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Modulation of $GABA_A$ receptors in Cerebellar Granule Neurons by Ethanol: A Review of Genetic and Electrophysiological Studies

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Abstract

Cerebellar granule neurons receive inhibitory input from Golgi cells in the form of phasic and tonic currents that are mediated by postsynaptic and extrasynaptic $GABA_A$ receptors, respectively. Extrasynaptic receptors are thought to contain $\alpha_6\beta_x\delta$ subunits. Here we review studies on ethanol (EtOH) modulation of these receptors, which have yielded contradictory results. Although studies with recombinant receptors expressed in *Xenopus* oocytes indicate that $\alpha_6\beta_3\delta$ receptors are potently enhanced by acute exposure to low (≥3 mM) EtOH concentrations, this effect was not observed when these receptors were expressed in Chinese hamster ovary cells. Slice recordings of cerebellar granule neurons have consistently shown that EtOH increases the frequency of phasic spontaneous inhibitory postsynaptic currents (sIPSCs), as well as the tonic current amplitude and noise. However, there is a lack of consensus as to whether EtOH directly acts on extrasynaptic receptors or modulates them indirectly; i.e. via an increase in spillover of synaptically released GABA. It was recently demonstrated that an R to Q mutation of amino acid 100 of the α_6 subunit increases the effect of EtOH on both sIPSCs and tonic current. These electrophysiological findings have not been reproducible in our hands. Moreover, it was shown the α_6-R100Q mutation enhances sensitivity to the motor-impairing effects of EtOH in outbred Sprague-Dawley rats, but this was not observed in a line of rats selectively bred for high sensitivity to EtOH-induced motor alterations (Alcohol Non-Tolerant rats). We conclude that currently there is insufficient evidence conclusively supporting a direct potentiation of extrasynaptic $GABA_A$ receptors following acute EtOH exposure in cerebellar granule neurons.

Keywords

GABA; ethanol; extrasynaptic; presynaptic; granule

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The Cerebellum as a Target of Ethanol

The cerebellum is involved in the control of balance as well as the coordination, planning, and fine regulation of voluntary movement. In addition, this brain region has been shown to be important for certain cognitive functions, including thought, behavior, and emotion (Schmahmann and Sherman, 1997). The cerebellum is composed of the cerebellar cortex, inner white matter, and deep cerebellar nuclei. Neurons of the cerebellar cortex are organized into three layers. The outermost is the molecular layer that contains the basket and stellate interneurons, the distal portion of the excitatory axons from the cerebellar granule neurons (CGNs), and the dendrites of Purkinje neurons. The Purkinje neuron layer is located below the molecular layer and it contains the large cell bodies of these neurons, which constitute the sole output of the cerebellar cortex and are inhibitory GABAergic neurons with axons that project to the deep cerebellar nuclei. The innermost layer of the cerebellar cortex is the granule cell layer, which contains the CGNs and the proximal segments of the ascending portion of their axons. This layer also includes Golgi interneurons and the mossy fibers. The CGNs receive excitatory glutamatergic input from mossy fibers and GABAergic inhibitory input from Golgi cells. This review focuses on the actions of ethanol (EtOH) on GABAergic transmission at CGNs and prior to discussing this topic, a brief overview of EtOH's effects on cerebellar function is provided.

Numerous studies have shown that short- and long-term EtOH exposure impairs normal development of cerebellar cortical neurons as well as their proper functioning at maturity. Investigators initially measured EtOH's effect on cerebellar type-A γ-aminobutyric acid receptor ($GABA_A$-R) function using $^{36}Cl^-$ flux assays in microsacs obtained from mature rodents. These studies consistently demonstrated that acute EtOH exposure enhances $^{36}Cl^-$ flux in this preparation (Allan and Harris, 1986;Harris et al., 1995;Proctor et al., 1992a;Proctor et al., 1992b). Most studies on the acute effects of EtOH on the function of specific populations of mature cerebellar neurons have focused on Purkinje neurons (Deitrich et al., 1989). These studies generally found that EtOH acutely depresses Purkinje cell firing (Basile et al., 1983;George and Chu, 1984;Siggins and French, 1979;Sorensen et al., 1980). It was shown that the benzodiazepine ligands, Ro 15-4513 and FG 7142 as well as and the $GABA_A$ receptor antagonist, bicuculline, antagonized the EtOH-induced depression of Purkinje neuron firing in anesthetized animals, thereby implicating $GABA_A$-Rs in the mechanism of action of EtOH (Palmer and Hoffer, 1990;Palmer et al., 1988). However, (Lee et al., 1995b) found that Ro15-4513 did not antagonize the acute effects of EtOH on Purkinje neuron firing. It was reported that EtOH did not always potentiate responses evoked by exogenous GABA and that it had a more reliable effect if β-adrenergic, $GABA_B$, or nicotinic receptors were concomitantly activated (Freund and Palmer, 1997;Freund et al., 1993a;Lee et al., 1995a;Lin et al., 1994;Lin et al., 1991;Lin et al., 1993;Palmer and Hoffer, 1990;Yang et al., 1999;Yang et al., 2000). More recently, it was reported that acute EtOH exposure increases quantal GABA release at Purkinje neurons in slices of the cerebellar vermis (Breese et al., 2006;Ming et al., 2006).

The acute effects of EtOH on other neuronal types of the mature cerebellum have only been investigated in a few studies. Single unit recordings showed that intraperitoneal injection of EtOH increases the discharge rate of rat inferior olivary nucleus neurons (Rogers et al., 1986). Acute EtOH exposure was shown to increase firing of inhibitory Golgi interneurons in cerebellar slices (Freund et al., 1993b). The authors of this study stated that this effect would be predicted to increase Golgi neuron input to CGNs and ultimately influence excitatory input to Purkinje neurons.

In the early 1990s, genetic, pharmacological, and functional studies suggested that $GABA_A$-Rs in CGNs are important targets of EtOH. These studies are discussed below in section 2.2.

Effects of EtOH on GABAergic Transmission at CGNs

Overview of GABAergic input to CGNs

CGNs relay excitatory input from mossy fibers to Purkinje neurons via divergent pathways and this process is profoundly modulated by GABAergic transmission. Golgi interneurons provide two types of GABAergic input to CGNs: tonic and phasic. Tonic GABAergic inhibition dominates over phasic inhibition (Hamann et al., 2002). The presence of a tonic GABAergic current in granule cells was first demonstrated by the existence of a bicuculline sensitive "background noise current" that persisted in the presence of glutamate antagonists, tetrodotoxin (TTX), or zero $[Ca^{2+}]_o$ (Kaneda et al., 1995). These findings indicated that the current persists in the absence of action potential-dependent GABA release. In rodents, this tonic current appears gradually during development; it is first apparent at P14 and reaches stable levels at ~P30 (Brickley et al., 1996;Tia et al., 1996;Wall and Usowicz, 1997). This current reflects activation of high-affinity extrasynaptic $GABA_A$-Rs by ambient GABA levels as well as spillover of synaptically-released GABA (Rossi et al., 2003). One factor that permits this accumulation of GABA is the unique configuration of the synapse between Golgi and granule cells. This synapse forms part of a glomerulus that contains four elements: 1) the glutamatergic mossy fiber afferent, 2) the axons from Golgi cells, 3) the granule cell dendrites, and 4) a glial sheath. Significantly, this sheath retains released GABA in the glomerulus, increasing the chances of extrasynaptic receptor activation. In addition to the protracted time course of glomerular GABA concentrations, the unique subunit composition of $GABA_A$-Rs present in cerebellar granule cells further contributes to the generation of tonic currents; i.e. extrasynaptic $GABA_A$-Rs contain $\alpha_6\beta_x\delta$ subunits, which are potently activated by GABA (Hamann et al., 2002). $\alpha_6\beta_x\delta$ receptors have a relatively low EC_{50} (~0.2-0.7 μM) for activation by GABA, which is in the range of the lowest level that transporters can maintain extracellular GABA concentrations (~0.16-0.4 μM) (Attwell et al., 1993;Santhakumar et al., 2006a;Saxena and Macdonald, 1996;Wallner et al., 2003). Moreover, $GABA_A$-Rs containing α_6 and δ subunits display relatively little desensitization (Saxena and Macdonald, 1994). All of these characteristics make these receptors ideal mediators of tonic currents triggered by low background levels of GABA.

The α_6 subunits have been the focus of research interest since they are almost exclusively expressed in the cerebellar granule cells. α_6 -/- knockout mice were generated, which also display complete loss of δ subunits due to the preferential receptor partnership of these subunits with α_6 subunits in CGNs (Brickley et al., 2001). In cerebellar slices from these mice, the tonic current was absent. Paradoxically, α_6 -/- mice exhibit normal motor function which raised questions about the physiological significance of the tonic current. However, deletion of the $GABA_A$ receptor subunits in these mice elicited an increase in a leak conductance mediated by the two-pore forming K^+ channel TASK-1 (Brickley et al., 2001). The increase in this current compensated for the loss of $GABA_A$ receptor subunits and normalized the response of CGNs to excitatory input. The impact of tonic inhibition on CGN excitability was also assessed by (Hamann et al., 2002). Reduction of this current with furosemide increased input resistance, lowered the threshold for action potential generation, and increased the number of action potentials triggered at different levels of current injection. Furosemide increased CGN excitability in response to mossy fiber-to-granule cell synaptic transmission. The studies of (Hamann et al., 2002) estimated that the tonic current mediates over 90% of inhibitory current in granule cells. These authors showed that reduction of the granule cell tonic current with furosemide has a significant impact on Purkinje cell excitability. Specifically, furosemide increases the excitability of Purkinje neurons in response to mossy fiber stimulation of CGNs. However, it does not affect excitability of Purkinje neurons in response to direct stimulation of the parallel fiber. Importantly, an elegant *in vivo* electrophysiological study recently demonstrated that tonic GABAergic inhibition of CGNs profoundly regulates the

transformation of mossy fiber somatosensory input into CGN output (Chadderton et al., 2004).

CGNs also receive phasic GABAergic inhibition in the form of inhibitory postsynaptic potentials (IPSCs) produced by conventional action potential- and Ca^{2+}-dependent vesicular GABA release from Golgi interneurons. This phasic inhibition is mediated in part by receptors containing $\alpha_1\beta_x\gamma_2$ subunits. These IPSCs superimpose on the background tonic current. This form of GABAergic inhibition mediates ~10% of the total inhibition of granule cells and is thought to be important for controlling synchronous rhythmic firing of granule cells (Hamann et al., 2002).

Modulation of GABAergic transmission at CGNs by EtOH

Relatively few studies have characterized the acute effect of EtOH on GABAergic transmission at CGNs. The first indication that EtOH might increase GABAergic input to this neuronal population came from *in vivo* studies performed with chloralose-anesthetized cats in which EtOH was shown to acutely inhibit responses of CGNs to auditory stimuli (Huang and Huang, 1992;Huang et al., 1991). More recently, a similar finding was reported for CGN responses elicited by visual stimuli (Huang and Huang, 2007). Collectively, these results are consistent with inhibition of mossy fiber-mediated excitation by EtOH. A possible mechanism mediating this effect could be an increase in GABAergic transmission at CGNs. (Engblom et al., 1991) assessed $GABA_A$ receptor function in cultured CGNs using the chloride-sensitive fluorescent probe, 6-methoxy-N-(3- sulfopropyl)quinolinium. These investigators found that EtOH at concentrations below 50 mM potentiated Cl^- efflux via $GABA_A$-Rs, particularly when low GABA concentrations were used to activate the receptors. In contrast, (Reynolds et al., 1992) found that currents evoked by pressure ejection of GABA onto cultured CGNs were potentiated by concentrations of EtOH between 1-50 mM only in 28% of the neurons examined. Several years later, a study focused on excitatory $GABA_A$-Rs expressed in the axonal rather than the inhibitory $GABA_A$-Rs present in CGN dendrites. (Schmid et al., 1999) found that 50 mM EtOH acutely potentiated the GABA-induced enhancement of [^{3}H]D-aspartate release in synaptosomes enriched for these axonal terminals. Interestingly, EtOH was effective in synaptosomes from Alcohol Non-Tolerant (ANT), but not Alcohol Tolerant (AT) rats.

The first study of EtOH's action on GABAergic transmission at CGNs using acute cerebellar slices was performed by (Carta et al., 2004), who found that acute EtOH exposure at concentrations as low as 20 mM reversibly increased the frequency of sIPSCs. The amplitude and decay time constants of these events were, however, unaffected. EtOH also increased the magnitude of the tonic GABAergic current mediated by $\alpha_6\beta_x\delta$ extrasynaptic $GABA_A$-Rs. Importantly, the effect of EtOH on the tonic current was abolished in the presence of the Na^+ channel blocker, TTX. Loose-patch cell-attached recordings revealed that EtOH increases spontaneous firing of Golgi cells. Taken together, these findings indicate that EtOH indirectly enhances the tonic current presynaptically via an increase in spillover of spontaneously released GABA from Golgi cells (see Fig 1 for a schematic representation). Our resultss are in general agreement with those of other investigators indicating that low EtOH concentrations do not potentiate currents mediated by extrasynaptic $GABA_A$ receptors in cultured CGNs (Casagrande et al., 2007;Yamashita et al., 2006).

Our finding that EtOH increases sIPSC frequency via an increase in Golgi cell firing has been independently reproduced by others (Hanchar et al., 2005;Santhakumar et al., 2006b). However, evidence has been put forward indicating that EtOH directly and very potently enhances the function of EtOH on $\alpha_6\beta_x\delta$ extrasynaptic $GABA_A$-Rs. (Wallner et al., 2003) reported that EtOH enhances the function of $\alpha_6\beta_2\delta$ and $\alpha_6\beta_3\delta$ recombinant receptors expressed in *Xenopus* oocytes at concentrations ≥30 mM and ≥3 mM, respectively; however, it should be noted that attempts to replicate these findings in a different expression system were

unsuccessful (Yamashita et al., 2006) (also see Borghese et al in this issue). Subsequently, (Hanchar et al., 2005) used site-directed mutagenesis and expression in *Xenopus* oocytes to demonstrate that a substitution from arginine (R) to glutamine (Q) at position 100 in the α_6 subunit dramatically enhances the potentiating effect of EtOH on currents mediated by $GABA_A$-Rs containing $\alpha_6\beta_3\delta$ subunits. These findings generated tremendous interest given that the α_6-R100Q polymorphism had long been implicated by studies with genetic rodent models in the mechanism of action of EtOH. Specifically, these initial genetic studies were performed with the AT and ANT rat lines. In the next sections, we discuss genetic, behavioral, and electrophysiological experiments of EtOH actions that have been performed with these rats.

Studies with AT and ANT Rats

α_6-R100Q and EtOH-related Behaviors in the AT and ANT Selected Lines

AT and ANT rats were selectively bred for differential ataxic sensitivity to EtOH as measured by performance on the tilting plane test (Eriksson and Rusi, 1981); see Korpi et al., this issue). In this test, each animal was given three trials in which it was placed on the raised end of a mechanical tilting plane that was elevated from a flat horizontal position to an 84-degree angle in 5 s. The average angle at which the animal slid to the base of the plane was recorded (baseline) followed by an intraperitoneal injection of EtOH (2.0 g/kg, 15% w/v in saline). After a 30-minute interval, the average angle obtained on 3 additional trials was recorded. Ataxic sensitivity was measured as the difference between the baseline and post-injection angle of sliding (Sellin and Laakso, 1987). Following more than 35 generations of selection pressure, ataxic sensitivity in the AT and ANT rats differed by over 15 degrees, which is probably the combined effect of at least two possibly genetically-independent sub-traits: a small, but significantly lower baseline angle and a significantly greater angle 30 minutes after 2.0 g/kg EtOH in the AT compared to the ANT rats (Radcliffe et al., 2004;Sarviharju and Korpi, 1993); R. Radcliffe, unpublished results). Other traits, such as anxiety, for which the AT and ANT rats also show a significant difference, may also contribute to the differential ataxic sensitivity (Tuominen et al., 1990).

The AT and ANT rats have been shown to be differentially sensitive to lorazepam and sodium barbital suggesting genetic differences in GABAergic function (Hellevuo et al., 1989) and receptor binding studies with the benzodiazepine ligand [^{3}H]Ro-15-4513 have also implicated GABAergic mechanisms. This ligand binds to two populations of $GABA_A$-Rs: diazepam sensitive and diazepam insensitive. The latter is revealed when the binding assay is conducted in the presence of high concentrations of diazepam. It was found that [^{3}H]Ro-15-4513 binding was normal in the AT, but a large proportion of the ANT rats did not possess the diazepam-insensitive binding sites; i.e., virtually all [^{3}H]Ro-15-4513 binding was blocked in the presence of diazepam (Uusi-Oukari and Korpi, 1990). It was subsequently shown that the binding difference was mediated by the α_6-R100Q single nucleotide polymorphism (SNP) (Korpi et al., 1993); also see Korpi et al., this issue). It is likely that this point mutation accounts for at least part of the genetic difference in benzodiazepine sensitivity in the AT and ANT rats (Korpi and Seeburg, 1993).

Given the known interaction between EtOH and $GABA_A$-Rs, it has been speculated by many investigators that the α_6-R100Q mutation might be at least partially responsible for the differential EtOH sensitivity of the AT and ANT selected lines, although in the original report it was noted that the mutation did not affect EtOH modulation of $\alpha_6\beta_2\gamma_2$ receptors expressed in a cell culture system (Korpi et al., 1993). Subsequent genotyping of a sample of 116 rats from generations 45-46 showed that the mutant (Q) allele occurred with a frequency of 0.86 in the ANT while the frequency was only 0.09 in the AT (Makela et al., 1995). Among AT and ANT breeders that were transferred to the University of Colorado in 1998 (generation 60), only

the mutant allele was found in the ANT, while both alleles were still present in the AT (Radcliffe et al., 2004). These results are consistent with a segregation of the Q allele in the ANT high-sensitivity phenotype. However, if a gene is important in a selected trait, it is expected that after many generations of selection (at least 34 for the AT and ANT; (Sarviharju and Korpi, 1993), alternate alleles of the gene should become fixed (i.e., homozygous) in the selected lines, especially considering the relatively limited number of breeding pairs that are typically maintained in such experiments. However, the alleles were not fixed in either the AT or ANT after at least 45 generations. It is possible that alleles for a gene could be present in a heterozygous state in one of the lines if one of the alleles has a dominant mode of action, although the recessive allele still would be expected to become fixed in the other line. Thus, it has to be concluded that the enrichment of the Q allele in the ANT and of the R allele in the AT, while certainly not proof of the null hypothesis, is not strong evidence in and of itself for an effect of the Q mutation on the tilting plane test, especially considering that a replication of the selection was not conducted (Crabbe et al., 1990).

Six generations of brother-sister matings were carried out with the University of Colorado rats to produce several inbred ANT and AT lines. Although inbreeding typically requires 20 or more generations, genotype testing indicated that the lines were already greater than 90% inbred when they arrived at the University of Colorado (Radcliffe et al., 2004). Care was taken to establish AT lines that carried one or the other of either the Q or R allele; this was not possible for the ANT since all of the University of Colorado ANT rats were homozygous for the Q allele. Studies of the inbred AT lines indicated that the Q mutation did not affect moderate or high dose EtOH sensitivity, including ataxic sensitivity on the tilting plane, consistent with the absence of an EtOH-modulating effect of the Q allele (Radcliffe et al., 2004). It is possible, however, that an interacting ANT allele at a different locus was required for the phenotypic expression of the Q mutation and therefore it would be without effect on a pure AT background.

One way to rule out the possibility of epistasis ("background effect") is to study the effect of a single gene mutation in a segregating population, or at least on several different strain backgrounds. With this in mind, Korpi and colleagues created an F2 intercross from the AT and ANT phenotypes to more adequately address the question of the involvement of the α_6-R100Q mutation in EtOH responses (Korpi and Uusi-Oukari, 1992;Sarviharju and Korpi, 1993). The original tilting plane test and several other behavioral tasks were examined in the F2 rats. [^{3}H]Ro-15-4513 binding in the presence of diazepam was measured as a surrogate marker for the α_6-R100Q mutation, which had not yet been discovered. None of the tilting plane measurements correlated to the [^{3}H]Ro-15-4513 binding, nor did measurements of high-dose EtOH sensitivity. Although not definitive, these results suggest that the *Gabra6* alleles did not segregate with the tilting plane phenotypes in the F2 generation and therefore were not important in the selected difference between the AT and ANT phenotypes, or in any of the EtOH-related responses that were measured (Korpi and Uusi-Oukari, 1992).

QTL Mapping Studies Related to α_6-R100Q in the AT and ANT Selected Lines

Following inbreeding of the AT and ANT lines at the University of Colorado, a large AT X ANT F2 intercross population was generated to conduct quantitative trait locus (QTL) mapping and other genetic studies (Radcliffe et al., 2004). The grandparents of the F2 rats carried both alleles of the α_6 mutation – the R allele in the AT and the Q allele in the ANT – to facilitate examination of its role in EtOH-related behaviors. QTL mapping is a type of linkage analysis in which members of a segregating population, such as an F2, are phenotyped for a trait of interest and then genotyped for informative DNA markers, typically throughout the entire genome. It is then determined at which genomic locations phenotype segregates with genotype. If a chromosomal region contains a gene that is polymorphic and that influences the phenotypic expression of the trait, then the markers in that region will correlate to the phenotype (Abiola

et al., 2003). Thus, a QTL is a chromosomal region containing a DNA sequence difference(s) that contributes to genetic variance for a trait (Mackay, 2001). We reasoned that if the α_6-R100Q mutation was at all responsible for the tilting plane difference in the selected lines, then a QTL should map at approximately 27.5 Mb on chromosome 10, the location of the rat *Gabra6* gene.

Over 1200 F2 rats were bred and tested (see (Radcliffe et al., 2004) for complete details of breeding and phenotype testing). In the first week of testing, the animals were assessed for EtOH-induced ataxia in exactly the same manner as that used for the selection of the AT and ANT phenotypes. In fact, the original apparatus was used, courtesy of Dr. Kalervo Kiianmaa. One week later, the animals were again tested on the tilting plane and during this test, the time at which the animal regained its original sliding angle was recorded. Finally, high-dose EtOH sensitivity (3.5 g/kg) was tested in the third week using the loss of righting reflex test (LORR).

QTL mapping is made more efficient by genotyping only the extreme responders in a segregating population, since it is these animals that capture the greatest proportion of the genetic variance (Darvasi and Soller, 1992). Thus, we genotyped 479 F2 animals (approximately equal numbers of males and females) that were in the upper and lower 20% of the distribution for ataxic sensitivity-the selection trait for the AT and ANT phenotypes described above. The average values for ataxic sensitivity for these groups of F2 rats are shown in table 1 along with the average values for the AT and ANT progenitors. A full genome scan was conducted using simple sequence-length polymorphism DNA markers (SSLP) that had been determined to be different between the inbred AT and ANT rats, and spaced at approximately 19 cM on average (see (Radcliffe et al., 2004) for a general description of genotyping procedures). Here we will focus on just chromosome 10, which is where *Gabra6* is located. The complete analysis is currently being prepared for publication.

The QTL analysis was conducted with the Rqtl package using the Haley-Knott regression method of interval mapping (Broman et al., 2003;Haley and Knott, 1992). Interval mapping is a method with which the location and significance of a QTL can be estimated in the region between markers. The chromosome 10 mapping results for baseline angle, angle at 30 minutes after 2.0 g/kg EtOH, and ataxic sensitivity (baseline angle minus 30 minute angle) are shown in Fig 2. Permutation methods were used to determine empirically-derived suggestive and significant likelihood of the odds ratio (LOD) thresholds; i.e., genome-wide probability of 0.05 and 0.63, respectively (Churchill and Doerge, 1994). These thresholds were very similar for each of the three traits, with LOD scores from 3.2 to 3.3 for significant and from 1.8 to 2.0 for suggestive. For clarity, only the lowest thresholds are shown in the figure. As can be seen, no single locus on chromosome 10 surpassed even the suggestive threshold for any of the three traits that were mapped. Importantly, this included the interval between markers at approximately 18 and 38 Mb within which the *Gabra6* gene is located. Interval mapping results for the other EtOH-related behaviors that were tested in weeks 2 and 3 were similar; i.e., all were below the suggestive level (data not shown). These results strongly suggest that the $GABA_A$-R α_6-R100Q mutation did not contribute to genetic variation for the behavioral tests that were examined in this study. It can be further concluded that the mutation does not contribute to differential EtOH-mediated ataxic sensitivity in the AT and ANT rats.

As mentioned above, an epistatic interaction may be necessary for the α_6 mutation to be involved in the AT/ANT difference; i.e., the Q allele requires another ANT allele at a different locus for its effect to be expressed. In a segregating population, unlinked alleles are randomly distributed meaning that some proportion of the animals will carry the necessary combination of alleles for the phenotype to be expressed if epistasis is involved. In such cases, QTL mapping procedures are capable of identifying the interacting loci. A pair scan analysis conducted in Rqtl did not reveal any loci interacting with any region of chromosome 10 (results not shown)

suggesting that epistasis was not a factor in this population, at least not involving chromosome 10.

It is possible for interval mapping procedures to overestimate a LOD score when the peak is situated somewhere near the middle of two flanking markers, but it is unlikely that it could be underestimated. Nonetheless, a randomly selected cohort of the F2 was genotyped specifically for the *Gabra6* SNP to ensure that this was not the case, since *Gabra6* is located between markers that were used for the QTL analysis. The Amplification Refractory Mutation System developed for use with the *Gabra6* SNP was used to genotype 72 F2 rats (Radcliffe et al., 2004;Saba et al., 2001). The rats were behaviorally tested as described above and the phenotypes were analyzed using one-way ANOVA with the influence of genotype as the factor to be tested. The results of this analysis are shown in Table 1. The distribution of genotypes in the F2 sample was not significantly different from the expected Mendelian ratio of 25%:50%: 25% (X^2, $p>0.9$) suggesting that the mutation did not influence fecundity. If the α_6-R100Q mutation conferred greater EtOH sensitivity, then it would be predicted that the angle 30 minutes after EtOH would have been smaller while ataxic sensitivity would have been larger in F2 animals with the Q allele compared to those with the R allele. In fact, just the opposite was observed, although there was not a significant effect of genotype for any of the measures (one-way ANOVA, $p> 0.3$). These results are consistent with the QTL mapping results and do not support the hypothesis that the α_6-R100Q mutation mediates any portion of the difference in tilting plane sensitivity in the AT and ANT rats.

Polymorphisms in the rat *Gabra6* regulatory region as well as in the *Gabra1*, *Gabrb2*, and *Gabrg2* genes have been found to be linked to the Q allele of *Gabra6* (Congeddu et al., 2003;Saba et al., 2005). It is not known if these polymorphisms have any effect on interactions between $GABA_A$-Rs and EtOH, although it has been postulated that the *Gabra6* regulatory region mutations influence α_6 expression, especially during the course of chronic EtOH administration (Saba et al., 2005;Sanna et al., 2004). The QTL mapping results further suggest that these or any other linked polymorphisms, if actually present in the ANT rats, do not contribute to the EtOH sensitivity difference between the AT and ANT phenotypes. Otherwise a QTL would have mapped to this region irrespective of which polymorphism was involved.

The α_6-100Q mutation has been found in other selected rat lines; specifically, the Sardinian Preferring and Non-Preferring rats (sP, sNP) and the Alko Alcohol and Alko Non-Alcohol lines (AA, ANA). These animals were selected for differential alcohol preference when presented with a choice between an EtOH solution or water (Colombo, 1997;Hilakivi et al., 1984). The non-preferring lines (sNP, ANA) both had a higher frequency of the Q allele compared to that of the preferring lines (sP, AA); the Q allele was not present in other rat lines selected for EtOH drinking behavior (Carr et al., 2003;Saba et al., 2001). There is a degree of consistency in these findings, although there are too few points to allow an actual correlational analysis. It should be noted that in the AT X ANT F2 study of (Sarviharju and Korpi, 1993) EtOH preference did not correlate to tilting plane sensitivity despite a preference difference in the AT and ANT rats. If the Q allele does indeed suppress EtOH preference, which is far from definitive at this time, then this result could be considered additional evidence, albeit indirect, for the absence of an effect of the α_6 mutation on the tilting plane test. However, it remains to be seen if any of the $GABA_A$ subunit gene polymorphisms that are clustered on chromosome 10, including α_6-R100Q, influence genetic variation in EtOH preference.

Electrophysiological Studies

(Valenzuela et al., 2005) investigated the effect of acute EtOH exposure on phasic and tonic GABAergic currents at CGNs in cerebellar slices from male (~30-day-old) ANT and AT rats that were inbred at University of Colorado HSC as discussed above (Radcliffe et al., 2004). Studies were performed on slices from a total of 4 ANT and 7 AT rats. All inbred ANT rats

were homozygous for the 100Q genotype and all inbred AT rats were homozygous for the 100R genotype. In slices from these rat lines, we did not detect any differences in the effect of EtOH on tonic GABAergic currents (Valenzuela et al., 2005). Basal tonic current amplitudes were not significantly different in slices from ANT versus AT rats. We also found no significant differences in the acute effect of EtOH on sIPSC frequency in CGNs from ANT and AT rats (Valenzuela et al., 2005). Finally, basal sIPSC frequencies and amplitudes were not significantly different in slices from ANT versus AT rats (Valenzuela et al., 2005). Our results indicate that neither differences in the basal properties of GABAergic transmission at CGNs nor differential modulation of this transmission by acute EtOH exposure are responsible for the phenotypes of ANT and AT rats. These findings are in agreement with the genetic studies discussed above.

Studies with Outbred Sprague-Dawley Rats

A significant effect of the α_6-R100Q mutation on EtOH-mediated impairment on the accelerating rotarod was observed in a population of outbred Sprague Dawley rats, which was recently found to carry both the R and Q alleles (Hanchar et al., 2005). This would appear to be at odds with the lack of such an effect on the tilting plane test and other behaviors in the AT and ANT rats, as described above. The authors suggested that the behavioral effect of the mutation is operative only at lower EtOH doses (< 1.5 g/kg), although it was shown that the most robust effect of the Q allele on EtOH enhancement of tonic GABA currents in cerebellar slices occurred at a concentration of 100 mM, which would be equivalent to a brain EtOH concentration produced by a high dose of EtOH (~3.5 g/kg) (Hanchar et al., 2005;Otis et al., 2005). While the effect of α_6-R100Q may be population-, dose- and/or task-dependent, the genetic evidence presented in Section 3 and in other reports cited above (especially Korpi et al., 1992) do not support a role for the α_6-R100Q mutation in EtOH-induced ataxia either in the AT and ANT selected lines or in segregating crosses derived from them. It is most parsimonious to conclude that the enrichment of the Q allele in the ANT was coincidental and was not because it contributes to genetic variance in the tilting plane test.

(Otis et al., 2005) argued that confounding differences likely arose during selective breeding of the AT and ANT rats and that this could explain the lack of an effect of the α_6-R100Q mutation on EtOH sensitivity in these animals as opposed to outbred Sprague- Dawley rats. Differences do arise during selective breeding, but these are minor in comparison to the random differences present in outbred rats. In a group of outbred animals such as the Sprague-Dawley rats used in the study of (Hanchar et al., 2005), it would be unlikely that any two animals would differ only at the α_6-100 locus.

In addition to testing behavioral responses to EtOH in the outbred Sprague-Dawley rats, (Hanchar et al., 2005) compared the effect of EtOH on CGN tonic GABAergic currents in slices from rats homozygous for α_6-100R versus α_6-100Q. These investigators reported that tonic GABAergic currents are enhanced by 30 and 100 mM EtOH to a greater extent in rats with the 100Q genotype. A similar result was found for the effect of these concentrations of EtOH on sIPSC frequency. In the presence of TTX, exogenous GABA, and a GABA transporter inhibitor, these investigators observed enhancement of tonic GABAergic currents in the presence of 10 mM EtOH and this effect was greater in slices from rats with the 100Q genotype. Based on these data, the authors concluded that the α_6-R100Q mutation increases both the presynaptic actions of EtOH on Golgi cell firing as well as the modulation of extrasynaptic $GABA_A$-Rs in CGNs. In light of the evidence discussed in Section 3, we attempted to replicate the findings of (Hanchar et al., 2005) using very similar experimental conditions to those used in their study (Botta et al., manuscript in preparation). We genotyped outbred Sprague-Dawley rats from Charles River (Hollister, CA; area H-41) and assessed the effect of EtOH on CGN tonic currents in parasagittal slices of the cerebellar vermis. We found that the effect of 25 mM

EtOH on tonic current amplitude and sIPSC frequency was not significantly different in slices from α_6-100R versus α_6-100Q rats (Botta et al., manuscript in preparation). Importantly, in slices from either α_6-100R or α_6-100Q rats, we failed to observe effects of 8 or 40 mM EtOH in the presence of TTX, exogenous GABA, and a GABA transporter inhibitor (Botta et al., manuscript in preparation). These findings are in agreement with our previously published data indicating that EtOH indirectly increases the tonic current via a presynaptic mechanism (Carta et al., 2004).

A possible explanation for the lack of consensus between our electrophysiological results and those of (Hanchar et al., 2005) is that there were methodological differences between our studies. One potentially significant difference was the choice of anesthetic. We used ketamine whereas (Hanchar et al., 2005) used the inhalational anesthetic halothane. Ketamine has been shown to enhance the function of $GABA_A$-Rs, and in particular that of CGN extrasynaptic $GABA_A$-Rs containing α_6 and δ subunits (Hevers et al., 2006;Lin et al., 1992). Another important difference is that (Hanchar et al., 2005) used slice cutting and storage solutions containing the NMDA receptor antagonist, D,L-APV, whereas we used cutting solution containing ketamine. Although we store our slices in ketamine-free ACSF and pefuse them with ACSF for at least 15 min prior to recording, it is possible that some anesthetic persists in the slices leading to occlusion of EtOH modulation of extrasynaptic $GABA_A$ receptors. We therefore compared the effect of EtOH on tonic GABAergic currents in slices prepared from 27-31 day-old male Sprague-Dawley rats using two different anesthetics and cutting/storage solutions. In protocol #1, rats were anesthetized with the inhalation anesthetic isoflurane (halothane is no longer available in our animal research facility) and brains were sliced in solution containing (in mM) 220 sucrose, 26 $NaHCO_3$, 10 glucose, 6 $MgSO_4$, 3 KCl, 1.25 NaH_2PO_4, 0.2 $CaCl_2$, and 0.001 D,L-APV. Immediately after the slicing procedure, slices were transferred to artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, 0.001 D,L-APV and 10 glucose equilibrated with 95% O_2 plus 5% CO_2, and allowed to recover at 36 °C for 45 min followed by storage at room temperature in the same solution. In protocol #2, rats were anesthetized with ketamine (250 mg/kg I.P.). Slices were cut in solution containing (in mM) 220 sucrose, 26 $NaHCO_3$, 10 glucose, 6 $MgSO_4$, 3 KCl, 1.25 NaH_2PO_4, 0.2 $CaCl_2$ and 0.43 ketamine (Carta et al., 2004;Shuttleworth and Connor, 2001). Immediately after the slicing procedure, slices were transferred to ACSF without D,L-APV and allowed to recover at 36 °C for 45 min, followed by storage at room temperature in the same ACSF. The tonic current was calculated by fitting all-point histograms to a Gaussian distribution (Hanchar et al., 2005) and was defined as the mean steady-state current recorded in the absence minus that recorded in the presence of 10 μM of the $GABA_A$-R antagonist SR95331. The tonic current noise was defined as the standard deviation of the steady-state current recorded in the absence minus that recorded in the presence of 10 μM SR95331. The effect of EtOH was calculated with respect to the average of control and washout responses. To match the conditions of (Hanchar et al., 2005), whole-cell patch-clamp electrophysiological recordings from CGNs were obtained in ACSF at 23 °C with an internal solution containing (in mM): 140 CsCl, 10 HEPES (pH 7.3), 1 EGTA, 4 magnesium ATP, 0.4 GTP, and 4 QX-314 at a holding membrane potential of -70 mV. As shown in Fig 3A-F, 40 mM EtOH increased the tonic current and the tonic current noise to a similar extent in CGNs from slices prepared using protocols #1 and #2. The magnitude of the tonic current was not significantly different between these slices (Fig 3G).

For comparison, we re-analyzed the data published in (Carta et al., 2004) using all-point histograms. These data were collected using different experimental conditions to those used above. First, the CsCl-based internal solution had a different composition; it contained in (mM) 140 CsCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES (pH 7.3), 2 Na_2-ATP and 4 QX-314. Second, recordings were obtained at 32 °C instead of 23 °C. Finally, 20 μM bicuculline was used instead of 10 μM SR95331 to block $GABA_A$-Rs. Under these conditions, the percent

change in tonic current and tonic current noise produced by 50 mM EtOH was similar from that observed in recordings obtained with 40 mM EtOH at 23 °C using slices prepared according to protocols #1 or #2 (Figs 3E-F). However, the magnitude of the tonic current in the experiments of (Carta et al., 2004) was greater (Fig 3G).

Neither basal frequency nor amplitude of the sIPSCs were significantly different in slices prepared using protocols #1 and #2 (Fig 4A-D). Moreover, EtOH equally increased sIPSC frequency (without affecting amplitude) in these slices (Fig 4A, E). The EtOH-induced percent change in sIPSC frequency shown in Fig 4E is similar to that we previously obtained with 50 mM EtOH (Carta et al., 2004).

Based on these findings, we conclude that the choice of anesthetic, neuroprotective agent, recording temperature, internal solutions or $GABA_A$-R antagonist have little influence on the effect of EtOH on sIPSCs and tonic current in CGNs.

Conclusions and Future Directions

EtOH is a difficult drug to study experimentally in part because it has multiple targets and its actions are dependent on a myriad of factors, including intracellular signaling pathways (Lovinger and Crabbe, 2005;Ron and Jurd, 2005). Moreover, EtOH is a solvent that can cause contaminants to be released from tubing, glassware, and other items used in laboratory experiments (Machu et al., 1998;Tully et al., 2000). Therefore, interpretations of EtOH research data are inherently complex and present a great potential for spurious associations. This issue has been particularly evident in investigations of the interaction between EtOH and GABAergic transmission (reviewed in (Criswell and Breese, 2005;Weiner and Valenzuela, 2006). Accordingly, experimental evidence in this area of research is generally received with skepticism, until important findings are replicated by multiple independent investigators using different experimental approaches and analytical methods. In the case of GABAergic transmission, inter-laboratory replication has contributed to the development of a strong case supporting a major presynaptic role in the mechanism of action of EtOH (Breese et al., 2006;Siggins et al., 2005;Weiner and Valenzuela, 2006). This body of evidence includes the demonstration by two independent laboratories that EtOH increases action potential firing of Golgi cells leading to an increase in phasic GABA release onto CGNs (Carta et al., 2004;Hanchar et al., 2005;Santhakumar et al., 2006b;Valenzuela et al., 2005). We have concluded that this effect indirectly increases the tonic current mediated by $\alpha_6\beta_x\delta$ receptors via enhanced GABA spillover onto extrasynaptic sites (see schematic in Fig 1) (Carta et al., 2004). Interestingly, it has been shown that these presynaptic actions of EtOH on Golgi cell firing are enhanced by the α_6-R100Q mutation via a complex circuit-type effect involving changes in CGN excitability (Hanchar et al., 2005;Santhakumar et al., 2006b). However, we have not been able to replicate these findings either in outbred Sprague-Dawley rats (Botta et al., Manuscript in preparation) or AT/ANT rats (Valenzuela et al., 2005). Importantly, the results of our genetic and behavioral studies with AT/ANT rats indicate that the α_6-R100Q mutation does not modify the motor-impairing effects of acute EtOH exposure (see Section 3 above).

In contrast to the presynaptic actions of EtOH on GABAergic transmission at Golgi cell- to-CGN synapses, there is a significantly greater degree of uncertainty regarding the possibility that EtOH directly modulates extrasynaptic $GABA_A$-Rs containing $\alpha_6\beta_x\delta$ subunits (see Sections 3-4). An important issue that has been ignored is that low EtOH concentrations preferentially enhance the function of receptors containing $\alpha_6\beta_3\delta$ subunits and it is unknown whether such receptors actually exist at extrasynaptic compartments in CGNs (Wisden et al., 1996). Indeed, extrasynaptic $GABA_A$-Rs in CGNs are unlikely to be a homogeneous population, with experimental evidence supporting the possibility that these contain more than

one α and/or β subunit subtype (i.e. α_1, α_6 , β_1, β_2 or β_3) (Jechlinger et al., 1998;Poltl et al., 2003;Sigel and Baur, 2000). Thus, the findings obtained with $\alpha_6\beta_3\delta$ receptors might not be physiologically relevant, particularly given that the β_2 subunit has been demonstrated to associate with δ subunit-containing cerebellar receptors and that it substantially decreases EtOH potency when co-expressed with $\alpha_6\delta$ subunits (Hanchar et al., 2005;Jechlinger et al., 1998;Poltl et al., 2003;Wallner et al., 2003).

In summary, data independently obtained in two different laboratories indicate that acute EtOH affects GABAergic transmission at CGNs via a presynaptic mechanism. However, there is no consensus as to whether EtOH directly affects CGN extrasynaptic $GABA_A$-Rs, and the reasons behind this discrepancy are unclear at the present time. To address these reasons, a number of studies could be performed. First, given that intracellular signaling cascades are well known to modulate $GABA_A$-R sensitivity to EtOH (Weiner and Valenzuela, 2006), future work should use less invasive experimental approaches (i.e. perforated-patch) to study sensitivity of native CGN extrasynaptic $GABA_A$- Rs to EtOH. Second, prior to pursuing additional studies with recombinant receptors, a more detailed characterization of the subunit composition of native CGN extrasynaptic receptors must be obtained. Finally, it should be independently verified that the α_6-R100Q mutation indeed increases behavioral sensitivity to EtOH in outbred rats. Clearly, only by the concerted efforts of several laboratories, will the controversies surrounding the interaction between CGN extrasynaptic $GABA_A$-Rs and EtOH be resolved.

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Abbreviations

ACSF

artificial cerebrospinal fluid

AA

Alko Alcohol

ANA
Alko Non-alcohol

AT
Alcohol Tolerant

ANT
Alcohol Non-Tolerant

CGN
cerebellar granule neuron

DL-AP5
DL-2-amino-5-phosphonovaleric acid

EtOH
ethanol

GABA
γ-aminobutyric acid

type-A GABA receptor
$GABA_A$-R

IPSC
inhibitory postsynaptic current

sIPSC
spontaneous IPSC

LOD
likelihood of the odds ratio

LORR
loss of righting reflex

QTL
quantitative trait locus

sP
Sardinian Preferring

sNP
Sardinian Non-Preferring

SNP
single nucleotide polymorphism

SSLP
simple sequence-length polymorphism

TTX
tetrodotoxin

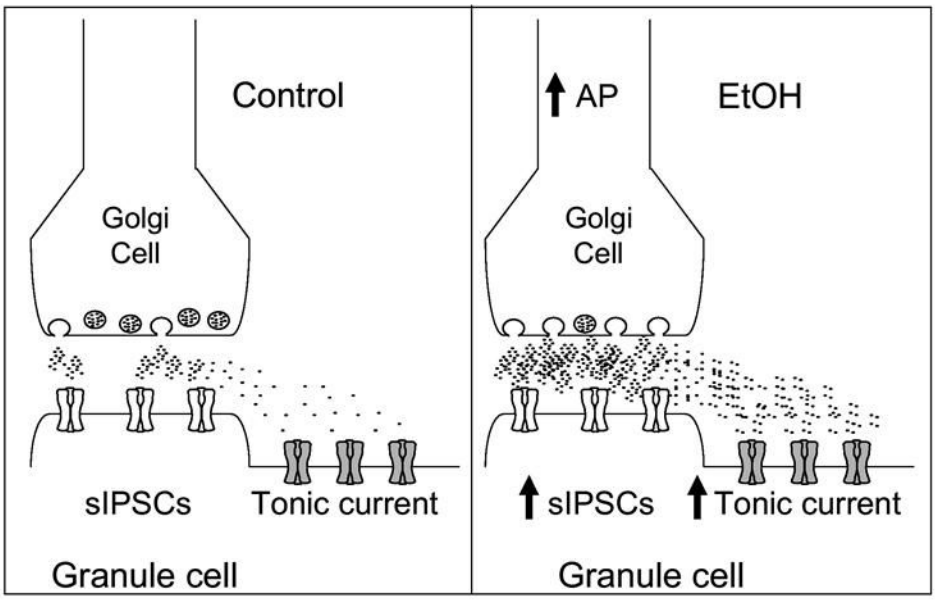

Figure 1.

Simplified model of the EtOH-induced enhancement of GABAergic transmission to CGNs. Under control conditions, spontaneous action potential firing of Golgi cells evokes synaptic GABA release and activation of postsynaptic $GABA_A$-Rs. Activation of these receptors generates sIPSCs. Extrasynaptic $GABA_A$-Rs are also activated via GABA spillover, contributing to the tonic current generation together with ambient GABA. Application of EtOH ($\geq$ 20 mM) increases spontaneous action potential (AP) firing in Golgi cells, which elevates GABA levels both at synaptic and extrasynaptic sites. This results in a parallel increase in both sIPSC frequency and the tonic GABAergic current.

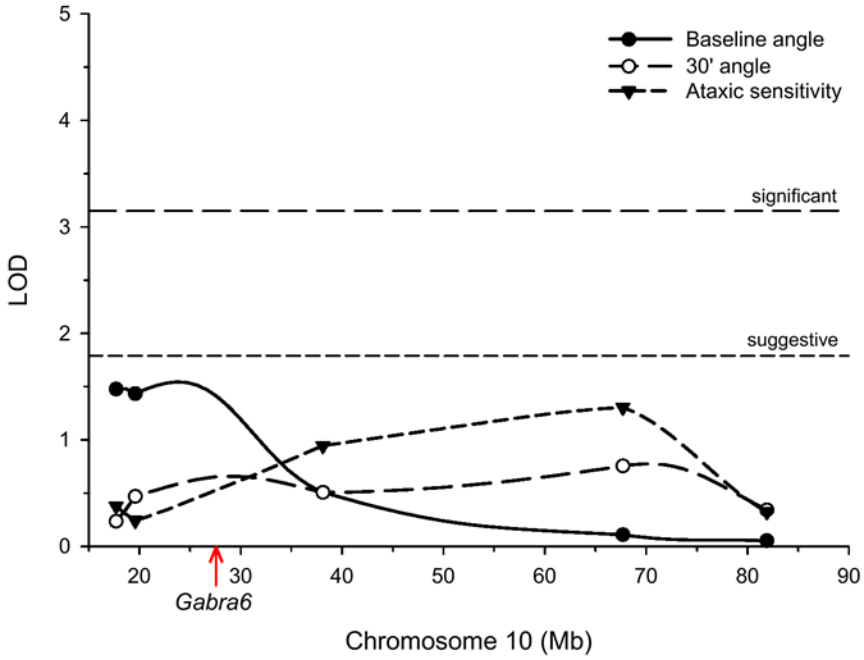

Figure 2.

Chromosome 10 QTL map of tilting plane test variables in F2 progeny derived from an AT X ANT intercross. Symbols indicate marker locations and horizontal lines show empirically-derived significance LOD thresholds. Physical location of *Gabra6* is indicated by the arrow.

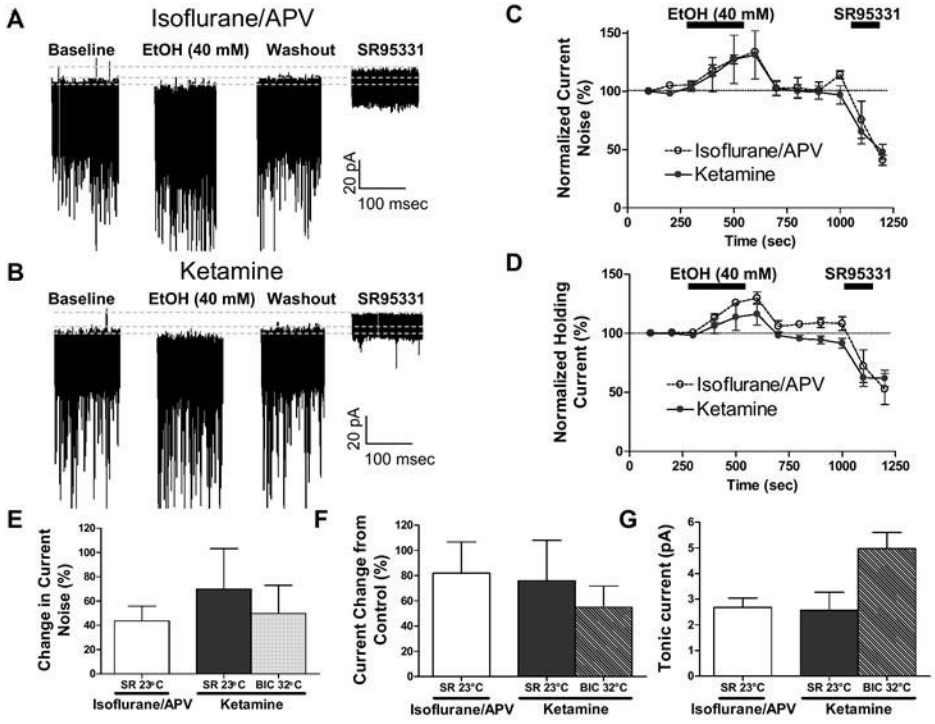

Figure 3.

The slice preparation protocol does not influence EtOH modulation of tonic currents in CGNs. **(A)** Sample trace illustrating the effect of EtOH on tonic currents recorded at 23 °C from slices prepared using isoflurane and D,L-APV (protocol #1-see text). **(B)** Sample trace illustrating the effect of EtOH on tonic currents recorded at 23 °C from slices prepared using ketamine (protocol #2-see text). **(C)** Time course of the effect of 40 mM EtOH on tonic current noise recorded at 23 °C from slices prepared as described in A-B (n = 4); data were normalized with respect to the values obtained during the first 100 s of recording. Bin size = 100 s. **(D)** Same as in C but for holding current amplitude. **(E)** Summary of the percent change in tonic current noise induced by 40 mM EtOH in slices obtained using isoflurane/ D,L-APV or ketamine; recordings were obtained at 23 °C and SR954331 (SR; 10 μM) was used to block $GABA_A$-Rs at the end of the experiment (n = 4). For comparison, four recordings from the study reported in (Carta et al., 2004) were reanalyzed as described in the text. Slices for those studies were prepared using ketamine (protocol #1); recordings were obtained at 32 °C using 50 mM EtOH and bicuculine (BIC; 20 μM) was used to block $GABA_A$-Rs at the end of the experiments. **(F)** Same as in E but for EtOH-induced percent change in tonic current. **(G)** Same as in E but for the basal magnitude of tonic current (steady state current recorded in the absence minus that recorded in the presence of the indicated $GABA_A$-R antagonist); $p < 0.05$ by unpaired t-test. See text for explanation of all-point histogram analysis used to calculate tonic current amplitude and tonic current noise. Bars represent the mean ± S.E.M.

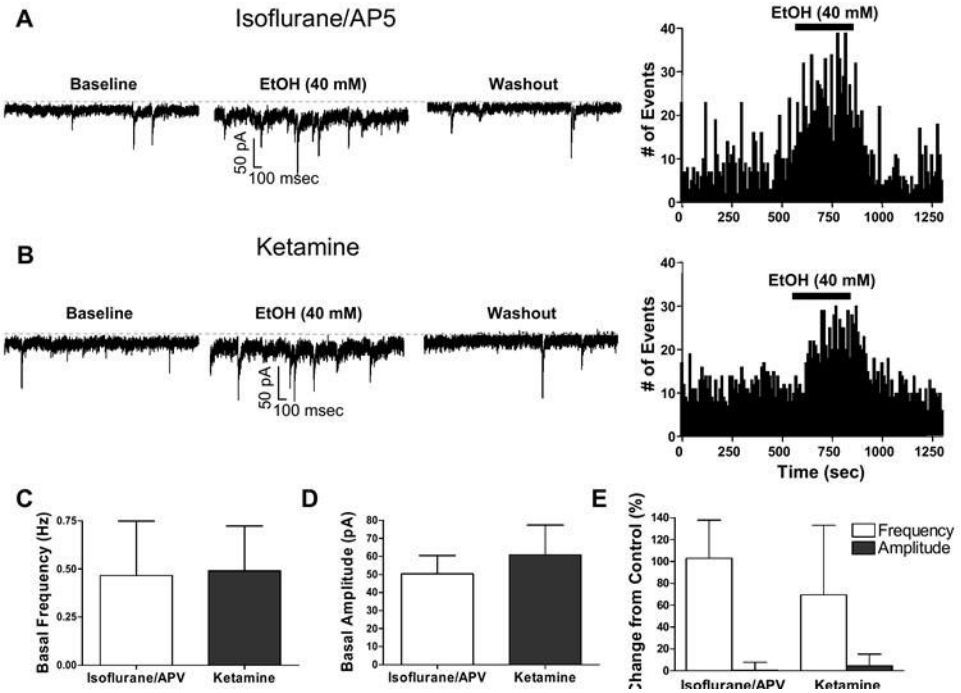

Figure 4.

The slice preparation protocol does not influence EtOH modulation of sIPSC frequency in CGNs. **(A)** Left panels: sample traces illustrating the effect of EtOH on sIPSCs recorded at 23 °C from slices prepared using isoflurane and D,L-APV (protocol #1-see text). Right panel: time course of the effect of EtOH corresponding to the same neuron. **(B)** Same as in A but for slices prepared using ketamine (protocol #2-see text). **(C)** Summary of the basal sIPSC frequency recorded from neurons in slices obtained using isoflurane/ D,L-APV (n = 4) or ketamine (n = 3). **(D)** Same as in C but for basal sIPSC amplitude. **(E)** Summary of the effect of 40 mM EtOH on sIPSC frequency and amplitude in slices prepared using isoflurane/ D,L-APV (n = 4) or ketamine (n = 3). Recordings were obtained at 23 °C in all cases. Bars represent the mean ± S.E.M.

Table 1

Mean phenotypic scores (± SEM) for the tilting plane test in the AT and ANT progenitors; all ATXANT F2 progeny that were generated for QTL mapping; the ATXANT F2 progeny that were in the upper or lower 20% of the distribution for ataxic sensitivity and that were used for QTL mapping; and additional F2 rats that were genotyped for the *Gabra6* mutation

[[Subjects|]]	[[Genotype|]]	[[n|]]	[[Baseline angle|]]	Angle at 30' after 2.0 g/kg EtOH	Ataxic sensitivity (baseline angle minus 30' angle)
[[AT|]]	[[--|]]	[[87|]]	81.2 ± 0.4 *	82.3 ± 0.3 *	-1.1 ± 0.5 *†
[[ANT|]]	[[--|]]	[[128|]]	[[83.2 ± 0.1|]]	[[74.3 ± 0.8|]]	8.9 ± 0.8 †
[[All F2|]]	[[--|]]	[[1168-1194|]]	80.3 ± 0.1 †	[[78.7 ± 0.2|]]	1.7 ± 0.2 †
F2 (lower 20% for ataxic sensitivity)	[[--|]]	[[239|]]	76.4 ± 0.3 *	81.4 ± 0.2 *	-5.0 ± 0.2 *
F2 (upper 20% for ataxic sensitivity)	[[--|]]	[[240|]]	[[81.1 ± 0.2|]]	[[71.7 ± 0.3|]]	[[9.5 ± 0.2|]]
[[Genotyped F2|]]	[[R/R|]]	[[19|]]	[[79.7 ± 1.1|]]	[[74.9 ± 1.8|]]	[[4.8 ± 2.3|]]
[[Genotyped F2|]]	[[R/Q|]]	[[35|]]	[[79.5 ± 0.8|]]	[[74.8 ± 1.1|]]	[[4.8 ± 1.2|]]
[[Genotyped F2|]]	[[Q/Q|]]	[[18|]]	[[80.6 ± 0.8|]]	[[77.6 ± 1.2|]]	[[3.0 ± 1.6|]]

* AT compared to ANT, or lower F2 compared to upper F2, $p<0.001$ (one-way ANOVA).

† These results were previously published in Radcliffe at al., 2004