

RESEARCH ARTICLE

Alcohol Withdrawal-Induced Seizure Susceptibility is Associated with an Upregulation of Ca_v1.3 Channels in the Rat Inferior Colliculus

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Abstract

Background: We previously reported increased current density through L-type voltage-gated Ca²⁺ (Ca_v1) channels in inferior colliculus (IC) neurons during alcohol withdrawal. However, the molecular correlate of this increased Ca_v1 current is currently unknown.

Methods: Rats received three daily doses of ethanol every 8 hours for 4 consecutive days; control rats received vehicle. The IC was dissected at various time intervals following alcohol withdrawal, and the mRNA and protein levels of the Ca_v1.3 and Ca_v1.2 α 1 subunits were measured. In separate experiments, rats were tested for their susceptibility to alcohol withdrawal-induced seizures (AWS) 3, 24, and 48 hours after alcohol withdrawal.

Results: In the alcohol-treated group, AWS were observed 24 hours after withdrawal; no seizures were observed at 3 or 48 hours. No seizures were observed at any time in the control-treated rats. Compared to control-treated rats, the mRNA level of the Ca_v1.3 α 1 subunit was increased 1.4-fold, 1.9-fold, and 1.3-fold at 3, 24, and 48 hours, respectively. In contrast, the mRNA level of the Ca_v1.2 α 1 subunit increased 1.5-fold and 1.4-fold at 24 and 48 hours, respectively. At 24 hours, Western blot analyses revealed that the levels of the Ca_v1.3 and Ca_v1.2 α 1 subunits increased by 52% and 32%, respectively, 24 hours after alcohol withdrawal. In contrast, the Ca_v1.2 and Ca_v1.3 α 1 subunits were not altered at either 3 or 48 hours during alcohol withdrawal.

Conclusions: Expression of the Ca_v1.3 α 1 subunit increased in parallel with AWS development, suggesting that altered L-type Ca_v1.3 channel expression is an important feature of AWS pathogenesis.

Keywords: alcohol withdrawal seizures, *Cacna1d* mRNA, *Cacna1c* mRNA, Ca_v1.2 α 1 subunit, Ca_v1.3 α 1 subunit

Introduction

Neuronal hyperexcitability following the cessation of chronic alcohol intake (i.e. alcohol withdrawal) often increases susceptibility to generalized tonic-clonic seizures. The inferior colliculus (IC) plays a central role in auditory information processing. In

rodents, the IC is critically involved in the initiation of acoustically-evoked reflex seizures (i.e. audiogenic seizures, or AGSs) during alcohol withdrawal (Frye et al., 1983; Eckardt et al., 1986; McCown and Breese, 1990, 1993; Faingold and Riaz, 1995;

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Chakravarty and Faingold, 1998). Electrophysiology studies revealed increased firing of IC neurons both prior to and during AGSs in rats subjected to alcohol withdrawal (Faingold and Riaz, 1995; Chakravarty and Faingold, 1998). Similarly, *in vitro* studies revealed elevated excitability of IC neurons following alcohol withdrawal (N'Gouemo et al., 1996; Evans et al., 2000; Faingold et al., 2000). These functional findings support the importance of IC neurons in the networks that underlie alcohol withdrawal-induced seizures (AWSs; Faingold et al., 1998).

Voltage-gated Ca^{2+} (Ca_v) channels play an important role in the mechanisms that underlie the neuronal hyperexcitability that leads to AWSs (Whittington et al., 1993). For example, blocking L-type Ca_v channels (i.e. the Ca_v1 family of Ca^{2+} channels) suppressed AGSs in rats subjected to alcohol withdrawal (Little et al., 1986). These findings suggest that remodeling of Ca_v1 channels—at least in the IC—is a key factor associated with AGS initiation during alcohol withdrawal. Consistent with this hypothesis, we found that the current density of Ca_v1 channels was elevated in IC neurons of rats subjected to alcohol withdrawal, and this increased current was associated with enhanced seizure susceptibility (N'Gouemo and Morad, 2003).

In the mammalian brain, Ca_v1 channels are comprised of two main isoforms, $\text{Ca}_v1.3$ and $\text{Ca}_v1.2$ channels; however, which isoform accounts for the increased current density in IC neurons during alcohol withdrawal is currently unknown. We previously reported that the increase in current density through Ca_v1 channels observed 24 hours after alcohol withdrawal (i.e. when AWS susceptibility peaks) follows a parallel time course with the occurrence of seizure susceptibility; moreover, by 48 hours after withdrawal (when the animal is no longer susceptible to AWS), Ca_v1 currents return to control levels (N'Gouemo and Morad, 2003). A temporal study of the onset of AGS found that seizure susceptibility is absent prior to the 7th hour of alcohol withdrawal, but progressively increases thereafter; thus, susceptibility peaks at 24 hours and is absent again at 48 hours (Faingold, 2008). Because alcohol withdrawal preferentially affects Ca_v1 channels, we examined whether the expression of $\text{Ca}_v1.3$ and/or $\text{Ca}_v1.2$ L-type channels is altered in IC neurons following alcohol withdrawal and whether these increases are correlated with the onset of AWS susceptibility.

Methods

Animals

Male Sprague-Dawley rats (250–300 g; Taconic) were used for these experiments. The animals were maintained in a temperature- and humidity-controlled room on a 12 hr light/dark cycle with food and water available *ad libitum*. All efforts were made to minimize the number of animals used in these experiments. All experimental procedures were approved by the Georgetown University Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Ethanol Administration

Ethanol (from a 95% stock solution) was administered by intragastric intubation in a 30% (v/v) solution in ISOMIL Infant Formula Concentrate (the ISOMIL formula was diluted 1:1 with water). Ethanol was administered as three daily doses delivered every 8 hours for 4 days, and the level of intoxication was evaluated using a standard behavior rating scale (N'Gouemo et al., 2006; Faingold, 2008). The first dose of ethanol was 5 g/kg body

weight, and subsequent doses were adjusted for each animal in order to achieve a moderate (i.e. accentuated staggering gait and considerable elevation of the pelvis) but not severe (i.e. lethargy without pelvic or abdominal elevation) degree of ataxia (Faingold, 2008); the amount of ethanol administered daily ranged from 9 to 15 g/kg. On the fourth day, ethanol treatment was terminated after the second dose. The behavioral signs of ethanol withdrawal include hyperactivity, tremors, tail spasticity, spontaneous seizures (myoclonus and forelimb clonic seizures), and acoustically-evoked startle response and seizures (N'Gouemo et al., 2006; Faingold, 2008). The control-treated animals were maintained under similar conditions but were given the ISOMIL formula without ethanol. Weight loss and mortality were negligible in both the ethanol-treated and control-treated groups.

Seizure Testing

To determine susceptibility to develop AGS, two animals from each group were randomly selected and tested for seizures 2–4 hours (the 3-hour group), 22–24 hours (the 24-hour group), and 46–48 hours (the 48-hour group) after ethanol withdrawal. The acoustic stimulus consisted of 100–110 decibel sound pressure level pure tones (Med Associates) or mixed sounds (delivered via an electrical bell) that were presented until seizure was elicited (or for 60 sec if no seizure activity was observed), as described previously (Riaz and Faingold, 1994). Animals that did not exhibit a seizure during the first trial were tested again two hours later. The animals used in the molecular studies were not subjected to acoustically-evoked seizure testing because evoked seizures can induce a long-lasting increase in extracellular GABA levels (Ueda and Tsuru, 1995), which in turn can alter the expression of Ca_v1 channels. In addition, using animals that were not exposed to stimulus-induced seizures prevented the effects of seizure severity and/or duration on the expression of Ca_v1 channels.

Blood Ethanol Levels

In a separate set of experiments, blood ethanol concentration (BEC) was measured in the control group and in the ethanol-treated group 3, 24, and 48 hours during ethanol withdrawal. The rats were anesthetized (50 mg/kg Nembutal; i.p.), and blood was extracted by intracardiac sampling using a 21-gauge needle. Serum ethanol concentration was measured using an Analox model GM7 analyzer (Analox Instruments).

Western Blot Analysis

At 3, 24, and 48 hours during ethanol withdrawal, the animals were deeply anesthetized with pentobarbital (100 mg/kg; i.p.), and the colliculi were immediately dissected and stored at -80°C until use. Tissue homogenates from each animal were lysed in 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 1% octylphenoxypolyethoxyethanol (Sigma-Aldrich), 10% glycerol, 1 mM ethylenediaminetetraacetic acid, and 1 mM Na_3VO_4 . The homogenates were cleared by centrifugation ($13\,800 \times g$, 4°C , 30 min). The supernatants were transferred to sterile microtubes and stored at -80°C until use. Protein concentration in the supernatants was measured using the bicinchoninic acid assay and a Bio-Rad Model 680 spectrophotometer. For each sample, 60 μg of total protein was separated by electrophoresis in a 7.5% sodium dodecyl sulfate-polyacrylamide gel and electro-transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences) for one hour and then probed overnight at 4°C with primary rabbit antibodies against either the $\alpha 1\text{D}$ subunit

(CaV1.3- α 1; 1:200; Alomone Labs) or the α 1C subunit (CaV1.2- α 1; 1:200; Alomone Labs). The membranes were also incubated with anti-glyceraldehyde 3-phosphate dehydrogenase antibody (1:10 000; Thermo Fisher Scientific) overnight at 4°C as an internal control. The membranes were probed with goat anti-mouse IRDye800 (1:10 000; LI-COR Biosciences) and goat anti-rabbit IR-Dye680 (1:10 000; LI-COR Biosciences) for 1 hour at room temperature, then scanned using an Odyssey Fc imager (LI-COR Biosciences).

RNA Isolation and Quantitative Real-Time PCR

To quantify the mRNA levels of the Ca_v1.3 and Ca_v1.2 α 1 subunits in the IC, rats were subjected to the 4-day ethanol intoxication paradigm (or control treatment), then anesthetized 3, 24, and 48 hours after ethanol withdrawal. The brains were removed, and the IC was immediately dissected and stored at -80°C until use. Total RNA was extracted from the IC using the RNeasy lipid tissue mini kit in accordance with the manufacturer's instructions (Qiagen). RNA concentration was measured using an Eppendorf Biophotometer, and the samples were stored at -20°C. Complementary DNA was synthesized from 1 μ g total RNA by reverse transcription using the Quantitect reverse transcription kit (Qiagen) and stored at -20°C. For each gene of interest, real-time polymerase chain reactions (PCRs) were performed in duplicate using the TaqMan fast universal PCR master kit (Applied Biosystems) in a StepOnePlus real-time PCR machine system (Applied Biosystems). The specific primers were as follows: CaV1.3 α 1 subunit (rat *Cacna1d*); Rn.PT.49a.11686797.g, GenBank accession no. NM_017298 and CaV1.2 α 1 subunit (rat *Cacna1c*); and Rn.PT.49a.9591539.g, GenBank accession no. NM_012517 (Integrated DNA Technologies). The amplification efficiencies of *Cacna1d* and *Cacna1c* were determined relative to a β -actin internal control using cycle conditions of denaturation at 95°C for 12 sec followed by 40 cycles of 95°C for 1 sec and 60°C for 12 sec. After amplification, a crossing threshold (C_t) value (representing the number of cycles required to reach a specific level of fluorescence) was determined for each sample.

Data Analysis

The protein levels of the Ca_v1.3 α 1 and Ca_v1.2 subunits were measured using densitometry of the corresponding bands (Odyssey Image Studio v3.2 software, LI-COR Biosciences). The integrated intensity of each band was normalized to the intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The mean of the control values was calculated, and the normalized intensity value of each control and alcohol-treated sample was expressed as a ratio relative to the mean control value. Fold change in mRNA expression was calculated using StepOne software with the equation $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ is the relative change of a given mRNA (Livak and Schmittgen, 2001). To obtain the $2^{-\Delta\Delta C_t}$ value for each sample, the C_t value for *Cacna1c* and *Cacna1d* mRNA was first normalized to β -actin, and the resulting ΔC_t was calculated as follows: $[\Delta C_t]_{Cacna1d} = C_{T,Cacna1d} - C_{T,\beta-actin}$ for *Cacna1d* (Ca_v1.3) and $[\Delta C_t]_{Cacna1c} = C_{T,Cacna1c} - C_{T,\beta-actin}$ for *Cacna1c* (Ca_v1.2). Changes in *Cacna1d* and *Cacna1c* mRNA during alcohol withdrawal were calculated relative to the control group using the following equation: $[\Delta\Delta C_t]_{Cacna1d} = (C_{T,Cacna1d} - C_{T,\beta-actin})_{ethanol withdrawal} - (C_{T,Cacna1d} - C_{T,\beta-actin})_{control group}$ or $[\Delta\Delta C_t]_{Cacna1c} = (C_{T,Cacna1c} - C_{T,\beta-actin})_{ethanol withdrawal} - (C_{T,Cacna1c} - C_{T,\beta-actin})_{control group}$. The final mRNA levels were determined by calculating $2^{-\Delta\Delta C_t}$. The prevalence of each type of seizure was analyzed using the chi-square (χ^2) test. To evaluate differences in the protein and mRNA levels, one-way analyses of variance (ANOVA; F) followed by a Bonferroni

or Student-Newman-keuls post hoc test were performed. Differences were considered significant at $p < 0.05$, and the summary data are presented as the mean \pm standard error of the mean or as a percentage (for the prevalence of wild running seizures [WRSs] and tonic-clonic seizures [clonus]).

Results

Ethanol Withdrawal Enhances the Susceptibility to Seizures

Figure 1 summarizes the susceptibility of each group to develop AGSs following control treatment or at 3, 24, and 48 hours during ethanol withdrawal. AGSs consisted of jumping and/or WRSs, which occasionally evolved into bouncing generalized clonus; 24 hours after ethanol withdrawal, these seizure types were observed in 100% ($\chi^2 = 196$, $p < 0.001$ vs. control) and 60% ($\chi^2 = 83$, $p < 0.001$ vs. control) of ethanol-treated rats, respectively. This susceptibility to AGS was transient, as these seizures were observed only in the 24-hour group, and not in the 3-hour or 48-hour group (Figure 1). No seizures were observed in the control group. ANOVA revealed that the BEC group means were significantly different ($F = 40$, $p < 0.001$). The BECs were significantly ($t = 9$, $p < 0.05$) elevated in the 3-hour group (0.26 ± 0.04 g/dL, $n = 4$) compared with those of the 24 (0.01 ± 0.001 g/dL, $n = 4$) and 48 (0.01 ± 0.001 g/dL, $n = 4$) hour ethanol groups and the control group (0.005 ± 0.001 g/dL, $n = 4$).

Ethanol Withdrawal Upregulates Ca_v1 Channels at the mRNA Level

Figure 2 summarizes the mRNA levels of the Ca_v1.3 (Figure 2A) and Ca_v1.2 (Figure 2B) α 1 subunits (*Cacna1c* and *Cacna1d* mRNA, respectively) in IC tissues obtained from the control and ethanol-treated groups. The expression of both *Cacna1d* ($F = 8$, $p < 0.001$) and *Cacna1c* ($F = 6$, $p < 0.01$) mRNA were increased significantly in the IC following ethanol withdrawal. Interestingly, the expression of *Cacna1d* mRNA was significantly increased at the 24-hour time point ($2^{-\Delta\Delta C_t} = 1.9 \pm 0.2$, $n = 6$, $t = 4$, $P < 0.05$), but not at the 3-hour time points ($2^{-\Delta\Delta C_t} = 1.4 \pm 0.1$, $n = 6$) or 48 ($2^{-\Delta\Delta C_t} = 1.3 \pm 0.2$,

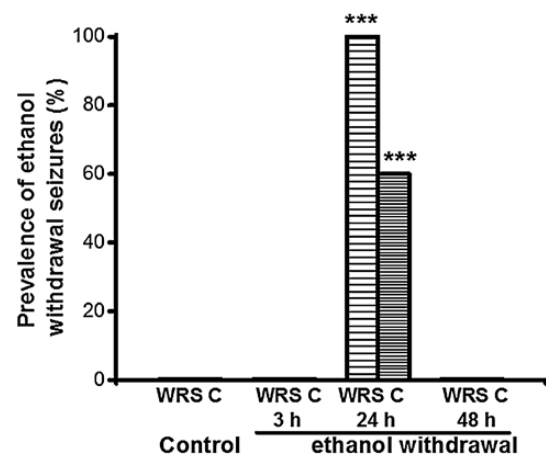


Figure 1. Prevalence of acoustically-evoked generalized seizures following ethanol withdrawal. These seizures consisted of wild running seizures (WRSs) or bouncing generalized tonic-clonic seizures (clonus [C]). At 24 hours after ethanol withdrawal, 100% and 60% of animals ($n = 10$) exhibited WRSs and clonus, respectively. No seizures were observed at 3 ($n = 6$) or 48 hours ($n = 6$) following ethanol withdrawal, and no seizures also were observed in the control group ($n = 6$, $p < 0.001$ versus control; chi-square test).

$n = 6$) during ethanol withdrawal, in comparison to the control group ($2^{-\Delta\Delta CT} = 1.0 \pm 0.1$, $n = 6$; Figure 2A). The level of *Cacna1d* mRNA was also significantly increased 24 hours during ethanol withdrawal compared to the 3-hour ($t = 3$, $p < 0.05$) and 48-hour ($t = 4$, $p < 0.05$) groups.

The expression of *Cacna1c* mRNA was significantly ($t = 4$, $p < 0.05$) increased 24 hours ($2^{-\Delta\Delta CT} = 1.5 \pm 0.1$, $n = 6$) and 48 ($2^{-\Delta\Delta CT} = 1.4 \pm 0.1$, $n = 6$) during ethanol withdrawal compared to the control group ($2^{-\Delta\Delta CT} = 1.0 \pm 0.1$, $n = 6$, Figure 2B). The expression of *Cacna1c* mRNA also was significantly increased in the 24-hour group compared to the 3-hour group during ethanol withdrawal ($2^{-\Delta\Delta CT} = 1.1 \pm 0.1$, $n = 6$, $t = 3$, $p < 0.05$). No change in the *Cacna1c* mRNA expression was observed 3 hours during ethanol withdrawal compared with the control group (Figure 2B). During ethanol withdrawal, the ratios of the mRNA expression levels of the *Cacna1d* to those of *Cacna1c* were 1.4:1.1, 1.9:1.5, and 1.3:1.4 at 3, 24, and 48 hours, respectively. Thus, a dominant expression of *Cacna1d* mRNA occurred at 3 and 24 hours following ethanol withdrawal.

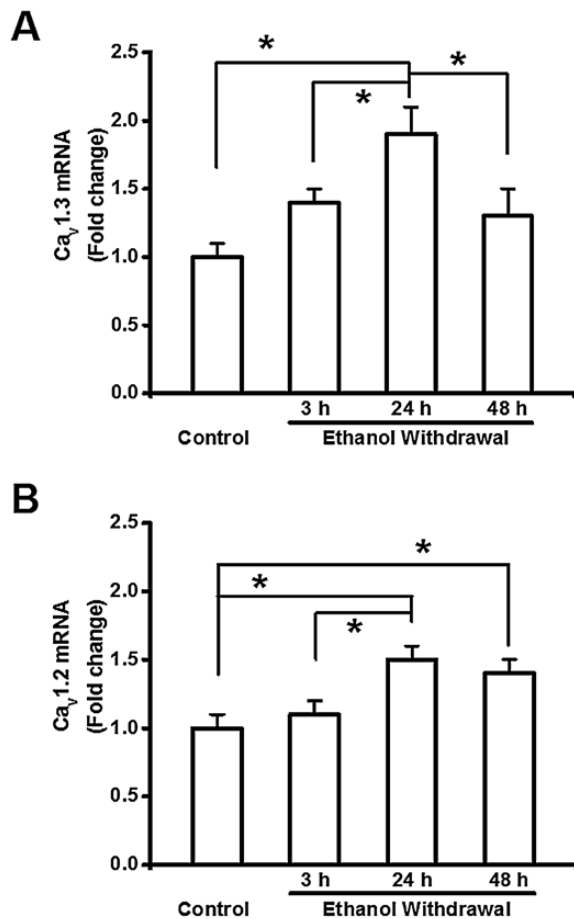


Figure 2. Ethanol withdrawal upregulates *Cacna1d* and *Cacna1c* mRNA expression in the inferior colliculus (IC). The levels of *Cacna1d* (*Ca_v1.3* $\alpha 1$ subunit; panel A) and *Cacna1c* (*Ca_v1.2* $\alpha 1$ subunit; panel B) mRNA were measured in the IC; the data are expressed as the fold change relative to the control group. (A) The level of *Cacna1d* mRNA was significantly higher in the IC 24 hours after alcohol withdrawal compared to the control group and the other alcohol-treated groups. (B) The level of *Cacna1c* *Ca_v1.2* mRNA was significantly higher in the IC 24 and 48 hours after alcohol withdrawal compared to the control group. The level of *Cacna1c* *Ca_v1.2* mRNA also was significantly elevated 24 hours after alcohol withdrawal compared to the 3-hour group. The data are shown as mean \pm standard error of the mean. $n = 6$ rats per group. * $p < 0.05$ versus control (ANOVA followed by a Bonferroni test or Student-Newman-Keuls).

Ethanol Withdrawal Upregulates *Ca_v1* Channels at the Protein Level

We next examined whether the increased expression of *Ca_v1* channels was reflected in a change in protein levels. Figure 3 summarizes the protein levels of *Ca_v1.3* (Figure 3A) and *Ca_v1.2* (Figure 3B) $\alpha 1$ subunits. Ethanol withdrawal significantly increased the protein levels of the *Ca_v1.3* ($F = 7$, $p < 0.001$) but not of *Ca_v1.2* $\alpha 1$ subunit in the IC. Consistent with the *Cacna1d* mRNA levels, the protein level of *Ca_v1.3* $\alpha 1$ subunits was increased significantly 24 hours ($152 \pm 8\%$, $n = 8$, $t = 4$, $p < 0.05$) after ethanol withdrawal, but not at the earlier (3-hour group: $102 \pm 10\%$, $n = 8$) or later (48-hour group: $116 \pm 12\%$, $n = 8$) time points compared to the control group ($100 \pm 8\%$, $n = 8$, Figure 3A). Comparison between ethanol-treated groups revealed that the protein expression level for the *Ca_v1.3*- $\alpha 1$ subunit was significantly elevated in the 24-hour group compared to the 3-hour ($t = 4$, $p < 0.05$) and 48-hour ($t = 3$, $p < 0.05$) groups during the withdrawal.

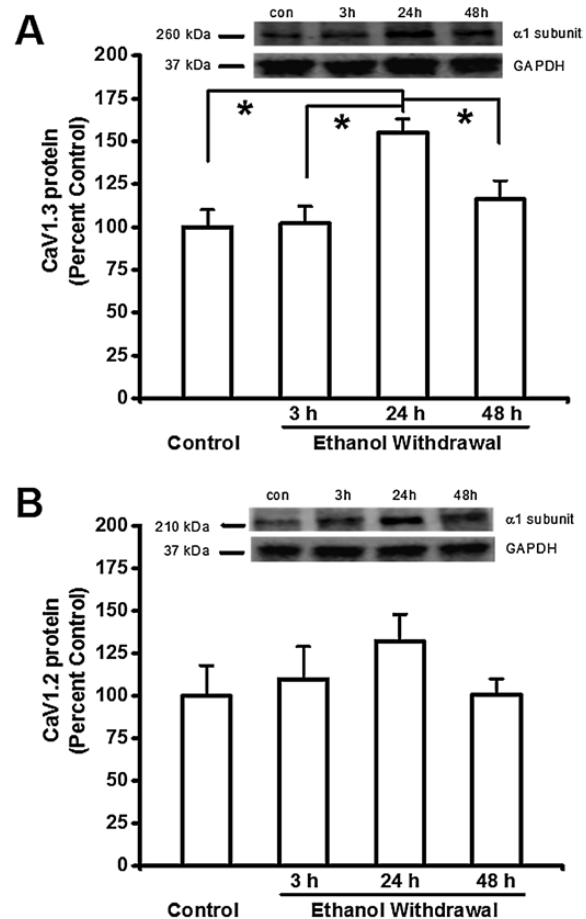


Figure 3. Ethanol withdrawal increases the protein levels of *Ca_v1.3* $\alpha 1$ subunits in the inferior colliculus (IC). Shown in insets are representative immunoblots of the (A-B) GAPDH, (A) *Ca_v1.3* and (B) *Ca_v1.2* $\alpha 1$ subunits measured from control IC samples and IC samples obtained at the indicated times after ethanol withdrawal. The bar graphs summarize the relative protein levels of *Ca_v1.3* $\alpha 1$ and *Ca_v1.2* subunits in the IC, expressed as a percentage of the control group. The density of the 260 kDa immunoreactive band (i.e. the *Ca_v1.3* $\alpha 1$ subunit; panel A) increased significantly in the IC 24 hours after ethanol withdrawal. The density of the 210 kDa immunoreactive bands (i.e. the *Ca_v1.2* $\alpha 1$ subunit; panel B) did not change significantly following ethanol withdrawal. The summary data are shown as the mean \pm standard error of the mean. $n = 8$ rats per group. * $p < 0.05$ versus control (ANOVA followed by a Bonferroni test or Student-Newman-Keuls).

In contrast, the protein expression levels of the Ca_v1.2- α 1 subunit were increased by 6% at 3 hours (106 \pm 19%, n = 8) and 32% at 24 hours (132 \pm 16%, n = 8) during ethanol withdrawal, and then returned to control levels (100 \pm 19%, n = 8) at 48 hours (100 \pm 10, n = 8, [Figure 3B](#)) later. The protein expression ratios of the Ca_v1.3- α 1 and Ca_v1.2- α 1 subunits were 100:106, 152:132, and 116:100 at 3, 24, and 48 h during ethanol withdrawal, respectively. Thus, ethanol withdrawal favored the protein expression of Ca_v1.3- α 1 subunit at 24 hours during ethanol withdrawal when the susceptibility to seizures peaked.

Discussion

Here, we report that the mRNA and protein levels of the Ca_v1.3 and Ca_v1.2 α 1 subunits increase in the IC during alcohol withdrawal, a condition that is associated with increased seizure susceptibility. In particular, expression of the Ca_v1.3 α 1 subunit was significantly increased 24 hours after alcohol withdrawal, when seizure susceptibility peaked. In contrast, expression of the Ca_v1.2 α 1 subunit increased 24 and 48 hours after alcohol withdrawal, when seizure susceptibility peaked and then resolved, respectively. Thus, expression of the Ca_v1.3 α 1 subunit changed in parallel with the occurrence of AWS, whereas upregulation of the Ca_v1.2 α 1 subunit outlasted the period of AWS susceptibility. At the protein level, Western blot analyses revealed that the Ca_v1.3 α 1 subunit—but not the Ca_v1.2 α 1 subunit—was significantly increased in the IC 24 hours after alcohol withdrawal. A significant increase in Ca_v1.3 α 1 subunit proteins was also observed 24 hours following alcohol withdrawal, as compared to the 3-hour group. These findings suggest that upregulation of Ca_v1.3 L-type Ca²⁺ channels might play an important role in the pathogenesis of AWS.

In the brain, Ca_v1 channels are located primarily in the dendrites and cell bodies; 80% and 20% of these channels are Ca_v1.2 and Ca_v1.3 channels, respectively ([Hell et al., 1993](#)). This predominance of Ca_v1.2 channels may also occur in the IC. Evidence suggests that Ca_v1.3 channels activate at more negative voltages and inactivate more slowly than Ca_v1.2 channels ([Koschak et al., 2001](#); [Xu and Lipscombe, 2001](#)). Given their biophysical properties, Ca_v1.3 channels may play an important role in the control of Ca²⁺-dependent firing, and these channels can help maintain the Ca²⁺ influx at membrane potentials at which Ca_v1.2 channels would remain closed. Here, we found that increased susceptibility to AWS was accompanied by an increase in Ca_v1.3 channels in the IC, consistent with a role for Ca_v1.3 channels in mediating neuronal hyperexcitability, leading to seizures. Analysis of the ratio of Ca_v1.3 to Ca_v1.2 channels reveals that alcohol withdrawal preferentially upregulates Ca_v1.3 channels (at 24 but not 3 hours during alcohol withdrawal when the susceptibility to seizures peaked) in IC neurons, consistent with the role of these channels in the pathogenesis of AWSs.

During alcohol withdrawal, the IC plays an important role in initiating AGSs ([Fyre et al., 1983](#); [Riaz and Faingold, 1994](#); [Faingold and Riaz, 1995](#); [Chakravarty and Faingold, 1998](#)). Pharmacological studies found that blocking Ca_v1 channels during alcohol withdrawal suppresses AGSs, leading to the hypothesis that these channels undergo remodeling, at least in IC neurons ([Little et al., 1986](#)). Consistent with this hypothesis, we previously reported increased currents through Ca_v1 channels in IC neurons during alcohol withdrawal at the time point at which susceptibility to seizures peaks ([N'Gouemo and Morad, 2003](#)). These findings suggest a possible causal relationship between changes in the composition of Ca_v1 channels in IC neurons and the occurrence of AWS. Here, we present evidence that the increase in Ca_v1

channels in IC neurons might not be a consequence of AWS, as Ca_v1.3 α 1 mRNA and protein levels increased in parallel with the occurrence of AWS. Thus, altered Ca_v1.3 channel expression may predispose the animals to develop AWS.

Alcohol withdrawal-induced clonus (i.e. bouncing generalized tonic-clonic seizures) originate primarily in the brainstem ([Faingold et al., 1998](#)). Whether the changes in Ca_v1.3 α 1 channels that we observed in the IC also occur in other epileptogenic sites in the forebrain (for example, in the hippocampus) is currently unknown. Increased levels of Ca_v1.3 α 1 subunits have been found in cortical neurons during alcohol withdrawal and are associated with increased seizure susceptibility ([Katsura et al., 2005](#)). Similarly, Ca_v1 channels are upregulated throughout the brain following alcohol withdrawal, and this upregulation occurs in parallel with the occurrence of seizures ([Watson and Little, 1999](#)). Generalized tonic-clonic seizures during alcohol withdrawal resemble seizures observed in the genetically epilepsy-prone rat ([Faingold et al., 1998](#); [Faingold, 1999](#)). In these rats, Ca_v1.3—but not Ca_v1.2- α 1 subunits were increased in IC neurons of both seizure-naïve and seizure-experienced animals ([N'Gouemo et al., 2010](#)). These findings suggest that the upregulation of Ca_v1.3 channels in IC neurons may be the molecular correlate of the enhanced susceptibility to generalized tonic-clonic seizures originating in the brainstem. In a Mongolian gerbil model of generalized tonic-clonic seizures, no changes in Ca_v1.3 or Ca_v1.2 α 1 subunits were found in pyramidal cells in the CA1 or dentate gyrus of seizure-prone animals ([Kang et al., 2004](#)). However, in the CA2 and CA3 regions of the hippocampus, both Ca_v1.3 and Ca_v1.2 α 1 subunits were significantly increased in pyramidal cells during the pre-seizure state, as well as postictally in seizure-sensitive gerbils ([Kang et al., 2004](#)). Whether these changes also occur in the brainstem is currently unknown. The increase in both Ca_v1.3 and Ca_v1.2 channels in the hippocampus may represent the molecular correlates of increased Ca²⁺-dependent excitability in an inherited model of generalized tonic-clonic seizures. Nevertheless, upregulation of Ca_v1.3 channels was reported in cerebellar Purkinje cells of seizure-sensitive gerbils ([Park et al., 2003](#)). In a kindling model of epileptogenesis, the mRNA level of Ca_v1.3—but not Ca_v1.2—channels was increased in the hippocampus during the non-convulsive limbic phase ([Hendriksen et al., 1997](#)). In contrast, no change in Ca_v1.3 mRNA levels was found in the hippocampus during generalized seizures ([Hendriksen et al., 1997](#)). Altered levels of Ca_v1 channels have also been reported in the thalamus. Notably, significant increases in the mRNA and protein levels of the Ca_v1.3 α 1 subunit were found in the dorsal part of the lateral geniculate nucleus of epileptic Wistar Albino Glaxo from Rijswijk rats; this brain section is a thalamic nucleus that is relevant in the pathophysiology of absence epilepsy ([Kanyshkova et al., 2014](#); [Dilger et al., 2011](#); [Lo et al., 2002](#)). Evidence suggests that altered Ca_v1.2 channels in hippocampal pyramidal cells may play a role in the pathogenesis of generalized tonic-clonic seizures in a model of febrile seizures ([Radzicki et al., 2013](#)). Finally, we posit that upregulation of both Ca_v1.3 and Ca_v1.2 channels in the forebrain is associated primarily with non-convulsive seizures, whereas upregulation of Ca_v1.3 channels in the brainstem and cortex is associated with increased susceptibility to generalized tonic-clonic seizures.

Although we did not specifically examine the mechanism underlying the upregulation of Ca_v1 channels in the IC following alcohol withdrawal, the consequence of this upregulation is likely an increase in Ca²⁺ influx. Alcohol exposure can suppress the activity of Ca²⁺ channels in IC neurons ([N'Gouemo,](#)

unpublished observations). Thus, an increase in the number of Ca_v1 channels following alcohol withdrawal might represent a compensatory mechanism for changes in the activity of IC neuronal circuits in response to decreased Ca_v1 channel activity during alcohol exposure. Among a myriad of other key functions, Ca_v1 channels control neuronal excitability, in part via their coupling with Ca^{2+} -dependent K^+ (K_{Ca}) channels (Morisset and Nagy, 1999). Notably, Ca^{2+} influx via $\text{Ca}_v1.3$ channels can induce a slow afterhyperpolarization by activating K_{Ca} channels, which in turn control neuronal firing (Shah and Haylett, 2000; Bowden et al., 2001; Goldberg and Wilson, 2005). Our finding of $\text{Ca}_v1.3$ channel upregulation suggests a role for currents that drive a slow afterhyperpolarization. However, both the abundance and current density of the large-conductance K_{Ca} channels that contribute to the slow afterhyperpolarization are decreased in IC neurons following alcohol withdrawal (N'Gouemo and Morad, 2014). Thus, the combination of $\text{Ca}_v1.3$ channel upregulation and large-conductance K_{Ca} channel downregulation might contribute to IC neuronal hyperexcitability and increase seizure susceptibility during alcohol withdrawal.

Our study has some limitations that bear mentioning. Notably, the relatively small sample size used in this study could, in principle, favor type II errors (i.e. a false negative result) over type I errors (i.e. a false positive result). For example, our finding that $\text{Ca}_v1.3$ $\alpha 1$ subunits are not upregulated 3 hours after ethanol withdrawal may have been due to a type II error. We also found a lack of positive correlation between the expression of *Cacna1d* mRNA and the levels of $\text{Ca}_v1.3$ $\alpha 1$ subunit proteins, which could be due to different mRNA and protein degradation rates. In the present study, total (cell surface and intracellular) expression of Ca_v1 protein was measured in IC neurons, which doesn't represent the functional (at the cell surface) Ca_v1 channels.

In summary, alcohol withdrawal differentially affects the expression of Ca_v1 channel subtypes in the rat IC neurons. The transient increase in the expression of $\text{Ca}_v1.3$ channels occurred in parallel with the occurrence of AWSs, whereas the increase in $\text{Ca}_v1.2$ channels outlasted the enhanced susceptibility to seizures. These findings suggest that $\text{Ca}_v1.3$ channels in IC neurons play an important role in the pathogenesis of AWSs.

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Statement of Interest

The authors have no conflicts of interest.

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