Behavioural Brain Research 217 (2011) 379–385

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

The sedative but not the memory-blocking properties of ethanol are modulated by α5-subunit-containing γ-aminobutyric acid type A receptors

Loren J. Martin[a], Agnieszka A. Zurek[b,1], Robert P. Bonin[b], Gabriel H.T. Oh[c], John H. Kim[b,c], Howard T.J. Mount[a,b,d], Beverley A. Orser[a,b,e,f,*]

[a] Institute of Medical Science, University of Toronto, Toronto, Ontario M5S 1A8, Canada
[b] Department of Physiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada
[c] The Krembil Family Epigenetics Laboratory, Centre for Addiction and Mental Health, Toronto, Ontario M5T 1R8, Canada
[d] Centre for Research in Neurodegenerative Diseases, Department of Medicine, Division of Neurology, University of Toronto, Toronto, Ontario M5S 1A8, Canada
[e] Department of Anesthesia, University of Toronto, Toronto, Ontario, Canada
[f] Department of Anesthesia, Sunnybrook Health Sciences Centre, Toronto, Ontario, M4N 3M5, Canada

ARTICLE INFO

Article history:
Received 1 October 2010
Received in revised form 29 October 2010
Accepted 1 November 2010
Available online 9 November 2010

Keywords:
Inhibition
GABA
Hippocampus
Ethanol
Memory
α5 subunit
Sedation

ABSTRACT

The precise mechanisms underlying the memory-blocking properties of ethanol are unknown, in part because ethanol targets a wide array of neurotransmitter receptors and transporters. The aim of this study was to determine whether the memory loss caused by ethanol is mediated, in part, by α5 subunit-containing γ-aminobutyric acid subtype A receptors. These receptors have been implicated in learning and memory processes and are targets for a variety of neurodepressive drugs. Also, since these receptors generate a tonic inhibitory current in hippocampal pyramidal neurons, we examined whether concentrations of ethanol that block memory *in vivo* increased the tonic current using whole-cell patch–clamp recordings in hippocampal neurons. Null mutant mice lacking the α5 subunit (*Gabra5*−/−) and wild-type mice were equally impaired in contextual fear conditioning by moderate (1 mg/kg) and high (1.5 mg/kg) doses of ethanol. The higher dose of ethanol also reduced auditory delay fear conditioning to the same extent in the two genotypes. Interestingly, wild-type mice were more sensitive than *Gabra5*−/− mice to the sedative effects of low (0.5 mg/kg) and moderate (1 mg/kg) doses of ethanol in the open-field task. Concentrations of ethanol that impaired memory performance *in vivo* did not increase the amplitude of the tonic current. Together, the results suggest that the α5-subunit containing γ-aminobutyric acid subtype A receptors are not direct targets for positive modulation by ethanol nor do they contribute to ethanol-induced memory loss. In contrast, these receptors may contribute to the sedative properties of ethanol.

1. Introduction

The ingestion of ethanol is associated with multiple behavioral effects including sedation, the inability to form new, long-term memories, ataxia and anxiolysis. The liability for memory loss depends on the amount and rate of consumption [1] as well as the genetic background of the subject [2]. The memory blocking properties of ethanol may arise from the interactions with specific molecular targets, [3] and the identification of such targets may help in the design of effective strategies to reverse this adverse effect.

Substantial evidence indicates that ethanol interacts with specific membrane-bound proteins, most notably several neurotransmitter-gated ion channels [3]. Ethanol decreases the release of glutamate and inhibits *N*-methyl-D-aspartate receptors in the brain [4–6]. Ethanol also depresses neuronal activity by increasing the release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and increasing the activity of GABA subtype A receptors ($GABA_AR$) in the brain [7] and that of glycine receptors in the spinal cord [8].

$GABA_ARs$ are the main inhibitory neurotransmitter receptors in the mammalian brain [9]. They are heteropentameric ion channels that are encoded from a family of 16 genes for 16 different subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ϕ, ε, π), but are typically composed of α, β, and γ subunits arranged in a 2:2:1 stoichiometry [10]. Populations of $GABA_ARs$ with distinct subunit composition differ in their sensitivity to pharmacological agents [14–16] and neuroanatomical and cellular localization [11–13].

* Corresponding author at: Department of Physiology, Room 3318, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario M5S 1A8, Canada.
Tel.: +1 416 978 0574; fax: +1 416 978 3687.
E-mail address: beverley.orser@utoronto.ca (B.A. Orser).
[1] These authors contributed equally to this manuscript.

0166-4328/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2010.11.008

In the hippocampus, $GABA_A$Rs containing the α5 subunit (α5$GABA_A$R) populate the extrasynaptic regions of CA1 and CA3 pyramidal neurons [13,17], where they generate a tonic inhibitory conductance [18–23]. Another extrasynaptic $GABA_A$ receptor subtype that contains the δ subunit also generates a tonic inhibitory conductance that has been shown to be positively up-regulated by ethanol *in vitro* [24–26]. Thus, we postulated that α5$GABA_A$Rs might also be relevant targets for the effects of ethanol *in vitro* and *in vivo*. Moreover, pharmacological up-regulation of α5$GABA_A$R activity is associated with deficits in learning and memory [22,23,27]. Behavioral studies in human volunteers suggest that α5$GABA_A$Rs are important for ethanol-induced memory impairment [28]. Specifically, the acute consumption of ethanol impaired the recall of word lists and the administration of a α5$GABA_A$R-selective negative modulator α5IA, attenuated the impairment of word recall without altering the breath alcohol levels, alterations in saccadic eye movements, or changes in blood pressure and heart rate.

To determine whether ethanol inhibits learning and memory via α5$GABA_A$R-dependent mechanisms, the effects of ethanol were studied with null mutant mice lacking the α5 subunit (*Gabra5*−/−) and wild-type (WT) mice using two fear conditioning paradigms. Also, since α5$GABA_A$R-selective inhibitors have been shown to reduce the motor-impairing and sedative effects of ethanol [29], we sought to determine whether the sedative properties of ethanol were mediated in part by α5$GABA_A$Rs. Finally, we examined whether a tonic inhibitory current generated by α5$GABA_A$Rs in cultured hippocampal pyramidal neurons is enhanced by various concentrations of ethanol using whole-cell patch clamp recordings.

2. Methods

2.1. Animal models

All experiments were approved by the Animal Care Committee at the University of Toronto. *Gabra5*−/− mice were generated as described previously [30]. For all behavioral tests, 3 month-old age-matched male *Gabra5*−/− mice and WT mice were studied. The experimenters were blinded to the genotype and the drug treatment group. For electrophysiological recordings, primary cultures of hippocampal neurons were prepared, as described previously [38], from Swiss Webster mice (Charles River, Montreal, Canada) on embryonic day 18. Neurons were maintained in culture for 14 to 21 days prior to recording.

2.2. Contextual and delay (cued) fear conditioning

In a Pavlovian fear conditioning task, mice were exposed to a tone (the conditioning stimulus) and a foot shock (the unconditioned stimulus) in a novel conditioning context. The unconditioned stimulus was presented during the last 2 s of presentation of the conditioning stimulus [31]. The conditioning chamber consisted of a Perspex acrylic arena with a light mounted in the lid (dimensions of chamber 350 mm × 200 mm × 193 mm; Technical and Scientific Equipment, Midland, Michigan). The floor consisted of stainless steel bars (4 mm diameter, 5 mm apart) that were connected to a computer, which controlled the duration of the test session and the timing, intensity, and duration of the shock. On day 1, each mouse received an intraperitoneal (i.p.) injection of 0.5, 1.0 or 1.5 g/kg ethanol (20%, v/v) or vehicle (physiological saline). Ethanol was administered 10 min before placement of the mouse in the fear conditioning chamber. Each mouse was allowed to explore the chamber for 180 s, after which a 2800-Hz tone (the auditory conditioning stimulus) from a frequency generator, amplified to 70 dB and lasting 20 s, was presented 3 times, at 60-s intervals. The last 2 s of each auditory conditioning stimulus was paired with a 0.7-mA electrical shot to the foot. On day 2, 24 h after the conditioning session, the mice were returned to the chamber for assessment of freezing, defined as the lack of any movement except that required for respiration; the freezing response was assessed every 8 s for 8 min (a total of 60 observations). On day 3, the conditioning chamber was modified as follows: the metal grid floor was covered with ceramic tiles, the walls were covered with black and white stripes and the ceramic tiles were wiped with a vanilla-scented cloth. On the same day, at 48 h after the conditioning session, the mice were again placed in the conditioning chamber and were monitored (every 8 s) for baseline freezing to the modified context for 180 s. After this baseline period, the auditory tone was presented continuously for 300 s and the freezing response was recorded every 8 s. For the purpose of analyzing freezing scores over the course of the entire session, the data were combined into 1 min bins.

2.3. Open-field test

We tested the sedative properties of ethanol by measuring spontaneous activity in an open-field arena made of acrylic glass with the dimensions 42 cm × 42 cm × 30 cm. WT and *Gabra5*−/− mice were given injections of ethanol (0.5, 1.0 or 1.5 g/kg i.p.) or saline and were then returned to their home cage. All mice were tested 10 min after ethanol injection for 5 consecutive minutes. The duration of time spent walking, rearing and grooming was monitored as an index of spontaneous locomotor activity in the open field. To score the time spent walking, rearing and grooming, a trained examiner used an event recorder. The floor and walls of the test chamber were cleaned with an ethanol solution between subjects.

2.4. Electrophysiological recordings

The concentration-dependent effects of ethanol on the tonic $GABA_A$R current recorded from cultured hippocampal neurons were studied using the whole-cell patch–clamp technique (at 20–23 °C). Electrodes were made from borosilicate glass pipettes and were fire polished. Whole-cell current was recorded with a Multiclamp 700B amplifier and headstage (Molecular Devices, Union City, California) with low-pass filtering at 10 kHz before digitization (Digidata 1200 data acquisition system; Molecular Devices). Compensation for series resistance, pipette capacitance and whole-cell capacitance was achieved electronically. A hyperpolarizing voltage step of +10 mV was applied periodically throughout each experiment to monitor series resistance. Only cells that demonstrated a stable series resistance (<20% change) were used for data analysis. Cells were perfused with a solution containing the following (in mM): 140 NaCl, 2.0 KCl, 1.3 $CaCl_2$, 25 HEPES, and 28 glucose, pH 7.4. The sodium channel blocker tetrodotoxin (0.3 μM) and the ionotropic glutamate antagonists 6-cyano-7-nitro-quinoxaline-2,3-dione (10 μM) and 2-amino-4-phosphonovaleric acid (40 μM) were added to the extracellular solution. During all the experiments, potassium currents were suppressed by using a CsCl-based internal solution that contained the following (in mM): 120 CsCl, 2.0 $MgCl_2$, 1.0 $CaCl_2$, 11 EGTA, 30 HEPES, 2.0 MgATP, and 2.0 tetraethylammonium at pH 7.3. The amplitude of the tonic current, under control conditions, was measured as the difference in the holding current measured before and during the application of bicuculline methiodide (100 μM). Ethanol was applied at concentrations of 30, 100, 300 and 1000 mM, which correspond to blood-alcohol levels of 0.14% (sobriety-impairing), 0.46% (anesthetic, potentially lethal), 1.38% (lethal) and 4.60%, respectively. The highest concentration (1000 mM) was selected as a positive control, although this concentration is cytotoxic. All drugs were acutely applied to the neurons using a multi-barrel fast perfusion system.

2.5. Statistical analysis

For all data analysis, Statistical Package for the Social Sciences software, version 11.0 (SPSS Inc., Chicago, Illinois) and GraphPad Prism software, version 4.0 (GraphPad Software, San Diego, California) were used. The fear conditioning results were subjected to a three-way analysis of variance (genotype × ethanol dose × time). The open-field data were analyzed using a two-way ANOVA (genotype × ethanol dose). *Post hoc* analysis for any main effects and interactions consisted of Tukey Honestly Significant Difference test for fear conditioning and the Bonferroni post-test for the open-field experiments. The amplitudes of the tonic current were analyzed by one-way ANOVA with the Dunnet post-test. The significance level was set at $p < 0.05$.

3. Results

3.1. Contextual and delay fear conditioning

To determine whether the activity of α5$GABA_A$Rs influenced ethanol-induced memory impairment, the performance of WT and *Gabra5*−/− mice in contextual and delay fear conditioning was assessed. Fear conditioning was selected because low doses of ethanol have been shown to disrupt the consolidation of fear memories [35]. Also, neurodepressive drugs, such as etomidate, that inhibit memory performance in this paradigm, are thought to do so in part through actions on α5$GABA_A$Rs [23].

3.2. Day 1: baseline freezing performance

The extent of baseline freezing on the conditioning day was examined to determine whether ethanol impaired the acquisition of fear conditioning in an α5$GABA_A$R-dependent manner. This initial analysis is important for distinguishing whether ethanol impairs the learning of an event or the memory of the event. For all mice, freezing increased as a function of the number of shock trials, which indicated the occurrence of learning (effect of time,

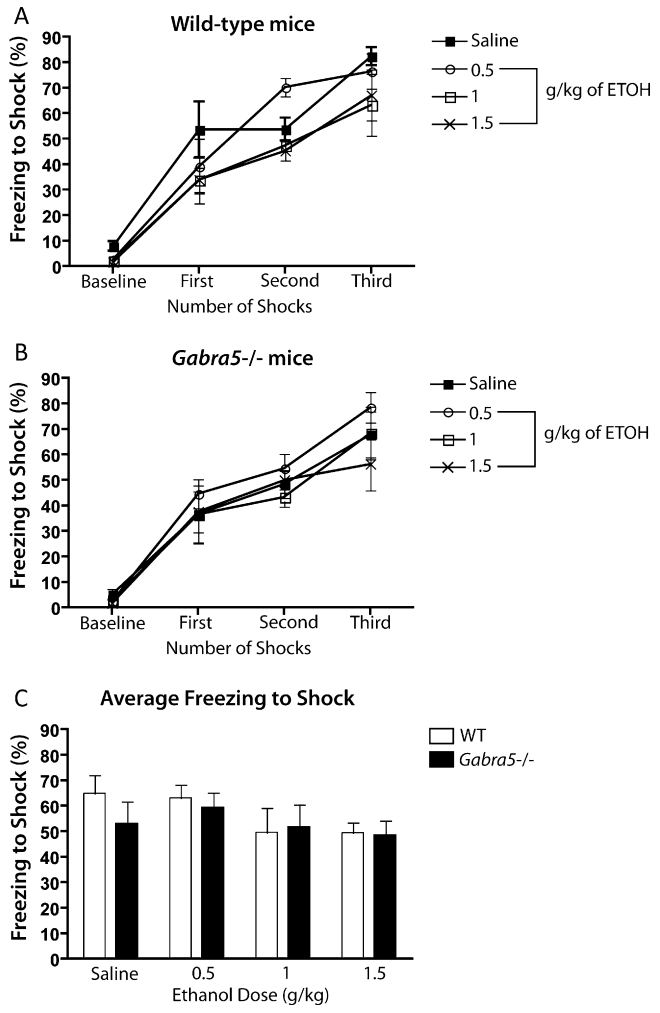

Fig. 1. Ethanol does not impair the acquisition of fear conditioning. Ethanol did not affect the percentage of time spent freezing during the baseline period (3 min) and freezing in response to the first, second and third foot shock (1 min time bins) in WT (A) and *Gabra5*−/− mice (B). (C) Post-shock data for the different doses of ethanol were further pooled and are represented as a bar chart for a direct comparison between the genotypes. Data are represented as mean ± SEM.

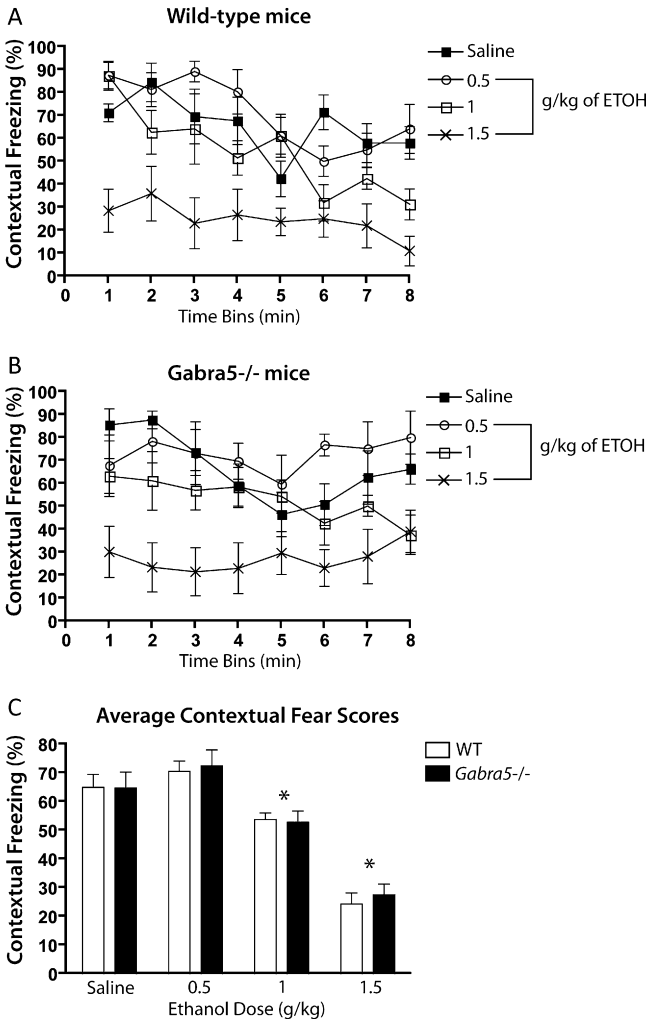

Fig. 2. Ethanol impairs contextual fear conditioning in WT and *Gabra5*−/− mice. The freezing scores of WT and *Gabra5*−/− mice are shown for each minute (1–8) during the contextual monitoring session. (A) The moderate dose of ethanol (1 g/kg) impaired performance of WT mice as the monitoring session progressed, as shown by a high freezing score during the first min and a low score during the last min ($p < 0.05$). The high dose of ethanol (1.5 g/kg) impaired the overall freezing performance of WT mice for the entire monitoring session. (B) Similar trends were observed for *Gabra5*−/− mice, with the moderate and high doses of ethanol impairing memory performance. (C) Contextual freezing data were pooled and are represented as a bar chart for a direct comparison between the genotypes. * denotes significantly different from saline-injected control mice ($p < 0.05$). Data are represented as mean ± SEM.

$F_{2,112} = 39.5$, $p < 0.001$; Fig. 1a and b). There were no interactions between genotype and the number of shock trials ($F_{2,112} = 0.188$, $p > 0.05$), the dose of ethanol and the number of shock-trials ($F_{6,112} = 0.501$, $p > 0.05$) or among the genotype, the dose of ethanol and the number of shock-trials ($F_{6,112} = 0.826$, $p > 0.05$). Together, these data indicate that the genotype and the dose of ethanol did not influence memory acquisition during the fear conditioning task. Overall baseline freezing performance did not differ between the genotypes (effect of genotype, $F_{1,56} = 0.175$, $p > 0.05$; Fig. 1) and was not influenced by the dose of ethanol (effect of ethanol dose, $F_{3,56} = 1.51$, $p > 0.05$; Fig. 1c). Furthermore, the interaction between genotype and ethanol was not significant, which indicates that ethanol did not affect baseline freezing irrespective of genotype (effect of genotype and dose interaction, $F_{3,56} = 2.02$, $p > 0.05$).

3.3. Day 2: contextual fear conditioning

The extent of freezing to contextual surroundings was examined to determine whether ethanol impaired hippocampus-dependent learning and memory processes via an α5GABA$_A$R-dependent mechanism. Over time, there was a general decrease in the level of contextual freezing in all groups (effect of time, $F_{7,392} = 6.19$, $p < 0.001$; Fig. 2a and b), which plateaued between 4 and 5 min. There were no differences in freezing scores between WT (Fig. 2a) and *Gabra5*−/− mice (effect of genotype, $F_{1,56} = 0.11$, $p > 0.05$; Fig. 2b). There was a significant effect of the ethanol dose on the overall percentage of time spent freezing (effect of ethanol dose, $F_{3,56} = 45.4$, $p < 0.0001$). The moderate (1.0 g/kg) and high (1.5 g/kg) doses of ethanol impaired contextual freezing to the same extent in both genotypes (Fig. 2c).

3.4. Day 3: delay (cued) fear conditioning

The conditioned fear response to an auditory stimulus (delay or cued fear conditioning) is highly dependent upon the amygdala [36]. We examined whether ethanol caused a differential sensitivity between genotypes for a memory behavior that depends on the amygdala, a brain region with a sparse distribution of α5GABA$_A$Rs [17].

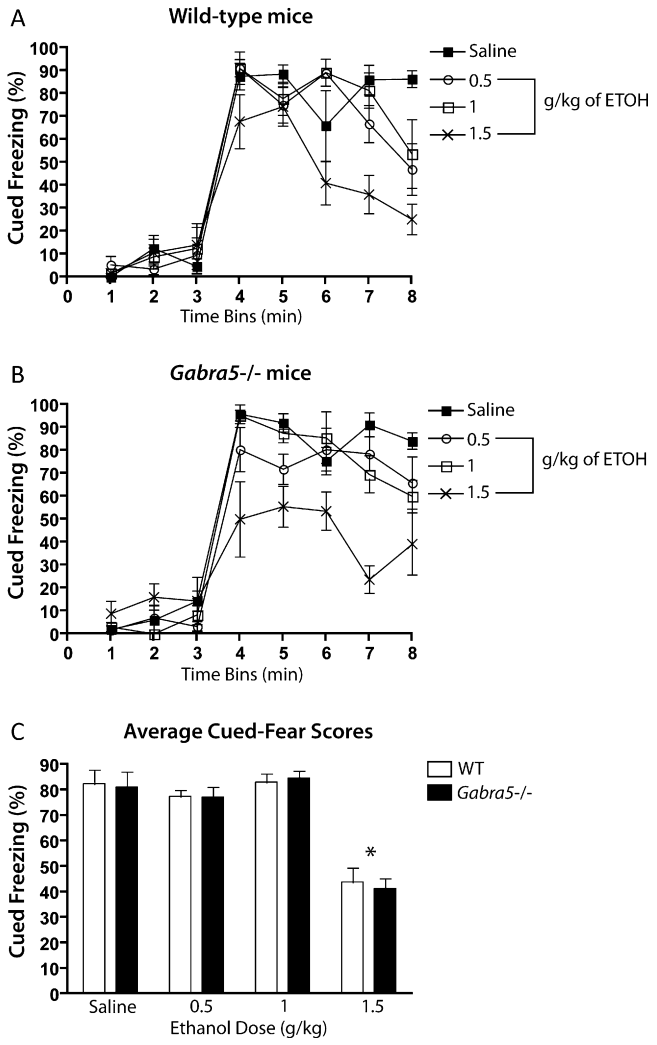

Fig. 3. Ethanol impairs delay/cued fear conditioning in WT and *Gabra5*−/− mice. The freezing scores of WT and *Gabra5*−/− mice are shown for each minute (1–8) during the monitoring session. Baseline freezing to the modified context was monitored for minutes 1–3 and the tone was subsequently presented continuously for minutes 4–8. (A) In WT mice, low baseline freezing scores were observed during the first 3 min. A dramatic increase in the freezing scores coincided with the onset of the auditory tone. The high dose of ethanol (1.5 g/kg) impaired the overall freezing performance of WT mice when the tone was presented. (B) A similar trend was observed for *Gabra5*−/− mice, with the high dose of ethanol impairing memory performance. (C) Cued freezing data were pooled and are represented as a bar chart for a direct comparison between the genotypes at the different doses of ethanol. * denotes significantly different from saline-injected control mice ($p < 0.05$). Data are represented as mean ± SEM.

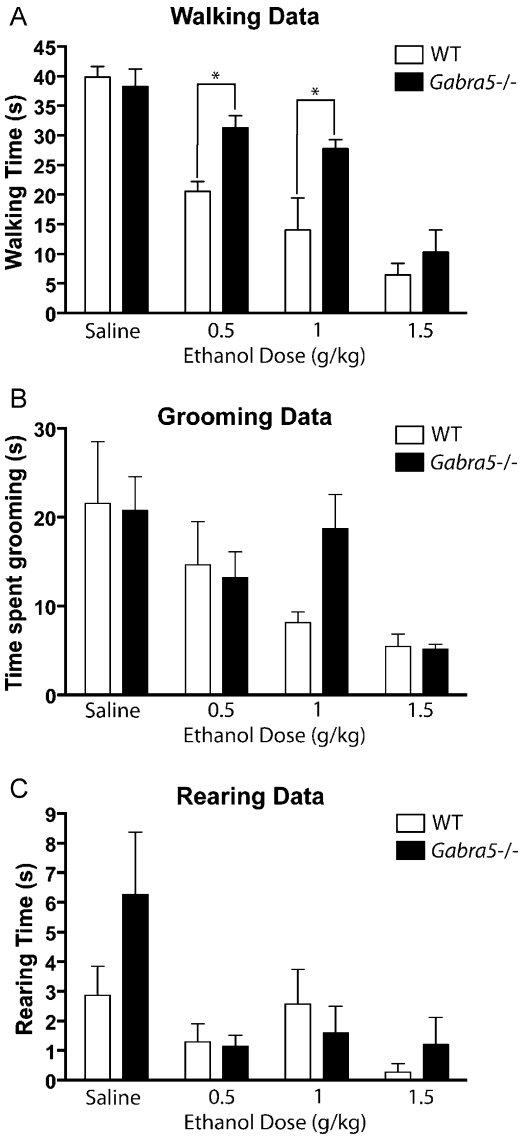

Fig. 4. *Gabra5*−/− mice are resistant to the sedative properties of ethanol. (A) Low (0.5 g/kg) and moderate (1.0 g/kg) doses of ethanol impaired spontaneous walking of WT mice in the open-field task. Ethanol, at the doses tested, had no significant effect on *Gabra5*−/− mice. In contrast, ethanol (1.5 g/kg dose) significantly impaired spontaneous walking in both genotypes. (B) All ethanol doses decreased the amount of time that WT and *Gabra5*−/− mice spent grooming in the open field. (C) All doses of ethanol decreased the amount of time that the WT and *Gabra5*−/− mice spent rearing. * denotes significantly different from saline-injected control mice ($p > 0.05$). Data are represented as mean ± SEM.

During the baseline assessment of freezing to the unconditioned, novel context, there were no differences between WT (Fig. 3a) and *Gabra5*−/− mice (effect of genotype, $F_{1,56} = 2.48$, $p > 0.05$) and no effect of the dose of ethanol (effect of ethanol dose, $F_{3,56} = 0.826$, $p > 0.05$; Fig. 3b). Freezing was greatest in all groups during the 5-min period coinciding with the onset of the conditioning stimulus tone (effect of time, $F_{7,392} = 164$, $p < 0.0001$). During the presentation of the conditioning stimulus (tone), there were no differences in freezing behavior between genotypes (effect of genotype, $F_{1,56} = 0.095$, $p > 0.05$). However, ethanol had a significant effect (effect of dose of ethanol, $F_{3,56} = 43.1$, $p < 0.0001$) but only at the high dose (1.5 g/kg) which reduced freezing in both genotypes (Tukey's HSD, $p < 0.001$; Fig. 3c). There was a decrease in freezing scores over the course of the 5-min exposure to the tone (effect of time, $F_{4,224} = 9.95$, $p < 0.0001$). There also was an interaction between time and dose of ethanol ($F_{12,224} = 2.24$, $p < 0.01$) resulting from a sharper decline in the freezing response over time in mice injected with 1.5 g/kg of ethanol (Tukey's HSD, $p < 0.001$). Fig. 3c summarizes the overall delay fear conditioning scores for the 5 min of testing with the tone presented.

3.5. Ethanol decreased locomotion in the open field test in an α5GABA$_A$R-dependent manner

To determine whether the impairment of fear conditioning by ethanol was confounded by immobility or sedation, the effect of ethanol on sedation was assessed in the open-field task. Ethanol decreased overall spontaneous locomotion and activity in both genotypes (two-way ANOVA, effect of genotype, $F_{3,56} = 61.2$, $p < 0.0001$; Fig. 4). Further analysis revealed an interaction between the genotype and the dose of ethanol ($F_{3,56} = 2.97$, $p < 0.05$), indicating that the two genotypes responded differently to the ethanol.

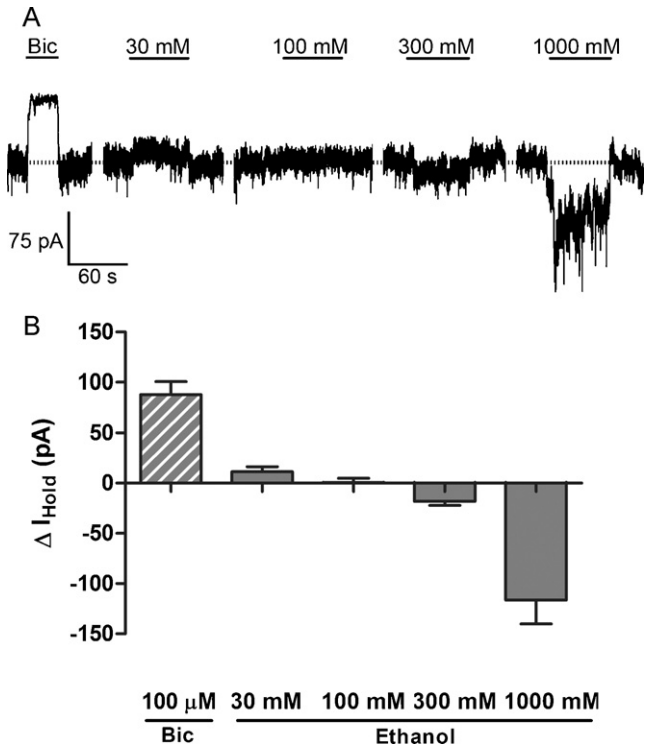

Fig. 5. Low clinically-relevant concentrations of ethanol do not potentiate a tonic inhibitory conductance in CA1 pyramidal neurons. (A) Current traces illustrate that low, moderate and high sobriety-impairing concentrations of ethanol do not potentiate a tonic conductance recorded in cultured hippocampal pyramidal neurons. (B) The change in holding current is presented for each concentration of ethanol. Only a lethal concentration of ethanol potentiated the tonic current (1 M). The number of cells for each concentration is represented above each bar and data are presented as mean ± SEM.

Interestingly, *Gabra5*−/− mice displayed a lower sensitivity than WT mice to ethanol at 0.5 g/kg ($p < 0.05$) and 1 g/kg ($p < 0.01$). Low (0.5 g/kg) and moderate (1.0 g/kg) doses of ethanol decreased the time spent walking in the open field in WT but not *Gabra5*−/− mice. In WT mice that received the low dose of ethanol, the duration of walking was 9 s (28%) less than the duration of walking for *Gabra5*−/− mice; with the higher dose, the duration of walking was 14 s (50%) less for WT mice. There were no differences between the genotypes when mice were injected with 1.5 g/kg ($p > 0.05$) ethanol (Fig. 4a).

Ethanol also reduced grooming ($F_{3,56} = 26.8$, $p < 0.001$) and rearing scores ($F_{3,56} = 20.1$, $p < 0.01$) in a similar, dose-dependent manner in the 2 genotypes (Fig. 4b and c).

3.6. Ethanol does not potentiate a GABAergic tonic conductance in cultured hippocampal pyramidal neurons

The effect of ethanol on a tonic inhibitory conductance was examined in cultured hippocampal pyramidal neurons. In these neurons, the tonic current is generated predominantly by α5GABA$_A$Rs [21]. The amplitude of the tonic current was determined by blocking GABA$_A$R activity with the GABA$_A$R antagonist, bicuculline methiodide (100 μM). This agent produced a reduction in the holding current as indicated by the outward shift in baseline. This change in holding current resulted from the inhibition of a large, persistent, inward GABA$_A$R current (88.1 pA ± 12.5 pA, $n = 7$, $p < 0.001$ versus baseline control holding current by Student's t test; Fig. 5). Ethanol applied to the neurons at 30, 100, 300 and 1000 mM produced an inward current which increased significantly only at the highest concentration tested (1000 mM) (Dunnett's post-test, $p < 0.001$) (one-way ANOVA, effect of ethanol, $F_{4,16} = 48.4$, $p < 0.0001$; Fig. 5). Notably, ethanol at 30 mM ($n = 5$), 100 mM ($n = 5$) and 300 mM ($n = 4$) ethanol did not significantly enhance the tonic current. Given that physiologically-relevant concentrations of ethanol did not enhance the tonic current in WT neurons and given that the amplitude of the tonic current is greatly reduced in *Gabra5*−/− neurons than in WT neurons [21], we did not examine the effect of ethanol on *Gabra5*−/− neurons. Together these data show that the tonic current in hippocampal pyramidal neurons is insensitive to low, sobriety-impairing concentrations of ethanol.

4. Discussion

Ethanol impaired contextual and delay fear conditioning in WT and *Gabra5*−/− mice to the same extent, suggesting that the memory-impairing properties of this agent are not mediated via the enhanced activity of α5GABA$_A$Rs. Interestingly, the total time spent walking in the open field was reduced by ethanol in WT but not *Gabra5*−/− mice. Additionally, at sobriety-impairing concentrations, ethanol did not enhance the tonic conductance in hippocampal pyramidal neurons. Together, the results of the current study suggest that α5GABA$_A$Rs do not contribute to the memory-impairment but may partially mediate the sedative properties of ethanol.

The fear-conditioning paradigm was chosen to study the role of α5GABA$_A$Rs in ethanol-induced memory impairment because the dorsal hippocampus has a high expression of α5GABA$_A$Rs [17] and is involved in contextual fear conditioning. In contrast, the expression of α5GABA$_A$Rs is low in the amygdala [17]. Therefore, as a control, we used delay fear conditioning, which requires the basal lateral nucleus of the amygdala [32]. The doses of ethanol used in the current study were carefully selected based on previous studies. The 0.5 g/kg dose of ethanol produced a blood alcohol concentration of 0.05%, which is considered non-impairing [37]; the 1.0 g/kg dose produced a blood alcohol concentration of 0.11%, which causes moderate impairment; and the 1.5 g/kg dose produced a blood alcohol concentration of 0.16%, which is twice the legal driving limit in the United States and is above the minimum concentration that has been shown to induce anterograde amnesia [33,34].

The acute injection of ethanol at moderate (1 g/kg) and high (1.5 g/kg) doses, but not at the low dose (0.5 g/kg) reduced contextual fear memory in both genotypes. This reduction in freezing scores was not the result of impaired acquisition during training by ethanol as all mice exhibited a progressive increase in freezing scores as the number of tone-shock pairings increased (Fig. 1a and b). The dose-dependent decrease in fear conditioning that we observed was similar to that described in previous studies, in which fear conditioning in rats and mice was impaired after administration of 1.0 or 1.5 g/kg of ethanol but not after administration of 0.5 g/kg [37,38]. In our study, moderate and high doses of ethanol reduced contextual fear conditioning whereas only the high dose impaired delay fear conditioning. Contextual fear conditioning, which relies on hippocampal function, appears to be more sensitive to the memory-blocking effects of ethanol than is delay fear conditioning.

We were surprised that the impairment of hippocampus-dependent contextual fear memory by ethanol did not depend on the expression of α5GABA$_A$Rs. Others have shown that in human subjects the α5GABA$_A$R-selective negative modulator α5IA, attenuated the memory-impairing effect of ethanol on a word-learning task [28]. The results of our study suggest that in the study by Nutt et al. [28], α5IA did not directly reverse the effects of ethanol on α5GABA$_A$Rs, but rather improved memory performance through an indirect mechanism. Additionally, the discrepancy between our

findings and the study in humans by Nutt et al. [28] may be attributable to differences in the effects of ethanol between human and rodent models or the types of memory that were studied. Furthermore, α5IA may have improved memory performance through non-selective actions since the concentration at which α5IA is selective for α5GABA$_A$Rs in humans is unknown.

Our results, along with those of others [29] suggest that α5GABA$_A$Rs are important for ethanol-induced sedation. We found that low and moderate doses of ethanol reduced spontaneous walking in the open-field assay in WT mice but not *Gabra5*−/− mice. Similarly, McKay et al. [29] previously demonstrated that RY 024, a α5GABA$_A$R-selective negative modulator, reversed the motor-impairing and sedative effects of ethanol in the absence of any baseline effect on behavior studied in the absence of ethanol.

The contribution of α5GABA$_A$Rs to sedation but not memory directly contrasts the properties of the anesthetic etomidate, which causes memory loss but not sedation via α5GABA$_A$R-dependent mechanisms [23]. A possibility is that ethanol reduces spontaneous movement in the open field by acting at the spinal cord as α5GABA$_A$Rs are highly expressed in the ventral horn [39].

Other populations of GABA$_A$Rs have also been shown to contribute to ethanol-induced sedation. For example, null mutant mice that lack the α1 subunit are resistant to the hypnotic actions of ethanol as compared to wild-type controls, these mice demonstrated increased locomotion in their home cage after receiving moderate to high doses of ethanol [40]. The α2 subunit has also been implicated in the sedative properties of ethanol as α2 null mutant mice showed resistance to ethanol-induced loss of the righting reflex [7]. Mice expressing a naturally occurring single-nucleotide polymorphism in the α6 subunit of the GABA$_A$R were also more sensitive to ethanol and exhibited a greater impairment in motor coordination on the rotarod test [41].

Our *in vitro* studies showed that low, moderate and high concentrations of ethanol did not enhance the tonic current in hippocampal pyramidal neurons (Fig. 5). In the hippocampus, a large proportion of the tonic conductance in CA1 and CA3 pyramidal neurons is generated by α5GABA$_A$Rs [18,21], whereas a smaller amplitude current has been attributed to δGABA$_A$Rs [19]. Others have reported that the function of δGABA$_A$Rs is up-regulated by low concentrations of ethanol [42]. The application of ethanol within the 10 mM to 30 mM range was shown to enhance the δGABA$_A$R tonic inhibitory conductance in cerebellar granule cells [41], dentate gyrus granule cells [24], hippocampal interneurons [19], and thalamic relay neurons [26]. Although the specific behavioral effects of ethanol that are mediated by δGABA$_A$Rs are unknown, α4 null mutant mice, which exhibit a reduced expression of δGABA$_A$Rs in the dentate gyrus, have normal behavioral responses to ethanol [43].

We observed that ethanol did not potentiate the tonic inhibitory current in hippocampal pyramidal neurons, suggesting appears that ethanol does not enhance either the α5GABA$_A$R- or the δGABA$_A$R-generated tonic conductance in cultured hippocampal pyramidal neurons. Given that the expression of δGABA$_A$R in pyramidal neurons is low, the lack of ethanol-induced enhancement of δGABA$_A$R-mediated tonic current was not surprising. However, the results of this study clearly demonstrate that the α5GABA$_A$R-mediated tonic inhibition in pyramidal neurons is not enhanced by ethanol.

The results reported here do not exclude the possibility that ethanol enhances an α5GABA$_A$R conductance in different neuronal populations in other regions of the brain. The differential effects of ethanol on α5GABA$_A$R activity in various brain regions may account for the absence of sedation after the administration of a low dose of ethanol observed in *Gabra5*−/− mice. The ability of ethanol to enhance an α5GABA$_A$R conductance in neuronal populations outside the hippocampus may depend on the expression of different cytosolic factors in different cell types, such as protein kinase C [44].

In summary, the current data show that ethanol does not impair fear conditioning by enhancing a tonic conductance in hippocampal pyramidal neurons and suggest that the role of α5GABA$_A$Rs in ethanol pharmacology relates mostly to ethanol-induced sedation, as evidenced by a reduction in locomotion.

Acknowledgements

This research was supported by grants from the Canadian Institutes of Health Research (to B.A.O.) and a Canada Research Chair Award (to B.A.O). L.J.M. was supported by a Doctoral Canada Graduate studentship from the Canadian Institutes of Health Research, A.A.Z. was supported by a Master's Canada Graduate Studentship from the National Sciences and Engineering Research Council and R.P.B. was supported by a Doctoral Postgraduate Studentship from the National Sciences and Engineering Research Council.

References

[1] White AM. What happened? Alcohol, memory blackouts, and the brain. Alcohol Res Health 2003;27(2):186–96.
[2] Nelson EC, Heath AC, Bucholz KK, Madden PAF, Fu Q Knopik V, Lynskey MT, et al. Genetic epidemiology of alcohol-induced blackouts. Arch Gen Psychiatry 2004;61(3):257–63.
[3] Crews FT, Morrow AL, Criswell H, Breese G. Effects of ethanol on ion channels. Int Rev Neurobiol 1996;39:283–367.
[4] Morrisett RA, Swartzwelder HS. Attenuation of hippocampal long-term potentiation by ethanol—a patch–clamp analysis of glutamatergic and gabaergic mechanisms. J Neurosci 1993;13(5):2264–72.
[5] Shimizu K, Matsubara K, Uezono T, Kimura K, Shiono H. Reduced dorsal hippocampal glutamate release significantly correlates with the spatial memory deficits produced by benzodiazepines and ethanol. Neuroscience 1998;83(3):701–6.
[6] Ron D. Signaling cascades regulating NMDA receptor sensitivity to ethanol. Neuroscientist 2004;10(4):325–36.
[7] Boehm SL, Ponomarev I, Jennings AW, Whiting PJ, Rosahl TW, Garrett EM, et al. γ-Aminobutyric acid A receptor subunit mutant mice: new perspectives on alcohol actions. Biochem Pharmacol 2004;68(8):1581–602.
[8] Mihic SJ. Acute effects of ethanol on GABA$_A$ and glycine receptor function. Neurochem Int 1999;35(2):115–23.
[9] Costa E. From GABA$_A$ receptor diversity emerges a unified vision of GABAergic inhibition. Annu Rev Pharmacol Toxicol 1998;38:321–50.
[10] Mohler H, Fritschy JM, Luscher B, Rudolph U, Benson J, Benke D. The GABA$_A$ receptors from subunits to diverse functions. Ion Channels 1996;4:89–113.
[11] Christie SB, De Blas AL. α5 Subunit-containing GABA$_A$ receptors form clusters at GABAergic synapses in hippocampal cultures. Neuroreport 2002;13(17):2355–8.
[12] Christie SB, Miralles CP, De Blas AL. GABAergic innervation organizes synaptic and extrasynaptic GABAA receptor clustering in cultured hippocampal neurons. J Neurosci 2002;22(3):684–97.
[13] Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. GABA$_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 2000;101(4):815–50.
[14] Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, et al. International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. Pharmacol Rev 1998;50(2):291–313.
[15] Harris RA, Mihic SJ, Dildy-Mayfield JE, Machu TK. Actions of anesthetics on ligand-gated ion channels: role of receptor subunit composition. FASEB J 1995;9(14):1454–62.
[16] Haas KF, Macdonald RL. GABAA receptor subunit gamma2 and delta subtypes confer unique kinetic properties on recombinant GABAA receptor currents in mouse fibroblasts. J Physiol (Lond) 1999;514(1):27–45.
[17] Sur C, Fresu L, Howell O, McKernan RM, Atack JR. Autoradiographic localization of α5 subunit-containing GABA$_A$ receptors in rat brain. Brain Res 1999;822(1–2):265–70.
[18] Glykys J, Mann EO, Mody I. Which GABA$_A$ receptor subunits are necessary for tonic inhibition in the hippocampus. J Neurosci 2008;28(6):1421–6.
[19] Glykys J, Mody I. Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABA$_A$ receptor α5 subunit-deficient mice. J Neurophysiol 2006;95(5):2796–807.
[20] Scimemi A, Semyanov A, Sperk G, Kullmann DM, Walker MC. Multiple and plastic receptors mediate tonic GABA$_A$ receptor currents in the hippocampus. J Neurosci 2005;25(43):10016–24.
[21] Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, et al. Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. Proc Natl Acad Sci USA 2004;101(10):3662–7.

[22] Martin LJ, Oh GH, Orser BA. Etomidate targets α5 $GABA_A$ receptors to regulate synaptic plasticity and memory blockade. Anesthesiology 2009;111(5):1025–35.
[23] Cheng VY, Martin LJ, Elliott EM, Kim JH, Mount HT, Orser BA. α5$GABA_A$ receptors mediate the amnestic but not sedative-hypnotic effects of the general anesthetic etomidate. J Neurosci 2006;26(14):3713–20.
[24] Wei W, Faria LC, Mody I. Low ethanol concentrations selectively augment the tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors in hippocampal neurons. J Neurosci 2004;24(38):8379–82.
[25] Yamashita M, Marszalec W, Yeh JZ, Narahashi T. Effects of ethanol on tonic GABA currents in cerebellar granule cells and mammalian cells recombinantly expressing $GABA_A$ receptors. J Pharmacol Exp Ther 2006;319(1):431–8.
[26] Jia F, Pignataro L, Harrison NL. $GABA_A$ receptors in the thalamus: α4 subunit expression and alcohol sensitivity. Alcohol 2007;41(3):177–85.
[27] Martin LJ, Zurek AA, MacDonald JF, Roder JC, Jackson MF, Orser BA. alpha 5GABA(A) receptor activity sets the threshold for long-term potentiation and constrains hippocampus-dependent memory. J Neurosci 2010;30(15):5269–82.
[28] Nutt DJ, Besson M, Wilson SJ, Dawson GR, Lingford-Hughes AR. Blockade of alcohol's amnestic activity in humans by an α5 subtype benzodiazepine receptor inverse agonist. Neuropharmacology 2007;53(7):810–20.
[29] McKay PF, Foster KL, Mason D, Cummings R, Garcia M, Williams LS, et al. A high affinity ligand for $GABA_A$-receptor containing α5 subunit antagonizes ethanol's neurobehavioral effects in Long-Evans rats. Psychopharmacology (Berl) 2004;172(4):455–62.
[30] Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Smith A, et al. Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the α5 subunit of the $GABA_A$ receptor. J Neurosci 2002;22(13):5572–80.
[31] Fanselow MS. Conditioned and unconditional components of post-shock freezing. Pavlov J Biol Sci 1980;15(4):177–82.
[32] Sigurdsson T, Doyere V, Cain CK, LeDoux JE. Long-term potentiation in the amygdala: a cellular mechanism of fear learning and memory. Neuropharmacology 2007;52(1):215–27.
[33] White AM, Best PJ. Effects of ethanol on hippocampal place-cell and interneuron activity. Brain Res 2000;876(1–2):154–65.
[34] Goodwin DW, Othmer E, Halikas JA, Freemon F. Loss of short term memory as a predictor of alcoholic blackout. Nature 1970;227(5254):201–7.
[35] Lattal KM. Effects of ethanol on encoding, consolidation, and expression of extinction following contextual fear conditioning. Behav Neurosci 2007;121(6):1280–92.
[36] Maren S. Neurobiology of Pavlovian fear conditioning. Annu Rev Neurosci 2001;24:897–931.
[37] Gould TJ. Ethanol disrupts fear conditioning in C57BL/6 mice. J Psychopharmacol 2003;17(1):77–81.
[38] Melia KR, Ryabinin AE, Corodimas KP, Wilson MC, Ledoux JE. Hippocampal-dependent learning and experience-dependent activation of the hippocampus are preferentially disrupted by ethanol. Neuroscience 1996;74(2):313–22.
[39] Ruano D, Letang V, Biton B, Avenet P, Benavides J, Scatton B, et al. Subunit composition of rat ventral spinal cord $GABA_A$ receptors, assessed by single cell RT-multiplex PCR. Neuroreport 2000;11(14):3169–73.
[40] Blednov YA, Walker D, Alva H, Creech K, Findlay G, Harris R. $GABA_A$ receptor α1 and β2 subunit null mutant mice: behavioral responses to ethanol. J Pharmacol Exp Ther 2003;305(3):854–63.
[41] Hanchar HJ, Dodson PD, Olsen RW, Otis TS, Wallner M. Alcohol-induced motor impairment caused by increased extrasynaptic $GABA_A$ receptor activity. Nat Neurosci 2005;8(3):339–45.
[42] Santhakumar V, Wallner M, Otis TS. Ethanol acts directly on extrasynaptic subtypes of $GABA_A$ receptors to increase tonic inhibition. Alcohol 2007;41(3):211–21.
[43] Chandra D, Werner DF, Liang J, Suryanarayanan A, Harrison NL, Spigelman I, et al. Normal acute behavioral responses to moderate/high dose ethanol in $GABA_A$ receptor α4 subunit knockout mice. Alcohol Clin Exp Res 2008;32(1):10–8.
[44] Choi DS, Wei WZ, Deitchman JK, Kharazia VN, Lesscher HMB, McMahon T, et al. Protein kinase C delta regulates ethanol intoxication and enhancement of GABA-stimulated tonic Current. J Neurosci 2008;28(46):11890–9.