH use in one spouse but not the other is a robust predictor of marital dissolution (Ostermann et al., 2005), which is often reported to result from increased negative interactions between partners and increased domestic violence (Marshal, 2003). However, living with a parent with an AUD can be especially damaging to children. Over 10% of American children live with a parent with an AUD (SAMHSA, 2012), and these children are at greater risk for physical and emotional abuse as well as neglect (Hindman, 1977). Additional long-term effects of growing up in a home with an AUD was identified in a prospective 40-year longitudinal study which found that the severity of the parent's AUD was directly correlated with their children's time in jail, sociopathy, and death in adulthood (Beardslee & Viallant, 1986). It is especially damaging that children raised by parents with AUDs

are at much larger risk for development of an AUD themselves, and this risk is determined by both genetic and environmental factors (Cadoret & Gath, 1978; Beardslee & Viallant, 1986; Cloninger et al., 1981; Enoch & Goldman, 1999; Prescott & Kendler, 1999; Hill, 2010). The large heritable AUD risk illustrates the necessity for individuals to gain access to accessible and effective treatment options. However, only about 8% of individuals with an AUD ever receive treatment (SAMHSA, 2012), which is down from 15% a few years prior (Cohen et al., 2007). Perhaps the low percentage of AUD sufferers enrolled in a treatment program is the result of relatively ineffective treatment options. In support, a mere 36% of treatment participants were considered low-risk for relapse (Dawson, 2005). The rest fall along a wide ranging recovery profile, but most remain at high-risk for relapse (Huebner & Kantor, 2011).

Much of the difficulty in developing effective long-term treatment strategies to combat AUD is a function of the heterogeneity of risk factors derived from the interaction between genetic and environmental influences. Furthermore, while great strides have been made in identifying many of the behavioral and cognitive correlates with risk for developing an AUD, the cellular substrates underlying many of these risk factors remain elusive. Identification of these cellular and molecular mechanisms would help direct the development of pharmacological treatments against AUD, helping to raise treatment enrollment, efficacy, and retention. One promising brain target for focused treatment effort is the cerebellum. Over the last few decades, evidence has accumulated suggesting heritable cerebellar variation in structure (Benegal, et al. 2007; Hill et al., 2011), functional connectivity (Herting et al., 2011; Cservenka et al., 2014), and response to acute EtOH (Schuckit, 1985; Schuckit, 1994) that is associated with increased risk for developing an AUD. Given the recent appreciation of the non-motor functions of the cerebellum and documented connections to reward centers, the cerebellum may influence predilection for enhanced EtOH consumption through mechanisms that extend beyond its traditionally accepted role in motor function. Accordingly, the cerebellum may serve to be a new

and promising experimental territory from which discovery of effective treatment options will help attenuate the harsh burden of EtOH on individuals and societies world-wide.

1.3. A brief history of traditional views of the cerebellum

The earliest recorded anatomical assessment of the human cerebellum came from the Brabantian anatomist, Andreas Vesalius who greatly amended and then criticized the description of his predecessor, Aelius Galen, in 1543 for generalizing his cerebellar description on that of an ox, not a human (reviewed by Glickstein et al., 2009). Thus began the common practice of overturning accepted cerebellar dogma in terms of anatomy, function, and perhaps even a role in addiction. Despite the fact that the traditional cerebellar role over the last half century has been limited to motor function, initial descriptions of cerebellar function were more deeply rooted in cognitive than motor function, albeit occasionally misguided and incorrect. The Italian anatomist, Vincenzo Malacarne, postulated in 1776 that the cerebellum was involved in plastic changes based on evidence of reduced folia in an “idiot” (Malacarne, 1776), perhaps suggesting a cognitive role, and further hypothesized that the variability in folia could be acquired by the degree of environmental enrichment (Glickstein et al., 2009).

Over a half a century later, phrenologists consigned sexual drive, or “philoprogenitiveness”, to the cerebellum (Combe & Combe, 1838), gaining anecdotal support from the skull’s shape of prison inmates incarcerated for sex offenses and additionally by successfully treating sex preoccupation in three individuals by placing ice on the ventral caudal region part of the head. This claim was soon debunked by Pierre Flourens who, after decerebellarating a rooster, observed that it remained sexually interested in the hens. Importantly, Flourens also noted severe motor impairment (Flourens, 1824), a claim that corroborated Luigi Rolando’s crude lesion studies in a variety of species (Rolando, 1809). It was Flourens who first argued that the cerebellum was involved in coordinated movement, correctly identifying that the production and coordination of movements were independent entities and

controlled by distinct brain regions. This cerebellar role in movement control has been consistently experimentally confirmed over the last two centuries and has become engraved as the primary cerebellar function. Rolando and Flourens' ablation experiments were some of the earliest examples of attempts to block function in order to expose its existence. I have used a similar approach where I pharmacologically inhibited neurons to reveal their role in EtOH consumption.

Like the ablation experiments, postmortem assessments, and more recently, magnetic resonance imaging (MRI), have been used to identify the primary question: is the cerebellum necessary for normal movement? Early reports of patients with cerebellar agenesis have been mixed and were often riddled with insensitive measures, incomplete assessment, and inconsistency in criteria (Anton and Zingerle, 1914; Glickstein, 1994; Sener & Jenkins, 1993; Tavano et al., 2007). However, a recent study used a weight dropping and catching procedure to parse the cerebellar role in predictive versus reactionary motor control. Subjects with cerebellar agenesis were significantly impaired in their ability to catch a weight that was expectedly dropped from their other hand, a measure of predictive motor control, but did not differ from controls in their reactionary ability to grasp a weight dropped unexpectedly (Nowak et al., 2007). This cerebellar role in coding predictive elements of motor behavior is thought to depend on the cerebellum's storage of an internal model, a neural representation of behavior comprised of forward and inverse elements (Ito, 2008). In this framework, the forward model processes the next stage of the movement based on the current state and the motor command, whereas the inverse model compares the current and expected states, correcting them with an altered motor command to obtain the desired state (Wolpert et al., 1998). Together, the internal model is constantly formed and adjusted as movements are repeated, promoting motor adaptations that have been modeled in robots (Kawato, 2009) and visualized with functional MRI (fMRI; Kawato et al., 2003).

It has been proposed that the cerebellum creates and compares similar internal models for proper execution of cognitive processes, thereby assessing and providing feedback to regions such as the motor cortices, the prefrontal cortex, the parietal and temporal cortices, and the anterior cingulate cortex (Ito, 2008). Similar to coordinated movement, executive functioning requires integration and correction of a series of rapidly coordinated cognitive steps. Within this framework, the internal models enable the cerebellum to predict the relationship between a series of cognitive (or motor) events. As these events transpire, the cerebellum generates predictions about subsequent events and initiates appropriate corrective steps, if necessary, via communication with the appropriate brain region (Allen & Courchesne, 2003; Ito, 2008).

Error learning and motor modification described by internal models likely require time tracking and management on the hundreds of millisecond level by the cerebellum. Ivry and Keele (1989) measured timing performance on a finger tapping task and on an interval discrimination task, finding that patients with isolated cerebellar neuropathy showed deficits in timing ability of hundreds of milliseconds, isolating their deficits in estimation and implementation to particular cerebellar regions (Ivry et al., 1988). These findings have been corroborated by studies in healthy controls where transcranial magnetic stimulation-disruption of the left lateral cerebellum caused a significant overestimation of perceived time intervals on a hundreds of millisecond time scale but not a longer multi-second time scale, which was disrupted by stimulation of the dorsolateral prefrontal cortex (Kock et al., 2007). Because proper execution of both cognitive and motor processes require accurate time maintenance on a millisecond time-scale, it's plausible that the cerebellum's role extends beyond just motor function and into the cognitive realm.

It's unclear why the overwhelming weight of the literature on the cerebellum is focused on motor control. A simple search on PubMed.gov using the terms "cerebellum" and "motor" yielded five times more hits than "cerebellum" and "cognition", which is a large reduction from the 24 fold increase when limiting the search to pre-1980 (assessed 8-21-14). There may be a

dual and complementary explanation for this discrepancy that led to the establishment of early models of cerebellar function that focused exclusively on motor control (Evarts & Thach, 1969). Anatomically, there are no direct monosynaptic connections between the cerebellum and the cortex. This created a problem because the early retro- and anterograde tracers failed to cross synapses, thus making the complete mapping of the polysynaptic cerebrocerebellar efferent and afferents difficult (Buckner, 2013). As a result, behavioral assessments of cerebellar function were limited to motor assessment. Experiments in animal models may have missed the influence of cerebellar manipulation on cognitive functioning because it either wasn't tested or because the deficits in cognitive function were attributed to other brain regions. But eventually, sufficient indirect evidence began to accumulate from extensive tracing and early anatomical imaging studies for cerebellar connections with both motor and non-motor association areas throughout the neocortex (Leiner et al., 1986; Leiner et al., 1989).

Recently, the development of functional brain imaging technologies has allowed researchers to more definitively assess the cognitive and behavioral consequences of these connections (reviewed by Buckner, 2013; Stoodley, 2012; Stoodley & Schmahmann, 2009). Peterson et al. (1989) used positron emission tomography (PET) to study the functional anatomy of basic single word processing, identifying distinct activation patterns in the right lateral cerebellar hemisphere for processing the word and activation in the anterior cerebellum for the motor control over producing the word, which supported a cerebellar influence over the cognitive aspects of speech. A further study of the cerebellar role in cognitive aspects of language using a word stem completion task confirmed that cerebellar activation contralateral to cortical activation is not merely the result of motor preparation, nor is it a slave system tied to cortical computations, and instead argues for a unique cerebellar contribution to the cognitive process (Desmond et al., 1998). fMRI enabled more spatially and temporally precise characterization of the cerebellum's cognitive role. Kim et al. (1994) found three to four times greater activation of the dentate nucleus, a cerebellar output nucleus, during a cognitively

demanding pegboard puzzle task than simply moving the pegs alone. Thus, since the mid-1990s, functional imaging has continued to support a cerebellar role in cognitive function.

The breadth of supporting functional imaging literature helps quell skeptics who argue that the cognitive impairment in patients with cerebellar pathologies are merely coincidental or result from undiagnosed cortical abnormalities. But despite the compelling evidence for a cerebellar role in cognitive processing, the traditional view of the cerebellum across the neuroscience community remains largely static. This is evidenced in a recent cerebellar agenesis case, where the authors note that, in addition to motor learning deficits, the patient also displayed impairment in IQ, visuospatial ability, planning behavior, memory, and attention, concluding that the neurpsychological deficits were, “more marked than one would expect in cerebellar disease” (Timmann et al., 2003). Such skepticism of a larger cerebellar influence is persistent in much of the behavioral literature, but with a growing body of evidence revealing cerebellar pathology in neuropsychiatric disorders (Konarski et al., 2005; Wang et al., 2014), a cerebellar role in a range of cognitive behaviors, including drug taking, may become an area of intense research focus in the near future.

1.4. Neuropsychiatric consequences of cerebellar dysfunction

This dissertation argues in favor of a cerebellar contribution to elements of cognitive processing that promote behaviors not traditionally ascribed to the cerebellum. In particular, the proposed case for a cerebellar role in EtOH intake is built on the premise that cerebellar functioning influences cognitive and motivational processes, that when disrupted, induce symptoms consistent with neuropsychiatric illness. Symptoms associated with cerebellar disturbance or defect can reveal realms of cerebellar involvement beyond motor control. But most importantly, cerebellar involvement in neuropsychiatric disorders characterized by impairments to cognitive and executive processing, including stimuli detection and motivated

approach versus avoidance behavior, can shed light on trajectories through which the cerebellum can modulate EtOH intake.

The link between neuropsychiatric disorders and cerebellar pathology has been surprisingly well documented (e.g., Konarski et al., 2005). Comorbid cerebellar abnormality with neuropsychiatric disorders characterized by severe cognitive deficit, such as Alzheimer's disease, broadly demonstrates an associative link between cerebellar functioning and cognitive processing (Cole et al., 1989; Klunk et al., 2004; Joachim, et al., 1989). Structural MRI revealed smaller right cerebellar hemisphere volumes in Alzheimer's disease patients compared to healthy controls that correlated with Mini-Mental Status Exam performance. These volume reductions were primarily driven by reductions in the posterior cerebellum which largely comprises the cerebellar hemispheres (Thomann et al., 2008). In support of cerebellar structural abnormalities in cognitive function deficits, a recent fMRI study similarly identified a strong correlation between the reductions in functional connectivity of resting state networks involving the cerebellum and Mini-Mental Status Exam performance in patients with Alzheimer's disease compared to healthy controls (Castellazzi et al., 2014). Thus, cerebellar dysfunction resulting from loss of gray matter volume could contribute to impairments in memory and executive function that are associated with Alzheimer's disease. In support, a study of nearly 4500 healthy aged individuals revealed that cerebellar gray matter volume was directly correlated with executive function and information processing speed (Hoogendam et al., 2014). However, because cerebral gray matter similarly correlated with these performance measures, it's difficult to tease apart the cerebellar role in cognitive performance from cerebral contribution. Nonetheless, it points to a cerebellar influence on age-related cognitive changes and supports many previous findings relating the cerebellum to cognition that have been extensively summarized (Buckner, 2013; Schmahmann, 1991; Stoodley, 2012; Stoodley & Schmahmann, 2009).

Exactly how the cerebellum contributes to cognitive processing remains unclear but may involve internal model generation for proper integration of cognitive steps. Impaired internal model generation due to cerebellar damage would attenuate performance on challenging cognitive tasks that require manipulations using working memory. Indeed, collapse of the cerebellar-generated internal models that include prefrontal cortical regions in patients with cerebellar degeneration is associated with impaired performance on executive function tasks such as the initiation and preservation subtests of the Mattis Dementia Rating Scale, processing-heavy memory tasks (Appollonio et al., 1993), and the Tower of Hanoi cognitive planning task (Grafman et al., 1992). These deficits may relate directly to loss of cerebellar neurons that integrate afferent information. If this is the case, then age related-changes in cognitive performance may be directly attributed to cerebellar gray matter volume. Support for this contention has come from canines, where 42% of the variance in cognitive performance, as assessed by a battery of 9 cognitive tasks, was explained by cerebellar Purkinje cell (PC) number independent of aging (Pugliese, et al., 2007). This considerable standardized effect size of cerebellar gray matter on cognitive tasks suggests that cerebellar cellular integrity indeed contributes to the cognitive performance of animals.

Arising from the cerebellum's role in executive function, there's strong rationale for considering a cerebellar involvement in a host of non-aging related neuropsychiatric disorders in which cognitive dysfunction can modulate approach/avoidance behavior motivated by environmental stimuli. For instance, schizophrenic patients have difficulty coordinating and monitoring information, which in part may contribute to the delusions and disorganized thought that largely define the positive symptoms of the disorder. Evidence for a cerebellar role in "cognitive dysmetria" among schizophrenics comes from PET scans, which revealed dysfunction in the prefrontal-thalamic-cerebellar networks recruited during recall of a fictional story (Andreasen et al., 1996) independent of recall performance (Wiser et al., 1998). This aberrant functional connectivity was largely restored by the antipsychotic medication,

olanzapine (Stephan et al., 2001), supporting strong predictive validity of functional measurements to delineate schizophrenia-associated abnormalities. Cerebellar gray matter abnormalities, detected using MRI-based texture analysis (a morphological characterization of cellular homogeneity), may underlie the aberrant functional connectivity detected among schizophrenics (Radulescu et al., 2014). These studies allude to cerebellar-seeded functional connectivity in organizing and executing cognitive processes, perhaps by establishing a temporal context, of which a cerebellar timing role has been ascribed (Ivry et al., 1988), to enable proper cognitive step integration.

Cerebellar pathology has also been implicated in Asperger's syndrome and Autism Spectrum Disorder (Radulescu et al., 2012). In fact, the largest non-heritable risk factor for developing Autism Spectrum Disorder is injury to the premature cerebellum (Limperopoulos et al., 2007; Limperopoulos et al., 2014; Wang et al., 2014). Focal cerebellar injury during development affects downstream brain development of regions associated with autism symptoms such as the dorsolateral prefrontal cortex, the sensorimotor cortex, premotor cortex, and middle temporal cortex (Limperopoulos et al., 2014). Thus, abnormal cerebellum may promote autistic-like phenotypes through downstream modulation of cortical development.

A meta-analysis conducted by Stanfield et al. (2008) identified that individuals with Autism Spectrum Disorder have larger cerebellar hemisphere volumes than controls, but smaller vermis volumes. In adolescent autistic subjects, vermis volume is negatively correlated with IQ, suggesting that abnormal cerebellar morphology may drive dysregulated downstream cortical development similar to that seen with focal cerebellar injury. Cerebellar volume differences may also reflect differences in cerebellar cell expression in autistic individuals compared to healthy controls. Intriguingly, postmortem studies, albeit with relatively small sample sizes, detected reduced PC numbers and size in both the hemispheres and vermis (Fatemi, et al., 2002; Ritvo et al., 1986). The chimeric lurcher mouse provides insight into the consequences of incomplete PC loss. The degree to which these mice lose PCs during the

second through fourth weeks of life is negatively correlated with stereotypic motor behavior characteristic of autism in humans (Martin et al., 2010). Furthermore, mice with genetically disrupted PC signaling and reduced PC excitability have autism-like behavioral phenotypes such as increased repetitive behavior, attenuated social approach and reduced preference for social novelty (Tsai et al., 2012). Together, these studies suggest that deficits in cerebellar processing are common in Autism Spectrum Disorder and contribute to behavioral manifestation of symptoms including cognitive deficits.

fMRI has provided valuable insight into the functional cerebellar activation differences during both motor and non-motor tasks in subjects with autism. Autistic subjects showed enhanced cerebellar activation during a motor task and lower activation during a selective attention task than controls that persisted when matched for performance (Allen & Courchesne, 2003). The attenuated cerebellar response among autistic subjects during an attention task is consistent with cerebellar abnormality in subjects with attention-deficit/hyperactivity disorder (ADHD; Giedd et al., 2001; Krain & Castellanos, 2006), suggesting that subjects with an abnormal cerebellum have impaired timing abilities that may commonly contribute to neuropsychiatric impairment across specific conditions (Gowen & Miall, 2005; Halloran et al., 2012).

Common cerebellar dysfunction and abnormal morphology across a variety of neuropsychiatric disorders (Berquin et al., 1998; Jacobsen et al., 1997) suggests a general cerebellar functional contribution to diverse cognitive disorders. Cerebellar impairment to internal model generation and assessment may be a common feature of neuropsychiatric disorder, and therefore, may not play a role in the discriminating feature of any particular disorder. Unarguably, cortical and subcortical abnormalities largely contribute to the range of symptoms across neuropsychiatric disorders (Banaschewski et al., 2005). Nonetheless, it's intriguing that among similar disorders largely defined by attention deficits, such as ADHD, autism and dyslexia, there are distinct cerebellar regions of gray matter loss for each disorder

within the hemispheres and vermis (Stoodley, 2014). These findings suggest that focal cerebellar deficits contribute to the specific behavioral and cognitive deficits associated with particular disorders.

In a broad assessment of the neuropsychiatric deficits associated with region-specific congenital cerebellar lesions, Tavano et al. (2007) concluded that the cerebellar vermis contributes to affective and social disruption (e.g., autism) whereas abnormalities in the hemispheres are associated with impairment of executive function, visuospatial abilities, and language. So if dysfunction in particular cerebellar subregions is responsible for the specific behavioral deficits associated with particular disorders (e.g., autism but not schizophrenia), then the question becomes: do aspects of a particular disorder's symptomology derive from specific cerebellar pathology? Despite the limited available evidence, data from children following removal of cerebellar tumors have provided insight into the role of particular cerebellar regions in neuropsychiatric disorders. In 7 to 13 year old children, removal of part of the right cerebellar hemisphere led to deficits in auditory sequential memory and language impairment whereas post-operative evaluation following left hemisphere surgeries found deficits in spatial and visual sequential memory. Children who underwent vermal surgeries presented with speech and language disturbances as well as behavioral disturbances on a battery of tests, consistent with symptoms expressed by autistic children (Riva & Giogi, 2000). These behavioral changes occur in otherwise normal children and likely reflect the cerebellar role in mediating these behaviors. In a case study of a patient with treatment resistant schizophrenia, transcranial magnetic stimulation of the vermis exacerbated his auditory verbal hallucinations (Garg et al., 2013). While the mechanism of this effect is unclear, the alteration in cerebellar output likely promoted hyperexcitability of the tempoparietal areas that are associated with auditory verbal hallucinations (Lennox et al., 2000). Together, these studies suggest that disruption to specific regions of the premature and mature cerebellum can cause or exacerbate symptoms of neuropsychiatric disorders characterized by abnormal cognitive processing.

Can normalizing cerebellar function or growth alleviate neuropsychiatric symptoms? In mice, restoration of PC excitability alleviated autism-like behaviors (Tsai et al., 2012). In humans, transcranial stimulation of the cerebellum vastly improved a variety of neuropsychiatric disorders in 10 out of 11 patients (Heath, 1977). However, consistent with similar studies, this study was underpowered, without controls, and assessed many different behavioral symptoms, thereby limiting the ability to isolate the cerebellar effect on a particular disorder or symptom. In a larger longitudinal assessment of 36 patients with ADHD, those who demonstrated behavioral improvement had similar developmental trajectories of cerebellar growth, particularly in the inferior-posterior hemispheres, than those who showed little improvement (Mackie et al., 2007). In a handful of studies reviewed by Schweren et al. (2013), ADHD treatment with methylphenidate normalized cerebellar structural and functional differences assessed by MRI. These findings are intriguing as they may reflect cerebellar-specific plasticity that's associated with neuropsychiatric disease status. Perhaps due to the prevalent underappreciation of the role of the cerebellum in non-motor function, cerebellar-specific assessments of neuropsychiatric disorders are uncommon, especially at the cellular level, and often provide only indirect measures of a neuropsychiatric disorder. In schizophrenics, restoring cortico-thalamo-cerebellar circuits by olanzapine, as discussed above, is associated with attenuated cognitive dysmetria (Stephan et al., 2001) perhaps through stabilization of cerebellar blood flow (Bellani et al., 2011). Although this suggests that restoration of functional connectivity involving the cerebellum attenuates impairment of executive function, the effects of olanzapine are not specific to the cerebellum, nor did the authors conduct a cerebellar-specific morphological or cellular assessment following treatment. In part, this is due to the spatial resolution limitations of fMRI combined with the complexity of cerebellar folia that make cortical thickness measurements and layer-specific assessment practically impossible in a human (Moulton et al., 2014).

To bypass the limitations of non-invasive imaging techniques in humans, animal models must be used. In a rat model of ADHD (spontaneously hypertensive rats display the major

symptoms of ADHD such as hyperactivity), treadmill exercise prevented the loss of PCs, which was associated with superior performance on the vertical bar test, a cerebellar-dependent measure (Yun et al., 2014). This behavioral improvement has strong construct validity, considering the well documented cerebellar-dependent motor deficits in ADHD children (Pitcher et al., 2003), but a stronger link could have been made for a direct cerebellar cell-specific role in ADHD had the authors also measured attention in these rats, for example using a five-choice serial reaction time test. Exercise can also enhance learning and memory that's paralleled by increases in hippocampal neurogenesis and lead to elevated gray matter levels in the prefrontal cortex (Erickson et al., 2013), therefore exemplifying the non-cerebellar-specific effects of exercise and hindering the ability to pin non-motor related behaviors of ADHD solely on PC rescue. Nonetheless, the evidence in support of cerebellar pathology involvement in neuropsychiatric disorders, as well as plastic changes associated with improvement in symptoms, provides substantial rationale for pursuing the cerebellum as an intervention target for intractable behavioral and cognitive disorders.

1.5. Cerebellar role in addiction and alcohol use disorder

The cerebellar role in cognitive impairments associated with neuropsychiatric disorders hints at potential involvement in other behavioral disorders such as drug addiction. Indeed, over the past two decades, significant cerebellar findings have been common across neuroimaging studies of addictive drugs (in part due to improved imaging software) and behaviors (e.g. internet gaming [Ding et al., 2013]), but they're often marginalized or even completely ignored in favor of promoting the better understood, and historically traditional, motivational circuitry (Moulton et al., 2014). This occurs despite known anatomical connections between the cerebellum and aspects of the reward circuitry including the ventral tegmental area (Ikai et al., 1992; Ikai et al., 1994), basal forebrain (Albert et al., 1985), and striatum (Delis et al., 2013) from which a cerebellar role in motivated behavior can be inferred. Nonetheless, support for a

cerebellar role in addiction has amassed from converging evidence from human clinical and neuroimaging studies associating cerebellar structural and functional variation with risk and acquisition of addiction behavior.

Similar cerebellar structural deficits are commonly detected in addicts of a variety of abused substances. Lower gray matter volumes, and often white matter volumes, are found primarily in the posterior cerebellum in addicts of cocaine (Sim et al., 2007), heroin (Lin et al., 2012), nicotine (Brody et al., 2004; Gallinat et al., 2006), and EtOH (Sullivan et al., 2000). One strategy to assess the relationship between the cerebellum and addiction is to correlate gray matter volume with addiction severity. Using this approach, Kuhn et al. (2012) identified a negative correlation between gray matter volume in the right cerebellar hemisphere cerebellar and severity of nicotine dependence in smokers. A similar approach found that cerebellar gray matter volume was inversely related to duration of cocaine addiction (Sim et al., 2007). Together, these studies suggest that structural differences in the cerebellum are reliably detected in drug addicts for a variety of drugs and the extent to which they deviate from normal is associated with severity of drug use. However, as with most retrospective human imaging studies, they cannot identify if the reduction in cerebellar volume is a consequence of drug use or a predisposing risk factor.

To gain insight into cerebellar contributions to development of drug addiction, independent of drug exposure, high risk populations are compared against low risk populations. Risk for developing a drug addiction is determined from an interaction between genetic and environmental factors (Jacob et al., 2003) that promote particular pre-drug exposure behavioral phenotypes such as high impulsivity, disinhibition, and under-control (Kreek et al., 2005; Tessner & Hill, 2010), many of which are directly influenced by cerebellar function (Hester & Garavan, 2004; Chambers et al., 2009; Moers-Hornikx et al., 2009). Cerebellar-dependent risk factors can promote abuse and dependence of a variety of abused substances (e.g., cocaine [Anderson et al., 2002]), and indeed many addicts are poly-substance abusers due to some

overlap between risk factors that promote drug use (Enoch & Goldman, 1999). However, my thesis work centers around EtOH, and so the remainder of this review will focus primarily on AUDs.

Twin and adoption studies have suggested that susceptibility to AUDs is 40-60% genetically determined (Enoch & Goldman, 1999; Prescott & Kendler, 1999; Hill, 2010), with the remainder attributed to environmental factors that are not shared by siblings. Some genetic risk is expressed in functional and morphological variation in the cerebellum. EtOH-naïve high-risk males with a family history of alcoholism (FH+; i.e., offspring of EtOH-dependent individuals and with two or more dependent first-degree relatives) had reduced cerebellar gray matter, which negatively correlated with the degree of externalizing behavioral risk factors (Benegal, et al. 2007). However, Hill et al. (2011) found the opposite; high-risk adolescents had greater gray matter volume than low risk adolescents, potentially driven by the interaction between particular alleles associated with the α2 subunit of the $GABA_A$ receptor ($GABA_AR$) and brain-derived neurotrophic factor. This contradiction could result from inconsistency across the studies in participant characteristics such as age range, EtOH use history, genotype, or different criteria for the “high risk” population, but collectively the studies suggest a strong genetic link between cerebellar morphology and family history of AUDs.

At a broader level, the contradiction in cerebellum volume studies may be indicative of the heterogeneity of risk factors that promote AUDs. Abnormal cerebellar volumes may promote risk for developing AUDs via different mechanisms depending on whether there’s increased or decreased volume compared to the low-risk population. In control subjects and alcoholics, vermis volume inversely correlates with general static ataxia (i.e., body sway, a form of cerebellar dependent motor impairment; Sullivan et al., 2006). Additionally, EtOH’s actions in the vermis contribute to ataxia (Dar, 2015) and thus, sensitivity to EtOH-induced static ataxia may be a behavioral marker of anatomical abnormality. Sensitivity to EtOH-induced static ataxia has been used as a measure of the cerebellar low-level of response (LLR) phenotype, which is

defined as requiring a higher dose of EtOH to achieve a given effect, and may reflect one functional correlate of risk for AUD development. The LLR phenotype is detected in about 40% of individuals with a FH+ for alcoholism compared to only 10% in those with a FH negative (FH-) for alcoholism (Schuckit & Gold, 1988). Indeed, young men with a FH+ for alcoholism were found to have lower EtOH-induced body sway scores at two different EtOH doses than those with a FH- for alcoholism (Schuckit, 1985). In a follow-up investigation eight years later, those who went on to develop an AUD, independent of FH, had lower EtOH-induced body sway scores during the initial test (Schuckit, 1994), suggesting that the cerebellar LLR is a genetically heritable trait that is associated with increased risk for developing an AUD in a subset of humans.

In support of the above contention, the magnitude of EtOH-induced cerebellar ataxia is inversely related to EtOH consumption and preference phenotypes in some inbred strains of mice (Gallaher et al., 1996) and in rats selected for differences in EtOH consumption (Malila, 1978; Bell et al., 2001). For example, C57BL/6J (B6) mice are behaviorally insensitive to EtOH-induced ataxia and have a high oral EtOH consumption phenotype, while DBA/2J (D2) mice are highly sensitive to EtOH-induced ataxia and have a low-EtOH consuming phenotype (Gallaher et al., 1996; Rhodes et al., 2007; Yoneyama et al., 2008). Based on human and rodent studies, the LLR phenotype has emerged as a validated model of AUD risk that encompasses cerebellar response phenotypes, as well as other response phenotypes, such as subjective responses to EtOH (Quinn & Fromme, 2011), which may or may not have a cerebellar contribution. According to this model, FH+ individuals generally report requiring more EtOH to achieve positive hedonic feelings than FH- individuals (Schuckit, 2009), and thus, end up drinking more to achieve the desired effect. While a cerebellar contribution to these additional components of the LLR has not been directly linked, it's intriguing that a polymorphism of the α6 subunit of the $GABA_AR$, found exclusively in cerebellar granule cells (De Blas, 1996; Laurie et al., 1992; Luddens et al., 1990; Pirker et al., 2000; Wisden et al., 1992) and the cochlear nucleus (Nusser et al., 1994;

Varecka et al., 1994), was linked to the LLR phenotype and alcoholism (Schuckit et al., 2009; the functional consequence of genetic polymorphism of the α6 subunit is described in section *1.10*). Thus, there appears to be genetic, structural, and behavioral evidence in favor of a heritable cerebellar-related contribution to the LLR phenotype that is associated with increased risk for developing alcoholism.

The differentiator model (DM) is an alternative model to describe heritable risk for alcoholism. The DM derives from the premise that the experience of EtOH's effects changes over time (King et al., 2002) and proposes that differences in the subjective responses to EtOH between FH+ and FH- individuals must be understood in the context of increasing or decreasing blood EtOH concentration (BEC). According to the DM, FH+ individuals are more sensitive to the positive stimulatory effects of EtOH that coincide with the ascending BEC, but are less sensitive to its negative sedative effects that coincide with a declining BEC (Newlin & Thomson, 1990). Thus, FH+ individuals are more likely to experience stronger rewarding effects and attenuated aversive or negative effects from EtOH. The LLR and DM models are not necessarily mutually exclusive, but could represent independent populations of heritable risk for AUDs (Morean & Corbin, 2010; Quinn & Fromme, 2011). Two independent pathways could help account for the contrasting cerebellar volume studies in FH+ individuals (Benegal, et al. 2007; Hill et al., 2011).

There is indirect evidence that exposes a cerebellar role in both the LLR and the DM. An intriguing fMRI study of controlled heroin exposure in opiate addicts identified a direct correlation between cerebellar activation and subjective report of "feeling tense" and "withdrawal feelings" (Sell et al., 2000). These heroin studies reveal a cerebellar influence in negative emotional processing, consistent with functional (Cservenka et al., 2014) and anatomical connectivity (Heath et al., 1978) between the cerebellum and the amygdala. In healthy individuals, unpleasant images and acute pain invoke distinct cerebello-amygdalar activation patterns (Moulton et al., 2011). Cerebellar connections with the amygdala enable a cerebellar

influence on the negative aspects of EtOH seeking and withdrawal, which has been shown to be disrupted in youth with a FH+ for alcoholism (Cservanka et al., 2014). Thus, distinct sensorimotor and emotional networks of aversion-related circuitry in the cerebellum are present before and after the development of addiction and may mediate genetic differences in negative aspects of drug exposure.

Additionally, functional connectivity deficits between the cerebellum and anterior prefrontal cortex networks are disrupted in FH+ individuals (Herting et al., 2011), likely further contributing to impairments in the assessment of striatal and amygdalar inputs to prefrontal regions for stimuli-related decision making (Ernst et al., 2009; Koechlin et al., 2003). The sensitivity of these networks to EtOH could differ between FH+ and FH- individuals in which the cerebellum contributes similarly to both LLR and DM models. Extending this idea, the cerebellar LLR may be a behavioral correlate of the subjective response to EtOH during the descending limb of the BEC. Research is needed into this cerebellar role as it may serve to link the genetic contributions to both the DM and LLR risk models. However, because the cerebellar link to the LLR phenotype has been well supported (e.g., Schuckit, 1985) and validated (Quinn & Fromme, 2011), the remainder of this dissertation will discuss heritable risk for developing AUDs in terms of the LLR model.

In summary, there is substantial heritable risk for developing AUDs that is associated with abnormal cerebellar anatomy and function. FH+ individuals have abnormal cerebellar volume and impaired functional connectivity with the prefrontal cortex and amygdala. The resulting aberrant processing may contribute to the subjective and functional components of the LLR. So while genetic contributions to risk for AUDs have been represented anatomically, functionally, and behaviorally, a clear understanding of the cellular substrates of the cerebellar LLR during acute EtOH exposure is lacking.

1.6. Cerebellar cortical circuitry

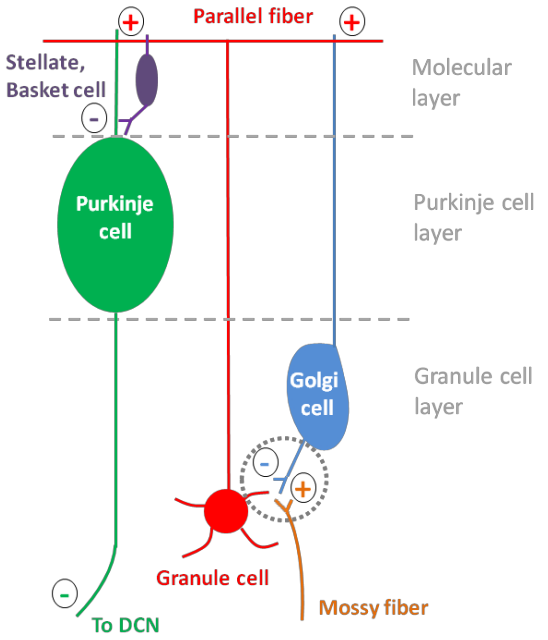

Figure 1. Schematic of the cerebellar cortex circuitry. Purkinje cells, Golgi cells, and the molecular layer interneurons (stellate and basket cells) are GABAergic. Mossy fibers, originating in the pontine nuclei of the brainstem, and granule cells are glutamatergic. Mossy fibers synapse in a glomerulus (dotted circle) with terminals from Golgi cells and granule cell dendrites. "+" signs represent glutamatergic excitation and "-" signs represent GABAergic inhibition.

The cerebellar circuitry (Fig. 1) is evolutionarily conserved across mammals (Hashimoto & Hibi, 2012), with differences among species being mainly of a quantitative nature rather than in the qualitative organization of the circuits (Hamori, 1992). Consequently, there is strong construct validity in using lower-order species such as mice and rats to understand human cerebellar circuits. The conservation of this circuitry is important since the cerebellum's ability to integrate sensory and predictive inputs, create internal models, and promote cognitive and emotional functioning (Ito, 2008) relies on highly structured and consistent cerebellar circuitry. The cerebellar cortex is the largest target of afferent information to the cerebellum. It is comprised of 10 lobules (lobes I-X; in both humans and rodents), each with three consistent cellular layers: the PC layer, the molecular layer, and the granule cell (GC) layer (Butler & Hodos, 2005). Afferent information to the cerebellar cortex reside in white matter tracks comprised of climbing fibers, originating in the inferior olive, and mossy fibers, originating in the pontine nuclei of the brainstem. The ultimate destination of these afferent signals is the PC, the primary or main integrator cell, and sole output of the cerebellar cortex. PC somas are oriented in a row of cells 2-3 cells thick, which parallels the topography of the pial surface of each lobe, thereby forming the PC layer. PCs have an extensive dendritic tree that branches 2-

dimensionally along the sagittal plane into the molecular layer. Here, they receive synaptic inputs from the GABAergic molecular layer interneurons, stellate and basket cells, and the glutamtergic parallel fibers emanating from the GCs. Parallel fibers are GC axons that have projected distally from the GC layer into the molecular layer, where they bifurcate, and run in both directions along the entire mediolateral length of the cerebellum, forming excitatory synapses on the sagittally oriented PC dendrites, molecular layer interneurons, and Golgi cell dendrites (Hamori, 1992). In turn, Golgi cell interneurons provide GABAergic feedback and lateral inhibition to GCs, and molecular layer interneurons provide lateral and feedforward inhibition to PCs. Each PC is influenced by ~10^5 GCs (De Shutter & Bower, 1994), the most numerous neuron type in the brain. GCs are the primary neuron type in the GC layer, but importantly, GABAergic Golgi cells (of which there's significant heterogeneity), serotonergic/GABAergic/glycinergic Lugaro cells (Simat et al., 2007), GABAergic/glycinergic globular cells, and glutamatergic unipolar brush cells (Rossi et al., 1995) are also diffusely distributed within the GC layer. Together, this consistent pattern of neuronal types throughout the three layers of the cerebellar cortex interact to process afferent input, culminating in integration by PCs to promote appropriate output responses.

PCs are pacemaker cells, in that in the absence of excitatory synaptic input, an oscillating interplay between voltage-gated calcium channels and calcium-activated potassium channels promote a tonic PC action potential (AP) firing rate greater than 50 Hz (Edgerton & Reinhart, 2003; Person & Raman, 2012b). However, the PC firing pattern can be altered by two sources of afferent input that originate outside the cerebellum. The first source comes directly from climbing fibers; each PC receives input from a single climbing fiber that makes approximately 1500 synapses onto its large proximal dendrites (Schmolesky et al., 2002). Each AP-driven synaptic input from the climbing fiber induces a powerful complex spike consisting of a cluster of calcium and sodium current-mediated APs that transiently but powerfully interrupts ongoing spontaneous AP firing, and plays an important role in synaptic plasticity associated with

cerebellar-dependent learning (Otsu et al., 2014; Wang et al., 2014; Yan & Lisberger, 2014). Thus, the climbing fiber input is considered a form of all or nothing error signal, with little flexibility for subtle modulation or integrative capacity.

The second source of afferent input is conveyed to PCs indirectly by glutamatergic mossy fibers which synapse in GC layer glomeruli to excite GCs and consequently promote parallel fiber glutamate release onto PCs and other molecular layer interneurons. Early description of the cerebellar glomerulus using electron microscopy identified that it is a complex synaptic region comprised of a centrally located mossy fiber terminal (~5µM in diameter) surrounded by a corona of granule cell dendrites and Golgi cell axon boutons (Hamori & Szentagothai, 1966). In one sample of a rat's vermis, there were approximately 50 GC dendrites and 230 synaptic junctions per glomerulus (Hamori et al., 1997). Thus, the cerebellar glomerulus is an example of synaptic divergence, in which a centrally positioned mossy fiber terminal (Hamori & Szentagothai, 1966) excites many GCs.

Parallel fiber activation of GABAergic stellate and basket cells in the molecular layer provide feed-forward inhibition of PCs, while providing negative feedback on the mossy fiber-GC relay by exciting Golgi cell basolateral dendrites in the molecular and GC layers (Eccles et al., 1966; Cesana et al., 2013). Importantly, PCs and Golgi cells have distinct sources of inhibitory input: stellate and basket cells inhibit PCs, while Golgi cells inhibit each other and GCs (Hull & Regehr, 2012). This enables GCs to drive distinct feed-forward and feedback inhibition mechanisms within the cerebellar cortex.

Activation of nicotinic acetylcholine receptors on Golgi cell terminals within glomeruli (presumably via paracrine transmission from diffuse cholinergic afferents) provides an additional source of inhibitory regulation within the cerebellar cortex (Eccles et al., 1966; Rossi et al., 2003). The multiple mechanisms of Golgi cell excitation enables temporally precise and controlled negative feedback on the mossy fiber-GC relay critical for normal cerebellar function. Indeed, simultaneous recordings of Golgi cells and nearby mossy fibers in awake squirrel

monkeys identified that Golgi cells show delayed and opposite directional tuning to the mossy fibers (Heine et al., 2010), suggesting that although mossy fiber and GC inputs drive Golgi cell activity, Golgi cell firing patterns are consistently out of phase relative to mossy fiber inputs, which may carry a motor command or cognitive process. Thus, Golgi cells likely sharpen temporal transitions of signaling frequency of each command through the cerebellar cortex. Their importance is demonstrated by the disrupted synaptic integration and severe cerebellar ataxia that follows acute Golgi cell ablation (Watanabe et al., 1998). The functional impact of negative feedback on mossy fiber-stimulated transmission through the cerebellar cortex will be discussed in further detail in Chapter 4.

1.7. Granule cells and $GABA_AR$ inhibition

Cerebellar GCs are the most numerous cell type in the brain (Llinas et al., 2004) and each GC is innervated by ~4 afferent mossy fibers, each generally forming high fidelity excitatory synapses, each capable of generating GC APs (Rancz et al., 2007). Even high frequency stimulation of presynaptic mossy fiber boutons at 500 Hz generates GC APs with only 30% depression in the net charge transfer, suggesting high-fidelity synaptic transmission between mossy fibers and GCs during presynaptic burst activity. However, an excitatory GC response to mossy fiber input only partly describes the fidelity of the mossy fiber-GC relay; a high firing frequency of the 50 billion GCs would impair the PCs' ability to three-dimensionally integrate peripheral input delivered via the mossy fiber-GC relay.

To maintain the fidelity of afferent transmission through the cerebellar cortex, GCs are inhibited by two forms of $GABA_AR$-mediated inhibition: 1) GCs exhibit traditional phasic inhibitory post-synaptic currents (IPSCs) mediated by synaptic $GABA_ARs$ containing α1β2/3γ2 or α6β2/3γ2 subunits. These phasic currents rapidly and transiently inhibit the GC, which is important for timing based signaling ("temporal fidelity"; Pouille & Scanziani, 2001) and synchronizing neuronal networks (Cobb et al., 1995), both of which are critical elements of

cerebellar processing. 2) GCs also exhibit a tonic form of inhibition mediated by high-affinity non-desensitizing extrasynaptic $\alpha_6\beta_{2/3}\delta$ subunit-containing $GABA_A$Rs (Hamann et al., 2002; Meera et al., 2011; Nusser et al., 1998; Rossi et al., 2003; Saxena & Macdonald, 1996; Stell et al., 2003; Wall & Usowicz, 1997). The δ-subunit is developmentally expressed in cerebellar GCs, establishing the presence of a tonic $GABA_A$R current around postnatal day 7 in rats (Wall & Usowicz, 1997). The δ-subunit confers high GABA affinity properties, and α6δ-subunit containing $GABA_A$Rs, found exclusively in the cerebellum and inferior colliculus (Laurie et al., 1992; Luddens et al., 1990; Saxena & Macdonald, 1996), are the most sensitive δ-subunit containing receptors in the brain (Mortensen et al., 2012). The high affinity of these receptors make them ideal for detecting low levels of ambient GABA, and generating a tonic inhibitory conductance carried by chloride ions. Indeed, enzymatic degradation of GABA attenuated the magnitude of the tonic inhibitory current (Wall & Usowicz, 1997), suggesting that the primary contributor of tonic inhibition is GABA as opposed to the spontaneous opening of extrasynaptic receptors seen in other brain regions (Birnir et al., 2000). Because of the compact nature of GCs (i.e. small soma volume, few dendrites), they have a high input resistance, so only a small change in membrane conductance is needed to powerfully influence the membrane potential. Consequently, the tonic $GABA_A$R conductance powerfully inhibits GCs, contributing three-times more of the total inhibitory charge of the GC than phasic IPSCs mediated by synaptic $GABA_A$Rs. This in turn reduces spontaneous GC activity (Hamann et al., 2002) and optimizes the contrast between spontaneous and mossy fiber activated GC input to PCs important for optimal cerebellar processing (Duguid et al., 2012).

Semyanov et al. (2004) reviewed the sources of ambient GABA that contribute to the GC tonic $GABA_A$R current, describing both AP-dependent and –independent mechanisms, some of which are still under debate. The generation of the tonic current is driven largely by vesicular GABA release from Golgi cells, which spills over from the synapse to provide a constant source of ambient GABA (Hamann et al., 2002; Rossi et al., 1998; Rossi et al., 2003). Further glial

encapsulation of the glomerulus during early adolescence in rats (~>35 days postnatal) increases the ambient GABA concentration and the magnitude of the tonic $GABA_AR$ current (Hamori & Somogyi, 1983; Wall & Usowicz, 1997). While AP-dependent vesicular GABA release may contribute to the ambient GABA concentration in the glomerulus, AP-independent GABA release can be modulated by acetylcholine (Rossi et al., 2003). Acetylcholine activates receptors on Golgi cell terminals, causing vesicular GABA release in a Ca^{2+}-dependent manner (Rossi et al., 2003). In these experiments, tetrodotoxin (TTX), a sodium channel antagonist, failed to block acetylcholine-stimulated GABA release, confirming its AP-independent nature. Nearly 85% of this acetylcholine-stimulated tonic current was driven by vesicular GABA release since concanamycin, a blocker of vesicular release largely attenuated the magnitude of the tonic current and completely abolished spontaneous IPSCs (sIPSCs). These studies suggest that a change in the magnitude of the tonic $GABA_AR$ current is coupled to a change in the frequency of sIPSCs. As the frequency in sIPSCs increases, so does the concentration of ambient GABA that spills over from the synapse and activates the extrasynaptic $GABA_ARs$ that mediate tonic inhibition. Thus, tonic inhibition of GCs is determined by multiple means of vesicular GABA release from GABAergic Golgi cells. However, a recent paper proposed that phasic spillover and tonic inhibition are mediated by distinct receptor mechanisms (Bright et al., 2011). While this report has yet to be independently replicated, one potential source of non-spillover derived ambient GABA could be through Best1 anion channels on neighboring glial cells, such as lamellar astrocytes and Bergmann glia (Lee et al., 2010). However, other groups failed to replicate these results, finding that Best1 channel antagonists actually potentiated the tonic $GABA_AR$ current amplitude (Diaz et al., 2012; Rossi et al., 2003). Thus, the sources of ambient GABA and their relative contributions remain a debate.

There are a few potential explanations that could underlie the inconsistencies in GABA sources that mediate the GC tonic $GABA_AR$ current across the literature. Some are experimental in nature such as the age of the animal and tissue health. The studies

investigating developmental regulation of glomeruli development (Hamori & Somogyi, 1983) and GABAergic transmission (Wall & Usowicz, 1997) using electron microscopy and patch-clamp electrophysiology in rats between postnatal days 6-40 have identified large developmental changes in glomerular structure that coincided with the transition from TTX-sensitive (i.e. AP-dependent) phasic sources of inhibition to TTX-insensitive (i.e. AP-independent) sources of tonic inhibition (Hamann et al., 2002; Rossi et al., 1998; Rossi et al., 2003; Wall & Usowicz, 1997). Therefore, within even a narrow few-day window during this developmental period, an experimenter could conceivably obtain drastically different results despite the same experimental protocol. This is a commonly studied animal age range for slice electrophysiology because *ex vivo* tissue health is better in younger animals, which allows for longer and more stable recording periods. Since most papers only report age ranges of the animals they used, and not mean ages, one potential explanation for the inconsistency across laboratories is simply due to subtle variation in the mean age of the animals used in their analyses which coincide with varying stages of glomerular development and δ subunit expression.

Another potential explanation stems from variation in slice tissue integrity across laboratories. A reduction in tissue integrity could subtly disrupt neurotransmitter transports, reuptake mechanisms, release machinery, and glomeruli structure. This, in turn, could bias conclusions regarding GABA release, binding, and reuptake. Tissue integrity can be highly variable as a result of differences in experimenter skill and procedure variation in brain removal, slicing, and post-slicing incubation. Neurons in the cerebellar cortex, in particular PCs, are highly sensitive to ischemic damage (Welsh et al, 2002), and factors such as young age and low temperature delay the onset of ischemic damage (Mohr et al., 2010). So the speed of tissue removal, the age of the animal, and the duration of hypoxia outside of the ice-cold preparation bath, are all factors that are rarely reported in methods sections but could contribute to slice tissue integrity. Additionally, the quality of the vibratome for slicing brain tissue can drastically alter tissue integrity by physically damaging cells and their connections. In particular, the

interaction between poor vibratome quality with the age of the animal could conceivably alter tissue quality. Although lacking experimental verification, poor vibratome quality during early investigations of tonic $GABA_AR$ currents may have contributed to the predominately TTX-insensitive (i.e. AP-independent) tonic $GABA_AR$ current detected in older animals. Consistent with this hypothesis, a recent assessment of adult mice (3-6 months), found that the most significant contribution to the ambient GABA concentration came from a TTX-sensitive source (Ye et al., 2013). Other factors such as neurosteroid levels and phosphorylation status of extrasynaptic $GABA_ARs$ may also be affected by subtle procedural variations, thereby influencing GABAergic transmission. These factors will be discussed in more detail below.

1.8. Neurosteroids and protein kinase C modulation of $GABA_ARs$

In addition to ambient GABA concentration, tonic $GABA_AR$ inhibition can be strongly modulated by receptor phosphorylation and neurosteroid binding. Neurosteroids are steroid derivatives that exert rapid effects as neuromodulators of ligand-gated ion channels. They can be either brain-derived or synthesized from peripheral precursors by developmentally-regulated enzymes in both neurons and glia (Compagnone & Mellon, 2000). Therefore, similar developmental regulation of glial encapsulation of the cerebellar glomerulus could affect neurosteroid levels and, in turn, GABAergic signaling. Common neurosteroids can be positive modulators of $GABA_ARs$, and the progesterone derivative, allopreganalone, is a potent positive allosteric modulator of δ-subunit containing $GABA_ARs$ (Barbaccia et al., 2001; Finn et al., 2004; Herd et al., 2007; Stell et al., 2003; Ye et al., 2013). Stressors such as EtOH exposure and withdrawal, forced swim, or simply injection of stress-related hormones CRH and ACTH, have been shown to increase allopregnanolone and its metabolic precursor, pregnenolone (Purdy et al, 1991; Tanchuck et al., 2009; Tokuda et al., 2011; Torres et al., 2001; Vallee et al., 2000). These findings have relevance in the context of pre-surgery handling-induced stressors, or the time-to-anesthesia onset, which can be highly stress-inducing. Furthermore, since neurosteroid

levels are highest in females, these factors can have a sex-specific impact on $GABA_AR$ transmission that varies across the menstrual cycle (Paul & Purdy, 1992). This was justification for restricting the investigations presented in Chapters 4 and 5 to male animals.

Importantly, the potency of neurosteroids and other drug actions at $GABA_ARs$ can be modulated by kinase-catalyzed phosphorylation of $GABA_ARs$, thus representing a dynamic mechanism regulating $GABA_AR$ transmission (Belelli & Lambert, 2005; Fanscsik et al., 2000; Kia et al., 2011). Kinases catalyze the transfer of a phosphate from ATP to protein molecules, such as neurotransmitter receptors, providing an important post-translational modification mechanism. Protein kinase C (PKC) is one such kinase that can regulate $GABA_AR$ activity (Song & Messing, 2005) in a manner that's dependent on PKC isoform and $GABA_AR$ subunit (Abramian, et al., 2010; Choi et al., 2008; Connelly et al., 2013; Kumar et al., 2005; Qi et al., 2007). Many types of kinases exist throughout the brain (cyclic AMP, tyrosine kinase, PKA), but due to its importance in mediating EtOH's effects, I'll be focusing on PKC in this dissertation.

PKC is expressed throughout the brain including the cerebellar cortex (Naik et al., 2000). One role of PKC-mediated phosphorylation is to regulate $GABA_AR$ membrane-surface density (Connolly et al., 1999). In cultured cerebellar GCs, enhancement of PKC activity increased receptor cycling and endocytosis (Balduzzi et al., 2002). However, this study did not identify the particular subunit composition of these $GABA_ARs$, thereby preventing us from isolating the contribution of PKC-mediated phosphorylation on extrasynaptic versus synaptic $GABA_AR$ cycling, which can be phophorylated by different PKC isoforms (Kia et al., 2011). Bright and Smart (2013) provided insight from the hippocampus and thalamus, finding that PKC activity reduced the number of α4β2δ subunit-containing $GABA_ARs$ from the membrane surface of dentate gyrus GCs and dorsal lateral geniculate thalamic relay cells. However, it's unclear if PKC similarly modulates $GABA_ARs$ comprised of different α,δ subunit combinations, and so it remains unknown if this effect would occur in the cerebellum where GCs express δ subunit-containing $GABA_ARs$ paired with the α6 subunit.

Studies looking at the role of PKC on neurosteroid- and EtOH-induced tonic $GABA_AR$s suggest that there are indeed differential effects of PKC-mediated phosphorylation, depending on the particular $GABA_AR$ subunits and specific PKC isoforms. There is a diverse set of at least 9 PKC isozymes (α, β, γ, δ, ε, η, θ, and ι; Popp et al., 2006) which have different expression profiles and phosporylation substrates. Among these, δ, ε, and γ can be activated by calcium and/or the second messenger signaling lipid, diacylglycerol (Song & Messing, 2005) and have been proposed to regulate drug and neurosteroid action in the central nervous system (Lee & Messing, 2008). Phosphorylation by the δ PKC isoform confers $GABA_AR$ sensitivity to the neurosteroid, pregnanolone (Choi et al., 2008). Importantly, PKCδ knockouts had similar tonic $GABA_AR$ current baseline amplitudes, suggesting that phosphorylation of PKCδ may be most important for regulating the effect of drug actions, and not necessarily basal $GABA_AR$s activity. In contrast, PKCε knockout mice showed increased sensitivity to allopregnanolone's sedative effects and enhancement of muscimol stimulated chloride uptake in cortical microsacs (Hodge et al., 1999; Hodge et al., 2002). Thus, phosphorylation of $GABA_AR$s by specific PKC isoforms can have opposing actions on the sensitivity to neurosteroids. As a result, modulation of $GABA_AR$ receptor properties induced by manipulating PKC activity will vary widely depending on expression of various PKC isoforms across brain regions. For instance, broad inhibition of PKC activity reduced the effect of allopregnanolone on the slow decay time constant of supraoptic nucleus magnocellular neurons (Fanscsik et al., 2000), while activation of PKC attenuated the effects of the deoxycorticosterone neurosteroid derivative, THDOC, on tonic currents in piriform cortex pyramidal cells (Kia et al., 2011). Together, these findings suggest that $GABA_AR$ phosphorylation by PKC can dynamically alter the response of $GABA_AR$s to various ligands and modulators that is dependent on specific regional expression of PKC isoforms and their interaction with $GABA_AR$s containing particular subunits (Harney et al., 2003; Kumar et al., 2009; Qi et al., 2007; Song & Messing, 2005; Vicini et al., 2002; Weiner et al., 1997). For *in vitro* slice recordings, PKC activity may differ across experiments due to a variety

of factors including genetic-regulated expression, ATP levels in the intracellular solution, animal stress *in vivo*, or abnormal neuronal firing patters during tissue extraction and slicing. Thus, even slight inter-lab variations in any of these factors may, via impacts on PKC activity, fundamentally alter $GABA_AR$ activity or their sensitivity to various modulatory ligands (including neurosteroids and EtOH), thereby contributing an additional factor to the inter-lab variability in the detection of tonic $GABA_AR$ current characteristics.

1.9. Interaction between PKC and EtOH

Many of EtOH's acute and chronic effects on behavior and cognitive function are mediated by $GABA_ARs$ (Kumar et al., 2009), and can be regulated by intracellular signaling pathways involving PKC (Newton & Messing, 2006; Stubbs & Slater, 1999). The interaction between PKC and EtOH is bidirectional; EtOH can stimulate PKC activity (Coe et al., 1996; Messing et al., 1991; Tokuda et al., 2011), but PKC can also alter $GABA_AR$ sensitivity to EtOH at the behavioral and electrophysiological levels, and in an isoform- and region-specific manner. For example, PKCγ knockout mice exhibited a shorter EtOH-induced loss of righting reflex and reduced EtOH and muscimol-stimulated chloride uptake in a cerebellar membrane preparation (Harris et al., 1995). One interpretive limitation with the muscimol stimulated chloride uptake preparation is that it is not cell type-specific, and while PKCγ mRNA may be limited to PCs (Brandt et al., 1987; Nishizuka et al., 1991), this study was unable to directly determine if the reduction in EtOH-stimulated chloride uptake in PKCγ knockouts was limited to PCs.

Additional isoform knockouts have found similar effects on EtOH-mediated behavior. PKCδ knockout mice were less sensitive to EtOH-induced impairment of rotarod performance, a measure of cerebellar ataxia, than wild-type mice. Because there were no genotypic differences in their sensitivity to benzodiazepines (which selectively modulate synaptic $GABA_ARs$ that do not contain the δ subunit), the impact of knocking out PKCδ appeared to be limited to δ-subunit containing $GABA_ARs$. Accordingly, genetically deleting PKCδ prevented EtOH enhancement of

tonic $GABA_AR$ currents in dentate gyrus GCs and thalamic relay neurons, mediated by δ-subunit containing $GABA_ARs$ (Choi et al., 2008). Thus, the PKCδ isoform confers sensitivity of $GABA_ARs$ to EtOH.

In contrast, PKCε knockout mice had enhanced sensitivity to benzodiazepine-induced ataxia and reduced EtOH-induced enhancement of $GABA_AR$ currents in transfected HEK293 cells by 40-60% (Qi et al., 2008). While this study identified that genetic deletion of PKCε enhances $GABA_AR$ sensitivity to EtOH, as opposed to a reduction in $GABA_AR$ sensitivity to EtOH from knocking out other PKC isoforms, one limitation is that it failed to directly demonstrate a behavioral correlate (i.e., enhanced behavioral sensitivity to EtOH-induced ataxia). One strength, however, was that the authors showed that PKCε phosphorylated the γ2 subunit of the $GABA_AR$, suggesting that PKC isoforms might only interact with specific $GABA_AR$ subunits and have composition-specific effects, but whether the reduction in PKCε-mediated phosphorylation of γ2 subunit-containing $GABA_ARs$ is responsible for reducing EtOH intake and preference in these knockouts (Hodge et al., 1999) remains unknown. Together, PKC isoforms can have specific and opposite effects on a range of EtOH-related phenotypes, thereby suggesting that the relative intracellular expression and activity levels of each isoform, perhaps genetically dictated, could contribute to variation in behavioral phenotypes.

Differences in isoform-specific effects likely result from variation in the targets and thereby contribute to different downstream effects. Therefore, the influence of PKC activity on EtOH sensitivity needs to be studied on a cell type and brain region-specific basis, and the interaction between EtOH and PKC activity in the cerebellum has been largely understudied. As mentioned above, the few studies that have looked at this interaction in the cerebellum have identified that PKCγ knockout mice had reduced muscimol and EtOH-stimulated chloride uptake (Harris et al., 1995) and that PKCδ knockout mice were less sensitive to EtOH-induced cerebellar ataxia (Choi et al., 2008). However, these studies failed to identify the specific cerebellar cell types that contribute to these phenotypic differences. For instance, how does

PKC activity in GCs impact EtOH sensitivity, and more specifically, how does it impact sensitivity to $GABA_AR$ inhibition? The above studies demonstrate that PKC activity can alter the sensitivity to EtOH at both the physiological and behavioral levels, and have thereby illuminated the importance of genetic regulation of PKC expression and activity in mediating EtOH-related behaviors that are associated with risk for developing AUDs.

1.10. EtOH effects on $GABA_AR$ inhibition of GCs

GCs are an integrator of afferent information to the cerebellum and provide a main source of glutamatergic input to PCs. Modulating their excitability, by altering tonic $GABA_AR$ inhibition can greatly impact transmission through the cerebellar cortex (Hamman et al., 2002). Thus, GC tonic $GABA_AR$ inhibition is a potential EtOH target that contributes to cerebellar dysfunction and behavioral impairment. EtOH increases the Golgi cell firing rate *in vitro* (Botta et al., 2010; Carta et al., 2004; Freund et al., 1993) and *in vivo* (Huang et al., 2012), thereby increasing vesicular GABA release onto GCs, which can be detected as an increase in the frequency of GC sIPSCs and the magnitude of the GC tonic $GABA_AR$. This EtOH-enhancement of tonic $GABA_AR$ inhibition is directly correlated with sensitivity to EtOH-impairment of rotarod performance (Hanchar et al, 2005), and it is consistent with an important role for α6βxδ $GABA_ARs$ in the development and maintenance of cerebellar motor control (Tia et al., 1996).

The mechanism by which EtOH increases Golgi cell activity to increase vesicular GABA release is not well understood but may involve modulation of nitric oxide signaling. There is concentrated nitric oxide synthase (NOS) expression in the GC and molecular layers of the cerebellum (Bredt et al., 1990). Similar to the EtOH response, reducing NO production depolarizes Golgi cells, increases their firing rate, and increases vesicular GABA release to GCs, thereby enhancing the frequency of GC sIPSCs and the magnitude of the tonic $GABA_AR$ current (Wall et al., 2003). Since EtOH inhibits NOS production both *in vitro* (Fataccioli et al., 1997; Persson & Gustafsson, 1992) and *in vivo* (Al-Regjaie & Dar, 2006), it's possible that

EtOH-inhibition of NOS activity depolarizes Golgi cells, perhaps by inhibiting the sodium-potassium ATPase or closing undetermined channels (Botta et al., 2010; Wall et al., 2003). One resulting hypothesis is that low baseline NOS levels would lead to less EtOH impairment and, as predicted by the cerebellar LLR, increase EtOH consumption. Consistent with this hypothesis, the magnitude of EtOH-induced reduction in NO metabolites correlated with functional ataxia (Al-Rejaie & Dar, 2006), and neuronal NOS (nNOS) knockout mice were less sensitive to EtOH-induced loss of righting reflex and consumed six times more EtOH (12% and 16%) than wild-type mice (Spanagel et al., 2002). Together, these findings suggest that genetically-regulated NOS expression levels may be an important factor in dictating neuronal sensitivity to EtOH impairment.

While there's broad agreement that millimolar EtOH concentrations dose-dependently enhance tonic inhibition, it remains contentious whether EtOH can enhance GC tonic $GABA_AR$ inhibition independent of increasing GABA release (Borghese & Harris, 2007; Botta et al., 2007b). In other words, does EtOH increase tonic inhibition only because it increases the ambient GABA concentration, or does it also result from direct enhancement of extrasynaptic $GABA_AR$ activity? Using an *in vitro* slice preparation, Fernando Valenzuela's group found that blocking AP-dependent vesicular GABA release with TTX effectively abolished the 50mM and 100mM EtOH-induced enhancement of GC tonic $GABA_AR$ inhibition holding current noise, suggesting that EtOH's action on GABAergic inhibition was solely mediated by presynaptic effects on Golgi cell activity, and not from direct activation of α6,δ subunit-containing $GABA_ARs$ (Carta et al., 2004). In opposition, Tom Otis and Martin Wallner's groups found that 10mM EtOH significantly enhanced GC tonic $GABA_AR$ inhibition by 8-15% in the presence of TTX and exogenous GABA, suggesting postsynaptic EtOH action likely through direct activation of extrasynaptic $GABA_ARs$ (Hanchar et al., 2005). Furthermore, the authors suggested that a naturally occurring single amino acid substitution, arginine to glutamine, at position 100 of the α6 subunit (α6-R100Q), significantly enhanced EtOH's action at the $GABA_AR$ to increase the

magnitude of the tonic current. Perhaps the animals used by Valenzuela and colleagues were genetically less sensitive to EtOH, thereby obscuring its direct action.

However, attempts to replicate Hanchar et al. (2005) have generally failed (Botta et al., 2007a), instead finding that EtOH did not enhance the tonic $GABA_AR$ current or holding current noise in the presence of TTX, while additionally failing to detect sensitivity differences to 25mM EtOH on tonic $GABA_AR$ inhibition between genotypes expressing either of the α6 polymorphisms (Botta et al., 2007). It's difficult to know what to conclude from these contrasting findings. Could laboratory-specific experimental conditions interact with genotypic variation to enable direct action by EtOH? The use of ketamine versus isoflurane during slice preparation did not influence EtOH modulation of tonic currents (Botta et al., 2007b), there was no interaction between δ subunit expression levels and exogenous GABA concentration on EtOH currents, and mild differences in zinc concentration was unlikely to underlie variation in EtOH-stimulated δ-$GABA_AR$ currents (Borghese & Harris, 2007). Other methodological differences were considered in a response letter from Tom Otis (Otis, 2008). For one, Botta et al. (2007) detected greater baseline tonic current amplitudes in the supposedly more sensitive α6-100Q genotype at trend levels and significantly higher sIPSC frequency compared to α6-100R genotypes, which were not detected by Hanchar et al. (2005) and perhaps point to differences in extracellular GABA concentrations. However, α6(100Q)β3δ $GABA_ARs$ expressed in oocytes were still potentiated by EtOH at high ambient GABA concentrations up to 10μM (Hanchar et al., 2005; Wallner et al., 2003), suggesting that any physiological changes in the ambient GABA concentration, which hovers around 160nM in slices (Santhakumar et al., 2006), would not be sufficiently large to obscure direct EtOH action in slices.

Another source of variation could come from higher extracellular potassium used by Valenzuela's group, which could affect sensitivity to EtOH in a genotype-dependent manner (extracellular solution contained 3mM KCl instead of 2.5mM KCl; In the experiments described in subsequent chapters, I have used the same 2.5mM KCl concentration as Otis and Wallner).

An additional argument proposed by Otis is that numerous studies of recombinant $GABA_AR$ have found that EtOH can directly potentiate α6β3δ $GABA_ARs$ (Hanchar et al., 2006; Wallner et al., 2003; Santhakumar et al., 2006) or α4β3δ (Hanchar et al., 2006; Sundstrom-Poromaa et al., 2002), so one would expect to see the same effect during slice recordings. However, as noted in a review by Lovinger & Homanics (2007), studies of recombinant $GABA_AR$ expression in heterologous cells have not always produced reliable and replicable results. Furthermore, many of these studies are from the same lab associated with the Hanchar et al. (2005) paper, so there's little external verification. Finally, post-translational processing may differ between slices and recombinant receptor expression in oocytes (Sivilotti et al., 1997) or immortal cell lines (Sap & Yeh, 1998), thereby limiting the conclusions that can be generalized from a recombinant system to the slice.

Among the potential explanations for these controversial results, the most promising is post-translational processing such as PKC-mediated receptor phosphorylation. PKC activity may deviate greatly between slice preparations and recombinant receptor expression in various host cells. In support of this contention, Yamashita et al. (2006) found that EtOH either had no effect or *suppressed* α6β2/3δ $GABA_AR$ currents expressed in Chinese hamster ovary cells using whole-cell patch-clamp procedures, which is the opposite of that detected by others in oocytes. However, when they used the perforated patch procedure to minimize the disturbance to the intracellular milieu, they detected a range of EtOH's effects, from potentiation to suppression of α6β2δ $GABA_AR$ currents. Together, these findings suggest that intracellular components can drive post-translational processing of $GABA_ARs$ to modulate the effect of EtOH on tonic $GABA_AR$ currents. Furthermore, they importantly identify that EtOH's effects on $GABA_ARs$ can range across the spectrum from potentiation to suppression. However, neither this study, nor any others, identified the specific factors that contribute to this range. The experiments described in Chapter 2 will aid in resolving this debate by describing the impact that PKC-mediated phosphorylation plays on EtOH's action at α6β2/3δ $GABA_ARs$. Identifying the

mechanisms by which EtOH impacts GC tonic $GABA_AR$ is critical for understanding how genetically-regulated EtOH sensitivity contributes to behavioral impairment and EtOH consumption.

1.11. EtOH effects on GABAergic transmission to PCs

EtOH effects on GC inhibition can impact glutamatergic input to PCs, but the integration of synaptic input by PCs can also be modulated by GABAegic input from molecular layer interneurons, which in turn are also excited by GC afferent parallel fibers. Each PC is innervated by approximately nine interneurons (Hausser & Clark, 1997). GABA release from a single interneuron can extend the PC interspike interval by a mean of 12%, reduce the membrane time constant, and reduce the input resistance, which together, impair integration of excitatory postsynaptic potentials (EPSPs) from GCs via shunting inhibition (Hausser & Clark, 1997). Thus, altering stellate and basket cell activity directly, or indirectly via changes in GC firing, may disrupt cerebellar processing and impair behavior (Mameli et al., 2008; Ming et al., 2005). EtOH dose-dependently increases GABAergic transmission to PCs. EtOH concentrations as low as 25mM to 50mM increase the frequency of miniature IPSCs in PCs without affecting the amplitude or decay time, suggesting that EtOH does not change the sensitivity of postsynaptic $GABA_ARs$. Furthermore, 50mM significantly increased molecular layer interneuron AP frequency measured directly on interneuron firing frequency and indirectly as an increase in PC sIPSC frequency that was greater than in the presence of TTX (Hirono et al., 2009; Mameli et al., 2008; Wadleigh & Valenzuela, 2012). The increase in GABA release at PCs reduced the PC evoked EPSP (eEPSP) and evoked APs from stimulation of parallel fibers (Mameli et al., 2008). Thus, moderate EtOH concentrations increase GABAergic transmission to PCs and attenuate the impact of evoked excitatory input.

However, the effect of EtOH on spontaneous PC APs remains controversial. Mameli et al. (2008) failed to detect a 50mM EtOH-induced change in the spontaneous AP firing in PCs, *in*

vitro, and Rogers et al. (1980) failed to detect an effect of four *in vivo* exposures ranging from 1g/kg to 4g/kg on PC firing, *in vivo*. These findings contrast with other studies that have detected an EtOH reduction in PC AP frequency *in vitro* (Freund et al., 1993; Sorensen, 1980; Urrutia & Gruol, 1992) and *in vivo* (Palmer et al., 1987). One methodological issue with these older studies is that they administered EtOH via pressure injection at concentrations between 750mM and 1M, which are well beyond the range of being physiologically relevant and may affect a host of non-$GABA_AR$ targets. These doses are significantly higher than the bath-applied 50mM EtOH concentration used by Mameli et al. (2008), which itself is at the high end of what animals will achieve *in vivo* (rodents and humans typically achieve BECs of 5-25mM during voluntary EtOH consumption). Although the most concentrated dose is not reaching the recorded PC, such high concentrations can alter the cell's membrane properties or afferent neurons in non-physiologically relevant ways. Additionally, it's been proposed that the strength of $GABA_AR$ inhibition in response to stimulation or pharmacological enhancement (e.g., EtOH), could depend on slice thickness (Jotty et al., 2015), suggesting the importance of feed-forward and feedback inhibition on EtOH's actions in an unperturbed cerebellum.

Still, it's possible for EtOH to enhance as well as suppress spontaneous PC firing (Ming et al., 2006). EtOH increased PC firing in the presence of a glutamate receptor antagonist, but also a GABA receptor antagonist. Furthermore, there was a direct correlation between the sensitivity to inhibition of PC firing by exogenous GABA with the EtOH-induced change in PC firing. These results suggest that both pre- and postsynaptic EtOH targets may affect spontaneous firing of PCs and the net effect of EtOH on the firing rate varies as a function of GABA sensitivity. But while stellate and basket cells are the only source of synaptically released GABA to PCs, no direct link has been made between EtOH-enhancement in molecular layer interneuron firing rates and behavioral impairment. Indirectly, genotypic differences in sensitivity to EtOH-induced reduction in PC spontaneous firing, *in vivo*, was directly correlated with the duration of EtOH-induced loss of righting reflex in rats (Palmer et al., 1987) and mice (Spuhler

et al., 1982). If EtOH enhancement of GABAergic transmission to PCs reduces their firing and this contributes to their behavioral impairment, then it strongly implicates a role for stellate and basket cells in EtOH-induced behavioral impairment. However, as some of the electrophysiology studies suggest, the effect of EtOH on PC firing may be subtle or even non-existent. Instead, EtOH may impair cerebellar processing and behavior by disrupting synaptic integration of PC afferents (Mameli et al., 2008). In summary, EtOH action on stellate and basket cell GABA release may be an additional source of EtOH disruption of cerebellar processing at low concentrations.

1.12. Focus of dissertation

The cerebellum is gaining recognition as a relevant brain structure in both motor and cognitive function. Evidence from the LLR and DM models has revealed a cerebellar contribution to risk for developing an AUD (Gallaher et al., 1996; Newlin & Thomson, 1990; Schuckit 1985). However, the genetically-determined underlying neural mechanisms for these risk factors remain unclear. Presumably, the cerebellar-dependent components of the LLR and DM models should have underlying neural substrates by which the cellular response to EtOH varies as a function of the phenotypic expression. Two promising targets are the EtOH-enhancement of GABAergic transmission from Golgi cells to GCs and molecular layer interneurons to PCs. Differing sensitivities to EtOH at these two sites may underlie the variation in behavioral phenotypes. Importantly, EtOH significantly affects $GABA_AR$ inhibition of GCs at a lower concentration than that of PCs (Botta et al., 2004; Hanchar et al., 2005; Ming et al., 2006; Mameli et al., 2008), suggesting that EtOH's impact on GCs largely underlie the changes in cerebellar-related behaviors seen at low blood EtOH concentrations.

Since the GC tonic $GABA_AR$ current is sensitive to EtOH enhancement and has the largest overall contribution to the inhibitory charge of the GC (Botta et al., 2004; Hanchar et al., 2005; Hamann et al., 2002), differing EtOH sensitivities across genotypes may contribute to

their divergent EtOH-related behavioral phenotypes. Before the studies presented here in Chapters 2 and 3, almost all research on EtOH enhancement of GC tonic $GABA_AR$ inhibition had been conducted in Sprague-Dawley rats (SDRs), and little attention had been given to how EtOH's actions on tonic $GABA_AR$ inhibition varies across species or divergent EtOH-consumption phenotypes. This neglect is a substantial problem because SDRs have a low EtOH-consumption phenotype (Khanna et al., 1990; Melchior & Myers, 1976), and therefore may not be relevant to understanding the cellular risk factors associated with AUD in humans. Instead, comparing EtOH responses across species or specific phenotypes could reveal cellular correlates associated with particular EtOH-related behaviors. Furthermore, a broader ensemble of genotypes could reveal genetic variation in factors, such as PKC activity, that are known to influence GABAergic signaling and may interact with EtOH effects in the cerebellum (Choi et al., 2008). Comparing these genetically-variable conditions may shed light on a fundamental controversy in the EtOH and $GABA_AR$ field: can EtOH directly act on α6β2/3δ $GABA_ARs$, and if so, what factors promote this action?

The aim of Chapter 2 was to characterize how EtOH differentially affects $GABA_AR$ inhibition of GCs across rodents with opposite EtOH-related behavioral phenotypes. I proposed that genetic variation in nNOS expression and PKC activity, factors known to affect $GABA_AR$ signaling, would underlie phenotypic variation in cellular responses to EtOH. Indeed, these factors were found to contribute to presynaptic and postsynaptic sources of EtOH response variation at GCs. The aim of Chapter 3 was to test the predictive validity of the hypothesis that the effect of EtOH on GC tonic $GABA_AR$ inhibition varies as a function of EtOH consumption phenotype in prairie voles (PVs). This hypothesis held up across species and illuminated the importance that EtOH's action on GC tonic $GABA_AR$ inhibition may have in promoting behavioral phenotypes associated with EtOH consumption. However, while Chapters 2 and 3 are GC-centric, Chapter 4 sought to characterize EtOH's actions on input to PCs. I specifically tested the hypothesis that EtOH's antipodal actions on GC tonic $GABA_AR$ in mice with opposite

EtOH consumption phenotypes would also differentially regulate signal transmission through the cerebellar cortex. This hypothesis was confirmed, and so in Chapter 5, I tested the hypothesis that EtOH's action on GC tonic $GABA_AR$ inhibition contributes to the animal's EtOH consumption phenotype. Together, this dissertation provides evidence that genetic influences on GC $GABA_AR$ inhibition's sensitivity to EtOH can differentially impact cerebellar cortical transmission and modify EtOH intake.

Chapter 2: Opposite actions of alcohol on tonic $GABA_A$ receptor currents mediated by nNOS and PKC activity

This chapter has been reformatted for inclusion in this dissertation from:

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2.1. Abstract

The molecular mechanisms that mediate genetic variability in response to EtOH are unclear. We find that EtOH has opposite actions (enhancement or suppression) on $GABA_AR$ inhibition in GCs of the cerebellum from behaviorally sensitive, low-EtOH consuming SDRs and D2 mice and behaviorally insensitive, high-alcohol consuming B6 mice, respectively. The impact of alcohol on GC $GABA_AR$ inhibition is determined by a balance between two opposing effects: enhanced presynaptic vesicular release of GABA via alcohol inhibition of NOS, and a direct suppression of the activity of postsynaptic $GABA_ARs$. The balance of these two processes is determined by differential expression of nNOS and postsynaptic PKC activity, both of which vary across rodent genotypes. These findings identify opposing molecular processes that differentially control the magnitude and polarity of $GABA_AR$ responses to alcohol across rodent genotypes.

2.2. Introduction

EtOH abuse is a leading cause of preventable death and illness, and the economic cost of alcohol abuse is estimated to be $185 billion annually in the USA alone (Harwood, 2000). Adoption and twin studies suggest that AUDs are 50-60% genetically determined (Hill, 2010; Prescott & Kendler, 1999). A growing body of research indicates that genetic differences in cerebellar processing and cerebellar responses to alcohol contribute to susceptibility to AUDs (Herting et al., 2011; Hill, 2010; Schuckit et al., 1996; Schuckit et al., 2005), but the mechanisms by which the cerebellum influences the development of AUDs are not known.

Insight into cerebellar contributions to AUD risk comes from studies of the LLR to EtOH phenotype, which is defined as requiring a higher dose of EtOH to achieve a given effect. EtOH-induced static ataxia (body sway), a form of cerebellar-dependent motor impairment, consistently shows LLR in individuals with a family history of AUDs compared to individuals without a family history of AUDs (Schuckit et al., 1996; Schuckit et al., 2005). Thus, low cerebellar sensitivity to EtOH may be a risk factor for AUDs. In support of this contention, the magnitude of EtOH-induced ataxia shows an inverse relationship with EtOH consumption and preference in some inbred strains of mice (Gallaher et al., 1996; Yoneyama et al., 2008) as well as lines of rodents selected for differences in EtOH consumption (Bell et al., 2001; Malila, 1978) or in EtOH-induced motor impairment (McClearn et al., 1981). Importantly, cerebellar specific injections of various drugs can inhibit systemically administered EtOH induced-ataxia (Al Rejaie & Dar, 2006), clearly indicating the central role of the cerebellum in mediating EtOH-induced ataxia.

Cerebellar GCs are the main integrators/processors of afferent input to the cerebellar cortex, making them powerful targets for pharmacological modulation of cerebellar processing (Duguid et al., 2012; Hamann et al., 2002). GCs exhibit traditional phasic $GABA_AR$-mediated inhibitory IPSCs, as well as the more recently discovered tonic form of $GABA_AR$ inhibition, mediated by extrasynaptic, α6,δ-subunit containing $GABA_ARs$ (Brickley et al., 2001; Hamann et

al., 2002; Meera et al., 2011; Rossi et al., 2003; Stell et al., 2003). The tonic form of $GABA_AR$ inhibition mediates 75% of total GC $GABA_AR$ inhibition, thereby powerfully controlling signal transmission through the cerebellar cortex (Hamann et al., 2002). Both the frequency of GABAergic sIPSCs and the magnitude of the tonic $GABA_AR$-mediated current are enhanced by behaviorally relevant concentrations of EtOH (Carta et al., 2004; Hanchar et al., 2005). Therefore, genetic variation in the sensitivity of GC $GABA_AR$ inhibition to EtOH is a candidate mechanism for mediating the relationship between cerebellar LLR and AUD in humans, or high EtOH consumption in animal models. Unfortunately, almost all research on EtOH-induced potentiation of GC $GABA_AR$ inhibition has been done on SDRs, and little attention has been given to how the sensitivity of GC $GABA_AR$ inhibition to EtOH varies across species or divergent genotypes (Botta et al., 2007). This neglect is a significant problem because SDRs have a high sensitivity, low EtOH consumption phenotype (Melchior & Myers, 1976), and thus may not be as relevant to AUD in humans.

We report here that EtOH can either increase or decrease $GABA_AR$ mediated inhibition of GCs, and the net impact across populations of GCs shifts, in a graded fashion, from strong enhancement in high sensitivity, low EtOH consuming rodents to suppression in low sensitivity, high EtOH consuming rodents. Furthermore, we found that the net impact of EtOH on GC $GABA_AR$ inhibition is determined by a balance between enhanced vesicular release of GABA (via EtOH inhibition of NOS) and a direct suppression of $GABA_ARs$. The balance of these two processes is determined by differential expression of nNOS and levels of postsynaptic PKC activity, both of which vary across rodent genotypes. These findings substantially alter the current dogma that the primary action of EtOH on $GABA_AR$ transmission is potentiation. Instead, our data indicate that EtOH can potentiate or suppress $GABA_AR$ transmission, and the polarity varies across rodent genotypes with divergent EtOH-related behavioral phenotypes.

2.3. Methods

2.3.1. Preparation of brain slices

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 Animal Care and Use Committee of the Oregon Health & Science University. Cerebellar slices were prepared acutely on each day of experimentation (Hamann et al., 2002; Rossi et al., 2003). Male and female rodents (18-28 days old), randomized for each experiment, were housed between 2-6 animals/cage and maintained on a standard light/dark cycle. Animals were anaesthetized with isoflurane and killed by decapitation. The whole brain was rapidly isolated and immersed in ice cold (0-2ºC) artificial cerebrospinal fluid (aCSF) containing (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 ice cold (0-2ºC) aCSF. Parasagittal slices (225µm) were made with a vibrating tissue slicer (Vibratome). Slices were incubated in warmed aCSF (33±1^{o}C) for one hour after dissection and then held at 22-23^{o}C until used. Kynurenic acid (1 mM) was included in the dissection, incubation and holding solution (to block glutamate receptors to reduce potential excitotoxic damage) but was omitted from the experimental solutions.

2.3.2. Electrophysiology

Slices were placed in a submersion chamber on an upright microscope, and viewed with an Olympus 60X (0.9 numerical aperture) water immersion objective with differential interference contrast and infrared optics. Slices were perfused with aCSF at a rate of ~7ml/min at room temperature (22-24º C). Drugs were dissolved in aCSF and applied by bath perfusion. Visually identified granule cells were voltage-clamped (Vh = -60mV) with patch pipettes, constructed from thick-walled borosilicate glass capillaries and filled with an internal solution containing (in mM): CsCl 130, NaCl 4, $CaCl_2$ 0.5, HEPES 10, EGTA 5, MgATP 4, Na_2GTP 0.5, QX-314 5. Solutions were pH adjusted to 7.2-7.3 with CsOH. Electrode resistance was 4 to 10

MΩ. Cells were rejected if access resistance was greater than 15 MΩ or if access resistance changed by > 15%, or if there were condition-independent changes in holding current. Note, the intracellular [Cl^-] sets E_{Cl^-} to ~0mV, which for the holding potentials used in all experiments (-60mV), results in $GABA_AR$ currents being inward (downward deflections in displays of the holding current). Accordingly enhancement and block/suppression of tonic $GABA_AR$-mediated currents are inward and outward, respectively. In all cases, only one cell was recorded from a given slice. In cases where the slice was exposed to more than one drug or different doses of the same drug, the order of drug application was randomized across slices. In all cases of multiple drug exposures to the same slice, a stable baseline was obtained following washout of drug for a minimum of 4 minutes. However, control experiments were never done on slices that had been exposed to TTX, GABAzine, or incubated in N^G-nitro-L-arginine (L-NA) as the time required to adequately wash such drugs was preclusive of additional unrelated experimentation.

2.3.3. Immunohistochemistry

Slices were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 17 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 1-2 days 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 Citifluor and imaged with confocal microscopy. See reagents below for source and dilution of antibodies used.

2.3.4. DAF-FM detection of NO production

We used DAF-FM as a fluorescent biochemical assay of cellular NO production (Kojima et al., 1998). Slices were incubated in the relevant experimental condition (control, control + 52mM EtOH, or control + 300µM L-NA) for a 30 minute equilibration period, followed by addition of the DAF-FM (1µM) + the relevant experimental condition for 30 minutes, followed by washout of DAF-FM in the continued presence of the relevant experimental condition for 30 minutes.

Following the exposure protocol, slices were fixed (as for immunohistochemistry, see above), and processed for confocal microscopy. Whole field emission analysis largely eliminates subjective bias, and so DAF-FM experiments were not performed blind to the conditions of the experiment.

2.3.5. Confocal microscopy

Images were acquired with a Zeiss confocal LSM780 laser scanning Microscope, using accompanying Zeiss software for acquisition, processing and subsequent analysis. A laser line falling within 20nm of the peak absorbance was used for each of the various fluorophores, with appropriate excitation, dichroic and emission filters. One of two objectives was used for all experiments: a 20X, 0.8 N.A. Plan Apochromat air objective (Fig. 4), or a 40X, 1.4 N.A. PlanApo oil-immersion objective (Figs. 1&6 and Supplementary Figs. 3&4). Pinhole diameter and slice step thickness were optimized for the objective used. For $GABA_AR$ subunit distribution studies (Fig. 1), quantification of nNOS and GAD 65/67 expression (Fig. 6D&G), and quantification of NO sensitive fluorophore (DAF-AM) fluorescence emission (Fig. 4) stacks of image planes were acquired, and then projected into a single stacked image (10µm thick). For a given fluorophore, digital gain and offset settings were identical for all images across all species and conditions. For quantification of GC encirclement by nNOS (Fig. 6A-D and Supplementary Fig. 3), 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 Supplementary Fig. 3). Values were obtained from at least 3 animals of each species, at least 2 slices from each animal and at least 5 distinct regions of each slice.

2.3.6. Analysis of $GABA_AR$ currents

Membrane currents were acquired at 20 kHz, filtered at 10 kHz, and analyzed with pClamp (v.6.3) software (Axon Instruments, Foster City, CA). For analysis and display of sIPSCs, data

were filtered at 2-5 kHz. The absolutely objective methodology used for analysis of $GABA_AR$ currents (automated measurement at fixed time points) obviated experimenter blinding, thus collection and analysis of all electrophysiological experiments were not performed blind to the experimental condition. When quantifying sIPSC occurrence, 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 and with a decay time constant at least 3-fold slower than the rise time. The tonic current was assessed by fitting the Gaussian distribution of all data points not skewed by synaptic events from a point 3 pA to the left of the peak value to the rightmost (smallest) value of the histogram distribution (see Supplemental Fig. 1 for examples). 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.

2.3.7. Statistics

All data are expressed as the mean ± the standard error of the mean. One-way ANOVA was used to detect significant main effects, and Student's t-tests were used for post hoc-analyses, while all other statistical comparisons were made with unpaired or one-sample t-tests. In all cases, statistical tests were two-tailed and we set the threshold for significance at $P < 0.05$. In some cases, parametric statistical techniques could not be used due to variable or antipodal responses that violated assumption of normally distributed data as determined by significant Shapiro-Wilk statistic ($P < .05$). In these cases (i.e. Figs. 2H, 3F, 6D,K&L, and 8D), we used non-parametric Kruskal-Wallis one-way analysis of variance tests with Dunn's method for pair wise comparison, or Mann-Whitney U tests where appropriate. In the figures, gray arrows point to an extended time scale of recording from different time points of the main trace. * signifies $P < 0.05$, ** signifies $P < 0.01$, and *** signifies $P < 0.001$. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in the field (Carta et al., 2004; Hamann et al., 2002; Hanchar et al., 2005; Rossi et al., 2003).

2.3.8. Reagents

All reagents were from Sigma Chemicals (St. Louis, MO) except AP5, bicuculline, gabazine, kynurenic acid, NBQX (all from Ascent scientific, UK), and iodoacetic acid (from Acros/Fisher). 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), α6 subunit of $GABA_AR$ (rabbit/Millipore AB5610/1:200), and δ subunit of $GABA_AR$ (rabbit/Millipore AB9752/1:200). Secondary antibodies were various excitation maxima Alexafluors (Invitrogen) from appropriate hosts diluted 1:500. For some antibodies (α6 and δ subunit of $GABA_AR$), specificity has been confirmed by lack of labeling in relevant knockout mice, for others (GAD 65 and 67) specificity has been confirmed by their labeling of a single band on Western blots (see manufacturer website for details, and links therein). For those antibodies where such confirmation of specificity was not already available (nNOS), we confirmed specificity ourselves, by examining slices from nNOS knockout mice (*Nos1*^{tm1Plh} homozygotes, backcrossed to C57BL/6J mice for >10 generations, from Jackson Laboratory), which did not display detectable nNOS staining (Supplementary Fig. 4). Furthermore, for all of the antibodies we used, the general qualitative expression pattern within the cerebellum is similar to what has been shown with other antibodies in other reports and to our own studies with alternative antibodies. Importantly, for all of the crucial immunohistochemical studies ($GABA_AR$ subunits and nNOS), we have conducted parallel electrophysiological studies of the relevant proteins' activity which confirm our histochemical assessments (see electrophysiological experiments in Figs. 1&6 for functional confirmation of $GABA_AR$ subunit and NOS expression levels, respectively).

2.4. Results

2.4.1. Mouse GC tonic current mediated by extrasynaptic $GABA_ARs$

To determine if EtOH consumption phenotype is associated with differences in GC $GABA_AR$ sensitivity to EtOH, we made voltage-clamp recordings (Vh = -60mV, with ECl= ~ 0mV, see methods) from GCs in cerebellar slices obtained from alcohol naïve, prototypical high and low EtOH consuming mice, B6 and D2 mice respectively (Yoneyama et al, 2008). First, we characterized the basal properties of $GABA_AR$-mediated inhibition in GCs from these two strains of mice. Similar to the well characterized SDR GCs, B6 and D2 GCs exhibit phasic sIPSCs and a powerful tonic current (tonic current amplitude = B6: 26.74 ± 4.27pA; D2: 22.90 ± 2.64pA, *n* = 12 cells from 5 animals each; see Supplementary Fig. 1 for current measuring methodology), both mediated by $GABA_ARs$ as evidenced by their blockade by the $GABA_AR$ antagonist, GABAzine (10µM, Fig. 1A,G). The GC tonic $GABA_AR$ current is thought to be mediated by extrasynaptic $GABA_ARs$ containing the α6 and δ subunit (Brickley et al., 2001; Hamann et al., 2002; Meera et al., 2011; Stell et al., 2003), but this has not been fully established in B6 GCs and has not been examined at all in D2 GCs. We used confocal microscopy to examine B6 and D2 cerebellar slices immunostained with antibodies to the $GABA_AR$ α6 and δ subunit, and found that both subunits are richly and exclusively expressed by GCs in both strains of mice, particularly on their dendritic terminals (Fig. 1B α6; 1D δ). Furthermore, in both strains of mice, the tonic current was reduced by furosemide (100µM, at which concentration it is specific for $GABA_ARs$ containing the α6 subunit (Hamann et al., 2002; Korpi et al., 1995); Fig. 1C,G, furosemide block of current **=** B6: 17.24 ± 2.16pA; D2: 13.99 ± 1.75pA, *n* = 17 cells each), and was enhanced by the $GABA_AR$ agonist THIP (500nM, which at concentrations up to 1µM is specific for $GABA_ARs$ containing δ subunits (Meera et al., 2011; Fig. 1E,G. THIP induced current= B6: 45.41 ± 3.93pA, *n* = 18; D2: 33.67 ± 7.40pA, *n* = 10 cells). Finally, diazepam (300nM), which potentiates the response of α1 containing receptors, but not α6 containing receptors, and does not affect the tonic $GABA_AR$ current in SDR GCs (Hamann et al., 2002; Saxena & Macdonald, 1996), did not affect the tonic $GABA_AR$ current in D2 or B6 mouse GCs (Fig. 1F,G; D2: -0.08 ± 0.34pA, *n* = 9 cells from 3 animals, *P* = .82; B6: -0.39 ± 0.57pA, *n* = 7

cells from 2 animals, *P* = 0.52, one-sample *t*-tests). In the same cells, diazepam increased the magnitude and decay time of sIPSCs (Supplementary Fig. 2), confirming its efficacy at

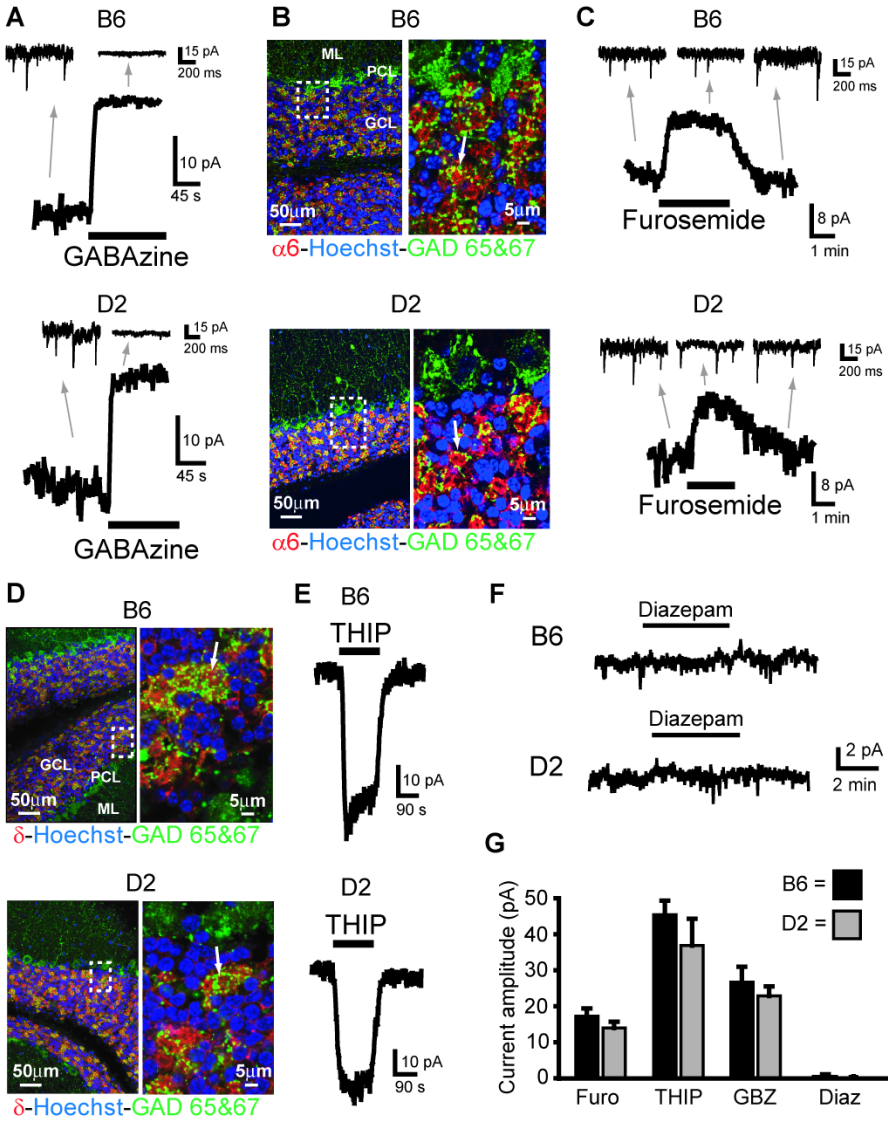


Figure 1. B6 and D2 mouse GCs exhibit similar magnitude tonic currents mediated by α6 and δ subunit containing $GABA_AR$s. **A.** Example traces showing block of tonic current by $GABA_AR$ antagonist GABAzine (10µM) in GCs from B6 (top) and D2 (bottom) mice. Note, in this and all other figures, the gray arrows point to an expanded time scale of recording from different time points of the main traces (coming from the region of the main trace that the back of the arrow extrapolates to), showing sIPSCs and block by GABAzine. **B.** Confocally acquired fluorescence images of cerebellar slices from B6 (top) and D2 (bottom) mice (representative scans from *n* = 8 slices from 4 B6s and *n* = 7 slices from 4 D2s) showing α6 subunit of the $GABA_AR$ (red) expression exclusively in the granule cell layer (identified by high density of GC nuclei, labeled blue with nuclear stain Hoechst, beneath the Purkinje cell layer, labeled green with antibody to GAD, left panels). Right panels are blow ups of boxed region showing α6 staining is around GC somas (labeled blue with nuclear stain Hoechst), and in GC dendrites located in the glomerulus (indicated by lack of nuclear stain, and colocalization with staining for GAD (green) which labels Golgi cell axon terminals). Arrow points to a representative α6 labeled glomerulus. GCL=GC layer, PCL=Purkinje cell layer, ML=Molecular layer. **C.** Example traces showing block of tonic current by $GABA_AR$ antagonist furosemide (100µM, at which concentration it is specific for $GABA_AR$s containing the α6 subunit) in GCs from B6 (top) and D2 (bottom) mice. Gray arrows point to expanded time scale of recording from different time points of main traces, showing lack of effect of furosemide on sIPSCs. **D.** Same as C&D except red immunostain comes from antibody for the δ subunit of the $GABA_AR$ (representative scans from *n* = 5 slices from 4 B6s and *n* = 9 slices from 6 D2s). **E.** Example traces showing enhancement of tonic current by $GABA_AR$ agonist THIP (500nM, at which concentration it is selective for $GABA_AR$s containing the δ subunit) in GCs from B6 (top) and D2 (bottom) mice. **F.** Example traces showing lack of effect of diazepam (300nM) on tonic $GABA_AR$ current in GCs from B6 (top) and D2 (bottom) mice. **G.** Plot summarizing mean tonic current induced by THIP (B6, *n* = 18 cells; D2, *n* = 10), and blocked by furosemide (B6, *n* = 17 cells; D2, *n* = 17) and GABAzine (B6, *n* = 12 cells; D2, *n* = 12), and lack of effect of diazepam (B6, n = 7 cells; D2, n = 9) in B6 and D2 mouse GCs. Furosemide-, THIP- and GABAzine-, but not diazepam-induced currents (*n* = 16 cells, *P* = .49), are all significantly different from zero $P < 0.05$ using one-sample *t*-tests, and none are significantly different between strains (furosemide: *P* = .25; THIP: *P* = .27; GABAzine: *P* = .45, unpaired *t*-tests).

diazepam-sensitive synaptically located α1 containing $GABA_A$Rs. These data indicate that, similar to previous reports in SDR GCs, both B6 and D2 GCs exhibit tonic $GABA_A$R currents mediated by extrasynaptic α6,δ-containing receptors. There were no detectable differences between B6 and D2 GC $GABA_A$R mediated tonic currents or sIPSCs (Fig. 1G and Supplementary Fig. 2, all $P > 0.05$).

2.4.2. Opposite actions of EtOH on GC tonic $GABA_A$R currents

Bath application of EtOH to SDR cerebellum increases the frequency of GC sIPSCs and the magnitude of the tonic $GABA_A$R current (Fig. 2A; Carta et al., 2004; Hanchar et al., 2005). Similar to SDR GCs, in some B6 and D2 GCs, bath application of 52mM EtOH caused an increase in the frequency of sIPSCs and an associated potentiation of the tonic $GABA_A$R current (Fig. 2B,E). However, in 41-45% of both B6 (16/39 cells) and D2 (14/31 cells) GCs, EtOH had no discernible effect on the tonic current (Fig. 2C,E) or the frequency of sIPSCs (discussed in greater detail below). Surprisingly, in 44% of B6 (17/39 cells) GCs and in 10% of D2 GCs (3/31 cells), EtOH actually suppressed the tonic $GABA_A$R current (Fig. 2D,E). Importantly, EtOH did not affect the holding current in any species of GC when $GABA_A$Rs were blocked by GABAzine (10μM, D2: $n = 8$ cells from 3 animals, B6: n = 11 cells from 4 animals, and SDRs: n = 32 cells from 8 animals, Fig. 2F,G), confirming that EtOH-induced inward and outward currents are due to potentiation and suppression of the tonic $GABA_A$R current respectively. To determine the overall impact on a population of GCs, and hence the output of the cerebellum (the sole output of the cerebellar cortex is via the PC, whose output is influenced by ~10^5 GCs (De Schutter & Bower, 1994; Hamann et al., 2002) we calculated the mean response of all cells from each type of rodent (Fig. 2H). The averaged impact of EtOH on the $GABA_A$R tonic current for a population of neurons was significantly different between strains ($H(2) = 49.54$, $P < .001$, by Kruskal-Wallis) with the average for SDR and D2 GCs being potentiation (increase in tonic current = SDR: 4.65 ± 0.51pA, $n = 47$ cells; D2: 2.51 ± 0.84pA, $n = 31$ cells), whereas, the average impact on B6 GC

tonic current was suppression (reduced by 2.32 ± 1.30pA, *n* = 39 cells). Thus, 52mM EtOH will suppress transmission through the cerebellar cortex in low EtOH consuming SDRs and D2 mice, but it will enhance transmission through the cerebellar cortex in high EtOH consuming B6 mice (Duguid et al., 2012; Hamann et al., 2002).

Although the correspondence between GC $GABA_AR$ response phenotype and EtOH consumption phenotype is quite striking, to determine if this potential relationship is generalizable, we examined GC $GABA_AR$ responses to EtOH in the first replicate line of Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) mice (Replicate lines 1: WSP1 & WSR1), which were bred for divergence in sensitivity to a chronic EtOH withdrawal phenotype but have a similar EtOH consumption phenotype that is intermediate between the low EtOH consuming D2 mice and SDRs and the high EtOH consuming B6 mice (Fig**.** 2I; Ford et al., 2011). While the GC $GABA_AR$ response to EtOH varied significantly across strains ($H(4) = 59.03$, $P < .001$, by Kruskal-Wallis), it was similar in WSP1 (reduced by 0.49 ± 1.12pA, *n* = 9 cells from 2 animals) and WSR1 mice (reduced by 1.50 ± 1.34pA, *n* = 7 cells from 2 animals, $P = .57$, unpaired *t*-test), and fell between SDR/D2 and B6 responses, being on average slightly suppressed (Fig. 2I). Thus, the net impact of EtOH on $GABA_AR$ transmission in a population of GCs varies from strong potentiation in low EtOH consuming SDRs and D2s, to strong suppression in high EtOH consuming B6s, with intermediate impact in moderate EtOH consuming WSR1 and WSP1 mice.

In the preceding experiments, we used a relatively high concentration of EtOH (52mM) to maximize our chances of discovering potentially subtle differences in responses across genotypes. However, while BECs reach 52mM in many non-choice rodent experiments (delivery of 2g/kg EtOH leads to BECs of 45-55mM, depending on species (Porcu et al., 2010), voluntary consumption of EtOH in rodents and humans more typically leads to BECs in the range of 5-25mM. To determine if the two types of response are activated by BECs achieved during voluntary EtOH consumption, we constructed dose response curves in B6 mouse and SDR

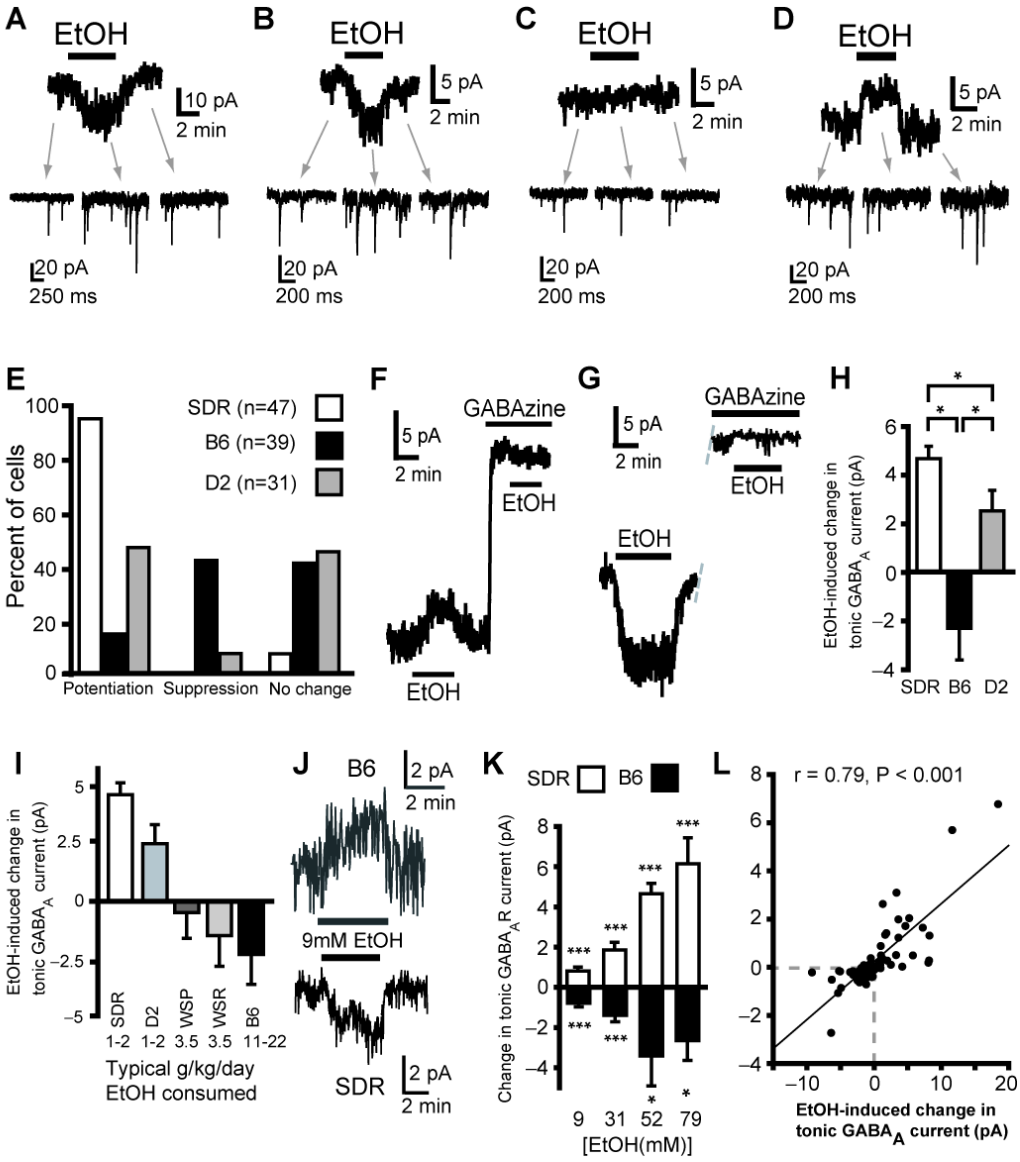


Figure 2. The impact of EtOH on GC tonic $GABA_AR$ currents varies in polarity and magnitude across rodent genotypes with divergent EtOH consumption phenotypes. **A.** Representative data showing that EtOH increases sIPSC frequency (insets indicated by gray arrows) and potentiates the tonic $GABA_AR$ current magnitude (main trace) in SDR GCs. **B-D.** Representative data showing that EtOH can either potentiate (**B**), suppress (**D**) or have no impact (**C**) on tonic $GABA_AR$ currents in D2 and B6 GCs. **E.** Plot of proportion of GCs showing potentiation, suppression or no effect of EtOH on tonic $GABA_AR$ currents in SDR, B6 and D2 GCs. **F,G.** Representative traces showing that both outward (**F**) and inward (**G**) currents induced by EtOH are abolished by the $GABA_AR$ antagonist, GABAzine (10μM). For cells that had shown significant EtOH-induced inward and outward currents under control conditions, in the presence of GABAzine, the mean EtOH-induced current was -0.09 ± 0.16pA and -0.13 ± 0.22pA, i.e. not significantly different to baseline for both (Inward: n = 13 cells, P = .55; outward: n = 14, P = 0.53, one-sample t-tests). **H.** Plot of the mean magnitude and polarity of the GC $GABA_A$ tonic current to EtOH for all cells tested from SDRs, B6s and D2s (* indicates significantly different, $P < 0.05$). **I.** Plot of the mean magnitude and polarity of the GC $GABA_A$ tonic current to EtOH for all cells from each type of rodent: SDRs (n = 47 cells), D2 mice (n = 31), WSP mice (n = 9), WSR mice (n = 7), and B6 mice (n = 39). Numbers at bottom of chart show the typical amount of EtOH consumed (in g/kg/day) for each type of rodent in published free access two or three bottle choice studies (from Ford et al., 2011; Mechior & Myers, 1976; Yoneyama et al., 2008). **J.** Representative traces showing that concentrations of EtOH as low as 9mM suppress and enhance tonic $GABA_AR$ currents in B6 mouse and SD rat GCs, respectively. **K.** Plot showing dose response relationship for EtOH-induced suppression and enhancement of tonic $GABA_AR$ current from baseline in B6 mouse (black) and SDR (white) GCs respectively (SDR: all ***P < .001; B6: ***P < .001 [9mM], ***P < .001 [31mM], *P = .028 [52mM]; P = .025 [79mM], one-sample t-tests). **L.** Plot of EtOH induced change in tonic $GABA_AR$ current magnitude versus EtOH-induced change in noise variance for each cell examined (all concentrations of EtOH tested 9-79 mM are included; SDR: n = 16 cells; B6: n = 27; D2: n = 36).

GCs, which respectively exhibit predominantly suppression and exclusively enhancement (Fig.

2J,K). As reported by others (Carta et al., 2004; Hanchar et al., 2005), in SDR GCs, concentrations of EtOH as low as 9mM (as would be achieved in an adult human consuming two units of alcohol) significantly enhance the magnitude of tonic $GABA_AR$ currents, and the degree of enhancement dose dependently increases until plateauing at about 52mM (Fig. 2J,K; 9mM: 0.81 ± .20pA, *n* = 21 cells; 31mM: 1.85 ± .38pA, *n* = 22 cells; 52mM: 4.65 ± .51pA, *n* = 47 cells; 79mM: 6.14 ± 1.29pA, *n* = 15 cells generated from a minimum 3 animals/condition). Conversely, EtOH significantly suppresses the magnitude of tonic $GABA_AR$ current in the majority of B6 GCs, starting at 9mM and plateauing at 52mM (Fig. 2J,K; 9mM: -0.79 ± 0.19pA, *n* = 23 cells; 31mM: -1.38 ± 0.31pA, *n* = 24 cells; 52mM: -3.37 ± 1.46pA, *n* = 39 cells; 79mM: -2.62 ± 0.96pA, *n* = 8 cells generated from a minimum 3 animals/condition). The magnitude of the EtOH-induced current in both directions was tightly and significantly correlated with the magnitude and polarity of the EtOH-induced change in noise variance (Fig. 2L; Pearson correlation, $r = 0.79$, $P < 0.001$, $n = 79$ cells from 5 SDRs, 10 D2s, and 10 B6s). Combined with the fact that EtOH does not induce any detectable currents in either direction in the presence of the $GABA_AR$ antagonist GABAzine (Fig. 2F,G), these data confirm that the EtOH-induced shifts in macroscopic current are mediated by enhancement and suppression of the opening of $GABA_AR$ receptor channels. Thus, both suppression and enhancement of $GABA_AR$-mediated tonic currents can occur in response to recreationally/clinically relevant concentrations of EtOH.

2.4.3. EtOH inhibition of nNOS increases Golgi cell firing

As has been shown previously in SDR GCs, blocking action potentials with TTX (500nM) abolishes EtOH-induced enhancement of GC sIPSC frequency and tonic $GABA_AR$ current (Fig. 3A,B; Carta et al., 2004). Thus, the increase in both sIPSCs and tonic $GABA_AR$ current are due to increased AP-driven, vesicular GABA release from Golgi cells (the GABAergic input to GCs), but the mechanisms mediating increased Golgi cell firing are not fully understood

(Botta et al., 2011; Carta et al., 2004). Biochemical and behavioral studies have determined that EtOH inhibits cerebellar NOS, and the inhibition of cerebellar NOS mediates EtOH-induced ataxia (Al Rejaie & Dar, 2006; Fataccioli et al., 1997), but the underlying mechanisms are not known. A recent study found that blocking NOS increases GC sIPSC frequency and tonic $GABA_AR$ current magnitude (which we confirm in Fig. 3C,D; Wall, 2003), very similar to what we and others observe for the response to EtOH (Fig. 2A). We therefore reasoned that the EtOH-induced increase in $GABA_AR$ transmission may be mediated by inhibition of NOS. In support of our hypothesis, blocking NOS with the substrate inhibitor L-NA (300μM) significantly inhibited the EtOH-induced increase in sIPSC frequency and tonic $GABA_AR$ current in both SDRs and D2s (Fig. 3E,F, all $P < .05$). To ensure that the block by L-NA is a true pharmacological blockade of EtOH actions at NOS, and not simply occlusion of the response to EtOH due to saturation of the vesicular GABA release machinery (since blocking NOS increases vesicle release, Fig. 3C), we conducted control experiments with nicotine. Nicotine (500nM) activates Golgi cell nicotinic acetylcholine receptors, which excites Golgi cells (Rossi et al., 2003), thereby increasing vesicular GABA release and resultant increases in tonic $GABA_AR$ currents (Fig. 3G). In contrast to the impact on EtOH, blocking NOS did not affect nicotine-induced increases in GC tonic $GABA_AR$ currents (Fig. 3G). Thus, the block by L-NA of EtOH-induced enhancement of IPSCs and tonic $GABA_AR$ currents is specific, and not due to occlusion by saturation of the system.

Our data suggest that EtOH-induced increases in GC IPSC frequency and tonic $GABA_AR$ current magnitude are mediated by EtOH suppression of a tonic production of NO that under control conditions limits the rate of vesicular GABA release. To further substantiate this notion, we used a fluorescent biochemical assay of cellular NO production, and tested whether NO is tonically generated in the GC layer, and whether EtOH suppresses such NO production (Fig. 4). DAF-FM diacetate is an NO-sensitive fluorophore that is cell permeant until

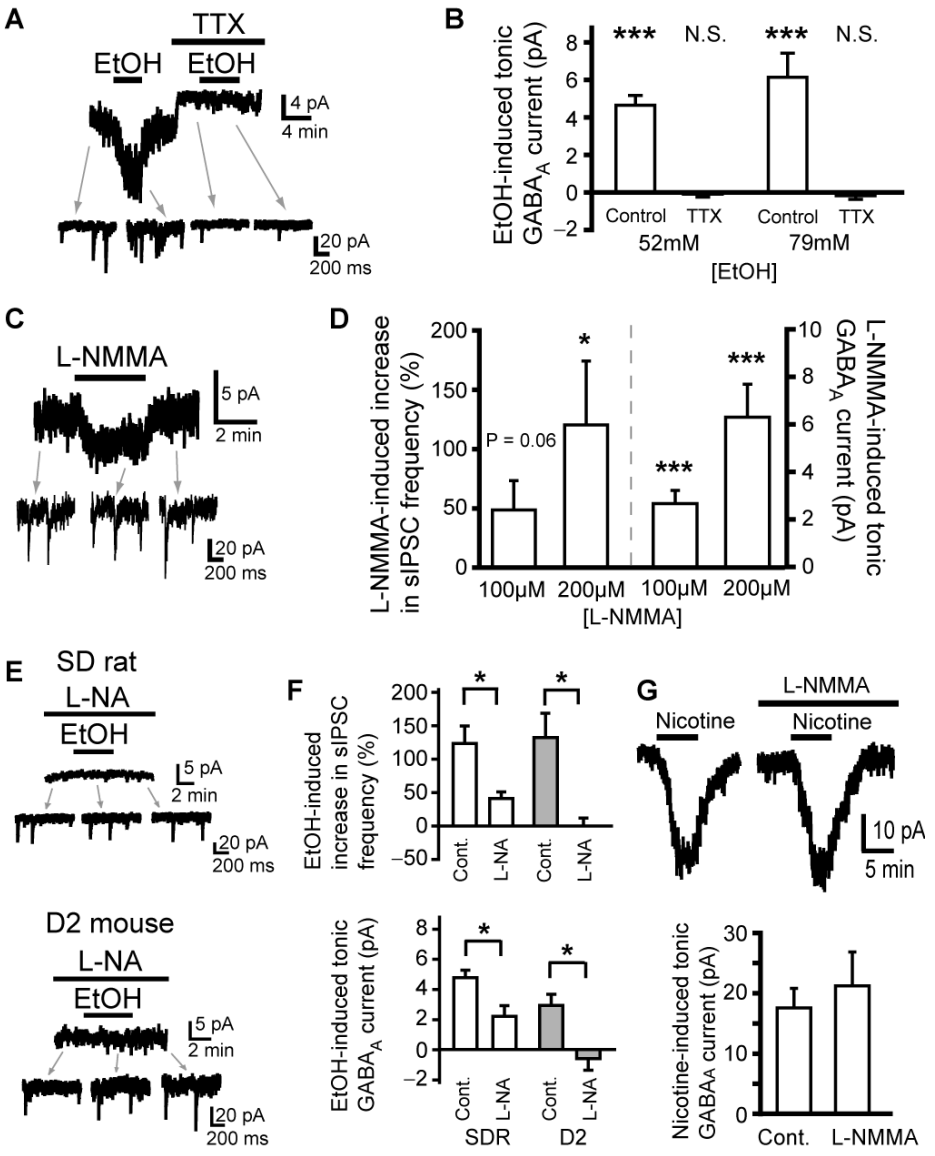


Figure 3. EtOH-induced potentiation of GC tonic $GABA_AR$ current is mediated by suppression of NOS and consequent increase in action potential dependent vesicular release of GABA in SDRs. **A.** Representative recording shows that blocking action potentials with TTX (500nM) abolishes the EtOH (52mM)-induced increase in sIPSCs and tonic $GABA_AR$ current magnitude. **B.** Chart depicts mean magnitude of EtOH-induced (52mM and 79mM) increase in tonic $GABA_AR$ current under control conditions (n = 47 cells for 52mM; n = 15 for 79mM) and in the presence of TTX (500nM; n = 11 cells from 3 animals for 52mM; n = 8 cells from 2 animals for 79mM). EtOH effects in TTX did not significantly differ from baseline, P = .54 for 52mM EtOH and P = .36 for 79mM EtOH; *** indicates significantly different from baseline P < 0.001 by one-sample t-tests. **C.** Representative recording shows that the NOS antagonist, L-NMMA (100µM) increases the frequency of sIPSCs and the tonic $GABA_AR$ current in SDR GCs. L-NMMA had no effect on GC currents in the presence of the $GABA_AR$ antagonist, GABAzine (10µM, n = 4 cells from 2 animals, not shown). **D.** Plot of mean increase in sIPSC frequency (left; 100uM: n = 22 cells from 10 animals, 48.34 ± 23.97%, P = .057; 200uM: n = 16 cells from 5 animals, 120.37 ± 53.94%, *P = .044, by one-sample t-tests) and tonic $GABA_AR$ current magnitude (right; 100uM: n = 22 cells, 2.56 ± 0.56, ***P < .001; 200uM: n = 16, 6.20 ± 1.38, ***P < .001, by one-sample t-tests) induced by L-NMMA in SDR GCs. **E.** Representative recordings showing that pre-incubation of slices and maintained presence in the NOS substrate inhibitor, L-NA (300µM), suppresses the EtOH-induced increase in sIPSCs and tonic $GABA_AR$ current in SDR (**top**) and D2 (**bottom**) GCs. **F.** Plots of mean increase in sIPSC frequency (**top**) and tonic $GABA_AR$ current magnitude (**bottom**) induced by EtOH (52mM) in SDR (white; n = 10 cells from 3 animals for L-NA treated slices, sIPSCs: *P = .015; tonic $GABA_AR$ current: *P = .026) and D2 (gray; n = 8 cells from 2 animals for L-NA treated slices, sIPSCs: *P = .015; tonic $GABA_AR$ current: *P = .029, comparisons between groups by Mann-Whitney U tests) GCs under control conditions and in slices treated with L-NA (300µM). **G.** Representative traces showing that L-NMMA (200µM) does not block nicotine (500nM)-induced increases in tonic $GABA_AR$ currents in SDR GCs. Mean increase in tonic $GABA_AR$ current magnitude induced by nicotine (500nM) in SDR GCs under control conditions = 17.56 ± 3.24 pA and in slices treated with L-NMMA (200µM) = 21.36 ± 5.61 pA (n = 5 cells from 2 animals, P = 0.46, paired t-test).

deacetylated by intracellular esterases, resulting in its intracellular accumulation. DAF-FM is essentially non-fluorescent until it reacts with NO, whereupon its fluorescence quantum yield increases 180 fold, making it an ideal intracellular sensor of [NO]. Cerebellar slices were soaked for 30 minutes in aCSF supplemented with DAF-FM (1µM; *n* = 20 slices from 2 animals) alone, DAF-FM + the NOS antagonist, L-NA (300µM; *n* = 22 slices from 2 animals), or DAF-FM + EtOH (52mM; *n* = 20 slices from 2 animals). Slices were then fixed, and analyzed with confocal microscopy. 3-D projections of confocally acquired images of DAF-FM treated slices revealed robust fluorescence throughout the GC layer and in the white matter axon tracks innervating the GC layer (Fig. 4A). Slices treated with DAF-FM + L-NA exhibited a similar pattern of fluorescence emission, but the intensity was significantly reduced compared to DAF-FM alone ($P < 0.001$; Fig. 4B,D), confirming that the fluorescence signal is generated by NO production by NOS. EtOH (52mM) also significantly reduced the intensity of fluorescence emission without noticeably affecting the pattern ($P < 0.001$ by unpaired *t*-test; Fig.

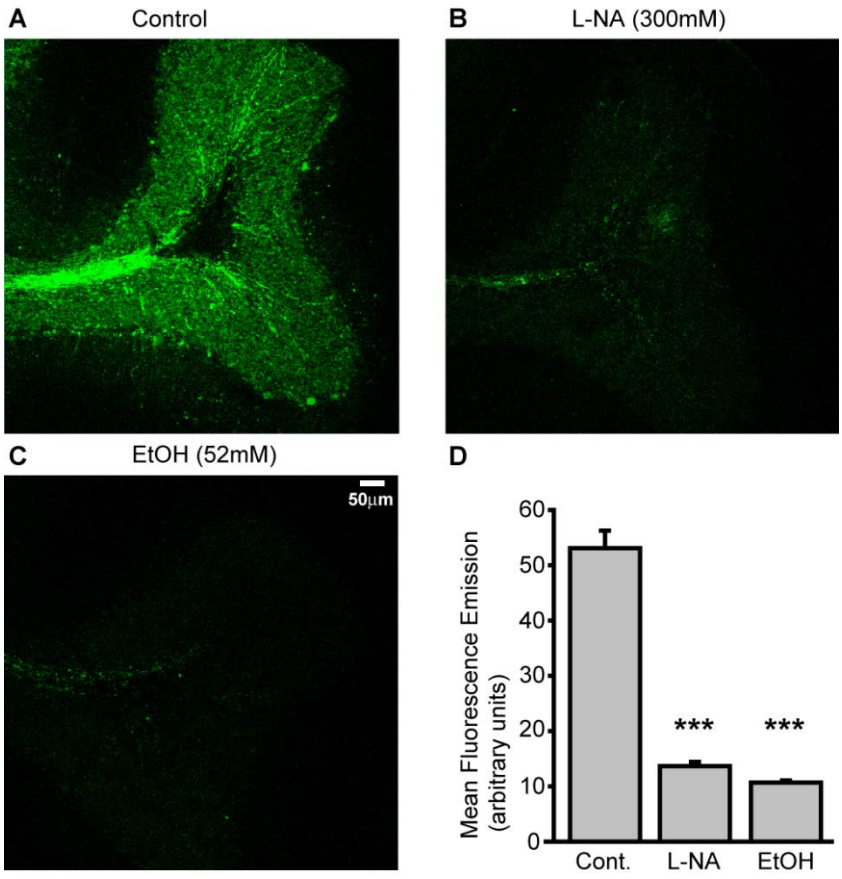


Figure 4. EtOH suppresses tonic production of NO in the granule cell layer. **A-C**. Confocally acquired images of fluorescence emission from SDR cerebellar slices that were pre-soaked in either the NO-sensitive fluorophore, DAF-FM alone (**A**), or combined with the NOS antagonist, L-NA (300µM; **B**) or EtOH (52mM; **C**). **D.** Plot of mean DAF fluorescence emission across the granule cell layer under control conditions (*n* = 20 slices from 2 animals), or when treated with L-NA (300µM, *n* = 22 slices from 2 animals) or EtOH (52mM, *n* = 20 slices from 2 animals). *** indicates significantly different from control slices, $P < 0.001$, unpaired *t*-tests.

4C,D). Thus, there is a tonic production of NO in the GC layer, and EtOH substantially inhibits that production.

Our data indicate that EtOH-induced increase in vesicular GABA release is triggered by blocking NO production, but is dependent on Golgi cell AP firing (since it is abolished by TTX, Fig. 3A,B). This raises the question of whether EtOH block of NOS causes the increase in Golgi cell AP firing that drives increased GABA release. To test this possibility, we made current-clamp recordings of Golgi cell responses to EtOH and NOS antagonists (Fig. 5). Under control conditions, with appropriate current injection (mean = 21.8 ± 18.6pA), Golgi cells stably fired APs (mean frequency = 0.7 ± 0.2Hz, n = 12 cells from 4 animals; Fig. 5A,B, top panels). Bath application of an NOS antagonist (L-NMMA, 200µM) or EtOH (52mM) significantly increased Golgi cell AP firing frequency (L-NMMA-induced increase = 26.7 ± 6.1%, *n* = 4 cells from 2 animals; EtOH-induced increase = 53.6 ± 14.1%, *n* = 8 cells from 4 animals; Fig. 5A,B,D). Pretreatment with another NOS antagonist (L-NA, 300µM) also increased Golgi cell firing frequency, and prevented further increases by EtOH (EtOH-induced change = -10.6 ± 8.3%, *n* = 9 cells from 3 animals; Fig. 5C,D). Thus, EtOH inhibition of NO production increases Golgi cell AP firing, thereby driving EtOH-induced increases in GC IPSC frequency and tonic $GABA_AR$ current magnitude.

Collectively, our data indicate that the EtOH-induced increase in GC sIPSCs and tonic $GABA_AR$ current is driven by increased Golgi cell AP firing activity, consequent to EtOH suppression of basal NO production. We therefore hypothesized that lower NOS expression and consequent NO production could underlie the relative lack of potentiation of $GABA_AR$ inhibition in B6 GCs compared to SDR and D2 GCs (Fig. 2E). Accordingly, we used neuronal NOS (nNOS) immunohistochemistry and confocal microscopy to examine the expression and distribution of nNOS in SDR, D2 mouse and B6 mouse cerebellum (Fig. 6A-G). nNOS expression is widespread and confluent in the granule cell layer of the SDR and D2 cerebellum,

primarily in a ring-like pattern, encircling GC nuclei stained with Hoechst (Fig. 6A,C), and in the cytoplasm of Golgi cell somas (identified by their larger size and expression of the GABA synthetic enzyme GAD65/67; Fig. 6E). Although similar ring-like staining was seen in B6 cerebellum, it was greatly restricted, and there were frequent dark patches, or lack of staining throughout the B6 GC layer (Fig. 6B),

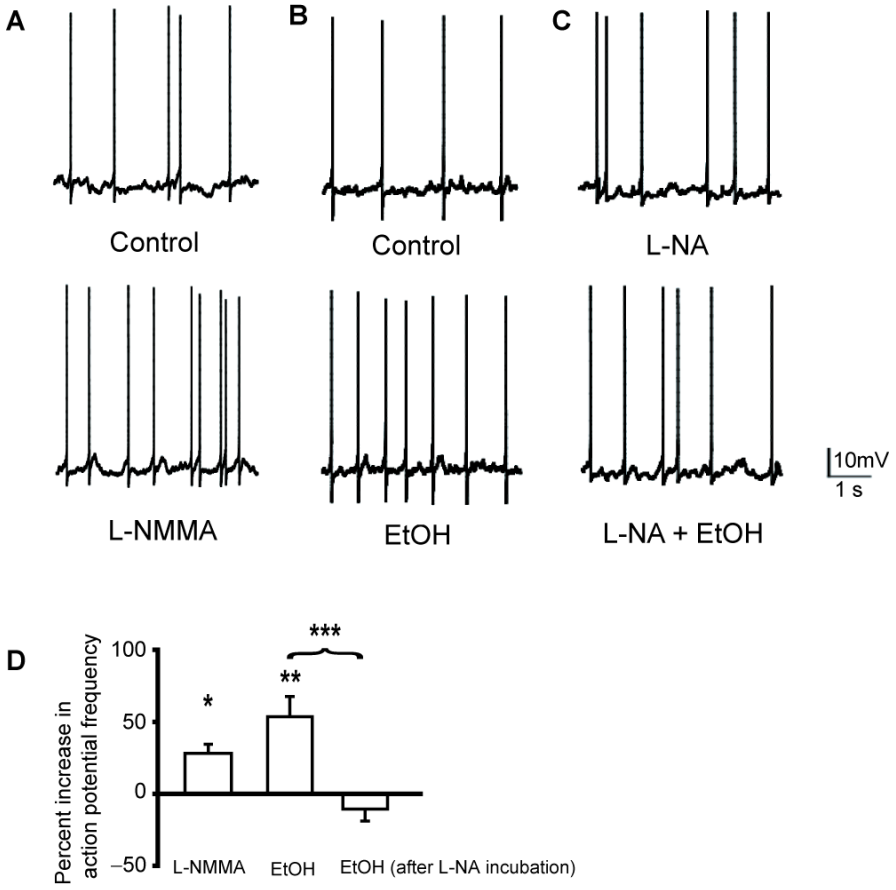


Figure 5. EtOH inhibition of NOS increases Golgi cell firing. **A-C.** Representative traces show action potentials (APs) in current-clamped Golgi cells under various conditions: **A.** Control (top) versus the NOS antagonist, L-NMMA (200µM, bottom) in the same cell, **B.** control (top) versus EtOH (52mM, bottom) in the same cell, and **C.** in the presence of the NOS antagonist, L-NA (300µM, top) alone or with EtOH (52mM, bottom) in the same cell. **D.** Plot of mean increase in Golgi cell AP firing frequency in L-NMMA (200µM; n = 4 cells, *P = .022, one-sample t-test), EtOH (52µM; n = 9, **P = .007), or EtOH after incubation and in the presence of the NOS antagonist, L-NA (300µM, n = 9, P = .24, one-sample t-tests). Unpaired t-test comparing conditions, **P = .001.

and the staining intensity in individual Golgi cell somas also appeared less intense (Fig. 6F). Importantly, nNOS signal was completely absent in slices from mice in which the nNOS gene was deleted, confirming the specificity of our antibody (Supplementary Fig. 4). Although interpreting differences in immunostaining intensity across preparations can be problematic, given the stark qualitative differences, we developed two methods to quantify nNOS expression levels around GCs and in Golgi cell somas, respectively. Since nNOS expression around GC somas appeared to reflect an all or nothing absence or presence of nNOS at various points around the GC soma perimeter, rather than a confluent difference in expression level at all

points, we quantified the degree of GC encirclement. To do this, we adjusted the gain of each image to equalize peak fluorescence intensities across slices, and then calculated the degree of GC encirclement for every GC in the image (see Supplementary Fig. 3 for details). The degree of GC encirclement by nNOS staining differed between groups ($H(2) = 2290.13$, $P < .001$) and was significantly smaller in B6 slices compared to SDR and D2 slices (Fig. 6D, both $P < 0.001$ by Dunn's method of multiple comparisons). Conversely, since the pattern of nNOS staining in Golgi cell somas was qualitatively similar across species, but appeared less intense in B6 mouse Golgi cells, we quantified overall Golgi cell nNOS staining intensity. To reduce potential variability caused by differences in antibody penetration or light scattering across preparations, we normalized nNOS staining intensity to the staining intensity of GAD 65/67 in the same cell. The ratio of the mean intensity (across the entire Golgi cell soma) of nNOS staining relative to GAD 65/67 staining is significantly smaller in B6 mouse Golgi cells compared to either D2 mouse or SDR Golgi cells (Fig. 6G; main effect of group, $F(2,18) = 31.03$, $P < 0.001$ by one-way ANOVA and $P < .001$ for both pairwise comparisons for SDR and D2 compared to B6). Thus, the level of nNOS expression in Golgi cell bodies, as well as in GC somas that densely surround Golgi cell somas, dendrites, and axons (Fig. 6E,F), is significantly reduced in B6 mice compared to D2 mice and SDRs. Given the diffusible nature of NO, both compartments could influence the Golgi cell target that suppresses Golgi cell AP firing, and thus the reduction in both compartments in B6 mice could explain the absence of EtOH-induced enhancement of GC sIPSCs and tonic $GABA_AR$ current (Fig. 2E,H).

To test whether the observed differences in nNOS immunostaining manifest as reduced NOS function, we used GC $GABA_AR$ current responses to pharmacological inhibition of NOS as a bioassay of NOS function (Fig. 6H-L). In keeping with reduced expression of nNOS in B6 cerebellum, blocking NOS directly, with the NOS substrate antagonist L-NMMA (200µM) produced a significantly smaller potentiation of sIPSC frequency and tonic $GABA_AR$ current in

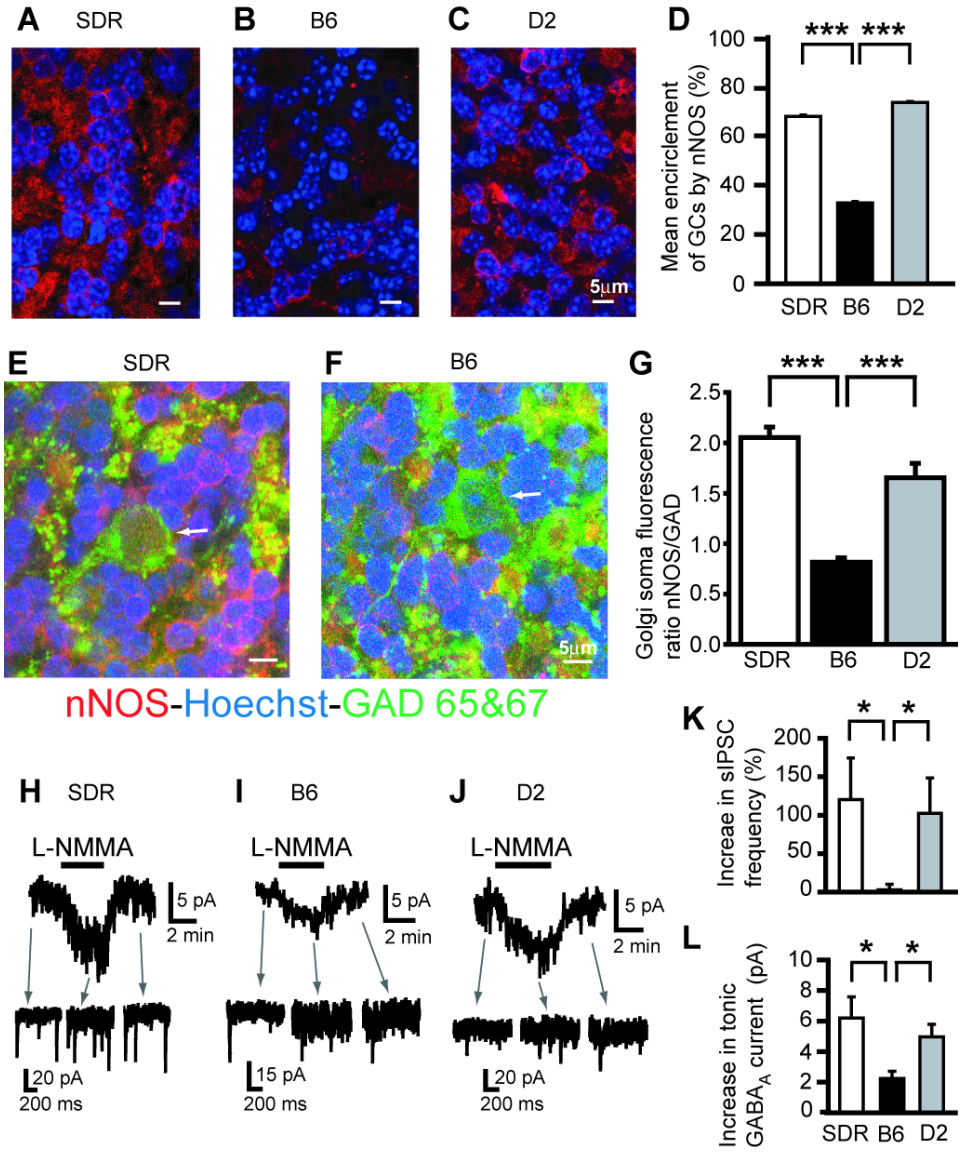


Figure 6. Differential expression of nNOS underlies differences in EtOH-induced potentiation of GC $GABA_AR$ transmission. **A-C.** Confocally acquired images of immunohistochemistry for nNOS (red) and nuclear stain Hoechst (blue) in the granule cell layer of SDR (**A**), B6 (**B**), and D2 (**C**) cerebellum. **D.** Plot of mean percent encirclement of GC soma/nuclei (as determined in **Supplementary Fig. 1**) for SDR (white; *n* = 2694 cells from 2 slices from 2 animals), B6 (black; *n* = 2316 cells from 6 slices from 2 animals) and D2 (gray; *n* = 3398 cells from 5 slices from 2 animals) cerebellum. *** indicates significantly different, *P* < 0.001. **E-F.** Confocally acquired images of immunohistochemistry for nNOS (red), GAD 65/67 (green) and nuclear stain Hoechst (blue) in the granule cell layer of SDR (**E**) and B6 (**F**) cerebellum. White arrows point to the cell body of a Golgi cell in each figure. **G.** Plot of mean ratio of fluorescence emission intensity for nNOS staining versus GAD 65/67 staining in individual Golgi cells in SDR (white, *n* = 6 slices), B6 (black, *n* = 6), and D2 (gray, *n* = 7, each from 2 animals) cerebellum (ratio in B6 = 0.82 ± 0.04, *n* = 6 SDR = 2.05 ± 0.10, D2 = 1.65 ± 0.14) *** *P* < 0.001 by unpaired *t*-tests. **H-J.** Representative recordings showing increase in tonic $GABA_AR$ current and sIPSC frequency induced by NOS substrate inhibitor, L- NMMA (200µM), in SDR (**H**), B6 (**I**) and D2 (**J**) GCs. **K,L.** Plots of mean increase in sIPSC frequency (**K**) and tonic $GABA_AR$ current magnitude (**I**) induced by L-NMMA (200µM) in SDR (white; *n* = 16 cells), B6 (black; *n* = 11), and D2 (gray; *n* = 17) GCs. * *P* < 0.05 by Mann-Whitney U tests.

B6 GCs (increase in sIPSC frequency = 2.76 ± 7.55%; tonic current = 2.25 ± 0.47pA, *n* = 11 cells from 4 animals) compared to SDR (increase in sIPSC frequency = 120.37 ± 53.94%; tonic current = 6.20 ± 1.39pA, *n* = 16 cells from 7 animals) and D2 GCs (increase in sIPSC frequency = 102.47 ± 46.08%; tonic current = 4.97 ± 0.82pA, *n* = 17 cells from 4 animals; *P* < 0.05 by Mann-Whitney U tests; Fig. 6K,L).

Although reduced expression of nNOS could alone account for the lack of physiological response to EtOH in B6 mice, another possible contribution is reduced activity of the nNOS that is expressed. To test this possibility, we treated slices with L-arginine, a rate limiting reactant in the production of NO by NOS. In SDR slices, L-arginine (100µM) enhanced EtOH-induced increases in sIPSC frequency and tonic $GABA_AR$ current magnitude (Supplementary Fig. 5A,C,D), suggesting that endogenous levels of L-arginine are rate limiting for NO production in the SDR cerebellum. In contrast, L-arginine did not affect the actions of EtOH on $GABA_AR$ currents in B6 mice (Supplementary Fig. 5B,C,D). Taken together, the data suggest that the absence of potentiation of GC $GABA_AR$ transmission in ~85% of B6 GCs (Fig. 2E) is primarily due to a lower level of expression of nNOS, the inhibition of which mediates EtOH-induced potentiation of $GABA_AR$ transmission in SDR and D2 GCs.

2.4.4. Postsynaptic EtOH actions suppress tonic $GABA_AR$ currents

While differences in nNOS expression explain the relative lack of potentiation of GC $GABA_AR$ transmission in B6 GCs, it does not explain the suppression of GC $GABA_AR$ transmission observed in ~45% of B6 GCs and ~10% of D2 GCs (Fig. 2E). Since EtOH-induced potentiation of GC tonic $GABA_AR$ current is due to action-potential dependent increased vesicular release of GABA (Fig. 3A,B), we considered that suppression of the tonic current might be mediated by suppression of vesicular release of GABA. However, the sIPSC frequency was not suppressed in B6 GCs that showed EtOH-induced suppression of the tonic $GABA_AR$ current (Fig. 7A). Furthermore, blocking APs with TTX did not affect the EtOH-induced suppression of tonic $GABA_AR$ current in B6 GCs (Fig. 7B,C). Interestingly, in D2 GCs, TTX not only blocked the EtOH-induced potentiation of tonic $GABA_AR$ current, but it converted potentiation into suppression, similar to EtOH actions in B6 GCs (Fig. 7B,C; EtOH-induced current in TTX in SDR: 0.09 ± 0.15pA, *n* = 11 cells from 6 animals; B6: 2.77 ± 1.26pA, *n* = 13 cells from 3 animals; D2: 2.71 ± 1.00pA, *n* = 9 cells from 2 animals). These data indicate that

EtOH can inhibit tonic $GABA_AR$ currents independent of vesicular GABA release, and inhibition is dominant in B6 GCs, absent in SDR GCs and present in D2 GCs but typically obscured by EtOH-induced increases in GABA release similar to what is observed in SDR GCs.

Suppression of tonic $GABA_AR$ currents without corresponding changes in vesicular GABA release is suggestive of a direct action of EtOH on the extrasynaptic $GABA_ARs$ that generate tonic $GABA_AR$ inhibition. However, changes in extracellular GABA concentration can occur independent of changes in vesicular release (Rossi et al., 2003). To circumvent potential actions of EtOH on GABA release or uptake mechanisms, and directly test whether EtOH suppression of tonic $GABA_AR$ currents is via direct actions in the postsynaptic cell, we conducted nucleated patch experiments, whereby the GC is extracted from the slice with the recording electrode (Fig. 7D-F). We then bath applied a low concentration of GABA (100nM, which should only activate high affinity α6,δ containing $GABA_ARs$; Meera et al., 2011) to isolated GCs, and examined responses to EtOH. In addition to visual determination of physical isolation, we confirmed that GC somas were functionally isolated from the slice by the disappearance of tonic $GABA_AR$ currents and sIPSCs (Fig. 7D). Using this approach, for B6 GCs in which EtOH suppressed the tonic $GABA_AR$ current under whole-cell recording conditions within the slice (% suppression of tonic $GABA_AR$ current = 15.50 ± 2.27%, *n* = 20 cells from 6 animals), EtOH also significantly suppressed subsequent responses of isolated GCs to exogenous GABA, and to a similar degree (% suppression of GABA-induced current = 20.29 ± 7.76%, *n* = 6 cells from 2 animals; Fig. 7E,F). Upon washout of exogenous GABA, subsequent application of GABAzine did not generate any detectable currents (Fig. 7E), confirming that physically isolated GCs were not affected by endogenous GABA release from the slice. These results unequivocally demonstrate that EtOH can act directly on the postsynaptic cell to suppress extrasynaptic $GABA_AR$ tonic current responses to a fixed concentration of GABA.

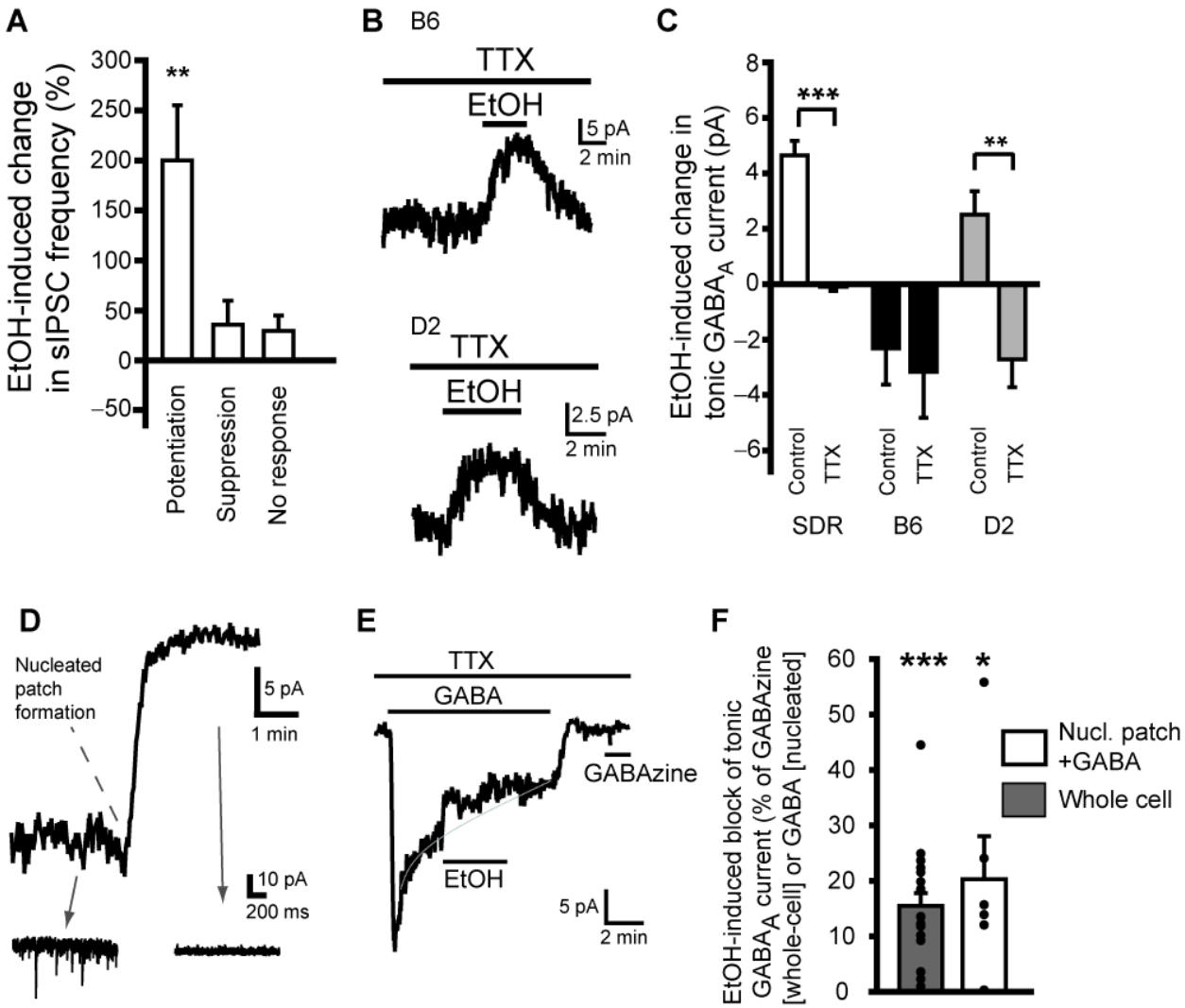


Figure 7. EtOH-induced suppression of tonic $GABA_AR$ current in B6 and D2 GCs is due to direct action on $GABA_ARs$. **A.** Plot of mean EtOH-induced change in sIPSC frequency in B6 GCs that exhibited EtOH-induced potentiation, suppression or no change of tonic $GABA_AR$ current. $^{**}P < .01$ by one-sample *t*-tests. **B.** Representative recordings showing that EtOH-induced suppression of tonic $GABA_AR$ current in B6 (top) or D2 (bottom) GCs 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 or in TTX (500nM) shows that TTX abolishes responses to EtOH in SDR GCs (white; $n = 47$ cells for control, $n = 11$ for TTX, $^{***}P < .001$), has no effect on responses to EtOH in B6 GCs (black; $n = 39$ for control, $n = 10$ for TTX, $P = .76$) and converts mean response to EtOH from potentiation to suppression in D2 GCs (gray; $n = 31$ for control, $n = 9$ for TTX, $^{**}P = .003$, by unpaired *t*-tests). **D.** Representative trace showing GC holding current during whole-cell recording configuration and upon transition to nucleated-patch recording configuration. Note loss of tonic $GABA_AR$ current and sIPSCs (insets) upon excision of the nucleated patch from the slice. **E.** Representative trace showing a nucleated patch recording from a B6 GC in the presence of TTX, during application of exogenous GABA (100nM) and subsequently EtOH (52mM), which suppresses the exogenous GABA evoked current. Note, after wash of exogenous GABA, subsequent application of the $GABA_AR$ antagonist, GABAzine (10μM) does not generate any current, confirming that the nucleated patch is isolated from endogenous sources of GABA from the slice. **F.** Plot of mean percent block by EtOH of tonic $GABA_AR$ currents in whole cell recordings (gray, $n = 20$ cells, $^{***}P < .001$) and of currents evoked by exogenous GABA applied to nucleated patches (white, $n = 6$, $^{*}P = .047$. All data from B6 GCs.

2.4.5. Low GC PKC activity enables postsynaptic EtOH action

Because tonic $GABA_AR$ currents in B6 and D2 mouse, and SDR GCs appear to be mediated by similar receptor subunits (α6βxδ; Fig. 1), but show a differential sensitivity to direct suppression by EtOH, we considered postsynaptic modulatory mechanisms that might alter $GABA_AR$ sensitivity to EtOH. Previous studies showed that genetic deletion of PKCγ reduced EtOH stimulation of $GABA_AR$-mediated Cl^- fluxes in a cerebellar microsac assay (Harris et al., 1995), but the microsac Cl^- flux assay does not provide any information about the cell types involved, the underlying type of $GABA_AR$ currents or the responsible $GABA_ARs$. In particular, with a mixed population of cells and $GABA_ARs$, it is possible that the reported global reduction of EtOH stimulation is achieved by increasing EtOH suppression of a subset of $GABA_ARs$, i.e. those mediating tonic $GABA_AR$ currents in GCs (PKCγ is heavily expressed in all cell layers of the cerebellum; Naik et al., 2000). To test this possibility, we determined whether blocking PKC in SDR GCs converted their normally insensitive tonic $GABA_AR$ currents to being sensitive to EtOH-induced suppression (Fig. 8A,C). In the presence of TTX (to isolate direct suppressive actions of EtOH), addition of the PKC inhibitor, Calphostin C (100nM), to the recording electrode solution, transformed SDR GCs such that EtOH reduced the magnitude of the tonic $GABA_AR$ current (-1.03 ± 0.41pA, n = 20 cells from 5 animals, P = .022) to a similar degree to what is observed in untreated B6 GCs (P = .47; Fig. 8A,C). Thus, insensitivity of SDR GC $GABA_AR$ tonic currents to direct suppression by EtOH is a consequence of ongoing postsynaptic PKC activity. We therefore tested whether activating postsynaptic PKC in B6 GCs prevents EtOH suppression of tonic $GABA_AR$ currents (Fig. 8B,C). As predicted, in recordings from B6 GCs (in TTX), including the phorbol ester PKC activator, PMA (100nM), in the recording electrode, abolished EtOH suppression of tonic $GABA_AR$ currents (0.02 ± 0.22pA, n = 8 cells from 2 animals, P = 0.94 by unpaired t-tests; Fig. 8B,C). The actions of PKC on $GABA_AR$ currents is specific to their sensitivity to EtOH, because neither inclusion of PMA in B6 GCs nor Calphostin C in SDR GCs affected the baseline magnitude of tonic $GABA_AR$ current (SDR control = 7.80 ± 1.42 pA; SDR Calphostin C = 7.75 ± 1.16pA; B6 control = 10.97 ± 0.66pA; B6 PMA = 8.30 ±

2.60pA; all $P > 0.05$). Our data indicates that EtOH can directly suppress tonic $GABA_AR$ currents in all genotypes tested, but such direct suppression is prevented by elevated postsynaptic PKC activity, whether it occurs naturally, as in SDRs, or it is pharmacologically induced in rodent genotypes with lower basal PKC activity.

To determine the relative importance of PKC regulation of EtOH direct suppression versus nNOS regulation of EtOH enhancement (via increased GABA release), we repeated the PKC experiments in B6 mouse and SDR GCs under control conditions (i.e., without TTX; Fig. 8D). In recordings of SDR GCs with Calphostin C in the recording electrode, although EtOH still enhanced the tonic $GABA_AR$ current, the enhancement was significantly less than for control cells (Calphostin C = 1.81 ± 0.60pA, $n = 16$

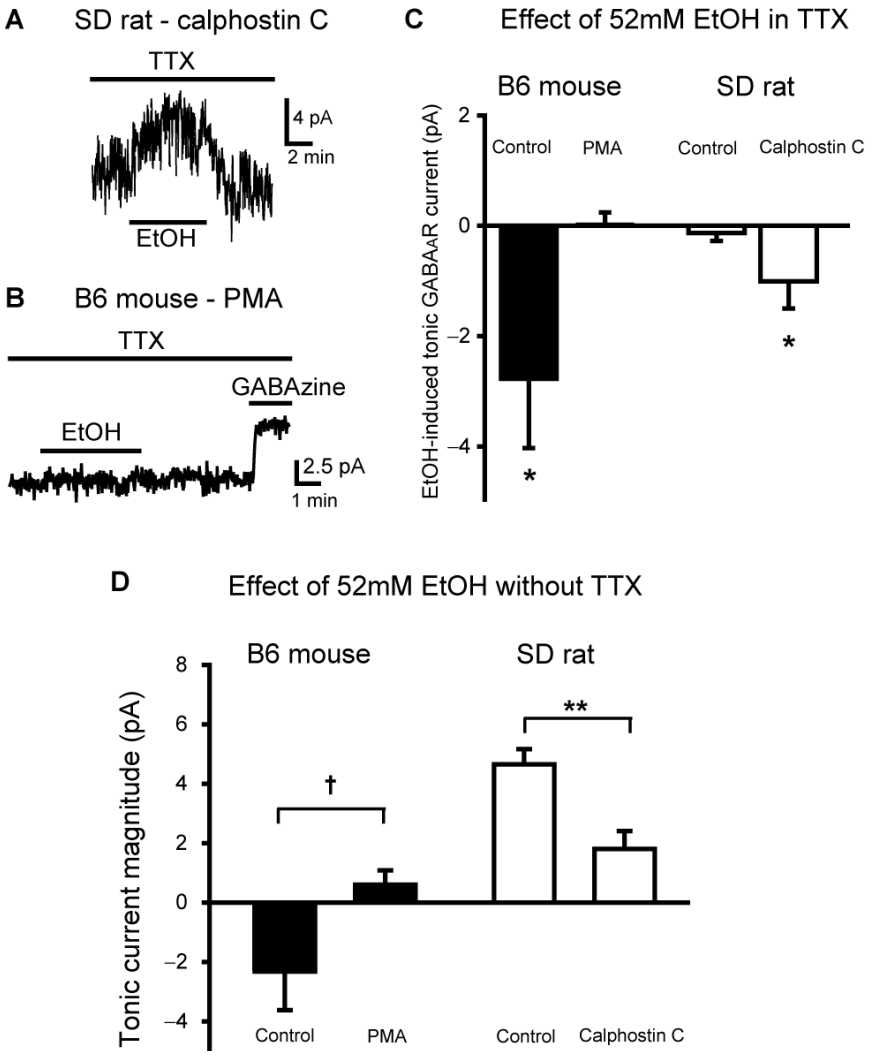


Figure 8. Direct suppression of tonic $GABA_AR$ current by EtOH is prevented by postsynaptic PKC activity. **A**. Representative recording from an SDR GC showing that in the presence of TTX (500nM) in the bath, and PKC inhibitor, Calphostin C (100nM), in the recording electrode, EtOH suppresses the tonic $GABA_AR$ current. **B.** Representative recording from a B6 GC showing that in the presence of TTX (500nM) in the bath, and PKC activator, PMA (100nM), in the recording electrode, EtOH does not suppress the tonic GAB_AR current. **C.** Plot of mean EtOH-induced change in tonic $GABA_AR$ current in the presence of TTX (500nM), in B6 GCs (black) with ($n = 13$ cells) or without ($n = 8$) PMA in the recording electrode, and in SDR GCs (white) with ($n = 20$) or without ($n = 11$) Calphostin C in the recording electrode. * indicates significantly different from baseline, $*P < 0.05$ by one-sample *t*-tests. **D.** Plot of mean EtOH-induced change in tonic $GABA_AR$ current in normal aCSF (no TTX added), in B6 GCs (black) with ($n = 6$ cells) or without ($n = 39$) PMA in the recording electrode, in SDR GCs (white) with ($n = 16$) or without ($n = 47$) Calphostin C in the recording electrode. $**P = .003$, † = .089, by Mann-Whitney U tests.

cells from 3 animals, *P* = .003; Fig. 8D). Conversely, in recordings of B6 GCs with PMA in the recording electrode, EtOH no longer suppressed the tonic $GABA_AR$ current, but rather slightly increased it (PMA = 0.67 ± 0.46pA, *n* = 6 cells from 2 animals, *P* = .089 by Mann-Whitney U tests; Fig. 8D). Combined with the nNOS experiments above, the data confirm that the net response to EtOH is determined by the balance between EtOH-induced increases in GABA release (via inhibition of nNOS) and direct suppression of postsynaptic $GABA_ARs$, with genetic variations in the two processes determining net response phenotype.

2.5. Discussion

2.5.1. Genetic variation in response to EtOH

Genetic contribution to AUDs must, at some level, manifest as a differential response to EtOH, but little is known about the underlying neural substrates. Here we used patch-clamp recording in brain slices to directly assess differences in EtOH-related neural mechanisms across rodent genotypes with documented divergent EtOH related behaviors. We focused on the cerebellum because the severity of EtOH-induced disruption of cerebellar dependent behaviors is inversely related to risk for developing AUDs in humans (Schuckit et al., 1996; Schuckit et al., 2005) and excessive EtOH consumption in many rodent models (Bell et al., 2001; Galaher et al., 1996; Malila, 1978; McClearn et al., 1981; Yoneyama et al., 2008). We found that the impact of EtOH on GC $GABA_AR$ transmission ranges from strong potentiation in high EtOH sensitivity/low EtOH consuming SDRs and D2s to strong suppression in low sensitivity/high consuming B6s, with intermediate impact in moderate sensitivity/moderate consuming WSR1 and WSP1 mice (Fig. 2I). While there are many factors that influence EtOH consumption by rodents, including taste (Fidler et al., 2011; McCool & Chappell, 2012), to our knowledge this is the first example of a cellular response to EtOH that varies in magnitude and polarity in parallel with EtOH behavioral phenotypes. And while it is unlikely that any one molecular process or brain region will fully explain a given EtOH phenotype, that a relationship between cellular response to EtOH and

EtOH consumption (Fig. 2I) holds across species (rats and mice), across inbred mice strains (B6 and D2), and across selected lines of mice (WSR and WSP) suggests that there is some overlap in the genes that influence EtOH consumption and GC $GABA_AR$ responses to EtOH. Importantly, in contrast to many brain regions such as the hippocampus (Liang et al., 2006), nucleus accumbens (Nie etal., 2000), thalamus (Jia et al., 2008), ventral tegmental area (Thiele et al., 2009), and substantia nigra (Peris etal., 1992), where EtOH only affects $GABA_AR$ transmission at concentrations far above those typically achieved during voluntary consumption by rodents or humans (≥ 50mM), both suppression and enhancement of GC $GABA_AR$ transmission occur at low, readily achieved concentrations of alcohol (9-31mM; Fig. 2J,K). Thus, to the extent that EtOH actions at $GABA_ARs$ influence acute EtOH sensitivity or voluntary consumption levels (Criswell et al., 2008; Kumar et al., 2009), the distinctively high sensitivity of GC $GABA_AR$ transmission and clear variation in response magnitude and polarity across rodent genotypes (Fig. 2I) highlights such actions as a potential cellular substrate for genotypic differences in EtOH phenotypes. Future studies should test whether experimental manipulations of GC $GABA_AR$ responses to EtOH influence EtOH sensitivity or consumption phenotype, and our identification of the molecular mediators of $GABA_AR$ responses (discussed below) should provide necessary targets for such endeavors.

Our findings also have important implications for interpreting behavioral data from knockout and transgenic mice, which are often created on a B6 background. In particular, genetic deletion of the α6 or δ subunit in B6 mice, which eliminates GC tonic $GABA_AR$ currents (Brickley et al., 2001; Stell et al., 2003), does not affect EtOH-induced disruption of rotarod performance (Korpi etal., 1999; Mihalek et al., 2001). However, studies in low EtOH consuming CD-1 mice clearly indicate that the cerebellum is the primary site of action for EtOH-induced disruption of performance on the rotarod (Al Rejaie & Dar, 2006), and, although controversial (Botta et al., 2007), studies in low drinking SDRs suggest that EtOH enhancement of tonic $GABA_AR$ inhibition in GCs is a primary mechanism of such disruption (Hanchar et al., 2005).

Our determination that EtOH has opposite actions on GC tonic $GABA_AR$ currents in B6 mice and SDRs may explain this apparent discrepancy. If the EtOH-induced suppression of tonic $GABA_AR$ currents observed in B6 mouse GCs is less disruptive of rotarod performance than the enhancement of tonic $GABA_AR$ currents observed in SDR GCs, then genetic deletions that eliminate tonic $GABA_AR$ currents in B6 mice would have less impact on EtOH disruption of rotarod performance than will manipulations of tonic $GABA_AR$ currents in SDR GCs. Importantly, B6 mice are less sensitive than D2 mice to EtOH disruption of rotarod performance (Gallaher et al., 1996), suggesting that indeed EtOH-induced suppression of $GABA_AR$ tonic currents in B6 mice is less disruptive of rotarod performance than potentiation of $GABA_AR$ tonic currents in D2 mice. Thus, accurate interpretations of past and future behavioral studies of EtOH in rodents with genetic or pharmacological alteration of α6 or δ subunits will require electrophysiological assessment of the actions of EtOH on GC tonic $GABA_AR$ currents specifically in the line or strain of rodent being tested. Here we provide this information for five frequently studied genotypes: B6 mice, D2 mice, WSR mice, WSP mice, and SDRs.

2.5.2. EtOH-induced enhancement of $GABA_AR$ transmission

In SDRs, EtOH-induced potentiation of GC $GABA_AR$ inhibition is mediated by an increase in AP-dependent, vesicular GABA release from Golgi cells (Fig. 3A,B; Botta et al., 2007; Carta et al., 2004; Hanchar et al., 2005). The increase in vesicular GABA release causes an increase in the frequency of GC sIPSCs and the magnitude of the tonic $GABA_AR$ current, due to accumulation of GABA in the extrasynaptic space. It has been suggested that EtOH also binds directly to GC extrasynaptic $GABA_ARs$ and increases their affinity for GABA, the degree of which is influenced by a nucleotide polymorphism in the $GABA_AR$ α6 subunit (Hanchar et al., 2005). Such a polymorphism could represent a mechanism for genetic control of GC $GABA_AR$ sensitivity to EtOH. However, in agreement with several other studies (Botta et al., 2007; Carta et al., 2004), in our hands, even at 79mM, EtOH did not cause detectable potentiation of GC

tonic $GABA_AR$ currents in the presence of TTX (Fig. 3A,B), arguing against direct potentiation of extrasynaptic $GABA_ARs$ by EtOH. Furthermore, since under physiological conditions (no TTX), EtOH-induced increased vesicular GABA release is the predominant cause of EtOH-enhanced GC tonic $GABA_AR$ currents (Botta et al., 2007; Carta et al., 2004), genetic control over that process is likely to be a more powerful control point. Indeed, here we demonstrate that the EtOH-induced increase in vesicular GABA release is mediated by suppression of NOS, and that genetic variation in nNOS expression across rodent genotypes powerfully controls the degree to which EtOH potentiates GC $GABA_AR$ transmission. Strikingly, based on our analysis of immunohistochemistry and bioassay, we estimate that the expression level of nNOS in the granule cell layer of B6 mice is about 25% of the levels expressed in SDRs and D2 mice (Fig. 6). Correspondingly, less than 20% of B6 GCs exhibited EtOH potentiation of GC $GABA_AR$ tonic currents, compared to >90% of SDR GCs and ~50% of D2 GCs (Fig. 2E). Our data do not address how blockade of nNOS leads to increased Golgi cell excitability, but recent studies suggest that suppression of the Na^+/K^+-ATPase and a K^+ conductance contribute to the EtOH–induced increase in Golgi cell firing (Botta et al., 2011), and both could be down-stream targets of NO. In particular, NO is known to increase Na^+/K^+-ATPase activity (White et al., 2008), so EtOH induced suppression of nNOS could reduce Na^+/K^+-ATPase activity, with resultant depolarization of Golgi cells. Similarly, while the molecular identity of the K^+ conductance that contributes to increased Golgi cell excitability is unclear (Botta et al., 2011), many K^+ conductances are positively modulated by NO, either directly or indirectly via NO-induced cGMP production (Salapatek et al., 1998; Wang et al., 1997). Thus, EtOH suppression of NOS could also contribute to the suppression of K^+ channels that increases Golgi cell firing.

2.5.3. EtOH-induced suppression of $GABA_AR$ transmission

It is widely accepted that enhancement of $GABA_AR$ transmission is a primary mechanism underlying the behavioral effects of EtOH (Criswell et al., 2008; Kumar et al., 2009).

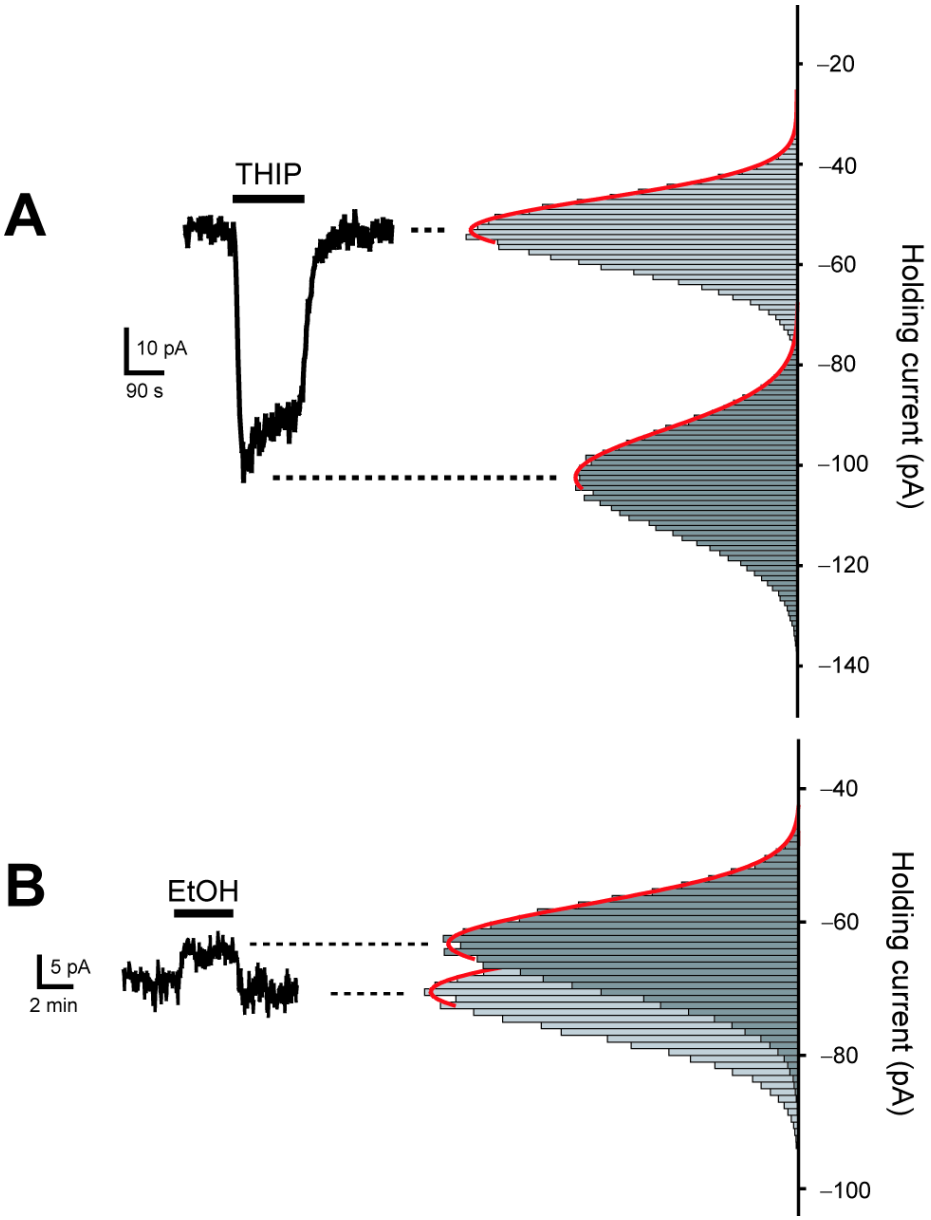
Contrary to this prevailing thinking, here we report that EtOH suppresses tonic $GABA_AR$ currents in several rodent genotypes (Fig. 2). The suppression of tonic $GABA_AR$ is not accompanied by a decrease in the frequency of sIPSCs, and is not blocked by TTX, and is observed in physically isolated B6 GC responses to exogenous GABA, suggesting a direct action on the extrasynaptic $GABA_ARs$ that mediate tonic inhibition (Fig. 7). Recent studies of recombinantly expressed $GABA_ARs$ with various point mutations indicate that $GABA_AR$ subunits may have multiple counteracting sites of action for EtOH, with the net effect of EtOH being determined by the balance of inhibitory and enhancement sites (Johnson et al., 2012; Mihic et al., 1997; Ueno et al., 2000). However, to our knowledge, this is the first report of EtOH suppressing native $GABA_ARs$ *in situ*. Studies of cultured rat GCs indicated that EtOH suppressed $GABA_AR$ currents in some GCs but enhanced it in others, and the proportion of cells showing suppression was increased in cells that were recorded with the perforated-patch technique (Yamashita et al., 2006), suggesting that endogenous kinases or other endogenous $GABA_AR$ modulators determine the polarity of action of EtOH. Indeed, we determined that elevated postsynaptic PKC activity occludes EtOH suppression of tonic $GABA_AR$ currents, and that genetic differences in PKC activity determine whether EtOH suppression of GC tonic $GABA_AR$ currents occurs in a given rodent genotype (Fig. 8).

2.5.4. Interactions among EtOH targets determine net response

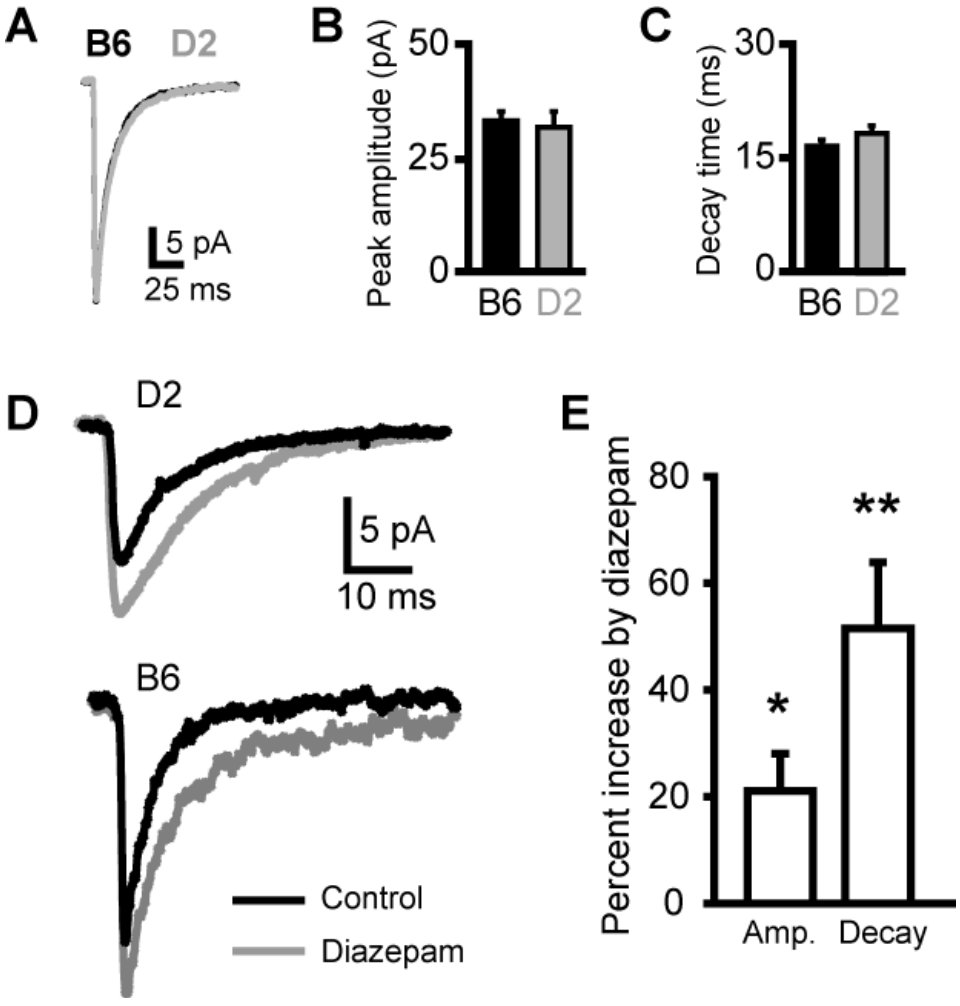
Taken together, our data demonstrate that there are two main mechanisms by which EtOH acts on GC $GABA_AR$ transmission: 1) inhibition of nNOS which results in increased AP-dependent vesicular release of GABA with a resultant increase in sIPSC frequency and tonic $GABA_AR$ current magnitude, and 2) a direct inhibition of α6,δ-containing $GABA_ARs$ and the tonic $GABA_AR$ current they generate in cells with low PKC activity. Thus, genetic or pharmacological control over two distinct and opposing molecular actions of EtOH enables a fine tuning of GC

tonic $GABA_AR$ responses along a spectrum from powerful enhancement to powerful suppression.

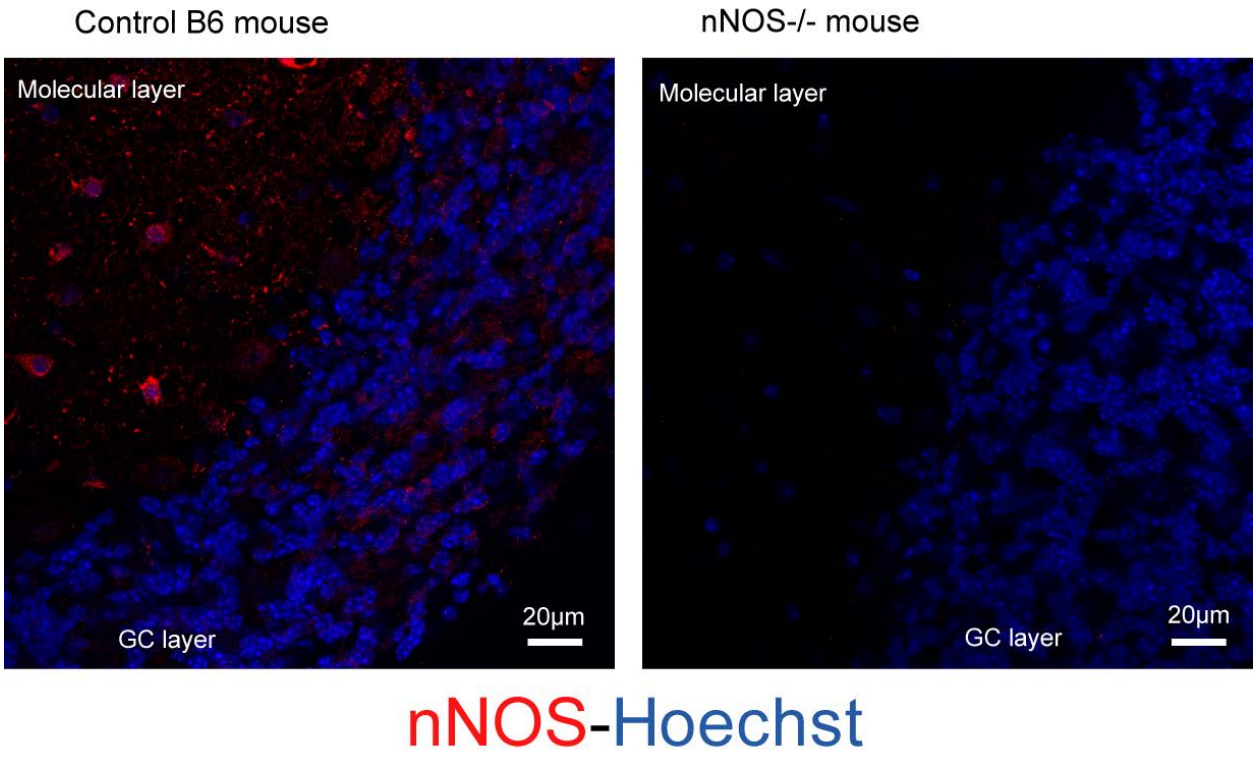
Supplementary Figure 1. Quantification of tonic current magnitudes. **A,B.** Representative examples of Gaussian fits to all points histograms of tonic current enhancement (**A,** induced by THIP, 500nM) and suppression (**B**, induced by EtOH, 52mM). For quantification of drug-induced changes in tonic current magnitude, all point histograms were generated for 20 second segments of the GC holding current, just prior to drug application, during the peak response to drug (**A**, only for THIP whose large currents showed desensitization) or steady state plateau just prior to wash (**B**, for all drugs other than THIP), and at a point with a post wash time interval equivalent to the time interval between pre-drug control and drug measurement. Such all point histograms were fitted with a single Gaussian function, from a point 3 pA to the left of the peak value to the rightmost (smallest) value of the histogram distribution, thereby excluding potential skewing of the tonic current measurement by overlapping sIPSCs. The magnitude of drug-induced change was quantified as the difference between the peak of the Gaussian fit of the all point histogram derived for the drug segment and the mean of the peak of the Gaussian fits of the all point histograms derived for the pre- and post-drug segments. Note, as for all figures in the manuscript, to enable clear presentation of the whole time course of such small currents, the traces shown are heavily filtered (1 Hz), but all quantification was done on minimally filtered (10 KHz) raw data traces.



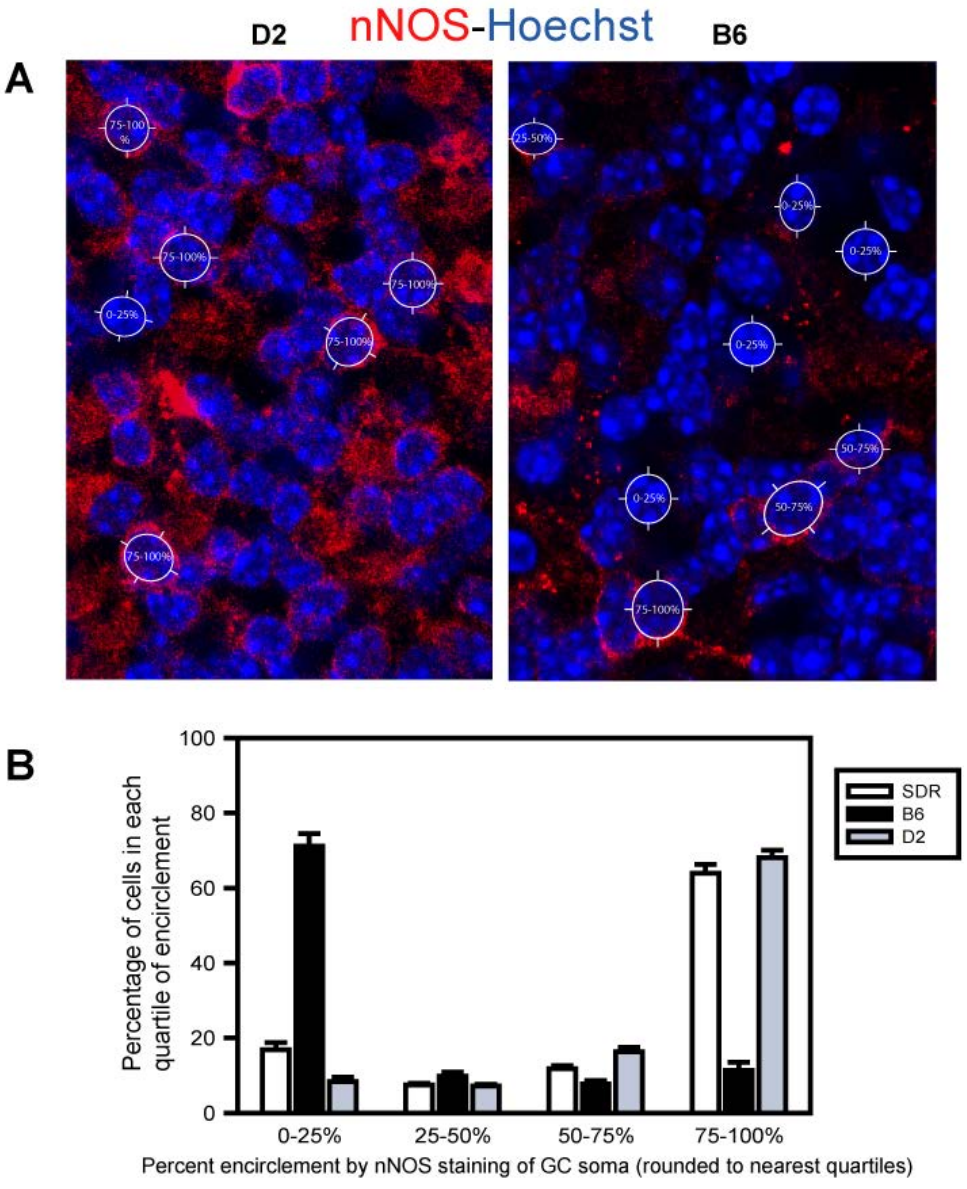
Supplementary Figure 2. $GABA_AR$ mediated sIPSCs in B6 and D2 mouse GCs have similar physiological properties and are enhanced by diazepam. **A.** Overlaid traces show the average of all sIPSCs from B6 (black) and D2 (gray) mouse GCs. **B,C.** Plots display mean amplitude (B6: 33.14 ± 2.07pA ; D2: 31.68 ± 3.47pA) and 10-90% decay time of sIPSCs (B6: 16.56 ± 0.82ms; D2: 18.22 ± 1.03ms) in B6 (*n* = 14) and D2 (*n* = 16) mouse GCs. **D**. Representative traces show that diazepam (300nM) increases the amplitude and decay time of sIPSCs in D2 (top) and B6 (bottom) mouse GCs. **E.** Plot of mean percent enhancement by diazepam of sIPSC amplitude and decay time. Percent enhancement was not significantly different between D2 (*n* = 6) and B6 (*n* = 7) GCs (amplitude: *P* = .73; decay *P* = .23, unpaired *t* tests) so data are combined (% increase in amplitude = 21.04 ± 7.02%, *P* = .01, signified by *; % increase in decay: 51.53 ± 12.36%, *P* = .001, *n* = 13, single-sample *t* tests, signified by **).



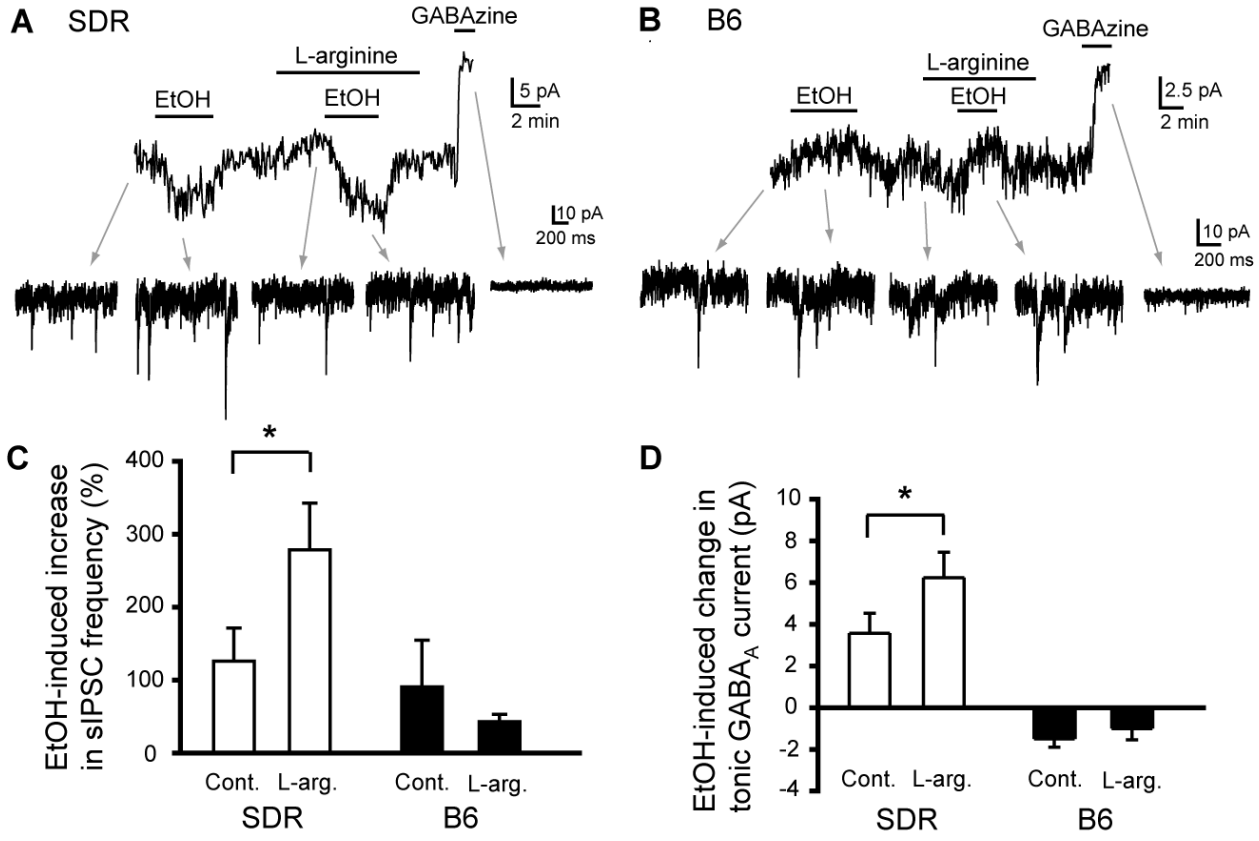
Supplementary Figure 3. Immunostaining signal for nNOS is absent in nNOS knockout mice. Representative examples of confocally acquired images of cerebellar slices stained with nuclear stain, Hoecsht (blue) and antibody for nNOS (red) from a wildtype B6 mouse (left) and from a mouse in which nNOS was genetically deleted (right; -/-). Images are representative of 3 separate slices for each genotype. Note, because there is relatively little nNOS signal in the GC layer in B6 mice (see main text and Fig. 6), the images include a portion of the overlying molecular layer which, in wildtype B6 mice showed more intense staining than the underlying GC layer, highlighting the complete lack of nNOS signal in the nNOS knockout mouse.



Supplementary Figure 4. Quantification of GC encirclement by nNOS. **A.** Representative confocal images of immunohistochemistry for nNOS in D2 (left) and B6 (right) cerebellar granule cell layer with examples of how the percent encirclement of GC somas by nNOS was determined by an experimenter blind to the animal's genotype. Circles divided into quartiles were stretched to closely circumscribe the GC nucleus (stained blue with nuclear stain Hoechst), and each nucleus in the image plain was categorized as being 0-25%, 25-50%, 50-75%, or 75-100% encircled by visibly detectable nNOS staining. Note, for each image the red channel gain was adjusted so that ~5% of the pixels were saturated, thereby normalizing fluorescence emission intensity across slices. **B.** Distribution of percent encirclement is skewed toward the top quartile for SDR and D2 GCs, but to the bottom quartile for B6 GCs. The mean percent encirclement (shown in main figure 6D) is significantly smaller for B6 GCs compared to SDR and D2 GCs.



Supplementary Figure 5. Providing exogenous NOS substrate increases the EtOH-induced enhancement of GC $GABA_AR$ currents in SDRs but not in B6 mice. **A**. Representative trace showing that adding the NOS substrate, L-arginine (100μM) to the bath increases the degree to which EtOH (52mM) enhances SDR GC sIPSC frequency (insets) and the tonic $GABA_AR$ current magnitude. **B.** Representative trace showing that L-arginine does not affect B6 GC responses to EtOH. **C,D.** Plot of mean percent change in sIPSC frequency (SDRS in white: 125.85 ± 45.41% in control, *n* = 14, versus 278.53 ± 63.97% in L-arginine, *n* = 14, **P* = .038; B6s in black: 90.56 ± 64.03% in control, *n* = 14, versus 42.75 ± 10.33% in L-arginine, *n* = 6, *P* = .48) and (**C**) mean increase in tonic $GABA_AR$ current magnitude (SDRs in white: 3.57 ± 0.96pA in control, *n* = 16, versus 6.24 ± 1.22pA in L-arginine, *n* = 16, **P* = .04; B6s in black: -1.50 ± 0.40pA in control, *n* = 7, versus -1.00 ± 0.53pA in L-arginine , *n* = 7, *P* = .48, by paired *t* tests) (**D**) induced by EtOH (52mM) under control conditions or in the presence of L-arginine (100μM) in the same cell, in SDRs and B6 GCs.



Chapter 3: Alcohol suppresses tonic $GABA_A$ receptor-mediated inhibition of cerebellar granule cells in the prairie vole

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Kaplan, J.S., Mohr, C., Hostetler, C.M., Ryabinin, A., Finn, D.A., & Rossi, D.J. (*in preparation*). Alcohol suppresses tonic $GABA_A$ receptor-mediated inhibition of cerebellar granule cells in the prairie vole: a neural signature of high alcohol consuming genotypes

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3.1. Abstract

A growing body of evidence has demonstrated a role for the cerebellum in EtOH and drug-related behaviors, suggesting that variation in EtOH-related behavioral phenotypes might be reflected in the cellular response to EtOH. We previously determined that EtOH had opposite actions (enhancement or suppression) on tonic $GABA_AR$ currents in cerebellar GCs in low and high-EtOH consuming rodents, respectively. We hypothesized that variation in GC $GABA_AR$ current responses to EtOH contributes to genetic variation in EtOH consumption phenotype. To explore the predictive validity of our hypothesis, we utilized another high EtOH consuming rodent, prairie voles (PVs), to test our hypothesis-driven prediction that alcohol would suppress their GC tonic $GABA_AR$ currents. Using voltage-clamped GCs in cerebellar slices from three month old PVs, we found that EtOH (52mM) predominately suppressed the magnitude of the tonic $GABA_AR$ current (12/21 cells) or had no effect (8/21 cells). Thus, the average impact of EtOH on PV GC tonic $GABA_AR$ currents was suppression. EtOH did not affect GC currents when $GABA_ARs$ were blocked with the $GABA_AR$ antagonist, GABAzine (10μM), confirming that EtOH-induced outward currents reflected suppression of tonic $GABA_AR$ currents. EtOH-induced suppression of tonic $GABA_AR$ currents was not blocked by the sodium channel blocker, TTX (500nM), and was independent of the frequency of phasic $GABA_AR$-mediated currents, suggesting that suppression was mediated by post synaptic actions on $GABA_ARs$, rather than a reduction of GABA release. Finally, the minimal EtOH enhancement of GC tonic $GABA_AR$ current was reflected in lower nNOS expression in the GC layer relative to low-EtOH consuming mice and rats of similar age. Combined, our data confirm our prediction that, similar to other high EtOH-consuming rodents, EtOH suppresses PV GC tonic $GABA_AR$ currents via direct actions in the postsynaptic cell. These findings add further support to our hypothesis that genetic variation in the magnitude and polarity of GC tonic $GABA_AR$ current responses to varies as a function of EtOH consumption phenotype, and they implicate GC tonic inhibition as a cellular substrate associated with enhanced EtOH consumption.

3.2. Introduction

AUDs are a leading cause of preventable death and illness that contribute to substantial emotional, social, and economic costs (Harwood, 2000). Adoption and twin studies have identified that genetic factors contribute 50-60% of risk for developing an AUD (Hill, 2010; Prescott et al., 1999). Among them, heritable variation in the cerebellar response to alcohol influences susceptibility to AUDs (Cservenka & Nagel, 2012; Cservenka et al., 2014; Herting et al.,2011; Hill, 2010; Schuckit, 1985; Schuckit, 1994; Schuckit & Smith, 1996). The mechanisms through which the cerebellum mediates risk for developing an AUD are unclear but may stem from a combination of alcohol's action on motor (Schuckit, 1985; Dar, 2015; Hanchar et al., 2005) and non-motor cerebellar functions (Strick et al., 2009; Stoodley et al., 2012) and related functional connections with areas involved in reward processing (e.g., ventral tegmental area [Ikai et al., 1992; Ikai et al., 1994], basal forebrain [Albert, Dempsey, & Sorenson, 1985], and striatum [Delis, Mitsacos, & Giompres, 2013]). These proposed mechanisms have been reflected in studies of the cerebellar LLR to EtOH phenotype, which is defined as requiring a higher dose of EtOH to attain a given effect. One aspect of the cerebellar LLR that is amenable to study is EtOH-induced static ataxia (body sway) in humans, and rotarod performance in rodents. The cerebellar LLR phenotype is most common in young men with a family history of AUDs (i.e., high genetic risk) (Schuckit, 1985) and predicts development of AUDs (Schuckit, 1996). It is also common in rodent strains with a high EtOH consumption phenotype (Gallaher et al., 1996; Yoneyama et al., 2008). Thus, the cerebellar LLR to EtOH phenotype is heritable and is associated with increased risk for developing an AUD in humans and high EtOH consumption in rodents. While cellular mechanisms that underlie the LLR behavioral phenotype are not well understood, we hypothesize the presence of a cellular target through which the LLR manifests as a genetically regulated response to EtOH that varies across behavioral phenotypes.

Cerebellar GCs are likely targets through which EtOH differentially affects cerebellar processing in high and low-EtOH consuming phenotypes. GCs are the most numerous neuron

type in the brain (~10^5 GCs [De Shutter & Bower, 1994]) and are the main relay of afferent information through the cerebellar cortex to PCs, the primary integrator and sole output of the cerebellar cortex. Thus, GCs are powerful targets of pharmacological modulation of cerebellar processing (Duguid et al., 2012; Hamann et al., 2002). Their activity is regulated by two forms of $GABA_AR$-mediated inhibition: the traditional phasic $GABA_AR$-mediated IPSCs, as well as a powerful form of tonic $GABA_AR$ inhibition mediated by extrasynaptic $GABA_ARs$ containing the α_6 and δ subunits (Brickley et al., 1996; Hamann et al., 2002; Wall & Usowicz, 1997). The tonic form of $GABA_AR$ inhibition mediates 75% of the entire inhibitory charge of the GC, thereby making it a powerful regulator of signal transmission through the cerebellar cortex (Hamann et al., 2002). Previous patch-clamp electrophysiological studies using SDR brain slice preparations have determined that acute EtOH increased afferent Golgi cell activity (Chapter 2; Botta et al., 2010; Carta et al., 2004; Kaplan et al., 2013), which enhanced GABAergic transmission to GCs through elevated vesicular GABA release (Chapter 2; Carta et al., 2004; Hanchar et al., 2005; Kaplan et al., 2013), leading to an increased GC sIPSC frequency and enhanced tonic $GABA_AR$ current magnitude. The magnitude by which EtOH enhanced the tonic $GABA_AR$ current was directly correlated with sensitivity to EtOH-induced cerebellar ataxia (Hanchar et al., 2005), a behavioral measure of cerebellar impairment. Importantly, enhancement of GC tonic inhibition was detected at EtOH concentrations as low as 9mM (Chapter 2; Kaplan et al., 2013), far below the sensitivity thresholds for other brain regions (e.g. hippocampus, nucleus accumbens, thalamus, ventral tegmental area, substantia nigra) (Jia et al., 2008; Liang et al., 2006; Nie et al., 2000; Peris et al., 1992; Theile et al., 2008) where EtOH only affected $GABA_AR$ transmission at concentrations above the threshold for binge consumption (~17mM; Crabbe et al., 2011). Therefore, genetic regulation of EtOH's effect on tonic $GABA_AR$ inhibition of cerebellar GCs may contribute to EtOH-related behavioral and neuronal phenotypes that promote risk for developing an AUD.

We recently provided insight into the relevance of tonic $GABA_AR$ inhibition of GCs in EtOH-related behavioral phenotypes by comparing GC tonic $GABA_AR$ current responses to EtOH in a range of genotypes. In low EtOH consuming rodents that do not express the cerebellar LLR (i.e., SDR, D2 mice), EtOH greatly enhanced the magnitude of tonic $GABA_AR$ inhibition, whereas in high EtOH consuming B6 mice that express the cerebellar LLR phenotype, EtOH suppressed tonic $GABA_AR$ inhibition of GCs (Chapter 2; Kaplan et al., 2013). The EtOH response in Rhesus Macaques, and WSR and WSP selected lines, all of which show intermediate EtOH consumption phenotypes, was between that of the high and low EtOH consuming genotypes (Chapter 2; Kaplan et al., 2013; Mohr et al., 2013). We also found that the net effect of EtOH on the magnitude and polarity of the tonic $GABA_AR$ current was dictated by two genetically regulated mechanisms: 1) the degree of nNOS expression in the GC layer determined the magnitude by which EtOH enhanced presynaptic vesicular GABA release to increase tonic $GABA_AR$ inhibition of GCs, whereas 2) low GC PKC activity enabled EtOH to postsynaptically inhibit the extrasynaptic α6,δ-containing $GABA_ARs$ that mediate the tonic $GABA_AR$ current (Chapter 2; Kaplan et al., 2013; Mohr et al., 2013). Thus, the high EtOH consuming, behaviorally insensitive B6 mice had low nNOS expression and low GC PKC expression that together, promoted EtOH-induced suppression of tonic $GABA_AR$ inhibition of GCs. We hypothesize that EtOH-induced suppression of tonic $GABA_AR$ inhibition of GCs is a common trait across many high-EtOH consuming genotypes mediated by low nNOS expression and low GC PKC activity levels.

In order to test the predictive validity of this hypothesis, we tested the effect of EtOH in another high EtOH-consuming species, the PV (Anacker et al., 2011a, 2011b; Hostetler et al., 2012). We conducted voltage-clamp recordings of GCs in cerebellar slices obtained from PVs and found that, similar to B6 mice, the predominant responses to EtOH were that of suppression (12/21 cells tested) or of no effect (8 out of 21 cells tested). As in B6 mice, EtOH suppressed the tonic $GABA_AR$ current in PV GCs via postsynaptic inhibition of extrasynaptic $GABA_ARs$

which, due to low nNOS expression in the GC layer, was not obfuscated by presynaptic EtOH actions. These findings provide support for the prediction that EtOH suppresses tonic $GABA_AR$ inhibition of GCs in genotypes with high EtOH consuming phenotypes and further implicates GC tonic inhibition as a cellular substrate of the cerebellar LLR phenotype that promotes increased EtOH consumption in rodents and increased risk for developing an AUD in humans.

3.3. Methods

3.3.1. Preparation of brain slices

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 Committee of the Oregon Health & Science University. Cerebellar slices were prepared acutely on each day of experimentation (Hamann et al., 2002; Rossi et al., 2003). Male and female prairie voles (68-97 days old; bred at the Veterinary Medical Unit of the Veterans Affairs Medical Center in Portland, OR), and age matched SDRs (Charles River; Seattle, WA), B6 mice and D2 mice (Jackson Laboratory-West; Sacramento, CA), were anaesthetized with isoflurane and killed by decapitation. The whole brain was rapidly isolated and immersed in ice cold (0-2˚C) aCSF containing (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 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^oC) for one hour after dissection and then held at 22-23^oC until used. Kynurenic acid (1 mM) was included in the dissection, incubation and holding solution (to block glutamate receptors to reduce potential excitotoxic damage) but was omitted from the experimental solutions.

3.3.2. Electrophysiology

Slices were placed in a submersion chamber on an upright microscope, and viewed with an Olympus 60X (0.9 numerical aperture) water immersion objective with differential interference contrast and infrared optics. Slices were perfused with aCSF at a rate of ~7ml/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, $CaCl_2$ 0.5, HEPES 10, EGTA 5, MgATP 4, Na_2GTP 0.5, QX-314 5. Solutions were pH adjusted to 7.2 with CsOH. Electrode resistance was 4 to 10 MΩ. Cells were rejected if access resistance was greater than 15 MΩ.

3.3.3. Immunohistochemistry

Brain slices were prepared from PVs, C57BL/6J and DBA/2J mice, and Sprague-Dawley rats in an identical manner as for electrophysiology experiments. After tissue removing and slicing, 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. See reagents below for source and dilution of antibodies used.

3.3.4. Confocal microscopy

Images were acquired with a Leica SP8-X point scanning confocal microscope using accompanying Zeiss 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 objective was used for all experiments: a 20X, 0.7 N.A. Plan Apochromat air objective. Pinhole diameter and slice step thickness were optimized for the objective used. For quantification of GC encirclement by nNOS (see Chapter 2; 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 who was blind to the experimental condition analyzed individual GC nuclei for percent encirclement by detectable nNOS signal (as in Chapter 2; 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.

3.3.5 Analysis of $GABA_AR$ currents

Membrane currents were acquired at 20 kHz, filtered at 10 kHz, and analyzed with pClamp (v.6.3) software (Axon Instruments, Foster City, CA). For analysis and display of sIPSCs, data were filtered at 2-5 kHz. When quantifying sIPSC occurrence, sIPSCs were defined as current deflections that had an amplitude (measured from the mean current) greater than the peak-to-peak amplitude of the current noise and with a decay time constant at least 3-fold slower than the rise time. The tonic current was assessed by fitting the Gaussian distribution of all data points not skewed by synaptic events from a point 3 pA to the left of the peak value to the rightmost (largest) 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.

3.3.6. Statistics

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

3.3.7. Reagents

All reagents were from Sigma Chemicals (St. Louis, MO) except GABAzine and kynurenic acid (Abcam, UK). 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 Alexa fluors of various excitation maxima (Invitrogen), with specificity against immunoglobulins of the hosts of the primary antibodies, diluted 1:500. Mounting medium was from Invitrogen as well.

3.4. Results

3.4.1. PV GCs exhibit phasic and tonic $GABA_AR$-mediated currents

PVs are a socially monogamous and high EtOH-consuming genotype (Anacker et al., 2010; Getz et al., 1981), but little is known about their properties of signal transmission 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 (Chapter 2; Kaplan et al., 2013; Mohr et al., 2013), we made whole-cell voltage-clamp recordings of GCs in sagittal slices (V_h = -60mV; E_{Cl} = ~0mV). PV GCs express both phasic spontaneous IPSCs and a tonic chloride conductance that are mediated by $GABA_ARs$, as evidenced by their block by the broad spectrum $GABA_AR$ antagonist, GABAzine (10µM; Fig. 1A,D; 12.26 ± 1.89pA, *n* = 23 from 5 animals). Extensive literature suggests that the GC tonic $GABA_AR$ current is mediated by α6 and δ-subunit containing extrasynaptic $GABA_ARs$ (Brickley et al., 1996; Hamann et al., 2002; Wall & Usowicz, 1997), but this has not been established in the PV. We used a pharmacological approach to determine if PVs express functional α6,δ-subunit containing $GABA_ARs$ that mediate GC tonic inhibition. Similar to reports from other species, the tonic current amplitude was reduced by the α6 subunit antagonist, furosemide (100µM, at which concentration it is selective for $GABA_ARs$ containing the α6 subunit [Hamann, et al., 2002; Korpi et al., 1995]; Fig 1B,D; 4.80 ± 1.12pA, *n* = 9 from 2 animals), 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 1C,D; 14.03 ± 1.67pA, *n*

= 10 from 2 animals). Together, these data confirm that PVs express phasic $GABA_AR$ inhibition as well as a tonic current that is mediated by α6,δ-subunit containing $GABA_ARs$.

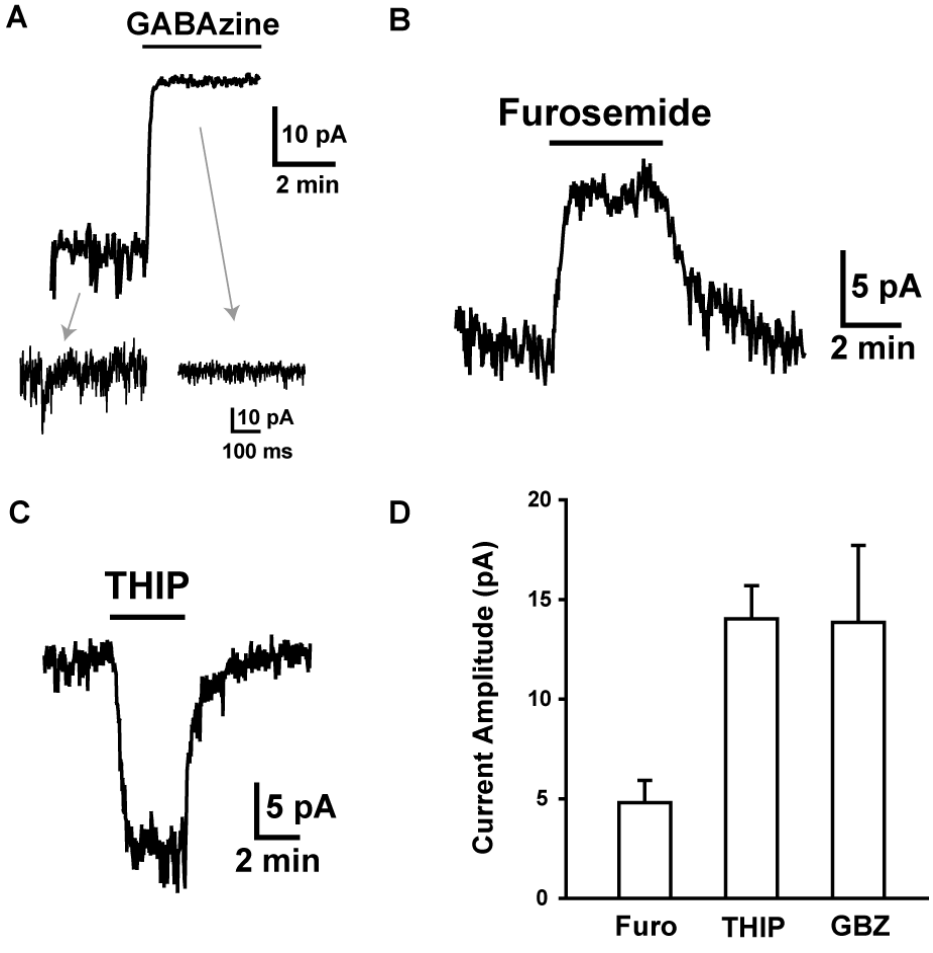

Figure 1. PV GCs exhibit a tonic current mediated by α6,δ-subunit containing $GABA_ARs$. **A.** Example voltage-clamp recording (V_h= -60mV, E_{Cl-} = ~0mV) of a PV GC showing block of tonic current by the $GABA_AR$ antagonist GABAzine (10μM). The gray arrows point to an expanded time scale of recording from different time points of the main traces (coming from the region of the main trace that the back of the arrow extrapolates to), showing sIPSCs and block by GABAzine. **B.** Example trace showing block of tonic current by $GABA_AR$ antagonist furosemide (100μM, at which concentration it is specific for $GABA_ARs$ containing the α6 subunit) in a PV GC. **C.** Example trace showing enhancement of tonic current by the $GABA_AR$ agonist THIP (500nM, at which concentration it is selective for $GABA_ARs$ containing the δ subunit) in a PV GC. **D.** Plot summarizing mean tonic current blocked by furosemide (Furo), enhanced by THIP, and blocked by GABAzine (GBZ).

3.4.2. EtOH suppresses tonic $GABA_AR$ inhibition of PV GCs

In high EtOH-consuming B6 mice, bath application of 9mM-79mM EtOH to cerebellar slices, on average, suppressed the magnitude of the tonic $GABA_AR$ current in GCs (Chapter 2; Kaplan et al., 2013). To test the prediction that EtOH would similarly suppress tonic $GABA_AR$ inhibition of PV GCs, we voltage-clamped GCs from slices and bath applied 52mM EtOH. Consistent with the previous report in the B6 mice, EtOH predominately suppressed or had no effect on the tonic $GABA_AR$ current (Fig 2A; suppression: 12/21 cells recorded; no effect: 8/21 cells recorded; enhancement: 1/21 cells recorded from 5 animals).

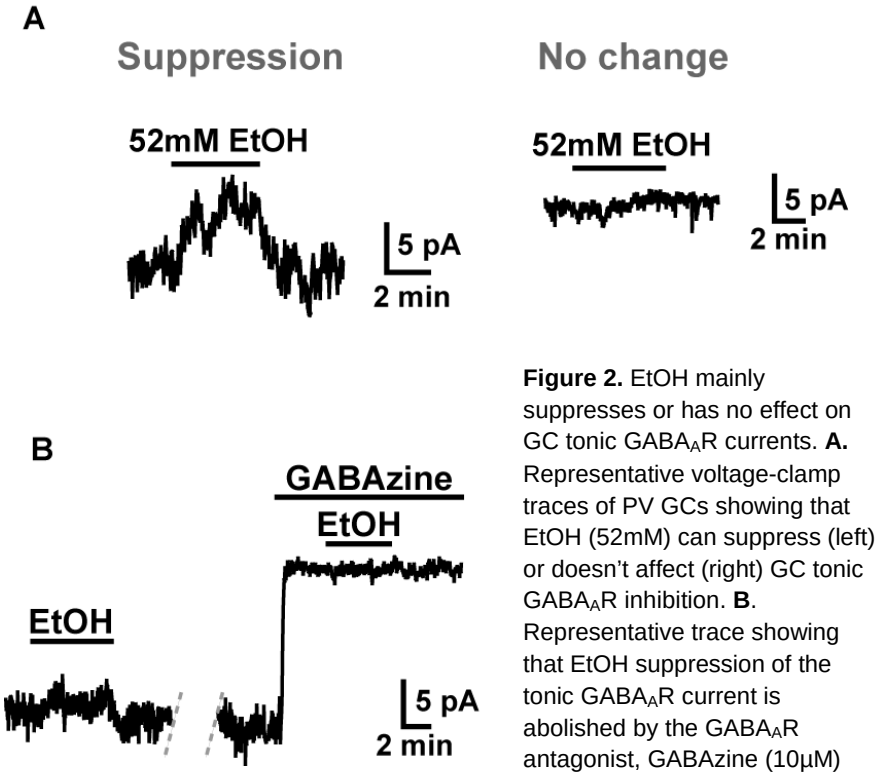

Figure 2. EtOH mainly suppresses or has no effect on GC tonic $GABA_AR$ currents. **A.** Representative voltage-clamp traces of PV GCs showing that EtOH (52mM) can suppress (left) or doesn't affect (right) GC tonic $GABA_AR$ inhibition. **B**. Representative trace showing that EtOH suppression of the tonic $GABA_AR$ current is abolished by the $GABA_AR$ antagonist, GABAzine (10μM)

GABAzine occludes EtOH-suppression of tonic $GABA_AR$ inhibition thereby indicating that it is indeed mediated via a $GABA_AR$ mechanism (Fig 2B; n = 10 from 4 animals). 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, likely impacts the efficacy of transmission from mossy fibers to PCs (the sole output of the cerebellar cortex). On average, EtOH significantly suppressed the magnitude of the tonic $GABA_AR$ current (-1.16 ± 0.34pA; t[20] = 3.39, P = .003, one-sample t-test) by 9.66 ± 2.35% of the total tonic $GABA_AR$ current as assessed by the GABAzine current amplitude. These data support the

hypothesis that EtOH suppresses tonic $GABA_AR$ inhibition of GCs in rodents with high EtOH consumption phenotypes.

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

We previously identified in mice that EtOH suppresses the tonic $GABA_AR$ current via postsynaptic inhibition of extrasynaptic $GABA_ARs$ that mediate the tonic current. However, was unclear if similar postsynaptic EtOH actions contributed to the EtOH suppression of GC tonic inhibition that was detected in over half of the PV GCs. EtOH could potentially be acting presynaptically to reduce Golgi cell activity, which would decrease vesicular GABA release, thereby reducing GABAergic transmission to the GC. To rule out presynaptic contributions to EtOH-induced suppression of tonic inhibition, we measured the EtOH-induced change in the frequency of sIPSCs, an indirect measure of Golgi cell activity. If EtOH reduced tonic inhibition by presynaptically attenuating Golgi cell activity, then we would detect a coinciding decrease in

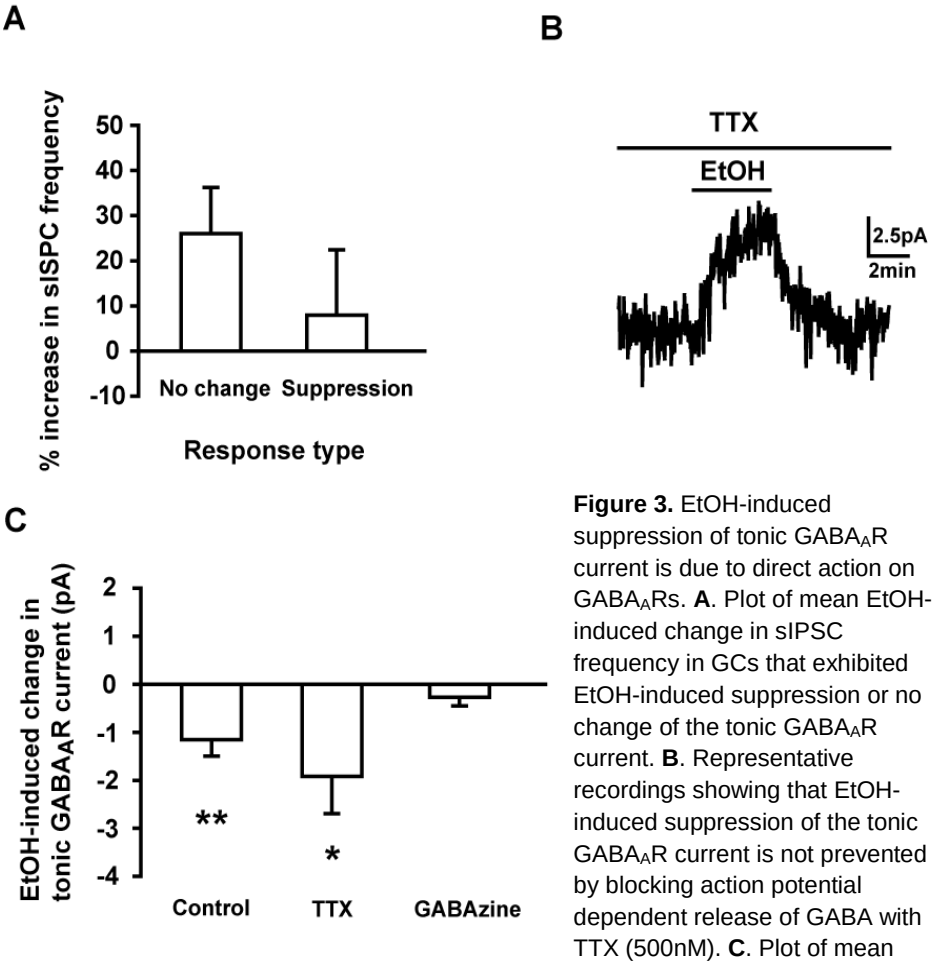

Figure 3. EtOH-induced suppression of tonic $GABA_AR$ current is due to direct action on $GABA_ARs$. **A**. Plot of mean EtOH-induced change in sIPSC frequency in GCs that exhibited EtOH-induced suppression or no change of the tonic $GABA_AR$ current. **B**. Representative recordings showing that EtOH-induced 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). **P* < .05, ***P* < .01.

sIPSC frequency in these cells. However, consistent with postsynaptic EtOH action, EtOH did not reduce sIPSC frequency in cells where EtOH suppressed the tonic $GABA_AR$ current (% change = 7.92 ± 14.50%) and 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,C), which blocks AP-dependent vesicular GABA release from Golgi cells, failed to block EtOH suppression of tonic $GABA_AR$ inhibition. These results are consistent with our previous findings in mice that EtOH suppresses tonic $GABA_AR$ inhibition by postsynaptically inhibiting extrasynaptic $GABA_ARs$.

3.4.4. Variable response to EtOH is mirrored by reduced expression of nNOS

Earlier reports demonstrated that EtOH enhanced GABAergic transmission to GCs by increasing Golgi cell activity (Carta et al., 2004; Botta et al., 2011; Chapter 2), which was generated by EtOH-induced inhibition of nNOS (Chapter 2; Kaplan et al., 2013; Mohr et al.,

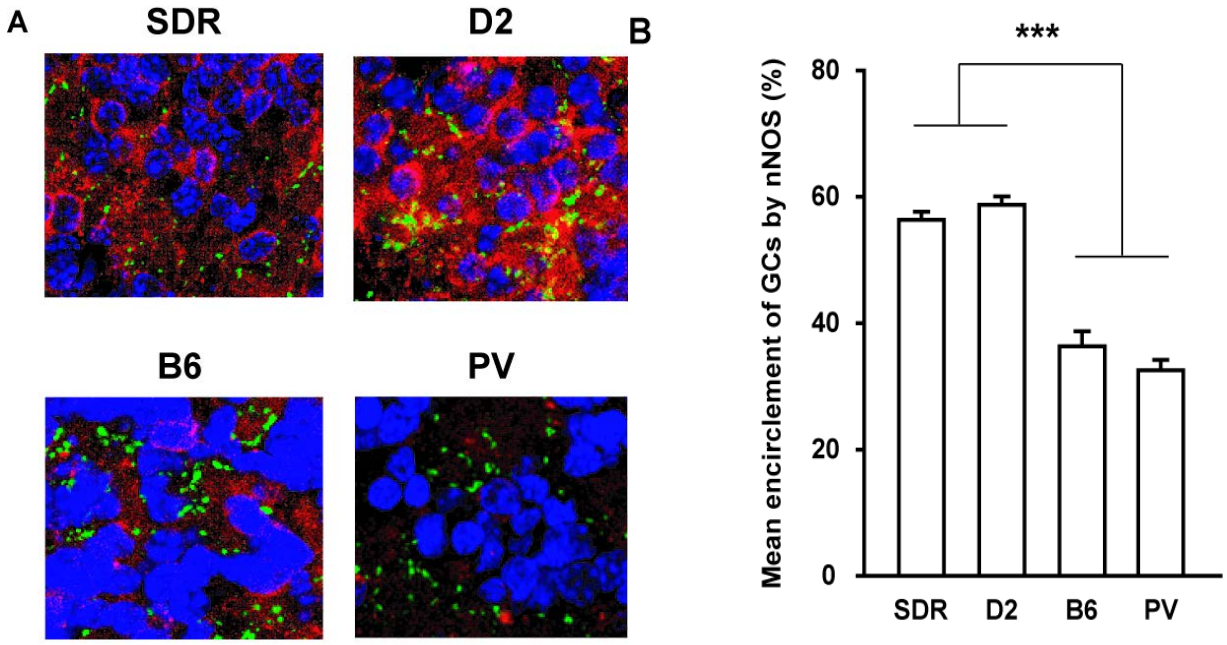

Figure 4. PVs and C57BL/6J mice have less mean encirclement of GCs by nNOS than Sprague-Dawley rats and DBA/2J mice. **A.** Confocally acquired images of immunohistochemistry for nNOS (red), GAD-65/67 (green) and nuclear stain Hoechst (blue) in the granule cell layer of Sprague-Dawley rat (SDR), DBA/2J 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 SDR and D2s both have significantly greater nNOS encirclement than B6s and PVs, $P < 0.001$.

2013). B6 mice, which show little EtOH enhancement of the GC tonic $GABA_A$ current, had reduced and variable nNOS expression, whereas D2 and SDRs, which show EtOH enhancement of GC tonic $GABA_AR$ inhibition, had greater nNOS expression in the GC layer (Chapter 2; Kaplan et al., 2013). We therefore reasoned that the minimal EtOH enhancement of PV GC tonic $GABA_A$ currents (enhancement in only 1/21 cells recorded) was reflective of reduced expression of GC layer nNOS and measured nNOS expression using immunohistochemistry with confocal microscopy in the GC layer of age-matched PVs, B6 and D2 mice, and SDRs. While nNOS expression was detected in all the genotypes, the nNOS expression pattern differed across rodents as a function of their EtOH-consumption phenotype and net EtOH effect on GC tonic $GABA_AR$ inhibition ($F(3,88) = 62.10$, $P < .001$, one-way ANOVA; Fig 4). nNOS expression, quantified as the mean percent GC encirclement (see Methods and Chapter 2), was similarly lower in the high-EtOH consuming PVs ($32.53 \pm 1.67\%$) and B6 mice ($36.32 \pm 2.39\%$) than the low EtOH-consuming D2 mice ($58.70 \pm 1.35\%$) and SDRs ($56.34 \pm 1.28\%$; all $P < .001$, pairwise comparisons by Student's *t* tests). Similar to previous description of B6 and non-human primates (Chapter 2, Mohr et al., 2013), the majority of PV GCs were not fully surrounded by nNOS, and many were entirely devoid of nNOS. Thus, the reduced nNOS expression in the GC layer of the PV is consistent with the predominant absence of EtOH-induced enhancement of GABAergic transmission to PV GCs.

3.4.5. EtOH effect on GC tonic $GABA_AR$ inhibition varies with consumption phenotype

PVs are one of a variety of mammalian genotypes in which we have measured the impact of 52mM EtOH on GC GABAergic inhibition. The observation that 52mM EtOH, on average, suppresses PV GC tonic $GABA_AR$ inhibition is consistent with EtOH enhancement of tonic $GABA_AR$ inhibition in low-consuming rodents and EtOH-suppression of tonic $GABA_AR$ inhibition in high-consuming rodents. To statistically confirm this pattern, we normalized the

EtOH response to the total tonic $GABA_AR$ current across genotypes, as determined by GABAzine current within each cell. A one-way ANOVA revealed a significant main effect of genotype on EtOH's impact on GC tonic $GABA_AR$ inhibition as a percent of the total $GABA_AR$ current ($F(4,74) = 21.00$, $P < .001$). Post-hoc comparisons confirmed that low-drinking SDRs (35.28 ± 6.08%, $n = 19$, from Chapter 2) and D2 mice (10.21 ± 5.41%, $n = 15$, from Chapter 2) significantly differed from high-consuming B6 mice (-10.67 ± 3.43%, $n = 15$, from Chapter 2) and PVs (-14.12 ± 2.63%, $n = 13$). Furthermore, EtOH had a significantly smaller effect on GC tonic $GABA_AR$ inhibition in non-human primates (0.75 ± 0.61%, $n = 12$, from Mohr et al., 2013), which express an intermediate EtOH-consumption phenotype, than in SDRs (all $P < .01$, pairwise comparisons by Student-Newman-Keuls t tests). This divergent EtOH effect across opposite consumption phenotypes illuminates the important contribution that GC sensitivity to EtOH can play in behavioral phenotypes that enhance intake in animals and risk for developing AUDs in humans.

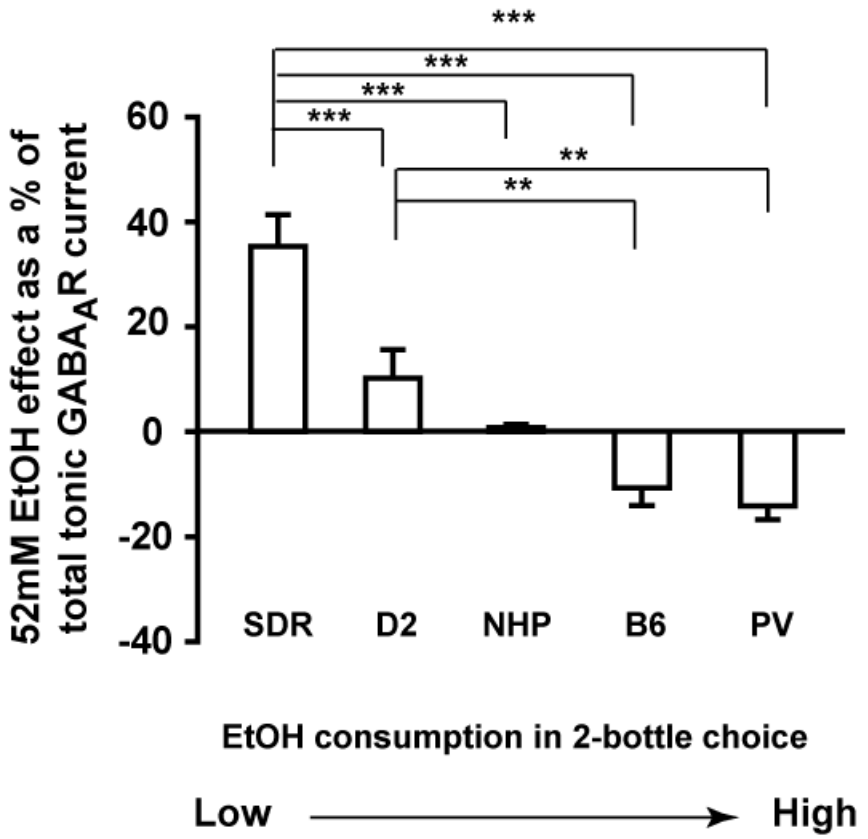

Figure 5. The mean magnitude and polarity of EtOH's (52mM) effect on the GC tonic $GABA_A$ current varies as a function of EtOH consumption phenotype during a 2-bottle choice access procedure. Sprague-Dawley rat (SDR), DBA/2J (D2), and C57BL/6J (B6) data from Kaplan et al. (2013). Non-human primate (NHP) data from Mohr et al. (2013). **$P < .01$, ***$P < .001$.

3.5. Discussion

Genetic contributions to developing an AUD in humans and enhanced EtOH consumption in rodents is likely reflected in differential responses to EtOH at the cellular level. We have focused our search to the cerebellum because previous reports have shown that a low sensitivity to EtOH-induced disruption of cerebellar-dependent behaviors was associated with increased risk for developing AUDs in humans (Schuckit 1985, Schuckit 1996) and enhanced EtOH consumption in rodents (Gallaher et al., 1996; Yoneyama et al., 2008). Consistent with behavioral variation manifesting at the cellular level, we identified that the effect of EtOH on the magnitude and polarity of tonic $GABA_AR$ inhibition of cerebellar GCs varied as a function of EtOH consumption phenotype, with EtOH causing enhancement in low EtOH consuming genotypes and suppression in high consuming genotypes (Chapter 2; Kaplan et al., 2013). Thus, we hypothesized that EtOH suppressed tonic $GABA_AR$ inhibition of GCs in high consuming genotypes. Here, we tested and confirmed the predictive validity of our hypothesis in the high EtOH-consuming PV, finding that EtOH predominately suppressed tonic $GABA_AR$ inhibition of GCs, which in part was determined by reduced nNOS expression in the GC layer compared to age-matched low EtOH consuming mice and rats.

Using patch-clamp recordings of brain slices and subunit-selective pharmacological ligands, we also determined that cerebellar GCs from PVs express functional α6,δ subunit-containing extrasynaptic $GABA_ARs$ that mediate a tonic inhibitory chloride conductance. Incorporation of α6 and δ subunits into $GABA_ARs$ enhance their sensitivity to low ambient levels of GABA (Saxena & McDonald, 1996) that results from spillover from neighboring synapses into the extracellular space (Rossi & Hamann, 1998). The magnitude of the tonic chloride conductance therefore reflects the general degree of feedback inhibition from afferent Golgi cells. Because the tonic $GABA_AR$ current contributes three-fold more to the overall inhibitory

charge of the GC compared to synaptically-mediated transmission (Hamann et al., 2002), it enables fine-tuning of neuronal gain and fidelity of excitatory transmission through the cerebellar cortex (Mitchell & Silver, 2003; Duguid et al., 2012). The conservation of an extrasynaptic $GABA_AR$-mediated inhibitory conductance across species such as rats (Rossi & Hamann, 1998), mice (Stell et al., 2003; Chapter 2), and non-human primates (Mohr et al., 2013) illuminates the importance of its role in modulating neuronal gain (Duguid et al., 2012; Mitchell & Silver, 2003).

3.5.1. Response to EtOH varies by consumption phenotype

PVs have become an increasingly utilized model for studying EtOH-related behavioral phenotypes because their high EtOH consumption and preference phenotypes (Anacker et al., 2011a; Anacker et al., 2011b; Hostetler et al., 2012) are comparable to commonly studied B6 mice (Yoneyama et al., 2008). Similar to B6 mice, 52mM EtOH suppressed ~10% of the PV tonic $GABA_AR$ current in GCs. This supports our previous observations from a variety of mammalian genotypes: that the magnitude and polarity of the EtOH effect on the tonic $GABA_A$ current in cerebellar GCs parallels their EtOH consumption phenotype, ranging from strong enhancement in low EtOH consumers to strong suppression in high EtOH consumers (Chapter 2; Kaplan et al., 2013; Mohr et al., 2013). While a 52mM EtOH dose falls along the high end of those achieved by humans (BEC = 2.45 mg/ml), we have previously identified an escalating dose-dependent increase in the magnitude of EtOH-induced suppression of tonic $GABA_AR$ inhibition in GCs from B6 mice ranging from 9mM through 79mM (Chapter 2; Kaplan et al., 2013). Thus, we predict that the EtOH-induced suppression of tonic $GABA_AR$ of PV GCs reported here would also be detected at lower concentrations regularly achieved during casual social drinking.

As was observed in B6 mice (Chapter 2; Kaplan et al., 2013), EtOH suppressed the tonic $GABA_AR$ current in more than half of the PV GCs recorded via postsynaptic EtOH

inhibition of extrasynaptic $GABA_A$Rs. Consistent with postsynaptic EtOH action, suppression of GC tonic $GABA_A$R inhibition was not blocked by TTX and did not coincide with a decrease in GC sIPSCs, implying that it is independent of AP-dependent vesicular GABA release from Golgi cells. However, the observation of EtOH suppression in approximately half of the recorded GCs indicates variability in the ability of EtOH to directly inhibit extrasynaptic $GABA_A$Rs. This variability in EtOH suppression of tonic inhibition of GCs has been observed in cultured neurons (Yamashita et al., 2006) and in slice recordings (Chapter 2; Kaplan et al., 2013) and likely reflects variation in the expression of an intracellular substrate that promotes postsynaptic EtOH action and facilitates direct inhibition of the extrasynaptic $GABA_A$Rs that mediate tonic inhibition. One likely candidate is PKC activity within the GC. Intracellular block of PKC activity within GCs of SDRs enabled EtOH suppression of the tonic $GABA_A$ current, which was not observed under control conditions, whereas activating PKC activity in B6 mice GCs prevented EtOH from suppressing the tonic $GABA_A$R current (Chapter 2; Kaplan et al., 2013). It's unclear whether the reduced activity of one or a variety of PKC isoforms promotes EtOH suppression of the tonic $GABA_A$R current. However, genetic deletion of PKCγ reduced EtOH-stimulated chloride uptake in cerebellar microsacs and was paralleled by lower sensitivity to EtOH's effects on the loss of righting reflex, an assay of cerebellar function (Harris et al., 1995). Although the current study does not further elucidate the mechanism of EtOH-induced suppression of tonic $GABA_A$ inhibition, future investigation into the role of specific PKC isoforms is warranted since pharmacologically targeting PKC-mediated phosphorylation of extrasynaptic $GABA_A$Rs may be a feasible strategy to curb behaviors that promote enhanced EtOH consumption.

3.5.2. Reduced NOS expression contributes to the lack of EtOH-induced enhancement of tonic $GABA_A$R inhibition

We detected a heterogeneous response to EtOH on GC tonic $GABA_A$R inhibition, which was predominately limited to suppression or no response. EtOH enhancement of tonic $GABA_A$R

inhibition, the predominant response in low-drinking rodent strains (Chapter 2; Kaplan et al., 2013), was only detected in one of the 21 cells from which we recorded in the present study. Although EtOH had been traditionally thought to increase GABAergic transmission to cerebellar GCs (Carta et al., 2004; Hanchar et al., 2005; Botta et al., 2007), this view was based largely on evidence collected from a low-EtOH consuming SDR model where EtOH consistently increased the frequency of synaptic IPSCs and the magnitude of GC tonic $GABA_AR$ inhibition. However, we failed to find that EtOH consistently increased GABAergic transmission to GCs in PVs and in other mammalian genotypes including mice and non-human primates (Chapter 2; Kaplan et al., 2013; Mohr et al., 2013). Collectively, the results point to variation in the genetic expression of an EtOH target that increases the activity of Golgi cells, the primary GABAergic input to GCs.

Directed by evidence that blocking NOS increased GABAergic transmission to GCs (Wall, 2003) and that EtOH inhibited NOS production (Persson & Gustafsson, 1992; Fataccioli et al., 1997; Al-Rejaie & Dar, 2006), we determined that EtOH increased Golgi cell activity, and consequentially increased vesicular GABA release, by blocking neuronal NOS production of NO (Chapter 2; Kaplan et al., 2013). Thus, low nNOS expression levels in the GC layer would likely be associated with a relative lack of EtOH-induced enhancement of GABAergic transmission to GCs. Indeed, the lack of EtOH-enhancement of GABAergic inhibition of PV GCs was paralleled by low nNOS expression. This was consistent with low nNOS expression in age-matched B6 mice and high nNOS expression in age-matched D2 mice and SDRs. The consistency of GC layer nNOS expression in the older mice and rats in the current study with the expression levels in the younger animals described in Kaplan et al. (2013) suggests that nNOS expression does not display a significant developmental shift, and in turn, implicates the importance of genetically regulated nNOS expression in mediating EtOH's impact on GC tonic $GABA_AR$ inhibition throughout one's lifespan.

The functional impact of low GC nNOS expression on EtOH impairment of cerebellar function has yet to be directly examined. NO signaling in the molecular layer plays a critical role

in synaptic plasticity at the parallel fiber/PC synapse (Shibuki & Okada, 1991) and the degree of NO production may dictate whether these synapses are strengthened or weakened (Wang et al., 2014). However, it remains unclear if diffusion of NO from the GC layer has any influence on plasticity in the molecular layer. Behaviorally, EtOH impairment of rotarod performance, a cerebellar-dependent task, was associated with a reduction in cerebellar NO nitrite+nitrate levels (Al-Rejaie & Dar, 2006). However, this study did not differentiate between molecular layer and GC layer NO, nor did it address whether low baseline NO levels afforded protection against EtOH-induced ataxia. The contrast between high GC layer nNOS expression in D2 mice, which are behaviorally sensitive to EtOH-impairment of rotarod performance (Gallaher et al., 1996), and low GC layer nNOS expression in behaviorally insensitive B6 mice provides strong support for the physiological relevance of nNOS expression levels in mediating EtOH-induced cerebellar impairment.

3.5.3. Limitations and future directions

The primary goal of the current study was to evaluate our prediction that EtOH suppresses GC tonic $GABA_AR$ inhibition in high drinking mammalian genotypes, such as the PV. We successfully confirmed this prediction, but our limited access to PV tissue did not allow for a detailed mechanistic analysis of the conditions that enable EtOH to suppress GC tonic inhibition. As mentioned above, we've concluded from rats and mice that low GC PKC activity enables EtOH to postsynaptically inhibit the extrasynaptic $GABA_ARs$ that mediate the tonic current (Chapter 2; Kaplan et al., 2013). Thus, it would be ideal to verify that low GC PKC activity confers EtOH suppression of tonic inhibition across PVs in future studies. So while our study is not as comprehensive as that carried out in rats and mice, the findings presented here provide important translational insight to the cerebellar effects of EtOH across species and should illuminate the potential relevance of EtOH action on GABAergic transmission of GCs in mediating behaviors that are associated with increased EtOH consumption.

It's important to note that while PVs are considered to be a high-EtOH consuming rodent strain, their outbred nature promotes a high degree of variability in individual EtOH consumption levels (Anacker et al., 2011). To be consistent with our previous studies, we recorded cells from EtOH-naïve animals, but this prevented us from being able to identify where each animal fell on the EtOH consumption spectrum. Future studies should directly investigate the relationship between EtOH consumption levels and EtOH's effect on GABAergic transmission to PV GCs.

PVs are socially monogamous and thus have been a widely used animal model for investigating the neurochemistry of social bonding and attachment (Getz et al., 1981, Carter et al., 1987; Carter et al., 1995). Only recently have PVs been used to model the social influence on EtOH-related behaviors (Anacker et al., 2011), and so a comprehensive battery of EtOH-related behaviors in the PV would be useful. In particular, the cerebellar sensitivity to acute EtOH-impairment remains unknown, thus preventing us from drawing any conclusions regarding the association between EtOH suppression of GC tonic inhibition and sensitivity to cerebellar impairment. This should be an emphasis of future studies as it could provide insight into the cellular mechanisms underlying the cerebellar LLR that in humans is associated with increased risk for developing an AUD (Schuckit, 1985; Schuckit, 1994; Schuckit & Smith, 1996).

3.5.4. Conclusions

This is the first known report of *in vitro* whole-cell patch-clamp recordings in the PV cerebellum. Consistent with other species, PV cerebellar GCs exhibit a tonic inhibitory conductance mediated by extrasynaptic α6, δ-containing $GABA_A$Rs that is a target of EtOH modulation. EtOH predominately reduced the magnitude of tonic $GABA_A$R-inhibition of individual PV GCs likely by postsynaptically inhibiting extrasynaptic $GABA_A$Rs. Reduced nNOS expression throughout the GC layer, as detected by immunohistochemistry with confocal microscopy, contributed to the relative absence of an EtOH-induced enhancement of GABAergic transmission to GCs. Together, our findings in the PV are consistent with other

previously investigated genotypes and further support our general hypothesis that the effect of EtOH on the magnitude and polarity of tonic inhibition of GCs varies as a function of EtOH-consumption phenotype, ranging from enhancement in low-EtOH consuming genotypes, to suppression in high-EtOH consumers. Future studies should investigate how EtOH's effects on GC tonic inhibition influence signal transmission through the cerebellar cortex, and in turn, behavioral intoxication and EtOH consumption.

Chapter 4: A low 9mM EtOH concentration has opposite effects on glutamatergic input to Purkinje cells in C57BL/6J and DBA/2J mice

4.1. Introduction

There are substantial cerebellar contributions to AUD risk. The cerebellum is among the most sensitive brain regions to EtOH modulation (Jia et al., 2008; Kaplan et al., 2013; Liang et al., 2006; Nie et al., 2000; Peris et al., 1992; Theile et al., 2009), and consistent reports of the LLR phenotype show that high-risk individuals are less sensitive to acute EtOH-induced cerebellar ataxia than their low-risk counterparts (Quinn & Fromme, 2011; Schuckit, 1985; Schuckit, 1994), which together implicate a common underlying neural target by which EtOH differentially affects cerebellar processing and EtOH consumption. PCs are the sole output of the cerebellar cortex, making them the final integrators of afferent information. Accordingly, disrupting or altering transmission to PCs will compromise signal transmission through the cerebellar cortex, and presumably disrupt cerebellar-dependent behaviors. In Chapters 2 & 3, I presented evidence that EtOH action on GC tonic $GABA_AR$ inhibition differed as a function of EtOH consumption phenotype, ranging from potentiation of tonic $GABA_AR$ inhibition in low-EtOH consumers to suppression in high consumers. These opposite actions on GC $GABA_AR$ inhibition might differentially influence cerebellar processing due to the consequent differential impact on GC glutamatergic input to PCs. However, the functional impact that the opposite actions of EtOH on GC tonic $GABA_AR$ inhibition has on glutamatergic input to PCs, and consequent PC output has not been directly tested.

Selective pharmacological manipulation of GC tonic $GABA_AR$ currents could provide insight into the impact that EtOH's actions on GC $GABA_AR$ inhibition has on transmission through the cerebellar cortex and consequent behavior. Reducing GC tonic inhibition with the α6-containing $GABA_AR$ specific antagonist, furosemide (specific at 100μM; Korpi et al., 1995), enhanced the excitability of GCs but not Golgi cells, Purkinje cells, or molecular layer interneurons (Hamann et al., 2002). Given the specificity of furosemide to selectively block GC tonic $GABA_AR$ inhibition, Hamann et al. (2002) used furosemide to characterize the role of GC

tonic $GABA_AR$ inhibition on transmission through the cerebellar cortex. They found that furosemide increased the mossy-fiber evoked GC firing frequency, which translated to enhanced mossy-fiber-evoked PC EPSP amplitudes and firing frequency. Therefore, reducing GC tonic $GABA_AR$ inhibition increased mossy fiber evoked transmission through the cerebellar cortex. On the other hand, enhancing GC tonic inhibition with the δ-subunit selective $GABA_AR$ agonist, THIP, reduced the spontaneous firing of GCs and suppressed the number of stimulus-evoked APs *in vivo* (Duguid et al., 2012). Taken together, selectively enhancing GC tonic $GABA_AR$ inhibition decreased evoked transmission through the cerebellar cortex while suppressing GC tonic inhibition enhanced transmission through the cortex. Thus, it seems likely that EtOH's opposite actions on GC tonic $GABA_AR$ inhibition, as described in chapters 2 and 3, will similarly exert a disparate effect on transmission through the cerebellar cortex.

EtOH also enhanced GABAergic transmission to PCs by increasing the firing rate of two types of molecular layer interneurons: stellate and basket cells (Hirono et al., 2009; Mameli et al., 2008; Wadleigh & Valenzuela, 2012). This increase in inhibitory transmission attenuated PC eEPSPs and evoked APs, but not PC spontaneous firing frequency (Mameli et al., 2008). Therefore, EtOH influenced $GABA_AR$ transmission in at least at two primary locations to disrupt cerebellar processing, GCs and PCs. Their relative contributions to EtOH-induced cerebellar impairment and whether they differ across rodents with opposite EtOH-related behavioral phenotypes is unknown.

Using whole-cell patch clamp from PCs in acutely prepared cerebellar slices, I tested the general hypothesis that EtOH differentially disrupts spontaneous and evoked signal transmission through the cerebellar cortex in B6 and D2 mice. I specifically hypothesized that EtOH's opposite effects on GC $GABA_AR$ inhibition between B6 and D2 mice differentially impacts spontaneous and mossy-fiber evoked excitatory input to PCs.

4.2. Methods

4.2.1. Preparation of brain slices

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 Committee of the Oregon Health & Science University. Cerebellar slices were prepared acutely on each day of experimentation (Hamann et al., 2002; Rossi et al., 2003). Male B6 and D2 mice (24-35 days old), were housed with 2-6 animals/cage, and maintained on a standard 12 hour light/dark cycle. Animals were anaesthetized with isoflurane and euthanized by decapitation. The whole brain was rapidly isolated and immersed in ice cold (0-2ºC) aCSF containing (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 ice cold (0-2^{o}C) aCSF. Parasagittal slices (225µm) were made with a vibrating tissue slicer (Vibratome). Slices were incubated in warmed aCSF (33±1^{o}C) for one hour after dissection and then held at 22-23^{o}C until used. Kynurenic acid (1 mM) was included in the dissection, incubation and holding solution (to block glutamate receptors to reduce potential excitotoxic damage) but was omitted from the experimental solutions.

4.2.2. Electrophysiology

Slices were placed in a submersion chamber on an upright microscope, and viewed with an Olympus 60X (0.9 numerical aperture) water immersion objective with differential interference contrast and infrared optics. Slices were perfused with aCSF at a rate of ~7ml/min at 31-34^{o}C. Drugs were dissolved in aCSF and applied by bath perfusion. Visually identified 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 9mM EtOH on PC sIPSC frequency in the presence of the ionotropic glutamate receptor antagonist, kynurenic acid (2mM), the internal solution for these voltage-clamp experiments contained (in mM): CsCl 130,

NaCl 4, $CaCl_2$ 0.5, HEPES 10, EGTA 5, MgATP 4, Na_2GTP 0.5, QX-314 5. Solutions were pH adjusted to 7.2-7.3 with CsOH. This solution was developed to optimize identification of $GABA_AR$-mediated Cl^- currents. Note, the intracellular [Cl^-] sets E_{Cl}- to ~0mV, which for the holding potentials used in these experiments (V_h = -60mV), results in $GABA_AR$ currents being inward. Separate voltage-clamp experiments assessed the effect of 31mM and 52mM EtOH on PC sIPSCs with V_h = +10. The internal solution for these voltage-clamp experiments was (in mM): Cs-gluconate 130, NaCl 4, HEPES 10, BAPTA 5, ATP Mg^{2+} salt 4, GTP Na^+ salt 0.5, QX-314 Cl 5, Paxilline 100nM, TEA 10. This solution was developed for stable PC recordings at a depolarized potential. Hence, paxilline and TEA were included to block Ca^{2+}-activated potassium channels. The internal solution for all current-clamp experiments was (in mM): K-gluconate 132.3, KCl 7.7, NaCl 4, $CaCl_2$ 0.5, HEPES 10, EGTA free acid 5, ATP Mg^{2+} salt 4, GTP Na^+ salt 0.5. Solutions were pH buffered to 7.2-7.3 with KOH. This solution doesn't include channel blockers and mimics a physiological intracellular solution. Electrode resistance was 1.5 to 3 MΩ. Cells were rejected if access resistance was greater than 15 MΩ or if access resistance changed by > 15%. In all cases, only one cell was recorded from a given slice. The stimulating electrode was filled with aCSF and lowered into contact with the white matter tracks of the same lobe as the recorded PC. To stimulate mossy fibers, the stimulating electrode was placed a mean of 480µm from the recording electrode. Stimulation intensity was adjusted to obtain PC eEPSP amplitudes (minimum amplitude 0.5mV) without activating climbing fibers. Stimulations that elicited complex spikes at the PC (from climbing fibers) were excluded in order to isolate the effects of stimulating mossy fibers. Mean mossy fiber stimulation intensities between strains were 341.82 ± 44.22µA for B6 mice and 354.71 ± 38.50µA for D2 mice. Cells with peak eEPSP amplitudes of less than 0.5mV were only analyzed for spontaneous activity. After acquiring a whole-cell patch recording, holding current was added to maintain the PC at a resting membrane potential between -60 and -62mV. There were no strain differences in holding current (B6: -243.04 ± 18.14pA; D2: -254.13 ± 9.90pA, *P* = .61).

In cases where the slice was exposed to more than one drug or different doses of the same drug, the order of drug application was randomized across slices. In all cases of multiple drug exposures to the same slice, a stable baseline was obtained following washout of drug for a minimum of 4 minutes. However, control experiments were never done on slices that had been exposed to GABAzine as the efficacy and time required to adequately wash GABAzine was preclusive of additional unrelated experimentation.

4.2.3. Statistics

All data are expressed as the mean ± the standard error of the mean. Percent change scores were calculated by comparing the eEPSP amplitude of the drug condition to the mean eEPSP amplitudes in the pre- and post-drug control conditions. To compare the effect of EtOH on PC eEPSP amplitudes from stimulations 2-10 in each train, the percent change by EtOH on the amplitude of each eEPSP# was taken as a ratio of the EtOH-induced % change in the first eEPSP of each train (ratio = % change eEPSP#1/% change eEPSP#2-10). Repeated measures ANOVA was used to detect significant interactions between EPSP# and strain effects. Between-strain post-hoc analyses were conducted using Student's t-tests. All other statistical comparisons were made with unpaired or one-sample *t* tests. In all cases, statistical tests were two-tailed. The threshold for significance was set at $P < 0.05$. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in the field (Carta et al., 2004; Hamann et al., 2002; Hanchar et al., 2005; Rossi et al, 2003).

4.2.4. Reagents

All reagents were from Sigma Chemicals (St. Louis, MO) except for GABAzine and kynurenic acid (Abcam, UK).

4.3. Results

4.3.1. A Low EtOH concentration differentially affects evoked excitatory input to PCs in B6 and D2 mice

I have shown that EtOH has opposite effects on GC tonic $GABA_AR$ inhibition in B6 and D2 mice: EtOH reduces inhibition in B6s and enhances it in D2s (Chapter 2; Kaplan et al., 2013). To determine the impact that these opposite EtOH effects have on mossy-fiber evoked polysynaptic transmission to PCs, I made current-clamp recordings of PCs in acutely prepared slices from EtOH naïve mice and measured the evoked EPSP (eEPSP) amplitude during a train of 10 mossy-fiber stimulations at 50Hz, elicited every 30 seconds. I measured the change in eEPSP amplitude in the presence of 9mM, 31mM, or 52mM EtOH (doses chosen for continuity with experiments presented in chapter 2). Analysis of the first stimulation in each train enabled me to assess the effect of EtOH on evoked transmission independent of network feedback. Bath application of the

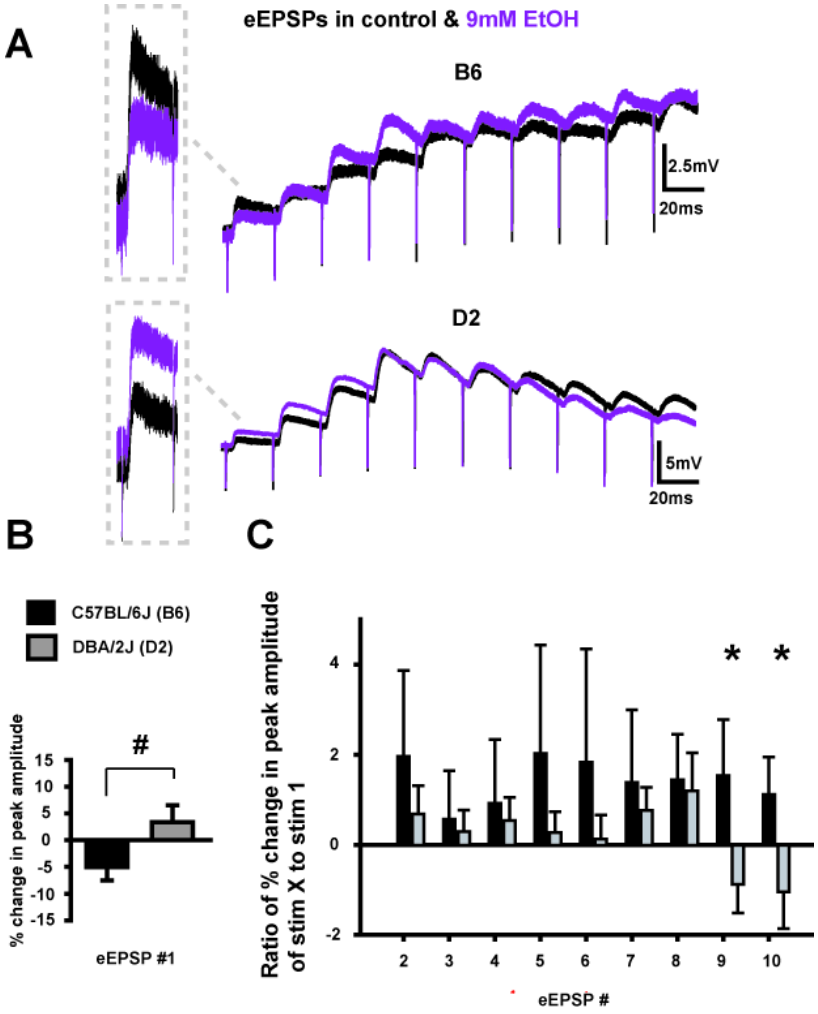

Figure 1. 9mM EtOH differentially affects PC eEPSP amplitudes in B6 and D2 mice. **A.** Representative current-clamp traces of PC eEPSPs from B6 (top) and D2 mice (bottom) in control (black) and 9mM EtOH (purple). The first eEPSP in each train is enlarged (dotted boxes). **B.** Summary bar chart displaying the EtOH-induced percent change in the first PC eEPSP peak amplitude in each train, from baseline, in B6 mice (black) and D2s (gray). # indicates that the two groups differ at trend level, $P = .055$. **C.** Summary bar chart showing the EtOH-induced percent change in subsequent PC eEPSPs in each train as a ratio of the % change of the first PC eEPSP. * indicates that the two strains significantly differ from each other at eEPSPs #9 and #10, $P < .05$.

low EtOH concentration, 9mM, a concentration at which D2 mice but not B6s show significant cerebellar ataxia (Gallaher et al., 1996), enhanced and reduced the amplitude of the first PC eEPSP in D2 and B6 mice, respectively, which differed at trend level ($t(24) = 2.01$, $P = .055$, unpaired *t* test; D2: 3.35 ± 3.19% increase, $n = 14$; B6: -5.02 ± 2.51%, $n = 12$; Fig. 1A,B). The effect of 9mM EtOH and the difference between these two strains was blocked by the broad-spectrum $GABA_AR$ antagonist, GABAzine (10μM; B6: 2.20 ± 6.47, $n = 9$; D2: -4.63 ± 4.81%, $n = 6$, $P = .46$, unpaired *t* test), suggesting that differences in 9mM EtOH's effects on mossy fiber-evoked transmission to PCs is mediated by $GABA_ARs$.

The EtOH-induced percent change in amplitude of the remaining stimulations in each train, stimulations 2-10, were analyzed as a proportion of the first stimulation (see Methods *4.2.3*). A mixed-factorial ANOVA revealed a significant interaction between strain and stimulation number ($F(8,197) = 2.14$, $P = .035$). Post-hoc analysis identified that EtOH differentially affected PC eEPSP amplitude for stimuli 9 and 10 between B6 and D2 mice and within D2 mice (eEPSP amplitude for stimulus 8 differed from 9 and 10 in D2s, all $P < .05$; Fig. 1C). Together, these results suggest that 9mM EtOH differentially affected evoked transmission through the cerebellar cortex in B6 and D2 mice.

4.3.2. Moderate-high EtOH concentrations reduce excitatory input to PCs in B6 and D2 mice

Many humans and mice, especially B6 mice, will voluntarily reach BEC levels that meet binge criteria (i.e., 80mg/dl or 17mM). To assess the effect of higher EtOH concentrations on evoked transmission through the cerebellar cortex, I repeated the same procedure as described above with 31mM, the concentration at which B6 mice show significant cerebellar ataxia (D2 mice show significant cerebellar ataxia at 9mM; Gallaher et al., 1996), and 52mM EtOH. Both 31mM and 52mM EtOH similarly attenuated PC eEPSPs from the first stimulation ($P > .05$; Fig. 2A) in B6 (31mM: -33.46 ± 10.05%, $n = 15$; 52mM: -15.45 ± 14.21%, $n = 16$) and D2 (31mM: -36.90 ± 23.10%, $n = 26$; 52mM: -22.97 ± 4.00%, $n = 14$) mice. There was also no difference in

the degree of attenuation of PC eEPSPs by 31mM and 52mM EtOH from stimulations 2-10 in B6 or D2 mice (all $P > .05$; Fig. 2B). Together, these results indicate that there are strain differences in EtOH's impact on PC EPSPs at an EtOH concentration where, *in vivo*, D2 mice but not B6 mice show cerebellar ataxia (9mM; Gallaher et al., 1996), but these strain differences disappear at higher EtOH concentrations in which both strains are ataxic (31mM and 52mM; Gallaher et al., 1996), possibly reflecting broader neural impacts of EtOH induced during more severe intoxication.

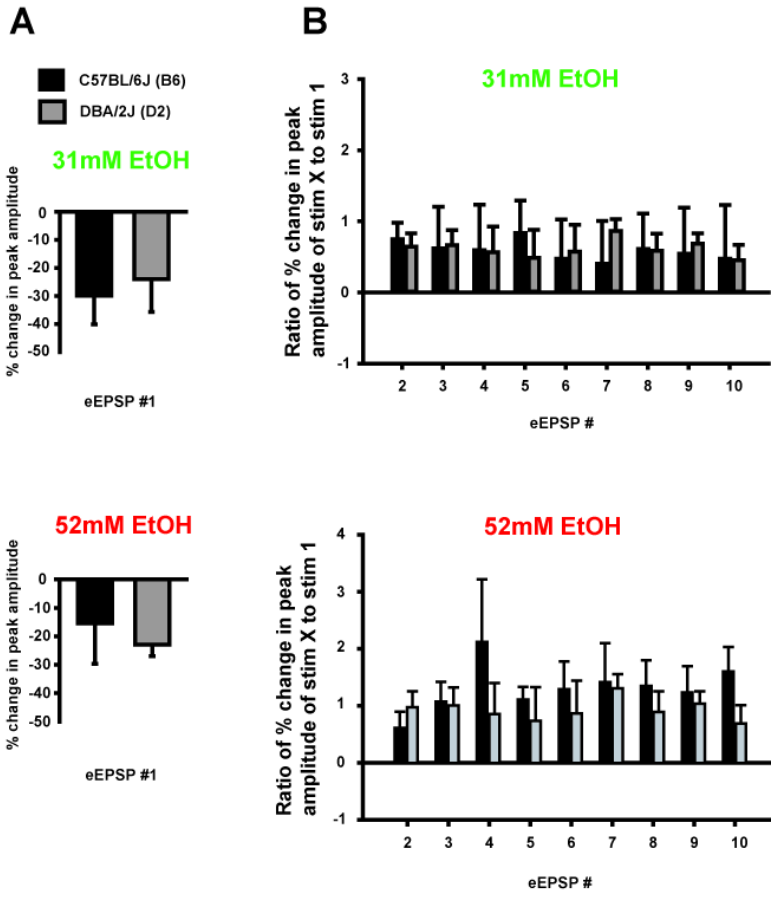

Figure 2. 31mM and 52mM EtOH dampen PC eEPSPs similarly in B6 and D2 mice. **A.** Summary bar charts displaying the percent change in the first PC eEPSP peak amplitude from 31mM EtOH (top) and 52mM EtOH (bottom), in B6 mice (black) and D2s (gray). **B.** Summary bar charts showing the percent change in subsequent PC eEPSPs in each train as a ratio of the % change of the first PC eEPSP from 31mM (top) and 52mM EtOH (bottom).

4.3.3. EtOH enhancement of $GABA_AR$ inhibition reduces spontaneous excitatory input to PCs in D2 but not B6 mice

9mM EtOH differentially affected evoked excitatory transmission to PCs between strains (Fig. 1), but while this electrical stimulation procedure is a valid method for exposing general EtOH-induced changes to the excitatory/inhibitory balance in the cerebellar cortex, the response

to large and indiscriminate electrical stimulation might obscure more physiologically relevant impacts of EtOH during more natural afferent activity. I therefore measured PC sEPSP frequencies to assess the effect of 9mM EtOH on non-electrically-evoked, spontaneous glutamatergic input to PCs. 9mM EtOH significantly reduced PC sEPSP frequency in D2 mice (-20.85 ± 7.79%, n = 16; $t(15) = 2.68$, $P = .017$, one-sample t test), but had no impact on sEPSP frequency in B6 mice (-0.56 ± 7.96%, n = 12, $P = .95$; Fig. 3A,C). GABAzine blocked 9mM EtOH selective enhancement of sEPSP frequency in D2 mice and had no effect in B6 mice (B6: -3.52 ± 15.28%, n = 12; D2: 0.35 ± 13.92%, n = 12, all $P > .05$, one-sample t test; Fig. 3C). Together, these findings suggest that GCs from D2 mice are more sensitive to the inhibitory effects of 9mM EtOH, which reduces spontaneous excitatory transmission to PCs. However, 31mM EtOH, the threshold concentration at which cerebellar ataxia manifests in B6 mice (Gallaher et al., 1996), reduced sEPSP frequency in both mouse strains (D2: -33.34 ± 8.40%, n = 7, $P = .007$, one-sample t test; B6: -18.38 ± 6.18, n = 9, $P = .018$; Fig. B,C). Interestingly, GABAzine reduced EtOH's effect on sEPSP frequency in D2 mice at trend level (-14.43 ± 6.93%, $t(6) = 2.12$, P = .078, paired t test), but had no effect in B6 mice (-16.37 ± 6.95%, $t(8) = 0.21$, $P = .84$; Fig. 3C). These results reveal that the onset of behavioral intoxication in B6 mice coincides with EtOH activation of non-$GABA_AR$ targets (see discussion below), while behavioral intoxication in D2 mice likely results from enhanced $GABA_AR$ inhibition of cerebellar GCs.

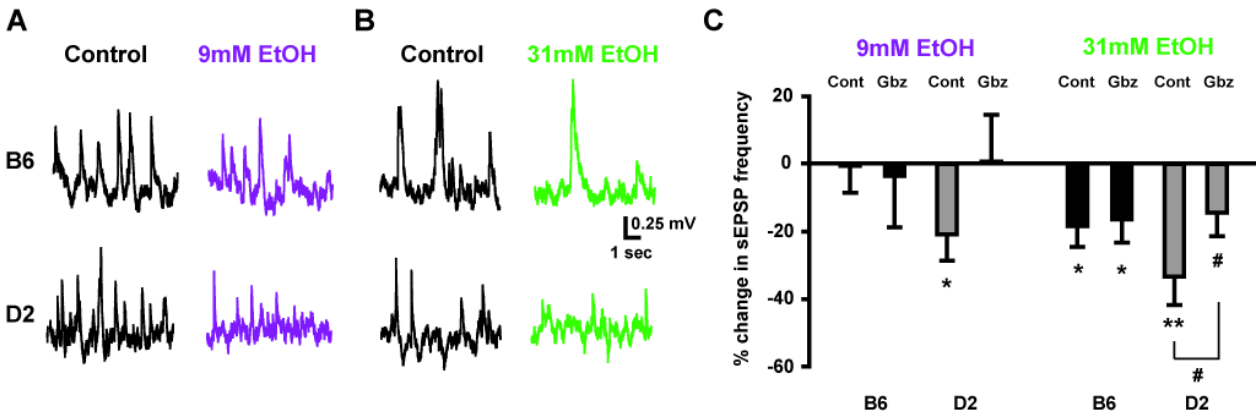

Figure 3. 9mM EtOH reduces PC sEPSPs in D2 but not B6 mice by enhancing $GABA_AR$ inhibition. **A,B.** Representative current-clamp traces of PC sEPSPs in control (black), 9mM EtOH (purple), and 31mM EtOH (green) in B6 mice (top) and D2 mice (bottom). **C.** Summary bar charts showing the percent change in PC sEPSP frequency by 9mM EtOH and 31mM EtOH under control conditions and in GABAzine in B6 (black) and D2 (gray) mice. * indicates significant difference from baseline, $P < .05$, ** $P < .01$; # indicates different at trend level $P = .08$.

Because EPSPs represent the net effect of excitatory and inhibitory input, an EtOH-induced enhancement in inhibitory input to the PC in D2 mice could have obfuscated excitatory

potentials. We therefore repeated the experiments using voltage-clamp, V_h = -60; E_{Cl} = -60, to isolate EPSCs. Again, 9mM EtOH reduced PC sEPSC frequency in DBA/2J mice but not in C57BL/6J mice (D2: -21.13 ± 5.79%, *n* = 6 cells, *P* = .015, one-sample *t* test; B6: 1.94 ± 7.31%, *n* = 6, *P* = .80; Fig 4A,D). This strain difference disappears at 31mM EtOH (D2: -23.41 ± 5.50%, *n* = 8 cells, *P* = .004, one-sample *t* test; B6: -23.97 ± 8.96%, *n* = 8, *P* = .032; Fig 4B,D), a concentration at which cerebellar impairment manifests in both strains (Gallaher et al., 1996). To confirm that an enhancement in GC tonic $GABA_AR$ inhibition reduces spontaneous GC input to PCs in both mouse strains, we increased GC tonic $GABA_AR$ inhibition with the selective δ-subunit $GABA_AR$ agonist, THIP (300nM). THIP predictably reduced sEPSC frequency in PCs from both D2 mice and B6 mice (D2: -25.62 ± 8.33%, *n* = 6 cells, *P* = .028, one-sample *t* test; B6: -18.00 ± 6.83%, *n* = 7, *P* = .039; Fig. 4C,D), providing further support that enhancement of GC tonic $GABA_AR$ inhibition by 9mM EtOH, which is present in D2 mice, but absent in B6 mice (Chapter 2; Kaplan et al., 2013), reduced glutamatergic input to PCs.

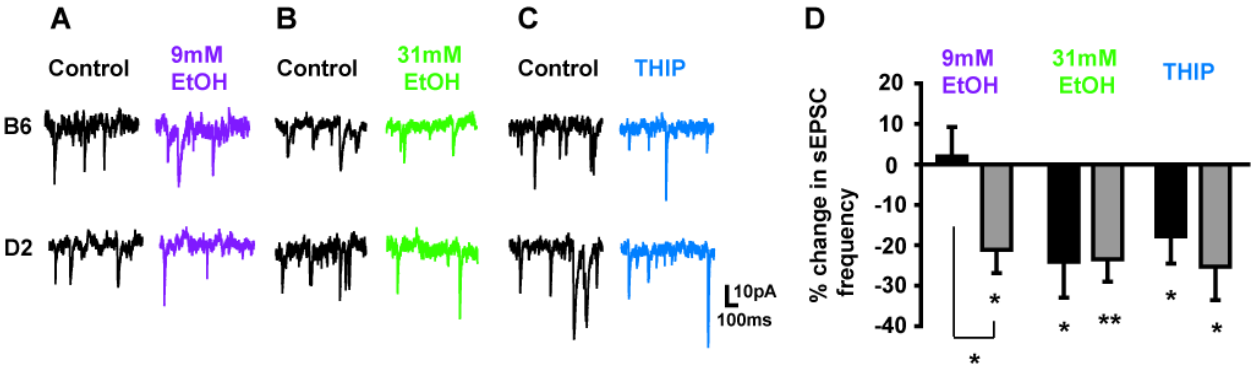

Figure 4. Enhancing GC $GABA_AR$ inhibition reduces PC sEPSC frequency. **A,B,C** Representative voltage-clamp traces of PC sEPSCs in control (black), 9mM EtOH (purple), 31mM EtOH (green), and THIP (300nM; blue) in B6 mice (top) and D2 mice (bottom). **D.** Summary bar charts showing the percent change in PC sEPSC frequency by 9mM EtOH, 31mM EtOH, and THIP in B6 (black) and D2 (gray) mice. * indicates significant difference from baseline, *P* < .05, ** *P* < .01.

4.3.4. 9mM EtOH does not differentially modulate molecular layer interneuron activity

An additional $GABA_AR$ target of EtOH-induced cerebellar disruption may come from enhancement in GABAergic transmission to PCs from stellate and basket cells, although previous reports failed to find robust increases in PC sIPSCs from EtOH concentrations below 50mM (Hirono et al., 2009; Mameli et al., 2008). To assess if stellate and basket cell sensitivity to EtOH differs between animals with opposite EtOH-related behavioral phenotypes, I first voltage-clamped PCs (V_h = -60mV, E_{Cl} = ~-0mV) and measured the effect of 9mM EtOH on PC sIPSCs in the presence of kynurenic acid (2mM) to block the effect of differential GC inputs. In both strains, 9mM EtOH had no effect on PC sIPSC frequency (all $P < .05$, from one-sample t tests; B6: -1.62 ± 10.05%, $n = 5$; D2: 2.32 ± 3.06%, $n = 5$; Fig. 5A,C). Therefore, molecular layer

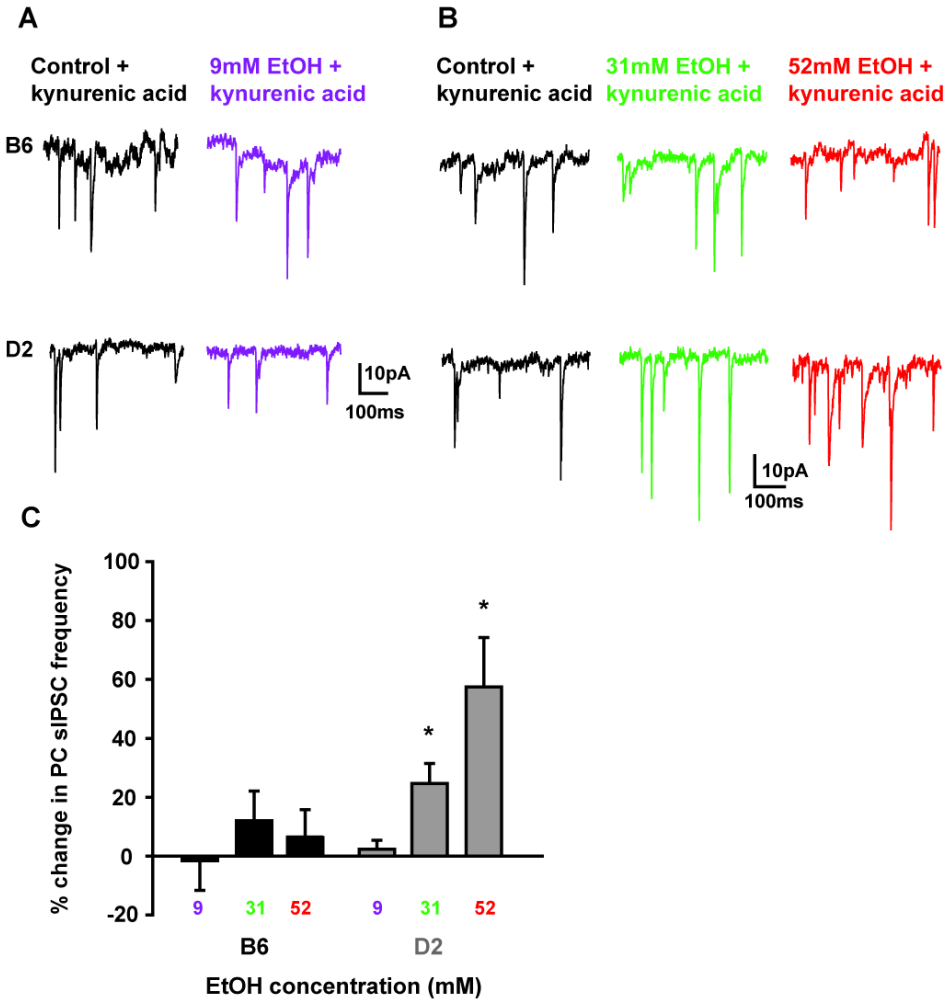

Figure 5. EtOH dose-dependently increases PC sIPSCs in the presence of the ionotropic glutamate receptor antagonist, kynurenic acid (2mM) in D2 mice. **A.** Representative voltage-clamp recordings (V_h=-60, E_{Cl}^- = 0mV) of PC sIPSCs in control (black) and 9mM EtOH (purple) in B6 (top) and D2 mice (bottom). **B.** Representative voltage-clamp recordings of PC sIPSCs in control (black), 31mM EtOH (green), and 52mM EtOH (red), from different cells than in A. **C.** Summary bar chart showing the 9mM, 31mM, and 52mM EtOH-induced percent change in PC sIPSCs frequency in B6 mice (left; black bars) and D2 mice (right; gray bars). * $P < .05$, one-sample t test.

interneurons are not sensitive to a low EtOH concentration in which differential effects on GC tonic $GABA_AR$ inhibition and excitatory transmission through the cerebellar cortex are observed. However, it's possible that molecular layer interneuron sensitivity to higher EtOH concentrations could contribute to behavioral intoxication. 31mM and 52mM EtOH increased PC sIPSC frequency in D2 mice (31mM: 24.63 ± 6.84%, n = 5; 52mM: 57.46 ± 17.77%, n = 5, all $P < .05$, one-sample t tests, Fig. 5B,C), but had no significant effects in B6 mice (31mM: 12.05 ± 10.01%, n = 4; 52mM: 6.34 ± 9.43%, n = 4, all $P > .05$, one-sample t tests; Fig. 5B,C). The effect of 52mM EtOH, but not 31mM EtOH, on PC sIPSC frequency significantly differed between strains ($P = .043$). Together, these results indicate that moderate-high EtOH concentrations enhance stellate and basket cell activity in D2 mice independent of glutamatergic input. Furthermore, they suggest that differences seen in 9mM EtOH's effect on mossy fiber-evoked transmission to PCs between strains are due to differences upstream of the PC-molecular layer interneuron synapse, presumably at the GC.

EtOH's effect on GC tonic $GABA_AR$ inhibition may influence parallel fiber driven molecular layer interneuron input to PCs. 31mM and 52mM EtOH similarly reduced mossy fiber-evoked parallel fiber input to PCs in B6 and D2 mice (Fig. 2), which presumably also attenuates excitatory input to stellate and basket cells. To assess if EtOH-induced dampening of excitatory transmission through the cerebellar cortex affects stellate and basket cell input to PCs, I similarly voltage-clamped PCs, but this time, manipulated the chloride gradient in order to isolate IPSCs (V_h = +10mV, E_{Cl} = ~-60mV) and tested the effects of 31mM and 52mM EtOH in the absence of kynurenic acid to incorporate EtOH's effects on network dynamics. 31mM EtOH caused a modest but significant increase in PC sIPSC frequency in D2 mice ($t(9) = 2.42$, P = .038, one-sample t test, n = 10, 9.78 ± 4.03%), while only increasing PC sIPSC frequency at trend level in B6 mice ($t(9) = 1.95$, $P = .083$, one-sample t test, n = 10, 7.76 ± 3.98%; there was no difference between B6 and D2 mice, $P = .73$, unpaired t test; Fig. 6). The higher 52mM EtOH concentration caused inconsistent and a non-statistically significant effect on PC sIPSC

frequency in B6 mice (5.37 ± 7.15% increase), but caused a much larger increase in PC sIPSC frequency in D2s (49.38 ± 23.17%, *n* = 6; Fig 6). In all cases, there were no statistically significant differences between EtOH's effects on PC sIPSC frequency in the presence or in the absence of kynurenic acid (all *P* > .05). This suggests that EtOH had similar effects on molecular layer interneuron activity regardless of EtOH-induced changes to GC activity. Together, these findings suggest that cerebellar molecular layer interneurons are insensitive to modulation by low EtOH concentrations and likely don't contribute to variation in EtOH-related behaviors observed at low BECs. However, elevated stellate and basket cell input to PCs by moderate-high EtOH concentrations may contribute to cerebellar impairment in D2 mice.

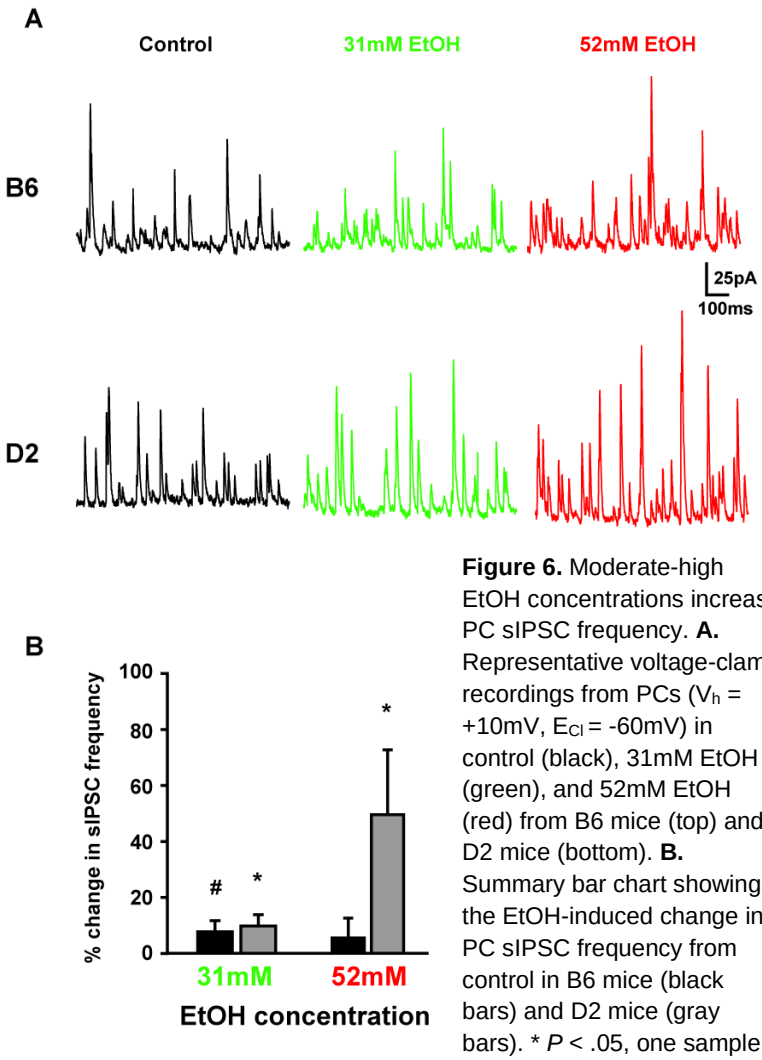

Figure 6. Moderate-high EtOH concentrations increase PC sIPSC frequency. **A.** Representative voltage-clamp recordings from PCs (V_h = +10mV, E_{Cl} = -60mV) in control (black), 31mM EtOH (green), and 52mM EtOH (red) from B6 mice (top) and D2 mice (bottom). **B.** Summary bar chart showing the EtOH-induced change in PC sIPSC frequency from control in B6 mice (black bars) and D2 mice (gray bars). * *P* < .05, one sample *t* test against baseline.

4.3.5. THIP and furosemide modulate evoked excitatory input to PCs

Enhancing GC tonic $GABA_AR$ inhibition with the selective δ-subunit $GABA_AR$ agonist, THIP, increases the probability of mossy fiber evoked GC spike failures (Duguid et al., 2012). In contrast, reducing GC tonic $GABA_AR$ with the α6-subunit $GABA_AR$ antagonist, furosemide,

increases the mossy fiber evoked excitatory transmission to PCs in SDRs (Hamann et al., 2002). To confirm that enhancement and suppression of GC tonic $GABA_AR$ inhibition similarly affected mossy fiber evoked transmission to PCs in B6 and D2 mice, I current clamped PCs and tested the effect of THIP (300nM) and furosemide (100µM) on PC eEPSP amplitude. THIP predictably reduced the PC eEPSP amplitude in D2 mice (-6.51 ± 2.21%, n = 7, P = .026, one-sample t test), but the reduction in the mean PC eEPSP amplitude in the B6 mouse (-10.12 ± 6.35%, n = 6) was not statistically significant (P = .17, one-sample t test), perhaps due to low statistical power. However, there were no differences in the effect of THIP on PC eEPSP amplitude between strains (P = .58, unpaired t test). On the contrary, furosemide's effect on PC eEPSP amplitudes was notably variable, causing neither consistent enhancement nor suppression, in either strain (B6: 12.44 ± 14.36%, n = 9; D2: 1.00 ± 7.92%, n =7; all P > .05, one-sample t tests). As a result, furosemide's effect on PC eEPSP amplitude was statistically similar between the strains (P = .57, unpaired t test). The reasons for this variability are unclear, but some potential explanations are discussed in detail below.

4.4. Discussion

Here, I demonstrate that opposite effects of 9mM EtOH on GC tonic $GABA_AR$ inhibition between B6 and D2 mice differentially modulate mossy fiber-evoked excitatory transmission to PCs, albeit in a more complicated manner than expected. 9mM EtOH reduced the frequency of spontaneous glutamatergic PC EPSPs and EPSCs in D2 mice but had no effect in B6s mice. These findings suggest that EtOH (9mM) enhancement of GC tonic $GABA_AR$ inhibition in D2 mice is sufficient to reduce GC firing, whereas EtOH (9mM) suppression of tonic $GABA_AR$ in B6 mice did not significantly affect GC firing. Importantly, the $GABA_AR$ antagonist, GABAzine, abolished the EtOH-induced reduction of PC sEPSPs, confirming that EtOH-induced changes in GC firing were mediated by EtOH enhancement of GC $GABA_AR$ currents. Interestingly, 9mM EtOH also caused differential effects on mossy fiber-evoked transmission through the cortex, as

expressed by the mossy fiber-evoked PC EPSPs: it enhanced transmission in the D2s and suppressed it in the B6s. However, moderate-high EtOH concentrations (31mM and 52mM), which had opposite effects on GC tonic $GABA_AR$ inhibition in B6 and D2 mice (Chapter 2), suppressed mossy-fiber evoked excitatory input to PCs in both B6 and D2s, and to a similar degree. The loss of differential impacts at higher EtOH concentrations mirrors the breakdown of differential behavioral responses at higher EtOH concentrations (Gallaher et al., 1996), presumably because higher concentrations of EtOH recruit additional non-genetically variable mechanisms that suppress transmission through the cerebellar cortex and thereby obscure differential actions at GC tonic $GABA_AR$ currents (Fig. 6). In further support of this conclusion, 31mM EtOH, in the presence of GABAzine, similarly suppressed PC sEPSP frequency in both strains, exposing a similar contribution of non-$GABA_AR$ EtOH targets in B6 and D2 mice at EtOH concentrations at which both strains are intoxicated (Gallaher et al., 1996).

Another aim of this chapter was to directly assess the influence of EtOH on $GABA_AR$ transmission from molecular layer interneurons to PCs. In both strains, 9mM EtOH had no effect on PC sIPSCs when ionotropic glutamate receptors were blocked with kynurenic acid, and there were no strain differences in the modest enhancement of PC sIPSCs by 31mM EtOH, regardless if kynurenate was or was not present. These findings indicate that the effect of low-moderate concentrations of EtOH on molecular layer interneuron inhibition of PCs ranges from nothing to small enhancement, but importantly, there were no significant differences between B6 and D2 mice. Instead, the relative lack of effect of 9mM EtOH on molecular layer interneuron activity and complete lack of difference between mouse strains suggests that the observed variation in mossy fiber-evoked PC response stems from the clear differences in EtOH's effect on GC inhibition (Chapter 2).

4.4.1. Proposed influence of the spatial relationship between activated GCs and recorded PC

An important finding from these studies is that 9mM EtOH differentially affected spontaneous and mossy fiber-evoked excitatory transmission to PCs between mouse strains with opposite EtOH-related behavioral phenotypes. This EtOH concentration is the threshold at which D2 mice, but not B6 mice, show significant cerebellar impairment of rotarod performance (Gallaher et al., 1996), making it tempting to speculate that a reduction in glutamatergic input to PCs by 9mM EtOH is the neural signature of high sensitivity to cerebellar impairment. 9mM EtOH reduced spontaneous glutamatergic input to PCs in D2 mice likely due to EtOH enhancing GC tonic $GABA_AR$ currents, which has a notably greater contribution to the inhibitory charge of the GC than phasic $GABA_AR$ currents (Hamann et al., 2002; Duguid et al., 2012). Furthermore, it is consistent with findings that THIP reduced PC sEPSC frequency reported here *in vitro* and previously by Duguid et al. (2012) who demonstrated that THIP reduces the spontaneous firing rate of SDR GCs *in vivo*. Thus, enhancement in GC tonic $GABA_AR$ inhibition by 9mM EtOH in D2 mice decreases GC excitability and reduces glutamatergic input to PCs.

Overall, the hypothesis that opposite EtOH actions on GC tonic $GABA_AR$ inhibition contribute to differential effects on mossy fiber-evoked transmission to PCs was supported. 9mM EtOH enhanced mossy fiber-evoked excitatory input to PCs from D2 mice while reducing it in B6 mice. However, the direction of this effect was paradoxical to those predicted based on the direction of EtOH's effects on GC tonic $GABA_AR$ inhibition (Chapter 2; Kaplan et al., 2013), and the reduction in sEPSP and sEPSC frequency by 9mM EtOH in D2 mouse PCs. One potential explanation for these paradoxical results derives from the spatial orientation between excited GCs and the recorded PC. These same spatial factors could also explain the variability in eEPSP amplitudes from the selective pharmacological agents, THIP and furosemide.

4.4.1a. Spatial factors may contribute to paradoxical effects of 9mM EtOH and variability in furosemide and THIP effects

In slice experiments, the mossy fiber-GC relay provides purely glutamatergic input to the PC directly above the activated GCs (Dizon & Khodakhah, 2011). In this circumstance, the PC firing rate increases in direct relation to the mossy fiber's firing rate. However, PCs positioned more lateral to a given population of GCs receive increasing amounts of inhibition from molecular layer interneurons that are activated by the parallel fibers from the given group of GCs (Dizon & Khodakhah, 2011; Park et al., 2012). This concept, known as "reciprocal signaling", suggests that the further lateral the recorded PC is from the stimulated mossy fibers, the greater amount of inhibition relative to excitation they will receive. Consequently, the variation in the magnitude of reciprocal signaling is a source of variability among the EtOH data, as well as the THIP and furosemide data described in this chapter. Thus, the proportional influence that a particular GC has on Golgi cells, molecular layer interneurons, and PCs may differ across GCs based on their relative spatial orientation within the slice (Dizon & Khodakhah, 2011). For instance, a particular GC may have a strong influence on PC EPSP amplitude, little impact on feedforward inhibition of PCs from molecular layer interneurons, and no influence on Golgi cell negative feedback. The influence of each of these components can vary across spatial parameters. Under this framework, the 9mM EtOH-induced change in PC sEPSP and sEPSC frequency, which is absent in B6 mice and reduced in D2 mice, may reflect a new GC steady state by EtOH, but may not be predictive of the response determined by a broad population of GCs, Golgi cells, and molecular layer interneurons in mediating mossy fiber-evoked PC eEPSPs. Mossy fiber-stimulation may recruit a different subset of GCs with differential proportional effects that contribute to enhanced feedforward inhibition of PCs in B6 mice and perhaps reduced feedforward inhibition of PCs but enhanced feedback inhibition of GCs in D2 mice. As a result, spatial factors likely contributed to reversing the polarity of 9mM EtOH's effects on PC eEPSP amplitude from my initial predictions.

Similar engagement of GCs with differential proportional influence on molecular layer interneurons on Golgi cells could explain the variable effects of the α6-$GABA_AR$ antagonist,

furosemide, in both strains, on mossy fiber-evoked PC eEPSP amplitude. In some cases, furosemide enhanced PC eEPSP amplitude while in others, it reduced PC eEPSP amplitude. In cases where it reduced PC eEPSP amplitude, furosemide may have enhanced the activity of a population of GCs that, due to a particular spatial orientation within the slice and innervation of interneurons, enhanced feedforward inhibition of PCs and feedback inhibition of GCs. Similar but opposite effects could have resulted during bath application of THIP, which by inhibiting GCs, may have selectively reduced glutamatergic input to PCs, or had a stronger impact on reducing feedforward inhibition of PCs.

The protocol used in the current study could be improved in the future by using optogenetic stimulation of mossy fibers. This would enable a more precise assessment of the spatial orientation between activated GCs and the recorded PC to ensure consistency across conditions and strains.

4.4.1b. Influence of Golgi cell firing rates on GC inhibition and input to PCs

Direct Golgi cell innervation of GCs produce fast IPSCs while spillover creates slow indirect IPSCs (Rossi & Hamann, 1998). We confirmed similar general GC IPSC amplitudes and decay times between B6 and D2 mice in Chapter 2, but it's important to note that the fast and slow IPSC components can vary across GCs because not all GC dendrites form synapses with Golgi cell terminals (Jakab & Hamori, 1988). Thus, the magnitude and temporal regulation of the inhibitory charge transfer to the GC differs as a function of direct versus indirect Golgi cell innervation, determined by the spatial relationship between activated GCs and innervating Golgi cells, which can have consequences on signal integration and GC firing. Both fast and slow forms of inhibition affect GC spike timing from mossy-fiber input, but in a differential manner. Fast inhibition has a stronger effect on decreasing the initial spike rate, while slow inhibition strongly suppresses the spike frequency later in the train (Crowley et al., 2009). Thus, fast and slow forms of inhibition differentially affect the input-output relationship of the mossy fiber-GC

relay. Importantly, the frequency of Golgi cell firing rates can influence the relative magnitude of the fast versus slow components. Fast and slow inhibitory components make a similar contribution to the inhibitory charge transfer to the GC at low Golgi cell firing rates (Holtzman et al., 2006), but an increase in the Golgi cell firing rate would increase the slow IPSC contribution and decrease that of the fast IPSC (Crowley et al., 2009). Consequently, the fast and slow components contribute in different ways to the inhibitory charge of the GC, which is determined by the characteristics of GC innervations by Golgi cells and potentially by the underappreciated heterogeneity of Golgi cell subtypes in the cerebellum (Simat et al., 2007). Because EtOH, on average, reduced GC tonic $GABA_AR$ inhibition in B6 mice (Chapter 2), it was predicted that 9mM EtOH would enhance spontaneous GC activity reflecting greater excitation. However, both the lack of effect of 9mM EtOH on spontaneous GC activity and the slight reduction in mossy fiber-evoked glutamatergic input to PCs by EtOH was unexpected. It is feasible that both of these unexpected findings could be attributed to a shift in the inhibitory balance from fast IPSCs to slow IPSCs as greater amounts of negative feedback spillover from neighboring synapses. This could prevent an increase in spontaneous GC firing and reduce mossy fiber-evoked transmission to PCs. Another non-exclusive possibility is that stimulating mossy fibers causes a greater degree of feedforward inhibition of GCs in B6 mice than in D2 mice. This resulting enhanced ratio of slow:fast IPSCs would have a stronger inhibitory effect on the GC and potentially reduce glutamatergic transmission to PCs, which was the average response detected in B6 mice.

Similarly, variability in the effects of THIP and furosemide on mossy fiber-evoked transmission to PCs could derive from shifts in the balance between slow versus fast forms of inhibition. Furosemide was expected to increase mossy fiber-evoked transmission to PCs, consistent with previous reports (Hamann et al., 2002). However, in my hands, furosemide enhanced PC eEPSP amplitudes in some recordings but suppressed them in others. Because furosemide directly enhances GC excitability by antagonizing α6-$GABA_ARs$ (Hamann et al.,

2002), there should be a coinciding increase in negative feedback from Golgi cells. Depending on the degree of Golgi cell innervations of GCs, and whether they provide fast versus slow inhibition of the GC, could determine whether furosemide's direct disinhibitory action on GCs is overridden by negative feedback from Golgi cells. Additionally, the extent of parallel fiber innervation of Golgi cells across slices could differentially determine the net effect of furosemide. Furosemide, under conditions of high parallel fiber innervation of Golgi cells would strongly increase negative feedback and elevate ambient GABA, thereby shifting the balance towards slow GC IPSCs. Consequently, mossy fiber-evoked PC eEPSP amplitude may be reduced if Golgi cell innervation by parallel fibers was high, Golgi cell spiking rates were increased, and the relative proportion of slow:fast IPSCs was enhanced. A similar but opposite phenomenon may have occurred for THIP application. Since THIP reduces GC excitation by enhancing δ-subunit $GABA_AR$ activity (Duguid et al., 2012), the coinciding reduction in negative feedback from Golgi cells highly innervated by parallel fibers within the slice may have been sufficient to have a slight disinhibitory effect, especially if there was a large degree of spillover-driven slow IPSCs, and enhance mossy fiber-evoked transmission to PCs in some circumstances. A better understanding of the effects of furosemide, THIP, and EtOH are needed on the firing rates of Golgi cells and whether these effects differ as a function of baseline firing rate. This could be accomplished using current-clamp of Golgi cells and comparing their baseline spike frequencies and the effect of the drug between mouse strains.

4.4.1c. GC axonal $GABA_AR$ likely don't contribute to EtOH's paradoxical actions

$GABA_ARs$ expressed on GC axons have been proposed to play an important role providing glutamatergic input to PCs (Pugh & Jahr, 2011; Pugh & Jahr, 2013). Unlike activation of $GABA_ARs$ at the synapse or neighboring extrasynaptic regions, activation of axonal $GABA_ARs$ is depolarizing due to a reduced chloride gradient in the axon (Pugh & Jahr, 2011; Pugh & Jahr,

2013). Whether EtOH interacts with these axonal $GABA_A$Rs is unclear, but could depend on unknown factors such as subunit composition and phosphorylation status.

While much of the emphasis of this dissertation has been on EtOH's actions at GC extrasynaptic $GABA_A$Rs, it may be proposed that GC axonal $GABA_A$Rs contributed to the paradoxical effects of EtOH on mossy fiber-evoked excitatory input to PCs. However, my data does not support this contention. In Chapter 2, I demonstrated that α6, δ-subunit containing $GABA_A$Rs were uniquely susceptible to direct postsynaptic EtOH action. Using immunohistochemistry, I confirmed receptor expression solely within the GC layer of the cerebellar cortex, suggesting that if a subset of these axonal $GABA_A$Rs is comprised of α6 and δ-subunits, then their expression is limited to the ascending axon within the GC layer. The spatial distribution of these axonal receptors is relevant because activation of rat axonal $GABA_A$Rs can cause antidromic spread of depolarization back towards the soma (Pugh & Jahr, 2011). Thus, if these axonal $GABA_A$Rs were present on the ascending axon, then their activation would have been detected in the voltage-clamp experiments described in Chapter 2, which represent the net effect of axonal and somatic $GABA_A$R-mediated currents. In these experiments, I detected similar magnitude responses to THIP and furosemide between strains, suggesting similar activation, or suppression, of $GABA_A$Rs containing α6 and δ-subunits. Similarly, the effect of EtOH on GC tonic $GABA_A$R inhibition represents the net consequences of EtOH actions on both axonal and somatic $GABA_A$Rs. The reduction in PC sEPSP and sEPSC frequency by 9mM EtOH in D2 mice and lack of effect in B6 mice is consistent with EtOH enhancement and suppression, respectively, of GC tonic $GABA_A$R inhibition detected at the soma. Thus, the net effects of EtOH on $GABA_A$R currents detected at the soma were preserved in GC output to PCs. It would then be unexpected that simulating mossy fibers would, through some unknown mechanism, differentially affect B6 and D2 mouse axonal receptors in a paradoxical manner to that predicted by the previous assessments in Chapter 2.

Nonetheless, identifying and characterizing the role of GC axonal $GABA_A$Rs in EtOH's actions would add important specificity to the description of EtOH's impacts on cerebellar $GABA_A$R inhibition described in this dissertation. It would first be important to identify the subunit composition of these axonal $GABA_A$Rs and their distribution along the ascending GC axon and parallel fibers using immunohistochemistry with confocal microscopy. Next, if these axonal receptors are expressed on the parallel fiber (as suggested by Pugh & Jahr, 2011), putative EtOH action on these axonal receptors could be assessed by measuring the amplitude of the parallel fiber evoked PC eEPSP following molecular layer pressure-injection administration of 9mM EtOH, a concentration that does not increase spontaneous molecular layer neuron activity, in the absence of afferent glutamatergic input, but was shown to directly inhibit α6, δ-subunit containing $GABA_A$Rs (Chapter 2; Kaplan et al., 2013).

4.4.2. Non-$GABA_A$R targets may have contributed to the effects of high EtOH concentrations

My initial hypothesis was that opposite actions of EtOH on GC tonic $GABA_A$R inhibition, detected at 9mM, 31mM, and 52mM EtOH in GCs from B6 and D2 mice (Chapter 2; Kaplan et al., 2013) would similarly contribute to opposite effects on excitatory input to PCs. However, GABAzine failed to block the reduction in PC sEPSP frequency by 31mM EtOH in B6 and D2 mice, and the opposite effects of EtOH on PC eEPSP amplitudes disappeared at 31mM and 52mM EtOH. Together, these findings expose contributions from EtOH's actions on non-$GABA_A$R targets known to be sensitive to higher concentrations of EtOH (Fig. 7). Importantly, these actions, many of which are discussed below, were only detected at EtOH concentrations at which both mouse strains show significant cerebellar impairment (Gallaher et al., 1996) and rarely obtain through voluntary EtOH consumption (for example, see Chapter 5). Therefore, it is consistent that strain differences in EtOH's effects on cerebellar processing disappear at EtOH concentrations at which behavioral differences are also absent.

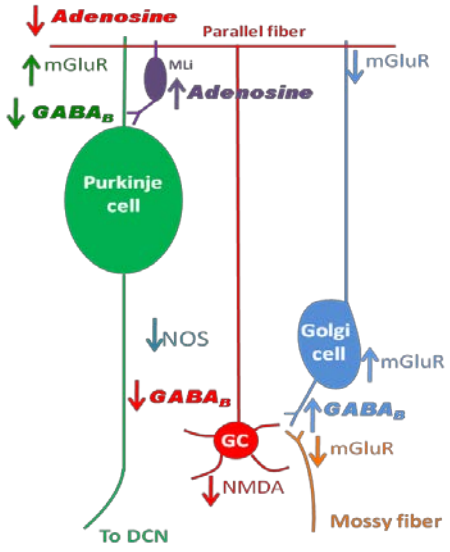

Figure 7. Schematic of the cerebellar circuitry illustrating proposed non-GABA$_A$R targets for EtOH impairment of cerebellar processing, described below. Neurotransmitters or receptors are color-coded with the neuron they're associated with. Targets in bold are enhanced by EtOH. The arrows indicate whether EtOH's effect on the particular target transiently leads to increased excitatory input to PCs (up arrow) or reduced excitatory input to PCs (down arrow) without taking into account additional sources of negative or positive feedback. MLi = molecular layer interneurons; GC = granule cell; DCN = deep cerebellar nuclei

4.4.2a. GABA$_B$R contribution

There is modest evidence that GABA$_B$Rs are directly activated by EtOH (Lewohl et al., 1999), but they have nonetheless been proposed to play an important role in modulating EtOH's effect on cerebellar processing dependent behaviors (Wiener & Valenzuela, 2006). GABA$_B$Rs are a distinct class of Gi/Go-coupled receptors that are expressed in the GC layer and highly expressed on PCs in the molecular layer (Bowery, et al., 1987). In the glomerulus, GABA$_B$Rs are expressed presynaptically on both Golgi cell (Kulik et al., 2002; Mapelli et al., 2009) and mossy fiber terminals (Mitchell & Silver, 2000; Thomsen et al., 2010), and postsynaptically on GC dendrites (Rossi et al., 2006). These respective receptors reduce GABA release from Golgi cell terminals, reduce glutamate release from mossy fiber terminals by attenuating presynaptic calcium signaling (Thomsen et al., 2010), and inhibit GCs by activating inwardly rectifying potassium channels (Lewohl et al., 1999). Since I've demonstrated that EtOH does not reduce GABAergic transmission to GCs, and GABAzine blocked EtOH's effect on the holding current (Chapter 2; Kaplan et al., 2013), EtOH's impact on GABA$_B$R signaling in the glomerulus is likely limited to reducing glutamate release from mossy fibers. Thus, moderate-high EtOH concentrations may be attenuating PC eEPSP amplitude by reducing glutamate release from mossy fiber terminals. To test the contribution that presynaptic GABA$_B$Rs expressed on mossy

fiber terminals play in the broad dampening of excitatory input to PCs by 31mM and 52mM EtOH, the same protocol described in this chapter could be implemented in transgenic mice with viral knockdown of $GABA_BRs$ in mossy fibers. If 31mM and 52mM EtOH had a reduced impact on PC eEPSP amplitude after viral knockdown of $GABA_BRs$, it would suggest that mossy fiber $GABA_BRs$ contributed to the reduction in mossy fiber-evoked excitatory input to PCs by moderate to high EtOH concentrations in wild type animals.

PCs also express $GABA_BRs$, which have an inhibitory effect and are important for inducing cerebellar long-term depression (LTD) of glutamatergic input from parallel fibers (Kamikubo et al., 2007). Ambient GABA that spills over from synaptic terminals of neighboring molecular layer interneurons activates these $GABA_BRs$. From this, one speculative explanation for the general reduction in evoked PC EPSP amplitude by 31mM and 52mM EtOH in both B6 and D2 mice is that enhanced stellate and basket cell activity, inferred from an increased PC sIPSC frequency, elevates ambient GABA concentrations and activates $GABA_BRs$. This would likely reduce EPSP amplitude by hyperpolarizing the membrane and reducing the input resistance of the PC, thereby tempering the impact of glutamatergic input. To test if elevated $GABA_BR$ activation by moderate-high EtOH concentrations is responsible for the general dampening of PC eEPSP amplitudes, the effect of EtOH could be measured in the presence of a $GABA_BR$ antagonist, CGP35348. A CGP35348 block of the EtOH (31mM and 52mM)-induced reduction in PC eEPSP amplitude would support a strong role for EtOH's effects on molecular layer interneurons and activation of PC $GABA_BRs$ in EtOH-induced dampening of glutamatergic input to PCs.

Intriguingly, EtOH only reduces PC AP frequency in a minority of cells (Lin et al., 1991), if at all (Mamelli et al., 2008), but $GABA_BRs$ may be important regulators of this sensitivity (Lin et al., 1991). In support of a $GABA_BR$ role in PC sensitivity to EtOH, the proportion of EtOH-sensitive PCs is reduced by CGP35348, and enhanced by the $GABA_BR$ agonist, baclofen (Yang et al., 2000). These findings suggest that $GABA_BR$ expression varies across PCs and is

important for EtOH dampening of PC excitability. Whether PC $GABA_BR$ expression differs between B6 and D2 mice is unknown. Although it's recognized that relative $GABA_BR$ subunit compositions could differ between strains, the overall $GABA_BR$ contribution could be determined electrophysiologically by recording from PCs in the presence of TTX and measuring baclofen current amplitudes. In these experiments, two important aspects would need to be considered. The first is the proportion of PCs that show responses to baclofen, and the second is the baclofen current amplitudes in the cells that do respond. Strain differences in either factor could explain the observation that, despite opposite effects of 31mM and 52mM EtOH on GC tonic $GABA_AR$ inhibition, they similarly reduced mossy fiber-evoked PC eEPSP amplitudes. If enhanced $GABA_BR$ activation is indeed responsible for the general dampening of excitatory input to PCs, then perhaps greater $GABA_BR$ expression in B6 mice explains why 31mM and 52mM EtOH reduced PC eEPSP amplitude, while enhanced $GABA_AR$ inhibition of D2 GCs underlies their reduction PC eEPSP amplitude.

4.4.2b. mGluRs

Metabotropic glutamate receptors (mGluRs) are found pre- and postsynaptically in the cerebellar cortex and can be inhibited by as low as 10mM EtOH (Carta et al., 2006), depending on the subtype (Netzeband & Gruol, 1995; Minami et al., 1998). Glutamate released from parallel fiber terminals activate mGluRs on Golgi cell dendrites and hyperpolarize the cell by enhancing an inwardly rectifying potassium current. Only 10% of Golgi cells are immunoreactive for the mGluR5 subtype in the rat cerebellum, while the remaining 90% of Golgi cells express the mGluR2 subtype (Neki et al., 1996). These subtypes are not directly sensitive to low concentrations of EtOH (Botta et al., 2004; Netzeband & Gruol, 1995; Minami et al., 1998), but could be affected by the higher 31mM and 52mM EtOH concentrations tested here in mice. Furthermore, EtOH suppression of Golgi cell dendritic mGluRs may have contributed to the sustained elevation of Golgi cell firing rates in regions expressing nNOS (Chapter 2; Botta et al.,

2004; Kaplan et al., 2013), and the consequential enhancement of GABAergic transmission to GCs, and general dampening of excitatory input to PCs.

In the cerebellum, the effect of mGluR2 activation has been more thoroughly characterized than activating mGluR5. Activation of mGluR2 receptors hyperpolarize the Golgi cell (Watanabe & Nakanishi, 2003) and play an important role in a transient suppression of Golgi cell activity from a high frequency train of GC glutamatergic input (Botta et al., 2004). In SDRs, this transient suppression of Golgi cell activity is reversed by 40mM EtOH, but not due to direct EtOH action on mGluR2s. Instead, high EtOH concentrations prevent the transient reduction in Golgi cell activity from high frequency glutamatergic input via a downstream mechanism. Thus, indirect effects of EtOH on mGluR2s expressed postsynaptically on Golgi cell dendrites lead to a net enhancement of GABAergic transmission to GCs via negative feedback from Golgi cells. Importantly, Botta et al. (2004) emphasized that higher EtOH concentrations, or genotypic variation, could render mGluR2s more sensitive to direct inhibition by EtOH. To test if mGluR2s have higher sensitivity to direct inhibition by low EtOH concentrations in mice, parallel fiber-evoked mGluR2 currents could be recorded in Golgi cells in the presence of AMPA/NMDA receptor antagonists (e.g. NBQX and APV). Reduced mGluR2 currents by EtOH would be interpreted as direct inhibition of the receptors that was blocked by the mGluR2 antagonist LY341495. In this Chapter, both 31mM and 52mM EtOH reduced mossy fiber-evoked PC eEPSP amplitude in B6 and D2 mice, despite opposite effects on GC tonic $GABA_AR$ inhibition (Chapter 2; Kaplan et al., 2013). To assess if this EtOH-induced dampening of excitatory transmission to PCs involves mGluR2s, the effect of EtOH could be tested in the presence of the mGluR2 antagonist, LY341495 (Botta et al., 2014). If the mGluR2 antagonist attenuated the EtOH-induced reduction in PC eEPSP amplitude, it would be interpreted that EtOH inhibition of mGluR2s contributes to the general dampening of mossy fiber-stimulated glutatmatergic input to PCs by moderate-high concentrations of EtOH.

Glutamatergic activation of mGluR2/3s on Golgi cell axon terminals (Ohishi et al., 1994) reduces GABA release during elevated mossy fiber activity (Mitchell & Silver, 2000). Spontaneous locomotor activity is more strongly suppressed by the mGluR2/3 agonist, LY379268, in EtOH-dependent rats than controls (Kufahl et al, 2011), but it remains unclear if cerebellar mGluR2/3s contribute to this effect and whether it's due to direct EtOH action on mGluR2/3s. To test the influence of EtOH on mGluR2/3-coupled signaling at presynaptic Golgi cell terminals, the effect of EtOH on GC sIPSCs could be measured following mossy fiber stimulation in the presence of AMPA/NMDA receptor antagonists, NBQX and APV, the $GABA_BR$ antagonist, CGP35348, and the NOS inhibitor L-NA. This cocktail of antagonists should eliminate any circuit and non-mGluR influences on Golgi cell activity. Any EtOH-induced change in GC sIPSC frequency or amplitude would be interpreted as EtOH action on presynaptic mGluR2/3 signaling. Enhanced GABA release from Golgi cell terminals resulting from direct presynaptic mGluR2/3 inhibition by 31mM and 52mM EtOH could partially explain the reduction in mossy fiber-evoked PC eEPSP amplitude in both B6 and D2 mice.

The mGluR1 subtype is expressed on both PC and GC dendrites (Carta et al., 2006; Masgrau et al., 2001). EtOH concentrations as low as 10mM reduce the mGluR1 component of the complex spike from climbing fiber input to PCs (Carta et al., 2006). However, 50mM, and not 17mM EtOH, blocked mGluR1-dependent LTD at the parallel fiber-PC synapse (Su et al, 2010), suggesting that EtOH sensitivity of mGluR1s may differ between synapses and also potentially across species. This EtOH inhibition of mGluR1 signaling may enhance glutamatergic transmission at the parallel fiber-PC synapse by blocking LTD formation. However, the stimulation protocol used in the current study was not an LTD-inducing protocol, and therefore, 31mM and 52mM EtOH did not likely affect long-term plasticity at the parallel fiber-PC synapse.

4.4.2c. NMDA receptors and calcium signaling

EtOH directly inhibits NMDA receptors throughout the brain (Kumari & Ticku, 2000). In cultured cerebellar GCs, NMDA receptors are inhibited by EtOH concentrations as low as 10mM in part due to reversible cytoskeletal rearrangement that impaired NMDA receptor function (Popp & Dertien, 2008). However, since GABAzine occluded the effect of 9mM EtOH on spontaneous glutmatergic input to PCs, it's unlikely that NMDARs played a role in EtOH's actions at this low concentration. Instead, inhibition of NMDA receptors expressed on GCs may be contributing to the reduction in mossy fiber-evoked PC eEPSP amplitudes by 31mM and 52mM EtOH in B6 and D2 mice. To empirically confirm that EtOH inhibition of GC NMDA receptors, independent of mGluR1s (EtOH inhibition of mGluR1s may also reduce NMDA currents [He et al., 2013; Skeberdis et al., 2001]), contributed to attenuated PC eEPSP amplitudes, the experiments could be repeated with sequentially added antagonists, starting with an mGluR1 antagonist, followed by the NMDA antagonist, APV. The effect of EtOH (31mM and 52mM) in the presence of each of the antagonists would be informative of the relative contributions that EtOH has on NMDA currents inhibited through G-protein coupled mGluR1s versus direct inhibition of NMDA receptors. The total NMDA contribution to an EtOH reduction in PC eEPSP amplitude could be determined by the difference between the EtOH reduction in PC eEPSP amplitude in the presence and absence of APV alone. Subsequently, the NMDA components can be compared between strains to assess if there are strain differences between B6 and D2 mice that could contribute to variation in their behavioral phenotypes.

In addition to EtOH suppression of calcium signaling by blocking NMDA channels, EtOH reduces calcium signaling by inhibiting voltage-gated calcium channels. Q-type calcium channels, expressed by cerebellar GCs (Walter & Messing, 1999), can be inhibited by EtOH at concentrations as low as 10mM in culture (Solem et al., 1997). The consequence of EtOH inhibition of voltage-gated calcium channels on GCs is not fully understood, but it could contribute to the general reduction in mossy fiber-evoked glutamate release from parallel fiber terminals by 31mM and 52mM EtOH in both B6 and D2 mice.

4.4.2d. Adenosine

Adenosine signaling in the cerebellar molecular layer has been proposed to be a target through which EtOH induces cerebellar impairment (Dar, 2000; Dar, 2006; Dar, 2015; Dunwiddie & Masino, 2001; Ruby et al., 2010). Adenosine receptors are expressed postsynaptically on PC and basket cell dendrites (Kocsis et al., 1984; Wall & Dale, 2007), and presynaptically on parallel fiber terminals (Rivkees et al., 1995; Wall & Dale, 2007). Adenosine receptors are coupled to G_i/G_o proteins and have an inhibitory effect by decreasing adenylyl cyclase and activating inwardly rectifying or ATP-sensitive potassium channels (Ebersolt et al., 1983; Heurteaux et al., 1995; Takigawa et al., 2002). Parallel fibers are the primary source of adenosine in the cerebellar cortex, which activate adenosine A1 receptors on PCs and basket cells (Kocsis et al., 1984; Wall & Dale, 2007) and exert feedback inhibition by activating adenosine A1 receptors on their own terminals to reduce glutamate release (Rivkees et al., 1995; Wall & Dale, 2007). EtOH, at concentrations as low as 5mM, enhances extracellular adenosine levels by inhibiting adenosine transporters (Ramadan et al., 2014), which in turn, contributes to cerebellar ataxia (Dar, 2015). Furthermore, EtOH block of adenosine transport accentuates the duration of inhibition at the parallel fiber-PC synapse by reducing glutamate release from the parallel fiber. Thus, increased adenosine signaling is an additional mechanism that may become engaged at moderate-high EtOH concentrations (31mM and 52mM), leading to dampened glutamatergic input to PCs and overriding the effect that differential EtOH action on GC tonic $GABA_AR$ inhibition has on mossy fiber-evoked PC eEPSPs. To assess how EtOH's effects on mossy fiber-evoked transmission to PCs are influenced by adenosine A1 receptor-mediated feedback inhibition on glutamate release from parallel fiber terminals, the effect of an adenosine A1 receptor antagonist can be tested on mossy fiber-evoked eESPCs in PCs voltage-clamped to the chloride reversal potential to eliminate molecular layer interneuron input on the holding current. If EtOH-enhancement of adenosine levels were indeed contributing to

the reduction in PC eEPSP amplitudes at 31mM and 52mM EtOH, then I would predict that the adenosine A1 receptor antagonist would reverse the EtOH-induced reduction in PC eEPSP amplitude.

Adensoine-induced reduction in GABA release from molecular layer interneurons may also contribute to ataxia (Dar, 2006). The high expression of adenosine A1 receptors on basket cells may be an influential target by which adenosine signaling contributes to cerebellar ataxia by reducing feedforward inhibition of PCs. However, previous studies (Hirono et al., 2009; Mameli et al., 2008; Wadleigh & Valenzuela, 2012) and the data presented here don't support this prediction. Instead, there's wide consensus that EtOH increases GABAergic transmission to PCs from molecular layer interneurons. However, activity-dependent enhancement in adenosine could lead to zones of reduced GABAergic transmission to PCs that's consistent with the spatial and temporal resolution of the parallel fiber activity profile (Wall et al., 2007). This is one such example in which non-$GABA_AR$ EtOH targets may interact with spatial factors to help explain the paradoxical reduction in GC spontaneous activity in D2 mice by 9mM EtOH, but enhancement of mossy fiber-evoked PC eEPSP amplitude. For instance, mossy fiber stimulation may have activated a population of GCs that contributed to a large degree of feedforward lateral inhibition of PCs under control conditions, but EtOH enhancement of adenosine A1 receptor activation (by increasing extracellular adenosine levels) could reduce GABA transmission to PCs, thereby enhancing mossy fiber-evoked PC eEPSP amplitude in some cases. Thus, experimental variation in the number of basket cells innervated by parallel fibers from activated GCs could contribute to variation across recordings. Furthermore, potential genotypic variation in adenosine A1 receptors could contribute to the differential effects of 9mM EtOH on mossy fiber evoked transmission to PCs. To test for genetic differences in adenosine signaling, parallel fiber stimulated glutamatergic input to PCs and molecular layer interneurons could be measured following adenosine transport block, and separately, with adenosine A1 receptor antagonists.

4.4.3. Molecular layer interneurons are not a source of differential 9mM EtOH responses

Here, I demonstrated that 9mM EtOH had no effect on the frequency of PC sIPSCs in the presence of kynurenic acid. These data suggest that GABAergic transmission from stellate and basket cells to PCs do not play a role in the behavioral differences detected at a low 9mM EtOH concentration at which D2 mice, but not B6 mice, show significant cerebellar ataxia (Gallaher et al., 1996). Instead, enhancement in PC sIPSC frequency by 31mM and 52mM EtOH was detected in D2 mice, but not B6 mice. Therefore, molecular layer interneurons may only contribute to behavioral intoxication seen at higher EtOH concentrations in D2 mice. Furthermore, the modest enhancement in sIPSC frequency by 31mM EtOH in D2 but not B6 mice is consistent with the relative insensitivity to EtOH modulation reported previously in SDRs (Hirono et al., 2009; Mameli et al., 2008). Since 31mM EtOH is the threshold at which B6 mice show significant cerebellar ataxia (Gallaher et al., 1996), it's unlikely that EtOH's actions on molecular layer interneurons contribute to this impairment, suggesting that they don't play a large role in behavioral phenotypes impacted by voluntarily consumed EtOH. However, it's interesting that 52mM EtOH enhanced PC sIPSC frequency to a much greater extent in D2 mice than B6 mice. The reason for this large enhancement in D2s but not B6s is unknown. One possibility is that, since EtOH-enhancement in PC sIPSC frequency is dependent on intracellular calcium stores, and not extracellular calcium concentration, there may be differences in intracellular calcium signaling between strains (Kelm et al, 2007). The behavioral relevance of this effect, though, is low, since D2 mice don't voluntarily reach such high BECs (i.e., 52mM EtOH is equivalent to a BEC of 245 mg/dl), and B6 mice do so only rarely (Finn et al., 2005; Finn et al., 2007; Chapter 5). Together these findings provide support that differential effects of EtOH on $GABA_AR$ inhibition of GCs more strongly contribute to the $GABA_AR$ sources

of genetic variation in EtOH-related behavioral phenotypes compared to $GABA_AR$ inhibition of PCs.

4.4.4. General conclusions

These data provide important insight into the consequence of a range of EtOH concentrations on excitatory and inhibitory transmission to PCs, and support the hypothesis that $GABA_AR$ inhibition of GCs is an influential target by which low EtOH concentrations may disrupt cerebellar processing. However, general inconsistency in responses to the various drugs tested, including EtOH, emphasizes the complexity of the circuitry's feedback and feed-forward mechanisms. Furthermore, the results expose a host of speculated, but unconfirmed non-$GABA_AR$ cerebellar EtOH targets that contribute to intoxication. Understanding the particular contributions of various EtOH targets in behavioral intoxication is an important area of future investigation.

Chapter 5: Microinjection of THIP into the cerebellar cortex reduces EtOH consumption in C57BL/6J mice

This chapter has been reformatted for inclusion in this dissertation from:

Kaplan, J.S., Nipper, M., Jensen, J., Rossi, D.J., & Finn, D.A. (*in preparation*). Microinjection of THIP into the cerebellar cortex reduces EtOH consumption in C57BL/6J mice.

5.1. Introduction

Chapters 2 and 3 described a novel EtOH-induced suppression of GC tonic $GABA_AR$ inhibition in high-EtOH consuming mammalian genotypes. Chapter 4 demonstrated that this EtOH-induced suppression of GC tonic $GABA_AR$ inhibition differentially regulates spontaneous and evoked excitatory transmission through the cerebellar cortex compared to EtOH enhancement in GC tonic $GABA_AR$ inhibition. However, it's unknown if EtOH suppression of GC tonic $GABA_AR$ inhibition contributes to an elevated EtOH consumption phenotype. Identifying a cerebellar $GABA_AR$ contribution to EtOH intake would reveal a new frontier for developing AUD treatments.

The GABA system has long been recognized as a promising pharmacological target to combat AUDs. Systemic administration of THIP, which is a selective agonist for δ-subunit containing $GABA_ARs$, strongly reduces EtOH intake in mice (Moore et al., 2007; Ramaker et al., 2011, 2012). However, the target brain regions that mediate this effect have not been fully established. Ramaker et al. (2014b) demonstrated that microinfusion of THIP into the nucleus accumbens shell attenuates EtOH consumption, while infusion into the infralimbic cortex enhances EtOH intake (Fritz & Boehm, 2014). Whether THIP's action within the cerebellum contributes to the large reduction in EtOH consumption from systemic administration is unknown.

The B6 mouse is a useful model to investigate the cerebellar contribution to EtOH intake due to their well-characterized high-EtOH consumption phenotype (Yoneyama et al., 2008) and propensity to achieve BEC levels above binge criteria (.8mg/ml or 17mM) during a two-hour limited EtOH access procedure (e.g., Ramaker et al., 2012; Ramaker et al., 2014b; Rhodes et el., 2005). Additionally, their relative insensitivity to EtOH-induced cerebellar ataxia (Gallaher et al., 1996) makes them a valid model of the cerebellar LLR phenotype. It's unclear how the cerebellar LLR phenotype modulates EtOH drinking behavior. Despite its association with increased heritable risk for developing an AUD (Schuckit, 1985; Schuckit, 1994), it's unknown

whether the cerebellar LLR phenotype directly enhances EtOH consumption or if it's merely a parallel phenotype with an negligeable impact on drinking behavior.

The experiments described in Chapters 2-4 propose that EtOH suppression of GC tonic $GABA_AR$ inhibition is a neural substrate of the cerebellar LLR phenotype. If suppression of GC tonic $GABA_AR$ inhibition were indeed contributing to the high EtOH consumption component of the LLR phenotype, then reversing it (i.e., enhancing tonic $GABA_AR$ inhibition) would be predicted to reduce EtOH intake. To investigate the contribution of GC tonic $GABA_AR$ inhibition on EtOH consumption, I measured the effect of cerebellar-specific THIP infusion on 10% EtOH consumption during a 2-hour limited access drinking paradigm. Consistent with the predictions made by the cerebellar LLR phenotype, I predicted that THIP would reduce EtOH intake.

5.2. Methods

5.2.1 Animals

Male B6 mice, approximately 9 weeks of age at the beginning of experiments, were purchased from Jackson Laboratory-West (Sacramento, CA). Mice were maintained on a 12-h reverse light-dark cycle (lights off at 0700), and all experiments were conducted during the dark cycle. Throughout the EtOH drinking study, mice were individually housed. Surgical and behavioral procedures were designed to minimize animal suffering and to reduce the number of mice used and in accordance with NIH guidelines. All procedures were approved by the Institutional Animal Care and Use Committee at the Portland VA Healthcare System.

5.2.2. Drugs

EtOH (200 proof; Pharmco Products, Brookfield, CT) was diluted in tap water to yield a 10% (*v/v*) EtOH solution. THIP (4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol hydrochloride) was purchased from Tocris Bioscience (Ellisville, MO) and dissolved in aCSF comprised of:

124mM NaCl, 26mM $NaHCO_3$, 1mM NaH_2P0_4, 2.5mM $CaCl_2$, 2mM $MgCl_2$, 10mM glucose, 2.5mM KCl.

5.2.3. 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 of the cerebellar cortex [A/P: -7.2, L/M: ±0.1, D/V: -0.8 mm (-1.8 mm with injectors)]. Mice were administered a single I.P. injection of ketoprofen (3mg/kg; Abbott Laboratories, North Chicago, IL) for post-operative analgesia. They were given a minimum of one week to recover before testing.

5.2.4. General drinking experiments

Both Experiments 1 and 2 were performed in the dark phase of the light/dark cycle to measure high levels of EtOH consumption (Rhodes et al., 2005). Beginning 3 hours into the dark cycle, mice had 2-hour access to a 25ml bottle containing a 10% EtOH solution and one 25ml bottle containing tap water; bottle positions were counterbalanced between the left and right side across subjects. At the conclusion of the 2-hour period, water and EtOH consumption was 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 mock-infusions where the injector was lowered through the cannula but no fluid was administered. Subsequent drug infusions were given five minutes prior to the EtOH access session in 200nl of solution delivered over one minute, with an additional 30 seconds allowed for diffusion. Infusions were spaced a minimum of three days apart to ensure that EtOH intake had returned to baseline for at least two days before subsequent infusions.

Experiment 1:

The purpose of Experiment 1 was to test whether microinjection of THIP into the cerebellar cortex impacted EtOH intake without reducing general consummatory behavior. Mice were given EtOH access 5 days/week (M-F) for 15 days prior to surgery. Following surgery, their drinking behavior stabilized (defined as <10% variation for three days) by the 7^{th} day of

EtOH access. Mice were administered an infusion of vehicle first followed by an infusion of THIP (500ng) three days later. Orbital blood samples were collected immediately following the 2-hour EtOH access period on the infusion days. After the THIP infusion and EtOH intake returned to baseline levels, mice were only given access to water during the 2-hour drinking session. After 5 days, their water intake had stabilized and they were again administered vehicle and then, three days later, THIP (500ng).

Experiment 2:

The purpose of Experiment 2 was to replicate THIP's effect on EtOH intake and to determine its dose-response properties. For this study, mice underwent surgery prior to receiving EtOH access, and EtOH intake was measured 7-days/week. Following drinking stabilization, mice received infusions of drug in the following order: Vehicle, 500ng THIP, 50ng THIP, 100ng THIP, 250ng THIP, and vehicle. Pre- and post-vehicle infusions were used to determine whether mechanical or psychological stress of the infusions altered EtOH intake differently after multiple infusions.

5.2.5. BECs

Immediately following the 2-hour EtOH access sessions on the vehicle and THIP infusion days in Experiment 1, blood samples (20µl) 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 EtOH concentrations (from 0.5 to 3.0mg/ml).

5.2.6. Histological confirmation

At the conclusion of studies, mice were euthanized 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 Imaging Solutions Ltd,

Buffalo Grove, IL). The morphology of the cerebellar cortex facilitated diffusion of drug into lobe VI in some animals. Because the overall aim of these experiments was to assess the impact of intra-cerebellar THIP on EtOH intake, and since there was no statistically significant difference between the subjects' drinking behavior where THIP diffused to lobe VI ($P < .05$), data from these subjects were included in the analysis.

5.2.7. Statistical analysis

All data are expressed as mean ± S.E.M. Repeated measures ANOVA was used to detect significant main effects, and paired *t* tests were used for comparisons between drug and vehicle when appropriate. Correlations between BEC and EtOH intake were assessed using a Pearson Correlation. In all cases, statistical comparisons were two-tailed and the significance threshold was set at $P < 0.05$. No statistical methods were used to pre-determine sample sizes, but sample sizes are similar to those recently reported following bilateral microinjection of THIP into the nucleus accumbens shell in B6 mice (Ramaker et al., 2014b).

5.3. Results

5.3.1a. Experiment 1: THIP microinfusion into the cerebellar cortex reduces EtOH intake

After completing experiments, cannulae placements into lobe IV/V/VI of the cerebellar cortex were correctly confirmed by histological and methylene blue confirmation (14mM diluted in aCSF) in 17 out of the original 23 animals (Fig. 1). Only data from subjects with confirmed placements were included in the analysis.

EtOH intake stabilized within three weeks from cannulation surgery (mean EtOH consumption = 2.78 ± .12g/kg, $n = 17$; data not shown). Following the stabilization period, subjects were microinfused with vehicle (aCSF) five minutes prior to their 2-hour EtOH access. Vehicle infusion did not affect EtOH intake (consumption = 2.62 ± .22, $n = 17$, $P > .05$, paired t-test, Fig 2). Analysis of orbital blood samples collected immediately following EtOH access revealed a significant positive correlation between EtOH intake and BEC ($r = .75$, $P < .001$,

Pearson correlation). This suggests that the high EtOH volumes consumed correspond to elevated BEC levels. However, since the high rate of EtOH metabolism in mice eliminated much of the EtOH consumed during the front-end of the EtOH-access period, the detected BECs (Mean BEC: .64 ± .08mg/ml, *n* = 17; Fig. 2) are likely an underestimate of the peak BECs obtained during the two-hour session.

To assess if tonic $GABA_AR$-mediated inhibition of cerebellar GCs can modify EtOH intake, I microinjected the δ- subunit selective $GABA_AR$ agonist, THIP (500ng), and measured EtOH consumption during the 2-hour access session. Consistent with our hypothesis, THIP attenuated EtOH consumption compared to vehicle by 26% ($t(16) = 2.47$, $P = .025$, paired *t* test; *n* =17; Fig 2) and reduced BEC levels by 34% ($t(16) = 2.66$, $P = .02$, paired *t* test;, *n* = 17). The correlation between EtOH intake and BEC levels remained significantly positively correlated in the presence of THIP, suggesting that THIP did not alter EtOH metabolism ($r = .54$, $P = .02$, $n = 17$, Pearson correlation, Fig 2A). The correlation between EtOH intake and BEC on the combined data also was significant ($r = .71$, $P < .001$, $n = 34$, Pearson correlation). THIP had no effect on the preference ratio (Supplementary Table 1), which reflected consistently low water volumes consumed during the EtOH access period. Together, these data suggest that enhancing

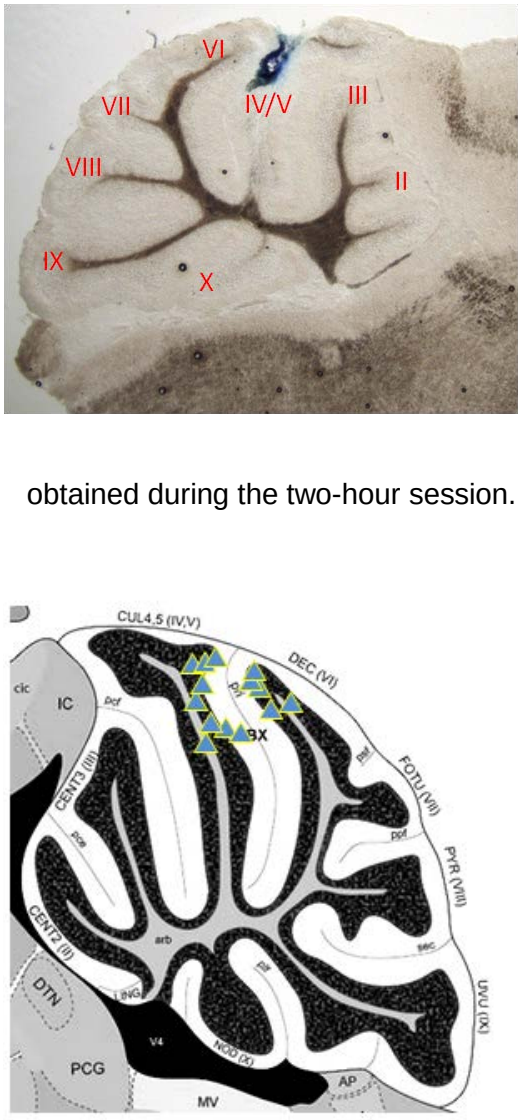

Figure 1. Cannula placement assessment. **A.** A representative sagital cerebellar section showing cannula placement in lobe IV/V of the cerebellar cortex. Following experiments, methylene blue (blue dye) was injected in the cannula for assessment of cannula placement. **B.** Sagittal reference image of the mouse cerebellum (0.225 lateral) taken from the Allen Brain Atlas. Injection sites are shown in blue triangles.

GABAergic inhibition of cerebellar GCs following a unilateral microinjection of THIP into layers IV/V/VI of the cerebellar cortex can reduce EtOH intake.

5.3.1b. Experiment 1: THIP does not affect water consumption

One possible explanation for the THIP-induced reduction in EtOH consumption is that it strongly impairs motor behavior thereby inhibiting the ability to consume EtOH. Another explanation is that THIP attenuates general consummatory behavior. To address these two explanations, mice were switched to consumption of water only for five days, and water consumption was measured during this two-hour period. When two water bottles were present, water consumption increased significantly, when compared to when EtOH was present in one bottle (water intake when given choice with EtOH: 0.07 ± .03ml; water intake without EtOH option: 0.65 ± .06ml, $t(16) = 8.71$, $P < .001$, $n = 17$). Microinfusion of

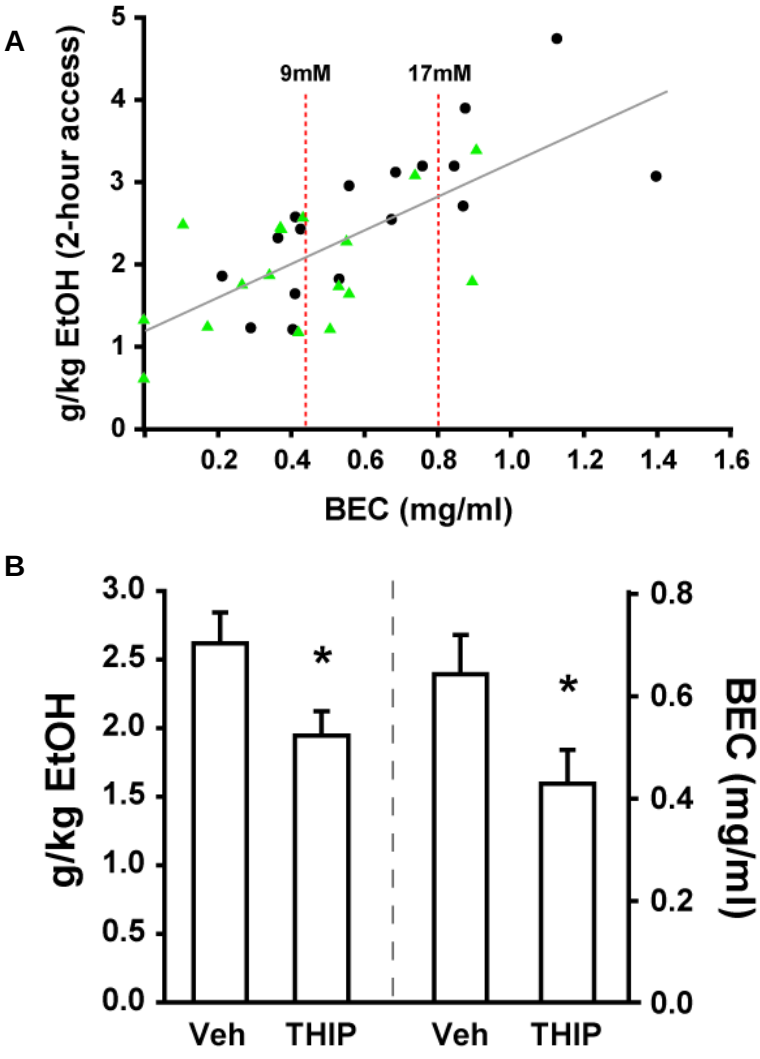

Figure 2. THIP reduces EtOH intake and BEC levels. **A.** A scatterplot showing that EtOH consumption during the 2-hour access period (g/kg) was strongly positively correlated with BEC levels after vehicle injection (black circles; $r = .75$; $P < .001$, $n = 17$), in the presence of THIP (500ng; green triangles, $r = .54$; $P = .02$, $n = 17$), and when combined ($r = .71$; $P < .001$, $n = 34$). Binge criteria (.8mg/ml or 17mM) and 9mM EtOH (for reference with *in vitro* experiments described in Chapters 2 and 4) are indicated with red dotted lines. **B.** Summary bar chart depicting that unilateral microinjection of THIP (500ng) into the cerebellar cortex attenuated EtOH intake (g/kg) and BEC levels (mg/ml). * indicate significant reductions by THIP compared to vehicle (all $P < .05$).

THIP (500ng) failed to alter water consumption, compared to vehicle infusion, during the two-hour period (P = .80, n = 17; THIP: 0.67 ± .08ml; Fig 3). These findings provide indirect evidence that cerebellar THIP infusion reduced EtOH consumption independent of its impact on motor function or general consummatory behavior.

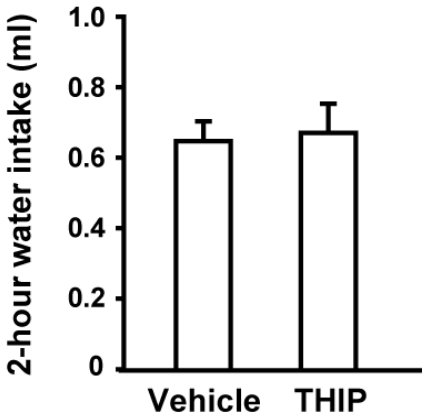

Figure 3. THIP does not affect water consumption. Summary plot showing that THIP (500ng) did not alter water consumption during the 2-hour recording period (P = .80, n = 17).

5.3.2a. Experiment 2: THIP dose-dependently reduces EtOH intake

The purpose of Experiment 2 was to replicate the effects of 500ng THIP and to reveal the threshold THIP concentration that reduced EtOH intake. Experiment 2 was similar to Experiment 1 except that animals had 7 days/week access to EtOH (instead of just 5 days/week) and included a THIP dose-response curve (vehicle, 50ng, 100ng, 250ng, and 500ng). Unfortunately, the longer experimental protocol was associated with an increased number of clogged cannulae or loose head mounts. Consequently, cannula placements were only confirmed in 12 out of 24 animals. Resulting from the coinciding reduction in statistical power, a repeated measures ANOVA only found a strong trend for a main effect of dose (F(4,11) = 2.49, P = .057; Fig 4). However, based on our a priori prediction that THIP would decrease EtOH intake and the results of Experiment 1, planned comparisons were conducted where each individual THIP dose was treated as an independent planned contrast against vehicle. Using planned contrasts, both the 250ng (n = 13) and 500ng THIP (n = 13) doses significantly reduced EtOH intake compared to vehicle (all P < .05; vehicle: 2.28 ± .14g/kg;

250ng THIP: ↓23%; 500ng THIP: ↓26%), while 100ng THIP differed from vehicle at trend levels (P = .077, n = 14; 100ng THIP: ↓15%) and 50ng THIP did not significantly reduce EtOH consumption (P = .14, n = 14; 50ng THIP: ↓12%). At the conclusion of the dose-response assessment, vehicle was tested for a second time on EtOH consumption. EtOH intake from the second vehicle infusion did not statistically differ from the first vehicle infusion (1.95 ± .35g/kg, P > .05, n = 12), confirming that the THIP-induced reduction in EtOH intake was not a consequence of a general attenuation in EtOH intake over the course of the experiment. Together, these findings confirm the conclusion from Experiment 1: enhancing GC GABAergic inhibition in only a small proportion of cerebellar neurons produces a 26% reduction EtOH drinking behavior.

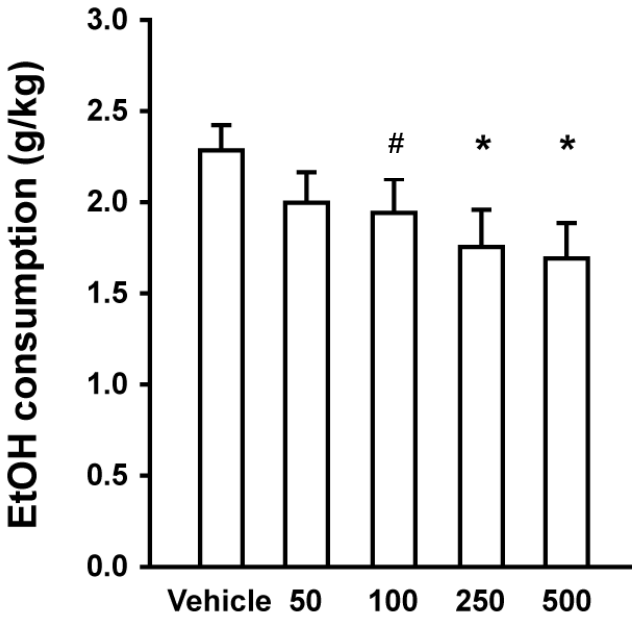

Figure 4. THIP dose-dependently attenuated EtOH consumption**.** Summary plot showing the effect of unilateral microinjection of vehicle (aCSF) and four THIP doses (ng) on EtOH consumption (g/kg). * indicates that both 250ng and 500ng THIP doses significantly reduced EtOH consumption compared to vehicle (all P < .05) while 100ng THIP reduced EtOH consumption at trend levels (# indicates P = .08). 50ng THIP: n = 14; 100ng THIP: n =14; 250ng THIP: n = 13; 500ng THIP: n =13.

5.4. Discussion

This is the first study to directly demonstrate a reduction in EtOH intake by selective, non-motor impairing, modulation of cerebellar processing. Specifically, the reduction in EtOH consumption by THIP suggests that enhancing GABAergic inhibition of cerebellar GCs, in only a small proportion of the cerebellar cortex, is sufficient to reduce EtOH intake. Furthermore, the fact that THIP similarly reduced EtOH intake regardless of whether the mice had 5 day/week or 7 day/week EtOH access increases the relevance of these findings, since problem EtOH use is

not always characterized by daily consumption (Knight et al., 2002). These findings are consistent with my hypothesis that the EtOH-induced suppression of GC tonic $GABA_AR$ inhibition contributes to the B6 mouse's high EtOH consumption phenotype and provide strong evidence for a cerebellar role in modifying EtOH drinking behavior independent of motor impairment or sedation. Together with the comprehensive electrophysiology assessment of EtOH's actions on $GABA_AR$ inhibition, these behavioral observations endorse the cerebellum as a novel target for pharmacological treatment and prevention of AUDs.

5.4.1. A cerebellar $GABA_AR$ contribution to EtOH consumption

The cerebellum has been proposed to play a modifying role in reward-based learning (Thoma et al., 2008) and drug addiction (Moulton et al., 2014), but has experimentally been ignored as a brain region that could modify EtOH intake. Recent anatomical and functional connectivity studies have identified cerebellar abnormalities in FH+ individuals with increased risk for developing an AUD (Benegal, et al. 2007; Cservenka et al., 2014; Herting et al., 2011; Hill et al., 2011). These abnormalities may also reflect differing sensitivity to acute EtOH characterized by the heritable cerebellar LLR phenotype (Schuckit, 1985; Schuckit, 1994), which also is associated with increased AUD risk. However, it is unclear how the cerebellar LLR contributes to enhanced EtOH intake.

There are at least two non-exclusive possibilities by which the cerebellar LLR may enhance EtOH consumption: 1) Impairment of cerebellar functioning (e.g. ataxia) is aversive and therefore, acts as a "shut-off switch" for subsequent consumption; 2) cerebellar output can modify reward processing, and the cerebellar LLR reflects a higher threshold before reward detection becomes impaired. These two possibilities, although not exclusive, describe distinguishable cerebellar contributions to EtOH drinking behavior. The first is dependent on the manifestation of ataxia, a behavior that increases the difficulty to obtain EtOH and thereby sufficiently increases the cost-benefit ratio to impede drinking, while the second is dependent on

the animal's ability to detect a range of reward magnitudes that can be modulated by cerebellar output. The THIP reduction in EtOH intake without affecting water consumption argues in favor of the second possibility: ataxia does not have to manifest in order for EtOH intake to cease. Importantly, it suggests that the link between the behavioral cerebellar LLR and risk for developing an AUD (Schuckit, 1985; Schuckit, 1994) may be the sensitivity to impaired cerebellar functioning that, in turn, disrupts reward processing or executive function relating to seeking and consuming a reward.

At this point, it remains unknown how disrupted cerebellar functioning influences reward processing. However, the cerebellum may achieve this function in a similar manner by which it shapes motor behavior, as a timing device that constructs and compares internal models of the actual motor performance with the expected (Allen & Courchesne, 2003; Ito, 2005; Ito, 2008). For motor control, feedback from the cerebellum to the motor cortices regarding the discrepancy, or error, between internal models enables appropriate correction in movement. It's been proposed that the cerebellum develops similar internal models for cognitive processing (Ito, 2008). Cognitive processing, like motor control, requires the proper alignment and execution of a series of time-dependent steps. The cerebellum provides a temporal metronome for step integration (Ivry et al., 1988) and develops a prediction of the appropriate cognitive action that is compared against the actual cognitive integration (Ito, 2005). Breakdown in this system may lead to cerebellar-dependent impairments in cognitive and executive function described in patients with schizophrenia (Stephan et al., 2001), ADHD (Mackie et al., 2007; Tavano et al., 2007), and autism (Riva & Giogi, 2000).

Functional imaging has revealed distinct cerebellar regions with strong functional connectivity to specific prefrontal cortical regions (Krienen & Buckner, 2009), which in conjunction with disynaptic connections to the ventral tegmental area (Ikai et al., 1992; Ikai et al., 1994), forebrain (Albert et al., 1985), and striatum (Albert et al., 1985; Delis et al., 2013), could enable a cerebellar contribution in the processing and assessment of reward-related

behaviors by similarly establishing and comparing internal models related to elements of reward. One such feature for cerebellar modification is the motivation to behaviorally approach reward-related stimuli, which is determined by an assessment of the reward conveyed to the prefrontal cortex by the striatum (Ernst & Fudge, 2009). Electrical stimulation of the cerebellar vermis causes a reduction in dopamine levels in the nucleus accumbens (Albert et al., 1985), and thus, could influence reward processing at the level of the striatum, by influencing elements of reward detection (e.g. salience), and the assessment by the prefrontal cortex, by failing to provide the temporal and predictive elements for appropriate integration of cognitive steps in decision making of reward seeking. This is intriguing in light of the inverse correlation between stimulated dopamine release in the ventral striatum and EtOH preference within the B6 mouse strain (Ramachandra et al., 2007), which, together with previous work, proposes that cerebellar output could alter dopamine levels in the reward circuitry and influence EtOH preference. Whether EtOH's effect on GC tonic $GABA_AR$ inhibition varies across the range of EtOH consumption levels within the B6 mouse strain, and if this suppression of GC tonic $GABA_AR$ inhibition impacts dopamine levels in the ventral striatum should be areas of future investigation.

5.4.2. THIP reduced EtOH intake independent of motor effects

Systemic THIP injections reduce EtOH intake (Moore et al., 2007; Ramaker et al., 2011, 2012) partially through direct actions in the nucleus accumbens shell (Ramaker et al., 2014b). Here, I demonstrate that THIP's actions in the cerebellum similarly reduce EtOH consumption, mimicking THIP's effect in a well-established region of the reward circuitry. Since there was no effect of THIP on water intake during the two-hour period where animals had previously been given access to EtOH, this reduction in EtOH consumption was independent of impairment to motor behavior and general consummatory behavior. This conclusion, however, does not preclude the possibility of THIP-induced impairment of microstructural motor control according to the topographical distribution of the cerebellar cortex (Lu et al., 2007). Pharmacological

manipulation of lobe IV/V attenuated EtOH-induced ataxia (Al-Ragaie & Dar, 2006a; Al-Ragaie & Dar, 2006b), suggesting that the regions manipulated in the current study are involved in motor behavior. While THIP may be inducing minor motor impairment, its effect is not significant enough to alter water consumption, and thus, would not contribute to a reduction in EtOH consumption.

An alternative explanation is that THIP could increase behaviors that compete with EtOH consumption. For instance, Ramaker et al. (2014b) observed an enhancement in locomotor activity from microinfusion of THIP into the nucleus accumbens shell. While a reduction in EtOH intake due to behavioral stimulation cannot be ruled out, there is no evidence that cerebellar modulation induces locomotor stimulation, while the nucleus accumbens is a critical brain region underlying locomotor stimulation (Sprow et al., 2014). Additionally, systemic THIP administration caused a slight increase in locomotor stimulation but failed to increase in the number of lever presses made on the previously inactive lever during operant EtOH reinstatement (Ramaker et al., 2014a), suggesting that animals were still able to discriminate and perform behaviors that previously led to reward delivery. Therefore, it's unlikely that the THIP-induced reduction in EtOH consumption resulted from an enhancement in competing behaviors.

Another alternative explanation is that the effects of THIP in lobes IV/V/VI integrate with the impairing effects of consumed EtOH in the other cerebellar lobules to induce ataxia. However, even in the absence of THIP, mice only obtained BEC levels around the threshold of meeting binge criteria (~.8mg/ml), but this level is still lower than the BEC threshold at which B6 mice show significant impairment of rotarod performance, a measure of cerebellar ataxia (~1.45mg/ml; Gallaher et al., 1996). After THIP injection, mice only reached BECs of ~.42mg/ml, well below the threshold to induce significant cerebellar ataxia. This BEC corresponds to approximately 9mM, which is the concentration at which I demonstrated in Chapter 4 that EtOH's influence on $GABA_AR$ signaling in the cerebellum and excitatory input to PCs is limited to GC tonic $GABA_AR$ inhibition, thereby largely limiting EtOH's actions to the same δ-subunit

containing $GABA_A$Rs that are enhanced by THIP. So despite their high EtOH consumption phenotype, on average B6 mice are not achieving BEC levels associated with significant cerebellar ataxia, arguing against a contribution of EtOH impairment in other cerebellar lobules to an ataxic response.

5.4.3. Site-specific GABAergic inhibition of GCs reduces EtOH intake

One of the most fascinating conclusions is that enhancing GABAergic inhibition of only a small subset of cerebellar GCs is sufficient to reduce EtOH intake. In the current experiments, only lobes IV/V/VI were targeted. These lobes were selected for two reasons. First, surgical implantation of cannulae prevented implantation into lobes VII-X without largely disrupting the cerebellar cortex along the way. Also, because of the animal's normal body movements and flexion point near the caudal end of the cerebellum, the more caudal cannulae were at greater risk of dislodging during the multi-week experimental protocol. Thus, choices for cannulae implantations were limited to the rostrally oriented lobules. The second reason for selecting these lobes came from previous cerebellar microinjection experiments by Al-Rejaie & Dar (2006a; 2006b), who successfully attenuated EtOH-induced ataxia through pharmacological modulation of lobes IV/V. Therefore, a primary reason that we targeted this cerebellar region was because, through its modulation, others have attenuated EtOH-induced behavioral impairment. While this study strongly supports further investigation into a cerebellar-based approach for pharmacological treatment to combat AUDs, targeting treatment to α6,δ-subunit containing $GABA_A$Rs in lobes IV/V presents a difficult challenge as these receptors are distributed throughout the cerebellar cortex. The treatment efficacy of a selective agonist for the α6,δ-$GABA_A$Rs that globally targets the cerebellum still needs experimental verification (see below).

5.4.4. Limitation

One primary limitation of the microinjection protocol is the inability to globally target extrasynaptic $GABA_A$Rs across the entire cerebellar cortex. The proportion of GCs affected by THIP within the cannula-targeted lobe is limited to the diffusion volume of the drug, which must be restricted to maintain pharmacological selectivity. THIP is a selective agonist for extrasynaptic δ-subunit containing $GABA_A$Rs at concentrations up to 1μM (Meera et al., 2011), and therefore enhances GC tonic $GABA_A$R without influencing phasic inhibition of GCs mediated by synaptic $GABA_A$Rs. However, the minimal dose of THIP found to attenuate EtOH consumption was 250ng, similar to the previously reported THIP dose thresholds in the nucleus accumbens shell (Ramaker et al., 2014b). 250ng, when administered in 200nL aCSF (see Methods), corresponds to a concentration of 7.08mM at its most concentrated point at the tip of the cannula. Consequently, the effect of THIP may range from activation of both synaptic and extrasynaptic $GABA_A$Rs at its most concentrated point, to selective enhancement of extrasynaptic $GABA_A$R-mediated currents within the penumbra. Maguire et al. (2014) found that the behavioral consequences of a 100ng (or 3mM) THIP dose injected into the nucleus accumbens core of wild-type mice were absent in α4-subunit knockouts, suggesting that the effect of a 100ng THIP dose was the result of THIP's action on δ-subunit containing extrasynaptic $GABA_A$Rs that mediate tonic inhibition. However, the selectivity of 250ng (7mM) and 500ng (14mM) THIP, doses that statistically reduced EtOH intake, for extrasynaptic $GABA_A$Rs is unclear. The inability to maintain consistent THIP concentrations and detect their range across all of the GCs is a limitation of microinjection experiments. To eliminate problems associated with a drug diffusion concentration gradient while maintaining lobule specificity, future studies should seek to utilize optogenetic technology, which would enable specific and consistent modulation of GC tonic $GABA_A$R currents. To test the effect of modulating GCs in the entire cerebellar cortex, designer receptors exclusively activated by designer drugs (DREADDs) would enable specific modulation of GC inhibition simultaneously in all cerebellar lobes. These

tools would also enable assessment of the impact that selectively suppressing GC tonic $GABA_AR$ inhibition would have on EtOH intake in D2 mice. Currently, the only pharmacological agent that selectively blocks α6,δ-subunit containing $GABA_ARs$ is the α6-subunit containing $GABA_AR$ antagonist, furosemide (Korpi et al., 1995). However, furosemide also blocks synaptically expressed α6-subunit containing $GABA_ARs$ as well as chloride transport in non-α6-subunit containing $GABA_ARs$ (Misgeld et al., 1986), which severely limits the interpretation of behavioral outcomes.

5.4.5. Conclusions

GC tonic $GABA_AR$ inhibition is a highly sensitive target for modulation by EtOH. Here, I've established that enhancing GC $GABA_AR$ inhibition can reduce EtOH intake in a high consuming mouse strain, representing the first demonstration that $GABA_ARs$ in lobes IV-VI of the cerebellum can regulate ethanol intake. Since α6,δ-subunit containing $GABA_ARs$ that mediate the tonic current are relatively confined to cerebellar GCs, GC tonic $GABA_AR$ inhibition is a promising and feasible target for pharmacologically combating problem EtOH use.

The THIP-induced reduction in EtOH intake confirms hypotheses derived from extensive assessment of genetically regulated EtOH influences on the cerebellar circuitry. Variance in EtOH sensitivity at the cellular level contributes to a range in EtOH consumption phenotypes, which I demonstrate can be pharmacologically manipulated. This is the first evidence for such cerebellar manipulation in affecting EtOH consumption, but it exposes likely benefit that further investigation of the cerebellum in reward processing may have in directing treatment and our understanding of reward networks.

Supplementary Table 1. THIP had no effect on EtOH preference ratio. Preference ratios represent the amount of a 10% EtOH consumed as a proportion of the total fluid consumption during the 2-hour EtOH access period (Preference ratio = ml EtOH/[ml EtOH + ml water]). There were no significant differences between the preference ratios in any of the conditions (all $P > .05$).

Preference Ratio

	Vehicle	50ng THIP	100ng THIP	250ng THIP	500ng THIP
Experiment 1	.94 ± .02	-	-	-	.93 ± .04
Experiment 2	.82 ± .05	.86 ± .05	.91 ± .04	.91 ± .04	.92 ± .04

Chapter 6: General Discussion

Our understanding of the role of the cerebellum has traditionally been limited to motor control, but evidence generated over the last few decades supports a broader cerebellar role in cognitive functioning and reward processing (Moulton et al., 2014; Schmahmann, 1991; Stoodley et al., 2012). Of particular relevance to my thesis, sensitivity to cerebellar impairment from acute EtOH exposure is a heritable behavioral phenotype that is associated with risk for developing an AUD in humans and elevated EtOH consumption in rodents (Gallaher et al., 1996; Schuckit, 1985; Schuckit, 1994). My research addressed the general hypothesis that genetically regulated neural substrates that contribute to EtOH impairment of cerebellar processing impact EtOH intake. Using slice electrophysiology in behaviorally well-characterized animal models, these experiments expose heritable contributions to EtOH's impact on cerebellar processing that vary as a function of EtOH-consumption phenotype. Furthermore, they are the first experiments to directly test the effect that modulation of cerebellar signal processing has on EtOH consumption, without the influence of substantial motor impairment. The overall findings of this dissertation identify and define the interaction between genetically regulated factors that are affected by low-EtOH concentrations and the consequent impact on cerebellar processing and its behavioral consequences. Together, they identify a significant cerebellar contribution to EtOH intake.

6.1. Summary of findings

GC tonic $GABA_AR$ inhibition is a sensitive target for modulation by low EtOH concentrations, but the specific impact varies considerably across mammalian genotypes, ranging from strong enhancement in low EtOH-consuming genotypes to suppression in high EtOH-consuming genotypes (Chapters 2 and 3). EtOH's overall effect on GC tonic $GABA_AR$ inhibition is determined by the relative expression of two genetically determined factors: nNOS expression in the GC layer determines the magnitude by which EtOH enhances GABAergic

transmission from Golgi cells to GCs, and low PKC activity in GCs enables EtOH to directly inhibit extrasynaptic $GABA_A$Rs that mediate the tonic current (Chapter 2). Low EtOH-consuming genotypes have greater GC layer nNOS expression levels than high-consuming genotypes (Chapter 2 and 3; Kaplan et al., 2013; Mohr et al., 2013), which results in EtOH more strongly increasing GABA release onto GCs in low EtOH consuming gentotypes. In higher EtOH-consuming genotypes, the balance shifts towards low nNOS expression and low GC PKC activity (Chapters 2 and 3), promoting EtOH-suppression of tonic $GABA_A$R inhibition. These opposite actions of EtOH on GC tonic $GABA_A$R inhibition fundamentally alter the effect of EtOH on signal transmission through the cerebellar cortex by differentially affecting spontaneous and mossy fiber evoked glutamatergic transmission to PCs, the sole output of the cerebellar cortex. In particular, low concentrations of EtOH (9mM) that are known to disrupt cerebellar-dependent behavior in low EtOH consuming D2 mice, but not high EtOH consuming B6 mice, suppress the frequency of sEPSPs in D2 but not B6 mouse PCs (Chapter 4). Furthermore, 9mM EtOH enhances/suppresses mossy fiber-evoked EPSPs in D2 and B6 mouse PCs, respectively.

I was not able to fully characterize all of the mechanisms mediating the observed specific effects of EtOH on transmission through the cerebellar cortex. However, since the differential impact that was initially discovered with respect to GC tonic $GABA_A$R inhibition appears to be the main target that is differentially modulated, and this differential impact was maintained at subsequent stages of transmission through the cortex, I tested whether pharmacologically counteracting EtOH suppression of tonic $GABA_A$R currents in high EtOH consuming B6 mice could reduce EtOH consumption. Targeted pharmacological enhancement of GC tonic $GABA_A$R with THIP microinjections into the cerebellum *in vivo* attenuates EtOH intake without largely impairing motor behavior or the ability to consume fluids (Chapter 5). Thus, EtOH suppression of GC tonic $GABA_A$R inhibition may not simply be associated with a high-EtOH consumption phenotype, but may also influence EtOH intake. Together, my findings identify genetically controlled molecular mechanisms (i.e., nNOS and PKC activity) and the

consequent neural mediator (i.e., GC tonic $GABA_AR$ and impact on excitatory input to PCs) by which the cerebellum influences EtOH consumption.

6.2.1. General interpretations – antipodal effect of EtOH on GC tonic $GABA_AR$ inhibition

Behavioral description of the cerebellar LLR phenotype has focused primarily on motor impairment (e.g. Schuckit, 1985), which is measured along a spectrum from no impairment (i.e., no ataxia) to severe ataxia. This range is inherently unidirectional, and when coupled to previous reports of the effect of EtOH, which were limited to enhancement of rat GC tonic $GABA_AR$ inhibition (Carta et al., 2004; Hanchar et al., 2005), bounded my initial hypothesis between a spectrum of EtOH-induced enhancement of GC tonic $GABA_AR$: I'd detect strong enhancement of GC tonic $GABA_AR$ in behaviorally sensitive rodents and little enhancement in behaviorally insensitive rodents. However, some prior evidence suggested the possibility, instead, of antipodal EtOH effects on tonic $GABA_AR$ currents. For instance, muscimol stimulated chloride uptake in cerebellar microsacs was reduced by EtOH in mice selectively bred for resistance to ethanol-induced hypnosis, measured by loss-of-righting-reflex duration, whereas it was enhanced in the selectively bred high-sensitivity strain (Allan et al., 1987). This was the first evidence, though indirect, that EtOH might actually reduce $GABA_AR$-mediated chloride influx. Later, patch-clamp recordings using perforated patch of Chinese hamster ovary cells with recombinant expression of α6,δ-containing $GABA_ARs$ revealed a range of responses to EtOH, from potentiation to suppression of α6β2δ $GABA_AR$ currents (Yamashita et al., 2006). Together, these studies suggested that genetically regulated intracellular factors have the capacity to enable either EtOH enhancement or suppression of α6,δ-containing $GABA_AR$ currents.

The experiments in Chapter 2 identified that the relative balance between nNOS expression in the GC layer and GC PKC activity dictates the range of EtOH's effect on GC tonic $GABA_AR$ inhibition, from enhancement to suppression. Thus, high nNOS expression in combination with moderate-high PKC activity leads to EtOH enhancement of GC tonic $GABA_AR$

inhibition. However, in the presence of TTX, EtOH suppresses GC tonic $GABA_AR$ inhibition in D2s to a similar extent seen in B6s, which suggests that sufficient nNOS expression can override direct inhibition of extrasynaptic $GABA_ARs$ by EtOH when PKC is low. One explanation is that EtOH alters $GABA_AR$ sensitivity directly at an allosteric site, consistent with previous reports (Hanchar et al., 2006; Santhakumar et al., 2007), and that phosphorylation by PKC either blocks EtOH from directly binding to this site or stabilizes the $GABA_AR$ complex in such a manner that prevents the binding of EtOH and subsequent influence on chloride conductance. Thus, much of the discrepancy regarding whether EtOH can directly affect α6,δ-containing extrasynaptic $GABA_ARs$ may stem from differing phosphorylation environments between labs (Carta et al., 2004; Hanchar et al., 2005). However, my results do not require direct EtOH binding to $GABA_ARs$. Instead, EtOH may be modifying $GABA_AR$ sensitivity through an alternative mechanism that ultimately affects $GABA_ARs$. The current studies, do not address EtOH binding characteristics or address the temporal regulation of $GABA_AR$ currents by dynamic PKC-mediated phosphorylation and phosphatase-mediated dephosphorylation. While EtOH enhancement or suppression of GC tonic $GABA_AR$ inhibition likely reflect a stable shift in the PKC/nNOS balance (i.e., high nNOS in cases of enhancement and low PKC with low nNOS in cases of suppression), responses that were categorized as “non-responders” (Chapters 2 and 3) may reflect either high PKC activity and low nNOS expression or low-moderate PKC activity with low-moderate nNOS expression that together negate each other’s impact. Thus, the range in behavioral sensitivity to EtOH impairment and EtOH consumption phenotypes may be determined across a spectrum bounded by strong enhancement to strong suppression of GC tonic $GABA_AR$ inhibition.

The direct consequence that these antipodal EtOH effects on GC tonic inhibition have on the cell’s firing characteristics and passive membrane properties *in vivo* can be inferred. I used voltage-clamp procedures to measure EtOH effects on α6,δ-containing $GABA_AR$ currents, but artificially set the driving force of chloride by setting the E_{Cl^-} to 0mV while holding the membrane

potential at V_h = -60mV. This creates a stronger driving force for chloride than that found *in vivo* or in an unperturbed cerebellar slice where the E_{Cl-} is approximately -75mV and resting membrane potential hovers around V_m = -70mV (Jorntell & Ekerot, 2006). The resulting smaller amplitude chloride currents carried by extrasynaptic $GABA_A$Rs contribute to GC inhibition by having a shunting effect (i.e., causing a decrease in the cell's input resistance) that increases the required excitatory amplitude to achieve spiking threshold (Mitchell & Silver, 2003). This shunting form of inhibition is also the primary consequence of activating extrasynaptic $GABA_A$Rs, which increases AP spiking thresholds in other brain regions such as the somatosensory cortex (Chance et al., 2002) and hippocampus (Semyanov et al., 2003). Based on Chapters 2-4 and the work by Mitchell and Silver (2003), EtOH-stimulation of GABAergic transmission, which increases both synaptic and extrasynaptic GC $GABA_A$R conductance, should attenuate the responsiveness to glutamatergic excitation from mossy fibers.

In hippocampal pyramidal neurons, sIPSCs can transiently inhibit neurons for 10-100ms *in vitro* (Pouille & Scanziani, 2001) and may provide a temporal gate for modulating transmission of specific signals. However, the small amplitude sIPSPs from a weak driving force had little impact on synaptic integration at cerebellar GCs *in vivo* (Pouille & Scanziani, 2001). Instead, the largest impact on neuronal gain of synaptic input to GCs is generated by tonic $GABA_A$R inhibition (Hamann et al., 2002), and modulating gain can lead to impairment in the fidelity of signal transmission through the cerebellar cortex (fidelity of signal transmission describes the relationship between stimulated mossy fiber-input to GCs and resulting GC activity as a function of the spontaneous GC activity; Duguid et al., 2012). Disruption of the fidelity in the mossy fiber-GC relay is associated with behavioral and cognitive dysfunction (Szemes et al., 2013) and, based on the THIP-induced attenuation in EtOH intake (Chapter 5), could impact reward processing and EtOH intake.

Suppression of GC tonic $GABA_A$R inhibition increases the input resistance of the GC while having a modest effect on the resting membrane potential. Importantly, *in vivo* patch-

clamp recordings reveal that both suppression and enhancement of GC tonic $GABA_AR$ inhibition cause deviations from the optimal GC "signal-to-noise" firing ratio (a comparison between the number of mossy-fiber evoked spikes to spontaneous spikes) and similarly lead to impairment in cerebellar processing (Duguid et al., 2012). Enhancing GC tonic $GABA_AR$ inhibition with THIP has minimal effects on spontaneous GC spiking while strongly reducing the sensory whisker-evoked spike output. Suppressing GC tonic $GABA_AR$ inhibition with GABAzine, which is a broad spectrum $GABA_AR$ antagonist that also inhibits synaptic $GABA_ARs$, increases the spontaneous firing rate while only modestly increasing the evoked spike output. The effect that suppressing or enhancing GC tonic $GABA_AR$ inhibition, either with selective pharmacology or with EtOH, has on GC spike output was never directly measured in this dissertation, but my indirect assessments are in partial contradiction with Duguid et al. (2012). Based on their report, I would have expected 9mM to enhance sEPSP frequency in the B6s and have no effect in the D2s. Instead, I found the opposite: 9mM EtOH had no effect on PC sEPSP frequency in B6 mice (where it suppresses GC tonic $GABA_AR$ inhibition), but reduced it in D2 mice (where it enhances GC tonic $GABA_AR$ inhibition; Chapter 4). The EtOH-induced reduction in PC sEPSP frequency was blocked by GABAzine, suggesting that it resulted from enhanced GC tonic $GABA_AR$ inhibition and was not due to EtOH action on non-$GABA_AR$ targets. The reasons for this discrepancy are unclear. One possibility is that the firing patterns in an *in vivo* anesthetized cerebellum differ from that in an *in vitro* slice. The differing levels of mossy-fiber glutamatergic input may shift the GC's input resistance and baseline firing probabilities to promote a greater reduction in spontaneous GC spiking when GC tonic $GABA_AR$ inhibition is enhanced in an *in vitro* slice preparation. Similarly, another possibility is that the impact of tonic $GABA_AR$ modulation on evoked and spontaneous firing patters may differ across species. D2 mice had a higher baseline firing rate than B6s (Chapter 4) and that reported by Duguid et al. (2012) in SDRs. The EtOH reduction in D2 PC sEPSPs may thus reflect greater sensitivity across the broader spontaneous GC firing rate, which was obfuscated in the SDR by a lower baseline firing

rate. Finally, it is likely that the impact of both enhancement and suppression of tonic $GABA_AR$ inhibition is dependent on the magnitude of such changes, and 9mM EtOH will almost certainly have a much more subtle impact than a high concentration of specific agonist or a saturating concentration of antagonist.

6.2.2 General interpretations – nNOS expression

Blocking NO production by pre-soaking slices in the non-selective NOS inhibitor, L-NA, attenuated the EtOH enhancement in Golgi cell firing and the increase in GC tonic $GABA_AR$ inhibition. Thus, EtOH enhances GABAergic transmission to GCs via an AP-dependent mechanism that requires a reduction in baseline NO levels. However, the electrophysiology experiments alone do not permit the specific conclusion that neuronal NOS is the EtOH target. Instead, this conclusion was based on the immunohistochemical observation of enhanced nNOS expression in genotypes where EtOH enhanced GABAergic transmission to GCs. It is therefore possible that EtOH's actions on inducible or endothelial NOS could also contribute to the electrophysiologically assessed NOS impact on GABAergic inhibition of GCs (discussed more in Future Directions below), although the endothelial NOS isoform has a considerably smaller contribution to cerebellar NO production than nNOS, and the inducible form is only activated under pathological conditions (Hall & Attwell, 2008; Stuehr et al., 2004).

Additionally, we used DAF fluorescence to visualize an EtOH-induced reduction of NO production (Chapter 2), confirming that EtOH indeed suppresses its activity. However, I am unable to directly confirm whether EtOH is acting directly on the NOS enzyme itself or on a precursor to NOS activation. The finding that L-arginine, an NOS substrate, enhanced the EtOH response in SDRs suggests that L-arginine is a rate-limiting substrate for NO production, but it doesn't directly address whether EtOH affected access to an NOS activator (e.g., calcium, diacylglerol; Newton, 1995). One possibility is that EtOH reduces NOS activation by blocking NMDA receptor-mediated calcium influx (Kumari & Ticku, 2000; Palmer et al., 1988; Wall,

2003), but this remains to be tested in the cerebellum. Therefore, my interpretations are limited to an EtOH-induced reduction in NO production that is mediated by reduced nNOS activity.

6.3. Cerebellar output

Behaviorally, enhancement of GC tonic $GABA_AR$ inhibition reduces EtOH consumption (Chapter 5), but the neural mechanism through which it achieves this behavioral outcome is an area for future exploration. PCs are the sole output of the cerebellar cortex and provide GABAergic input to the deep cerebellar nuclei (DCN). The DCN, in turn, make polysynaptic connections across the brain. Thus, modifying PC activity will have a functional consequence via modulation of DCN outputs in response to afferent signaling.

However, inferences drawn in Chapter 4 regarding EtOH's downstream effect on DCN output, when only single PCs were assessed, are limited. A single PC has a less reliable influence on DCN neuron activity than a population of PCs (Catz et al., 2008). Furthermore, spike timing and firing rates of populations of PCs, firing in synchrony, play important roles in information coding (De Zeeuw et al., 2011) and influence DCN output (Catz et al., 2008; Person & Raman, 2012b). Thus, both PC spike timing and firing rate across a population of PCs needs to be considered to comprehensively assess the impact that modulation of GC $GABA_AR$ inhibition directly has on the outcome of cerebellar cortical processing at the level of the DCN.

GCs play an important role in generating and synchronizing PC simple spike activity (Galliano et al., 2013; Heck et al., 2007). Specifically, blocking glutamatergic input to PCs from a majority of GCs attenuates GC-evoked PC simple spike activity (Galliano et al., 2013), and by enhancing GC tonic $GABA_AR$ inhibition, EtOH may similarly reduce or impair PC simple spiking patterns. Reducing PC simple spiking in a subset of PCs may have a disinhibitory influence on DCN neurons, but due to the 50:1 PC to DCN neuron convergence ratio, the effect of one or a few PCs on DCN neuron output may be negligible (Person & Raman, 2012b; Sugihara et al., 2009). Therefore, EtOH's effects on the firing synchrony in a larger population of PCs may

directly affect cerebellar-dependent behaviors through downstream deviant output from the DCN.

Since GCs can synchronize spiking from a population of PCs (Galliano et al., 2013; Heck et al., 2007), then enhancement in the activity of a population of GCs can synchronize population PC spiking that affects DCN neuron activity. On the other hand, suppression in the activity of a population of GCs may lead to the failure to synchronize PC spiking and impair modulation of DCN output. My data suggests that nNOS expression determines if EtOH dampens GC activity, which in turn, may impede their role in synchronizing PC firing. Immunohistochemical and electrophysiological assessment of nNOS expression revealed clustered expression patterns (especially in B6 mice, PVs, and NHPs) throughout the sagittal orientation of the GC layer (Chapters 2 and 3; Kaplan et al., 2013; Mohr et al., 2013). These nNOS rich and poor domains may promote similar GC responses to EtOH within each domain (i.e., suppression of tonic $GABA_AR$ inhibition or insensitivity in nNOS poor domains and enhancement of $GABA_AR$ inhibition in nNOS rich domains). In the presence of EtOH, GCs in nNOS poor domains may be more effective at synchronizing a population of PCs within parasagittal zones (up to 500µm) or microzones (up to 100µm) than GCs in an nNOS rich domain (Person & Raman, 2012b).

However, whether PC spiking rates are enhanced or reduced by GC-mediated firing synchrony depends on the spatial relationship between the PCs and afferent GCs (Dizon & Khodakhah, 2011). Photostimulation of glutamate from subsets of GCs directly beneath PCs, in the parasagittal plane, has a purely excitatory effect on PCs, while GCs positioned 340-400µm laterally from the target PC have a purely inhibitory effect via feedforward inhibition by molecular layer interneurons (Dizon & Khodakhah, 2011). Thus, activated GCs positioned laterally to a group of PCs will provide reciprocal signaling through feedforward inhibition to the PC, and although perhaps synchronized with surrounding PCs, may cause a reduction in output to the

DCN. This contrast between excitation and inhibition through reciprocal signaling is an important component of cerebellar processing (Person & Raman, 2012b).

EtOH may impair cerebellar-dependent behaviors by reducing the contrast between excitation and inhibition through reciprocal signaling. Since the effect of GCs on PCs ranges from excitation to inhibition, depending on their respective spatial locations, the impact of EtOH on PC output to a given neuron in the DCN is largely determined by the spatial distribution of nNOS expression patterns (both beneath and lateral to a given PC) relative to the particular population of PCs that converge onto the specific DCN neuron. Under this framework, GCs in nNOS rich domains are most strongly inhibited by EtOH, and as a result, provide weaker signaling to PCs. This may be reflected in attenuated excitation of the PCs directly above these PCs during afferent mossy fiber signaling, compared to in the absence of EtOH, and weaker inhibition of PCs positioned laterally to these GCs. Since proper cerebellar function requires strong rate-coding contrast (Person & Raman, 2012b), the reduced contrast may be reflected in an indistinguishable change in PC input to the DCN. Consequently, the DCN output won't change, leading to a failure in conveyance of the appropriate corrective step to downstream forebrain targets (Ito, 2005), the result manifesting in impaired cerebellar-dependent behavior or cognitive function.

Studies that have experimentally manipulated PC firing patterns and DCN outputs have largely limited their assessment to motor behavior. The behavioral experiments in Chapter 5 indirectly modulated PC activity by affecting GC firing properties (inferred from Chapters 2-4). Intriguingly, one study similarly modulated PC activity by substantially diminishing the glutamatergic output from a majority of GCs, by rendering $Ca_V2.1$ channels inactive. This manipulation reduced PC simple spike frequency, but had no effect on performance of cerebellar-dependent tasks such as rotarod, the Erasmus Ladder, and occulomotor assessments such as the vestibulo-ocular reflex (Galliano et al., 2013). In Chapter 5, THIP microinjection presumably also did not affect all PCs in a given lobe. Therefore, it is not

surprising that THIP didn't affect water consumption, our indirect measure of motor impairment. However, one could argue that the water consumption test is not a sensitive measure of motor impairment, and thus, fine motor impairments would go undetected. The Galliano et al. (2013) study argues against that postulation by demonstrating that a reduction in GC signaling, in the majority but not all GCs, has no effect on more sensitive acute motor assessments. Instead, the GC contribution to motor behaviors may be limited to complex procedural cerebellar-dependent motor learning (Alba, et al., 1994; Galliano et al., 2013; Steuber et al., 2007), and so a THIP-induced impairment of motor behavior may have been detected under alternative experimental protocols. Since pharmacological manipulation of lobes IV/V/VI lobes by Dar (2006) blocked EtOH-induced ataxia, it suggests that they significantly contribute to cerebellar-dependent motor behavior. Therefore, it's more likely that THIP didn't impair motor function and water intake due to incomplete suppression of GC activity, consistent with Galliano et al. (2013).

An important interpretational consideration from the Galliano et al. (2013) study, however, is that the unaffected GCs still had normal membrane properties (e.g., input resistance), while THIP enhancement of GC tonic $GABA_AR$ inhibition via microinjection is presumed to reduce the GC input resistance. The consequential reduction in gain of the mossy fiber-GC relay to PCs (Mitchell & Silver, 2003) was not affected in the GCs with active $Ca_V2.1$ channels. Thus, it remains possible that reducing glutamatergic input to PCs by pharmacologically enhancing GC tonic $GABA_AR$ may have a differential effect on motor behavior than if input was reduced by blocking vesicular glutamate release by genetic deletion of $Ca_V2.1$ channels.

The THIP-induced reduction in EtOH intake (Chapter 5) is likely dependent on cerebellar outputs to reward circuitry in the forebrain (see Future Studies below for more discussion). But how EtOH's effects on PCs affect downstream DCN neuron output is unclear. For instance, it's not simply that increasing the firing rate of a single PC input to a DCN neuron will reduce its output (McDevitt et al., 1987). Instead, DCN neurons produce short-latency APs that

immediately follow synchronous IPSPs and enable the coding of spike-timing information conveyed by PCs firing in synchrony (Person & Ramann, 2012b). Thus, the firing rate of DCN neurons can vary as a function of the spiking rate of synchronous PC inputs and the number of PCs in synchrony. Sensory stimuli thought to increase PC activity, via enhancement of the mossy fiber-GC relay, reduced DCN neuron spiking rates (Rowland & Jaeger, 2005) similar to that detected by stimulating parallel fibers, *in vitro* (Person & Raman, 2012a). In B6s, reduced mossy fiber-evoked input to PCs by 9mM EtOH could, in turn, contribute to reduced PC input to DCN neurons, more gaps in afferent IPSPs, and a resulting higher DCN output firing rate. In light of this, it's intriguing that the DCN support intracranial self-stimulation, an operant assessment of reward-related behavior (Corbett et al., 1982). Additionally, vermis stimulation at 100Hz for one hour increased dopamine turnover in the rat nucleus accumbens (Albert et al., 1985), suggesting that increasing PC spiking frequency above normal levels (~60Hz; Person & Raman, 2012b) can also increase the gain of reward processing. However, both the PC firing rate and stimulation intensity are beyond physiologically relevant ranges, further emphasizing the complexity in trying to predict DCN output to forebrain regions from EtOH's effects on individual PCs.

6.4. Evolution of hypotheses and understanding of EtOH's actions

These studies began with the simple hypothesis: EtOH will more strongly increase GABAergic transmission to GCs in low EtOH consuming rodents that are behaviorally sensitive to cerebellar ataxia (e.g., D2 mice and SDR) than high EtOH consuming, behaviorally insensitive rodents (e.g., B6 mice). This hypothesis was formulated from human (Schukit, 1985; Schukit, 1994) and animal studies (Gallaher et al., 1996; Yoneyama et al., 2008) of the cerebellar LLR phenotype. It seemed necessary that behavioral sensitivity to acute EtOH would have a neural substrate by which measureable differences in response to EtOH exposure could be detected; i.e., I should be able to find a genetically regulated cellular LLR. However, it quickly

became apparent that EtOH's effects did not simply range along a unipolar spectrum from strong enhancement to no enhancement. Instead, the neural LLR is actually a response to EtOH that ranges along a bipolar distribution, from enhancement to suppression of GC tonic $GABA_AR$ inhibition.

In keeping with the LLR framework, I next proposed that suppression of GC tonic $GABA_AR$ inhibition would be less disruptive of transmission through the cerebellar cortex. In some regard, this prediction was supported. EtOH-induced suppression of GC tonic $GABA_AR$ inhibition in the B6 mice did not affect spontaneous glutamatergic input to PCs, while as predicted, EtOH significantly reduced sEPSP frequency in D2 mice. However, when mossy fibers were stimulated, EtOH enhanced the excitatory input to PCs in D2 mice, and that contrasted with a slight reduction in excitatory input to PCs in B6 mice. The specific mechanisms mediating this effect are not fully understood at present, but hypothetically may relate to variation in PC input resistance driven by EtOH-induced dampening of spontaneous GC activity in the D2 mice, and consequent decrease in both AMPA receptor activation and feed-forward GABAergic inhibition. The impact that raising the PC input resistance has on signal integration and its effect on input to the DCN was not tested. Regardless of the mechanisms, the opposite impact of EtOH, between D2 and B6 mice, persisted at the PC output.

For genetic variation in EtOH's effect on GC tonic $GABA_AR$ inhibition to be the primary mechanistic explanation for the cerebellar LLR phenotype, it assumes that there aren't any other highly sensitive cerebellar EtOH targets that show genotypic diversity. Ancillary targets for differential EtOH sensitivities include, but are not limited to, molecular layer interneurons (Hirono et al., 2009; Mameli et al., 2008; Wadleigh & Valenzuela, 2011), NMDA receptor signaling (He et al., 2013), metabotropic glutamate receptors (Carta et al., 2006), NO's interaction with nicotinic acetylcholine receptor-signaling (Taslim & Dar, 2011), and neurosteroid signaling (Tokuda et al., 2011). While some of these targets could contribute to differing sensitivity to EtOH-induced cerebellar impairment, only EtOH's effect on molecular layer interneurons was

explicitly assessed (Chapter 4) and found to be similarly insensitive to EtOH modulation in both B6 and D2 mice at low EtOH concentrations, relative to its effect on tonic $GABA_AR$ inhibition. There also was no variation in the GC response to bath application of the synthetic neurosteroid, ganaxolone (personal observations), although baseline levels or EtOH-stimulated neurosteroid concentrations were never measured. Nonetheless, the striking differences in EtOH's effect on GC tonic $GABA_AR$ inhibition and downstream effect on glutamatergic input to PCs strongly suggests that EtOH's action on GC $GABA_AR$ inhibition is a principal contributor to the cerebellar LLR and associated EtOH consumption phenotype.

Another hypothesis derived from Fernando Valenzuela's laboratory (e.g., Carta et al., 2004) and my own preliminary work in the SDR model (Chapter 2) was that EtOH's actions on GC tonic $GABA_AR$ inhibition would be solely presynaptic. These early experiments were conducted in SDRs, and TTX blocked the effect of 52 and 79mM EtOH on the magnitude of GC tonic $GABA_AR$ inhibition and the holding current noise. Adding B6 and D2 mice to the assessment of EtOH's actions revealed postsynaptic inhibition of GC tonic $GABA_AR$ currents by EtOH when PKC activity was low, which could be induced in SDRs by pharmacologically blocking GC PKC activity. PVs (Chapter 3) and non-human primates (Mohr et al., 2013) confirmed the predictive validity of these findings. The case for postsynaptic EtOH action, and in particular postsynaptic suppression of $GABA_ARs$ by EtOH remains controversial. However, it's supported here by a reduction in the root mean square of the membrane potential noise, a response in the presence of TTX under whole-cell conditions or after nucleated patch formation, and induction or block by modulating GC PKC activity. Together, these findings reveal the importance of considering EtOH's actions on GABAergic inhibition of GCs in a dynamic matter that ranges from exclusive presynaptic action to postsynaptic action.

6.5. Future directions

These studies highlight a number of directions through which EtOH's actions on cerebellar processing can be better understood. First off, the findings that nNOS and GC activity dictate the effect of EtOH on GC tonic $GABA_AR$ inhibition only partly predict EtOH's action *in vivo*, as they omit the complex interaction between environmental factors and phosphorylation/dephosphorylation events, and NO production. *In vitro*, diverse EtOH responses were detected within animals, ranging from pure enhancement of tonic $GABA_AR$ inhibition to suppression. What factors promote the GC conditions that facilitate this range? Because calcium can activate PKC activity (Newton, 1995), *in vivo* cerebellar activity patterns may differentially affect PKC activity. Similarly, nNOS signaling may vary as a function of calcium signaling levels (Palmer et al., 1988; Wall, 2003), suggesting that the precise effects of acute EtOH may be state-dependent that's bounded by a genetically-determined range. Identifying how particular activity states influence EtOH responses may be critical in developing efficacious cerebellar-centric AUD treatments with minimal consequences on other cerebellar-dependent functions.

The use of broad-spectrum PKC agonists/antagonists, and non-specific NOS antagonists prevented the assessment of particular PKC and nNOS isoform contributions to EtOH's effects on GC tonic $GABA_AR$ inhibition. Future studies should work to uncover the relative contributions of the host of PKC isoforms active within GCs (Naik et al., 2000) in order for more precise genetic screening or to direct pharmacological treatment strategies. Similarly, non-selective NOS inhibitors don't distinguish between inducible, endothelial, or neuronal forms of NOS. So while the results derived from immunohistochemistry of nNOS support the electrophysiological assessment, the possibility for contributions from other minor NO sources (i.e., inducible and endothelial NOS isoforms) on the EtOH response can't be ruled out. Thus, future studies should systematically test whether nNOS is the sole NOS EtOH target in the cerebellum by ruling out contributions from inducible and endothelial NOS.

Another question that remains unresolved is how EtOH is actually interacting with α6,δ-subunit containing $GABA_A$Rs. The long debate over EtOH's direct action may wane since I've shown that it depends on the phosphorylation state of the receptor, but the manner in which EtOH actually interacts with α6,δ-subunit containing $GABA_A$Rs to reduce their open probability for a given concentration of GABA remains unresolved. How it achieves this should be an area of future investigation. Is it a direct action of EtOH on the extrasynaptic $GABA_A$Rs, or is it via EtOH actions on $GABA_A$R active second messengers? Furthermore, it's unclear why some groups (e.g. Hanchar et al., 2005) detect direct postsynaptic enhancement of GC tonic $GABA_A$R inhibition by EtOH, while only suppression was reported here. Could this difference be due to differential phosphorylation characteristics, such as isoform expression differences across species? Phosphorylation targets on the $GABA_A$R, such as at the α or β subunits (Abramian et al., 2010; Jovanovic et al., 2004), may differentially contribute to direct EtOH action occurring at the interface of these subunits (Wallner et al., 2014). Exactly how EtOH suppresses these receptors is an important area of future research as EtOH sensitivity may be predicted based on genotypic variation of particular subunits (Hanchar et al., 2005).

Finally, the cerebellar outputs that directly affect EtOH drinking behavior are a critical next stage of assessment. I've proposed above that cerebellar input to areas of the reward circuit, such as the ventral tegmental area, the nucleus accumbens, and the prefrontal cortex, are candidate regions through which the cerebellum could influence elements of EtOH consumption. One quick and basic strategy to direct extra-cerebellar investigation could be to microinject alcohol into the cerebellum, similar to the method described in Chapter 5, and then search for regions of elevated forebrain cFOS expression. Alternatively, cerebellar-specific projections that impact EtOH consumption can be assessed by injecting an anterogradely expressing halorhodopsin into the cerebellum and then systematically inactivating the hypotheses-directed forebrain regions (e.g. nucleus accumbens, ventral tegmental area) with

light. Reductions in EtOH consumption using this method would provide further insight into the cerebellar outputs that influence EtOH intake and potentially other reward-related behaviors.

6.6. Relevance to preventative and treatment strategies to combat AUDs

Genomics has become a popular strategy for exploring the interaction between gene expression with the environment in EtOH sensitivity and AUD risk (Morozova et al., 2014; Schuckit, 2014). These studies have revealed substantial genetic networks that influence elements of EtOH-related phenotypes ranging from EtOH metabolism to neurotransmitter signaling (Morozova et al., 2014). However, understanding the functional consequence of candidate genes within the nervous system is a critical element in establishing pharmacological treatment strategies or devising diagnostic preventative techniques. Here, I established that both GC PKC activity and nNOS expression in the GC layer contribute to EtOH's impact on GC tonic $GABA_AR$ inhibition, but that nNOS expression most strongly correlated to consumption phenotype across animals (Chapters 2 and 3). Thus, genetic screening of nNOS-related genes may provide added diagnostic insight and accuracy into risk for developing an AUD.

To date, there is only limited evidence supporting the utility in genetic screening for the nNOS gene. Mice deficient in the α splice variant of the nNOS gene drank nearly six times more EtOH than wild-type mice and showed lower sensitivity to EtOH's impairment of the righting reflex (Spanagel, et al., 2002). Intriguingly, this finding contrasts with the effects of acutely inhibiting nNOS on the neurobehavioral effects of EtOH. Acute administration of a variety of NOS inhibitors, instead, reduces EtOH consumption (Calapai et al., 1996; Rezvani et al., 1995) and preference in dependent rats (Lallemand & De Witte, 1997). Together, these studies propose that a large reduction of nNOS activity, either by EtOH or pharmacological inhibition, serves to limit EtOH consumption. On the other hand, when nNOS activity is already low, no such drug-induced contrast is perceived. These behavioral studies, therefore, are consistent with the results from Chapters 2 and 3 that associate lower nNOS expression levels with higher

EtOH-consuming genotypes. As a result, the potential efficacy of nNOS inhibitors in reducing EtOH intake may be dependent on baseline nNOS expression levels.

Beyond nNOS expression, the findings here expose the cerebellum as a novel target with promising efficacy for combating AUDs. This awareness is the first step in orienting our attention to devising appropriate interventional therapies which may be employed at very young ages. The Danish Longitudinal Study of Alcoholism identified cerebellar-dependent biomarkers, such as muscle tone at five days, unassisted sitting, and walking at one year, that were associated with higher risk for developing EtOH dependence within 30 years (Manzardo et al., 2005). This study highlights the relevance of the cerebellum as a diagnostic target for AUD risk and proposes the importance of early interventional strategies, similar to that in Autism (Corsello, 2005). Thus, early diagnosis of AUD risk from cerebellar-dependent measures may direct functional interventions to attenuate risk.

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