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Alcohol Withdrawal Is Associated with a Downregulation of Large-Conductance Ca²⁺-Activated K⁺ Channels in Rat Inferior Colliculus Neurons

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Abstract

Rationale—Large conductance calcium-activated potassium (BK_{Ca} or K_{Ca1.1}) channels are well-known molecular targets for the action of alcohol and therefore may play an important role in the pathogenesis of alcohol withdrawal syndrome.

Objectives—We evaluate the modifications of total outward K⁺ currents and protein expression of BK_{Ca} channels α -subunit in inferior colliculus (IC) neurons obtained from controls and rats subjected to alcohol withdrawal associated with enhanced susceptibility to seizures.

Methods—Outward K⁺ currents and BK_{Ca} channel proteins were measured using the whole cell configuration of patch clamp techniques and western blot analysis, respectively.

Results—Total outward K⁺ current density was significantly reduced in IC neurons at 24 h and 48 h during the alcohol withdrawal period when the susceptibility to seizures was maximal and absent, respectively. The iberiotoxin-sensitive (BK_{Ca}) current density and conductance also were significantly reduced at 24 h following alcohol withdrawal. Consistent with functional data, the levels of protein expression of α -subunit associated with BK_{Ca} channels also was significantly reduced in IC neurons at 24 h and 48 h following alcohol withdrawal.

Conclusions—The downregulation of BK_{Ca} channels that outlasts the finite period of elevated susceptibility to alcohol withdrawal seizures. These findings indicate that BK_{Ca} channels, *per se*, may not be fundamentally important for the generation of alcohol withdrawal seizures.

Keywords

alcohol withdrawal; BK_{Ca} channels; iberiotoxin; outward current density; protein; seizures

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Introduction

Alcohol withdrawal-induced neuronal hyperexcitability can lead to generalized tonic-clonic seizures in many species including rodent and humans. In rodents, the susceptibility to acoustically evoked generalized seizures (audiogenic seizures) occurs during a finite period following abrupt cessation of chronic alcohol intoxication. Multiple lines of evidence indicate that inferior colliculus (IC) neurons, known for their role in the processing of auditory information are critical in the initiation of audiogenic seizures following alcohol withdrawal (Frye et al. 1983; Eckardt et al. 1986; McCown and Breese 1990; Caird 1991; Faingold and Riaz 1995; Chakravarty and Faingold 1998). Electrophysiological studies reported increases in IC neuronal firing immediately before the onset of and during audiogenic seizures in rats subjected to alcohol withdrawal (Faingold and Riaz 1995; Chakravarty and Faingold 1998). Similarly, *in vitro* studies have reported that IC neurons exhibited elevated bursting activity following alcohol withdrawal associated with enhanced seizure susceptibility (Evans et al. 2000). The mechanisms underlying alcohol withdrawal-induced increases in IC neuronal excitability that lead to seizures are not fully understood. We have previously reported increases in the current density of the L- and P-type of the high-threshold voltage-gated Ca^{2+} (Ca_V) channels in IC neurons of rats subjected to alcohol withdrawal and exhibiting enhanced susceptibility to audiogenic seizures (N'Gouemo and Morad 2003). The enhanced Ca_V channel currents should lead to higher intracellular Ca^{2+} activity, providing the mechanism to activate the large ($\text{K}_{\text{Ca}1}$ or BK_{Ca}) and small ($\text{K}_{\text{Ca}2}$ or SK_{Ca}) conductance Ca^{2+} -activated K^+ (K_{Ca}) channels that initiate repolarization and the ensuing afterhyperpolarization (Lancaster and Adams 1986; Storm 1987). Activation of K_{Ca} channels may therefore serve as an intrinsic inhibitory mechanism of IC neurons. Loss of K_{Ca} channel activity would therefore contribute to neuronal hyperexcitability that can lead to seizures. Although IC neurons exhibit K_{Ca} channel conductances (Sivaramakrishnan and Oliver 2001), their role in the pathogenesis of alcohol withdrawal seizures remains unknown. Nevertheless, evidence suggests that neuronal BK_{Ca} channels may be important molecular targets of alcohol-action in various brain sites (Davies et al. 2003; Cowmeadow et al. 2005). Alcohol exposure potentiates BK_{Ca} channel open probability in the hypothalamic-neurohypophysial system and nucleus accumbens (Dopico et al. 1996; Martin et al. 2004; Pietrzykowski et al. 2004), while its chronic exposure decreased BK_{Ca} current density (Knott et al. 2002; Pietrzykowski et al. 2004). Thus, the effect of alcohol on BK_{Ca} channels may have potential consequences on the excitability of IC neurons. Here, we report on the functional and molecular expression of neuronal BK_{Ca} channels, and find that alcohol withdrawal is accompanied by downregulation of BK_{Ca} channels in IC neurons.

Materials and methods

Ethanol administration and seizure testing

We used a convenient model of ethanol dependence that mimics alcohol withdrawal seizures seen in humans (Faingold 2008). Ethanol (95%) was given by intragastric intubation to male Sprague-Dawley rats (180–230 g) in 30% v/v solution of diluted (1:1 with water) ISOMIL Soy Protein Infant Formula (Abbott Laboratories, Abbott Park, IL). Ethanol administration protocol consisted of three daily doses every 8 h for 4 days, and the spectrum of

intoxication, which is broken down into six stages (neutrality, sedation, moderate ataxia, severe ataxia, loss of righting reflex, and coma) was evaluated based on the standard behavior rating scale (Faingold 2008). The priming dose of ethanol was 5 g/kg, resulting in blood alcohol levels of 0.3 ± 0.01 g/dL ($n=4$). Subsequent ethanol doses were adjusted for each rat, to maintain a moderate but not severe degree of ataxia. The daily amount of administered ethanol varied from 9 to 15 g/kg for each rat, which allowed for intoxication to be maintained at a consistent moderate degree of ataxia. Ethanol was withdrawn on the fourth day after the second daily dose. Rats were sacrificed in two groups at about 24 h and 48 h, respectively, after the last ethanol administration on the basis of previous study indicating that blood ethanol levels at that time were negligible, while audiogenic seizure susceptibility was higher at 24 h but absent at 48 h (N'Gouemo et al. 1996; N'Gouemo and Morad 2003). Control animals were maintained under similar conditions and schedule but fed only with the ISOMIL diet without ethanol. To determine the incidence of audiogenic seizures, rats were subjected to acoustic stimulation every hour (until seizures occurred) for three hours, starting at the 23rd hour for the 24 h group and at the 47th hour for the 48 h group, during the ethanol withdrawal period. Two out of four rats per group, when no spontaneous seizure activity was observed, were subjected to acoustic stimulation (bell, 100 dB), which was presented until wild running seizures were elicited or for 60 sec (Riaz and Faingold 1994). The remaining rats, those not exposed to acoustic stimulation that did not exhibit seizures were used for electrophysiological and molecular studies. The seizure severity score was evaluated according to the scale of audiogenic seizures (Jobe et al. 1973). Weight loss and the incidence of mortality were negligible in this study

Cell preparation

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.). The rats' brains were then perfused with an oxygenated (95% O₂ and 5% CO₂) solution containing (in mM): 110 choline chloride, 2.5 KCl, 1.2 NaH₂PO₄, 26 NaHCO₃, 2 sodium/pyruvate, 1 L-ascorbic acid, 20 dextrose, 0.5 CaCl₂, and 1.5 MgCl₂ (290–300 mOsm with sucrose). The brains were removed and immersed in a cutting sucrose solution containing (in mM): 205 sucrose, 5 KCl, 1.2 NaH₂PO₄, 26 NaHCO₃, 2 sodium/pyruvate, 1 L-ascorbic acid, 20 dextrose, 0.2 CaCl₂, and 1 MgSO₄, (osmolarity 290–300 mOsm with sucrose), and bubbled with 95% O₂ and 5% CO₂. Coronal brainstem slices (300 μm thick) at the level of the IC were sectioned using a Leica Vibratome 1000S. The IC external cortex was microdissected and placed in an oxygenated (100% O₂; 30–32°C) Leibowitz (L-15) medium (Life Technologies, Grand Island, NY) containing papain (20 U/ml; Worthington Biochemicals, Lakewood, NJ) for 60 min. The enzyme was washed with L-15 medium containing trypsin inhibitor (1mg/ml) and albumin bovine serum (1mg/ml). IC neurons were then dissociated by gentle trituration with a fire-polished Pasteur pipette in Neurobasal-A (Life Technologies, Grand Island, NY) medium supplemented with 2% B27 (Life Technologies, Grand Island, NY); 1% horse serum; 1% fetal bovine serum; and penicillin (100 U/ml)-streptomycin (0.1 mg/ml). Dissociated IC neurons were then plated onto concavalin (50 μg/ml) coated-glass coverslip for at least 1 hr before whole cell recordings.

Electrophysiology

Total outward K^+ currents were recorded at room temperature from the soma of IC neurons using the whole cell configuration of the patch clamp techniques (Hamill et al 1981). The patch electrodes were made from borosilicate glass capillaries and had 2–4 Ω M resistances when filled with solution containing (in mM): 0.5 EGTA (100 nM free Ca^{2+}) 140 KCl, 5 $MgCl_2$, 4 Na-ATP, 0.4 GTP, 14 phosphocreatine, 10 HEPES, and 10 glucose (pH 7.3 with KOH). After establishment of the whole cell configuration, currents were recorded using extracellular solution containing (in mM): 2 $CaCl_2$, 140 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 0.001 tetrodotoxin (pH 7.4 with NaOH; 305 mOsm with sucrose). Voltage clamp experiments were performed with a Dagan 8900 patch clamp amplifier (Dagan Corporation, Minneapolis, MN). Currents were filtered at 10 kHz, and whole cell capacitance and series resistance were compensated with the amplifier circuitry. Leak and residual capacitance currents were not subtracted for any voltage protocols. Outward currents were evoked by voltage steps (50 ms duration), from the holding potential of -50 mV to $+60$ mV by 10 mV increments, and measured at 15 ms (sustained currents). For pharmacological studies, outward currents were evoked by test potential to $+20$ mV and monitored until a steady state was reached before applying BK_{Ca} channel blocker. To isolate BK_{Ca} channel currents, iberiotoxin (100 nM), a potent blocker of these channels, was used. Outward currents were recorded before and during iberiotoxin exposure. The iberiotoxin-sensitive current was obtained as difference current in the presence and absence of iberiotoxin.

Western immunoblotting

Rats were deeply anesthetized with pentobarbital (100 mg/kg; i.p.). Their brains were removed, and IC tissues were quickly dissected (the area corresponding to the periaqueductal grey was discarded) and stored at $-70^\circ C$ until processed. Tissue homogenates from each rat were prepared in a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Protein concentrations in the supernatant were determined using bicinchoninic acid assay reagent (Thermo Fisher Scientific, Rockford, IL), and each sample was diluted to a protein concentration of 5 mg/ml in the TE buffer. Homogenate proteins were separated using a 7.5% sodium dodecyl sulfate-polyacrylamide gel (Life Technologies, Grand Island, NY) electrophoresis, and transferred to a polyscreen polyvinylidene fluoride membrane (PerkinElmer, Waltham MA) in the blotting buffer (25 mM Tris-HCl, 192 mM Glycine, 20% methanol). The blots were processed using methods described by Towbin et al. (1979). Briefly, the blots were incubated in a TBST buffer (20 mM Tris-HCl, pH 7.4; 140 mM NaCl and 0.1% v/v Tween 20) containing 5% nonfat dry milk (BLOTTO buffer) with BK_{Ca} channel α -subunit antibody (Alomone labs, Jerusalem, Israel) at concentrations of 1.0 μ g/ml, respectively overnight at $4^\circ C$. The blots were then washed (30 min) with a TBST buffer and incubated with a donkey anti-rabbit antibody (GE HealthCare Biosciences, Piscataway, NJ) at a dilution of 1:2000 in a BLOTTO buffer for 30 min at room temperature. After several washes of the membrane, individual bands were visualized on film using the enhanced chemiluminescence Super Signal West Pico reagents (Thermo Fisher Scientific, Rockford, IL), and analyzed.

Data analysis

Data were analyzed using Clampfit 10 software (Molecular Devices, Sunnyvale, CA) and Origin 8.0 (Originlab, Northampton, MA). Membrane potentials were corrected for the junction potential, and outward currents were normalized relative to the membrane capacitance as an estimate of current density. The ethanol intoxication/withdrawal paradigm did not alter IC neurons cell capacitance. To generate steady-state activation curves, the conductance ($G=I/V-V_{rev}$) was determined at each potential (V) and the reversal potential (V_{rev}) of the current was measured for the I-V curves (Fig. 3C). Normalized conductances were plotted as a function of test pulse and was best fitted with a Boltzmann equation, $G/G_{max}=1/(1+e^{(V_{1/2}-V)/k})$, where G_{max} is the maximum conductance, generating values for half-maximum conductance (G) at potential ($V_{1/2}$) and the slope factor (k). To quantify the abundance of BK_{Ca} channel α -subunit protein, individual bands were quantified by computer-assisted densitometry using the Bio Image Analysis Intelligent Quantifier software (Bio Image System, Inc, Jackson, MI), which calculated the integrated intensity of a band using both the density and the area of the band. The integrated intensity of the BK_{Ca} channel α -subunit protein band was compared to a standard curve of known protein concentration from rat hippocampus, as done previously in our laboratory (N'Gouemo et al. 2009). The integrated intensities of the bands from IC tissues determined at the same time and on the same piece of film were compared with the interpolated standard curve to yield a value of subunit expression relative to the standard hippocampus tissue. The utilized integrated intensities were always in the linear part of the standard curve. For total protein quantification, β -actin was used as loading control. To determine differences in current density and protein expression, one-way or two-way analysis of variance was performed. Differences were considered significant if $P<0.05$. The illustrated current traces represent the average of 3–5 consecutive traces. All data presented were mean \pm S.E.M.

Results

Total Outward K⁺ currents and enhanced susceptibility to seizures

Audiogenic seizures following ethanol withdrawal consisted of wild running seizures that evolved into tonic-clonic seizures; this seizure phenotype was observed in all rats tested at 24 h following ethanol withdrawal. The susceptibility to seizures was transient such that the susceptibility to audiogenic seizures was present at 24 h but absent at 48 h following ethanol withdrawal. Figure 1A shows a representative trace of total outward K⁺ current evoked at +20 mV in IC neurons obtained from a control animal. Quantification reveals that the mean peak current density evoked at +20 mV was significantly decreased in IC neurons (32.2 ± 1.8 pA/pF, n=23) obtained from rats subjected to 24 h ethanol withdrawal and exhibiting seizure susceptibility, as compared to the control group (53.0 ± 1.6 pA/pF, n=21) and to the 48 h group (43.9 ± 1.9 , n=12, $F=41$, $P<0.001$, Fig. 1b). The decreases in outward peak current density persisted, surprisingly, at 48 h following ethanol withdrawal (43.9 ± 1.9 , n=12, compared to 53.0 ± 1.6 pA/pF, n=21, $F=13$, $P<0.001$) even when the seizure susceptibility was no longer present (Fig. 1).

Figure 2 quantifies the voltage-dependence of total outward K⁺ currents in the control group and in rats subjected to ethanol withdrawal. The original traces of total outward K⁺ currents

from three representative neurons from each group showed significant reduction of the current density 24 h (middle traces, Fig. 2a) and 48 h (lower traces, Fig. 2a) following ethanol withdrawal as compared to the control group (upper traces, Fig. 2a). Figure 2b quantifies the current density at larger range of voltages (-50 mV to $+40$ mV). Activation of K^+ channels occurs around -35 ± 2 mV ($n=12$) in the control group (filled squares), but shifts to less negative potentials of -27 ± 2 mV ($n=13$) and -29 ± 2 mV ($n=9$) in the 24 h group (filled circles) and 48 h group (filled triangles) following ethanol withdrawal, respectively. Comparison of the I-V relations of total outward K^+ currents between the control group (filled squares) and 24 h following ethanol withdrawal (filled circles) showed a reduction of the current density, on the average, by $48 \pm 4\%$ at voltages positive to -40 mV without significant alterations in their voltage dependence (Fig. 2b, upper curves). The current density at 24 h following ethanol withdrawal (filled circles) was also decreased compared to 48 h following ethanol withdrawal (filled triangles); this decrease, on the average, by $39 \pm 4\%$, was seen at voltages positive to -30 mV (Fig. 2b, middle curves). Quantification also shows that the current density at voltage positive to -20 mV was significantly decreased, on the average, by $34 \pm 5\%$, at 48 h following alcohol withdrawal (filled triangles) compared to the control group (filled squares; Fig. 2, lower curves). However, this decreased outward K^+ current was not accompanied by ethanol withdrawal seizure susceptibility.

Contribution of BK_{Ca} channels to reduced total outward K^+ currents

The possible contribution of BK_{Ca} channels to the reduced total outward K^+ currents in IC neurons at 24 h following ethanol withdrawal was determined using iberiotoxin (100 nM), a potent blocker of BK_{Ca} channels. Figure 3a shows representative iberiotoxin-sensitive currents (BK_{Ca} channel currents) in IC neurons obtained from the control (upper traces) and following ethanol withdrawal (lower traces). Quantification of this effect shows that iberiotoxin-sensitive current density was significantly decreased in IC neurons (5.8 ± 1.1 , $n=8$, $F=10.6$, $P<0.01$, Fig. 3b) of rats subjected to ethanol withdrawal and exhibiting seizure susceptibility, as compared to the control group (15.6 ± 2.8 , $n=8$, Fig. 3b). Figure 3c quantifies the voltage-dependence of iberiotoxin-sensitive currents in controls ($n=6$) and rats subjected to ethanol withdrawal ($n=6$). The iberiotoxin-sensitive current was significantly reduced following ethanol withdrawal at voltages between -10 mV and $+20$ mV, on average by $50 \pm 3\%$, compared to controls. Because of the long lasting decrease in the levels of protein expression BK_{Ca} channel (see below), no attempt was made to evaluate the iberiotoxin current density in IC neurons at 48 h following ethanol withdrawal. Figure 3d shows that the mean conductance was markedly reduced, on average by 2.5 fold, in IC neurons following ethanol withdrawal (filled circles) compared to controls (square circles). Ethanol withdrawal also significantly reduced the mean value of G_{max} (0.2 ± 0.1 pS/pF, $n=6$ compared to controls: 0.5 ± 0.1 pS/pF, $n=6$; $F=8.4$, $P<0.01$; Fig. 3e) without affecting activation parameters $V_{1/2}$ (ethanol withdrawal: -0.5 ± 5 mV, $n=6$; controls: -9.8 ± 4 mV, $n=6$; Fig. 3f) and k values (ethanol withdrawal: 11.3 ± 2 mV/e-fold, $n=6$; controls: 9.7 ± 2 mV/e-fold, $n=6$; Fig. 3f).

Downregulation of BK_{Ca} channels

Figure 4 shows plots of total BK_{Ca} channel α -subunit proteins and their immunoreactive bands in IC tissues obtained from the control group, following ethanol withdrawal. Total

BK_{Ca} channel α -subunit proteins were recognized by the antibody as 125-kD (bands in IC neurons. Quantification shows that total protein levels of BK_{Ca} channel α subunits were significantly ($F=5.5$, $P<0.05$) decreased in IC neurons at 24 h following ethanol withdrawal ($83\pm3\%$, $n=9$) compared to the control group ($100\pm6\%$, $n=9$; Fig. 4a). Significant ($F=11$, $P<0.01$) downregulation of total BK_{Ca} channel α subunit proteins was also seen at 48 h following ethanol withdrawal ($80\pm4\%$, $n=8$) compared to controls ($100\pm2\%$, $n=8$; Fig. 4b).

Discussion

The major finding of this report is that total outward K⁺ currents were reduced in IC neurons following alcohol withdrawal. The fraction of outward K⁺ currents carried by BK_{Ca} channels was also significantly reduced in IC neurons following alcohol withdrawal. BK_{Ca} channels conductance was also markedly reduced in IC neurons following ethanol withdrawal. Consistent with functional data, total protein abundance of BK_{Ca} channel α -subunit was reduced in IC neurons at 24 h and 48 h following alcohol withdrawal when the seizure susceptibility is present and absent, respectively. These findings provide direct evidence that BK_{Ca} channels are downregulated in IC neurons during alcohol withdrawal, and this effect outlasts the finite period of enhanced susceptibility to seizures. Decrease in Ca²⁺-activated K⁺ currents following ethanol withdrawal Acute alcohol application has been reported to increase BK_{Ca} channel open probability and the Ca²⁺-dependent afterhyperpolarization potentials, resulting in the inhibition of neuronal activity (Carlen et al. 1982; Niesen et al. 1988; Dopico et al. 1996). Consistent with these findings, spontaneous and acoustically evoked firing in IC neurons were suppressed following acute administration of alcohol in awake animals (Faingold and Riaz 1995; Chakravarty and Faingold 1998). In our study, even though the Ca_V channel currents were found to be enhanced in IC neurons following alcohol withdrawal, a significant decrease in total outward K⁺ (including BK_{Ca}) current density was observed. These findings are consistent with the observed decrease in afterhyperpolarization of dentate gyrus neurons following alcohol withdrawal (Carlen et al 1990), since both the large and small conductance K_{Ca} currents are known to contribute respectively, to the generation of fast and slow afterhyperpolarization potentials (Sah 1996; Vergara et al 1998). Activation of K_{Ca} currents are also known to terminate bursting activity, and reduction of these current should increase the propensity for bursting (Alger and Nicoll 1980). In this respect, a greater incidence of bursting activity has been reported in IC neurons during the alcohol withdrawal period, consistent with the downregulation of BK_{Ca} channels found in the present study (Faingold and Riaz 1995; Evans et al. 2000; Faingold et al., 2000). The mechanisms underlying the decrease in total outward K⁺ currents following alcohol withdrawal is not yet fully understood. In our study, IC neurons also exhibited large outward K⁺ channel currents despite the presence of Ca²⁺ chelator (EGTA) in the patch pipette (data not shown). Since EGTA is known to suppress the diffusion of free Ca²⁺ into the dendritic shaft and the cell soma, Ca²⁺ influx is likely to be restricted to the immediate vicinity of the Ca²⁺ entry sites (Stern 1992; Deisseroth et al 1996). K_{Ca} channels and the Ca²⁺ transporting proteins must therefore be colocalized in the same microdomain. In this respect, we have previously reported that the levels of expression of Ca_V2.2- α_1 pore-forming subunit protein, in particular, were significantly downregulated in IC neurons following alcohol withdrawal (N'Gouemo et al. 2006). Thus, Ca_V2.2 channels may be

preferentially colocalized in the same microdomain containing BK_{Ca} channels. This idea is supported by recent findings that BK_{Ca} channels are activated by Ca²⁺ influx via Ca_v2.2 channels in brain and spinal cord neurons (Loane et al. 2007; Ye et al. 2012). It would be interesting to characterize the functional coupling of BK_{Ca} channels and Ca_v2.2 channels in IC neurons, and evaluate how alcohol intoxication/withdrawal alters this coupling.

Remodeling of BK_{Ca} channels distribution may also account for the downregulation of BK_{Ca} channels in IC neurons following alcohol withdrawal. BK_{Ca} channels are mainly expressed in clusters and chronic ethanol exposure causes a declustering (and internalization) of membrane BK_{Ca} channels and also reduced the density of BK_{Ca} channels in the remaining clusters in hypothalamic-neurohypophysial explants (Pietrzykowski et al 2004). Change in the expression of specific BK_{Ca} channel splice variant may also contribute to the downregulation of the channels. Multiple neuronal BK_{Ca} channels splice variants have been described including ALCOREX (Pietrzykowski et al 2008). Interestingly, alcohol exposure dramatically reduced the expression of mRNA levels of ALCOREXBK_{Ca} channels splice variant in hypothalamic-neurohypophysial explants via a microRNA-miR-9-dependent postranscriptional mechanism (Pietrzykowski et al 2008). Whether alcohol withdrawal alters the expression of ALCOREX-BK_{Ca} channel splice variant in IC neurons remain unknown.

We have reported that alcohol withdrawal was associated with an increased current density of L- and P-type of Ca_v channels (N'Gouemo and Morad 2003). Preliminary studies demonstrated increases in intracellular Ca²⁺ transients in IC neurons following alcohol withdrawal (N'Gouemo, unpublished data). An increase in intracellular Ca²⁺ transient would be expected to activate K_{Ca} channels; however, in the present study, we found that BK_{Ca} current density and conductance are reduced during alcohol withdrawal. This effect was not accompanied by changes in V_{1/2} and k values suggesting that alcohol withdrawal did not alter the voltage sensing properties of BK_{Ca} channels. Thus, the downregulation of BK_{Ca} channels may have differential sensitivity to Ca²⁺ during alcohol withdrawal. However, in this study, no attempt to characterize the sensitivity of BK_{Ca} channels to Ca²⁺ was made. It has been reported that H⁺ and Ca²⁺ compete for the same binding site on BK_{Ca} channels (Laurido et al. 1991). Interestingly, acidification reduces the activity of BK_{Ca} channels under high levels of intracellular Ca²⁺ conditions such as following alcohol withdrawal (Hou et al. 2008; N'Gouemo, unpublished data; Song et al. 2010; Su et al. 2010). Thus, if H⁺ binding to BK_{Ca} channel during alcohol withdrawal was to occur, then the BK_{Ca} currents would decrease despite the increase in intracellular Ca²⁺ seen in IC neurons following alcohol withdrawal (N'Gouemo, unpublished data).

Downregulation of BK_{Ca} channels and alcohol withdrawal seizure susceptibility

The IC neurons play an important role in the pathogenesis of alcohol withdrawal seizures (Frey et al. 1983; Riaz and Faingold 1994; Faingold and Riaz 1995; N'Gouemo et al. 1996). L-type Ca²⁺ channel blockers are known to suppress audiogenic seizures following alcohol withdrawal (Little et al. 1986), and our previous data suggests that alcohol withdrawal associated with seizure susceptibility is accompanied by increases in high threshold Ca_v channel currents in IC neurons (N'Gouemo and Morad 2003). The precise mechanisms of

how enhanced Ca_V channel current density contributes to alcohol withdrawal seizures are not fully understood. The enhancement of Ca_V channel currents should provide sufficient Ca^{2+} for Ca^{2+} -dependent inactivation of Ca^{2+} currents and concomitant activation of K_{Ca} channels, leading to termination of bursting activity that accompany seizure activity. These mechanisms, however, appear to be significantly altered, as both Ca^{2+} channels inactivation is suppressed (N'Gouemo and Morad 2003), and BK_{Ca} α -subunit channel expression is downregulated (present study) in IC neurons following alcohol withdrawal. In this study, we found no seizure susceptibility at 48 h following alcohol withdrawal, despite reduced total outward K^+ current density and downregulation of α -subunit associated with BK_{Ca} channels in IC neurons. Interestingly, no enhancement of total Ca_V channel current density was reported in IC neurons at 48 h following alcohol withdrawal (N'Gouemo and Morad 2003). These findings suggest that *i*) downregulation of BK_{Ca} channels, per se, may not play a critical role in the generation of alcohol withdrawal seizures, *ii*) potential remodeling of other ion channels expression and/or neuronal networks may occur and contribute to suppress membrane hyperexcitability related to the reduction of BK_{Ca} channel function in IC neurons, and *iii*) the coupling between upregulation of Ca_V channels and downregulation of BK_{Ca} channels may play an important role in IC neuronal hyperexcitability that leads to alcohol withdrawal seizures. In this study, we found that BK_{Ca} current density and channel protein was decreased by approximately 50% and 20% following alcohol withdrawal, respectively. These findings suggest that in addition to BK_{Ca} channels, iberiotoxin suppresses other outward K^+ currents in IC neurons.

The role of BK_{Ca} channels in the pathogenesis of seizures and epilepsies is complex (N'Gouemo 2011). BK_{Ca} channels are thought to provide an intrinsic inhibitory mechanism of neuronal hyperexcitability that could otherwise lead to seizures. Surprisingly, activation of BK_{Ca} channels can facilitate burst firing in hippocampus neurons (Gu et al. 2006), and cause generalized epilepsy in humans (Du et al. 2005; Diez-Sampedro et al. 2006). In the same line, blockade of BK_{Ca} channels suppressed tonic-clonic generalized seizures in presensitized animals (Shruti et al. 2008; Sheehan et al. 2009). Genetic deletion of BK_{Ca} channel $\beta 4$ subunits that results in elevated cell surface expression of BK_{Ca} channels and larger currents triggered the occurrence of limbic seizures (Brenner et al 2005; Shruti et al 2012). Interestingly, BK_{Ca} channel $\beta 4$ subunit knockout mice consumed significantly more alcohol than their wild type counterparts and therefore are likely to experience alcohol withdrawal episodes (Martin et al 2008). The role of BK_{Ca} channel $\beta 4$ subunits in the etiology of alcohol withdrawal seizures remains to be determined. In a fly model of ethanol intoxication/withdrawal, a blockade of *slo* gene (encoding for BK_{Ca} channel) neural promoter prevented the occurrence of ethanol-induced enhancement of electrographical seizure susceptibility (Ghezzi et al. 2010, 2012). However, the present study shows that downregulation of BK_{Ca} channels outlasted the finite period of alcohol withdrawal seizures suggesting that these channels, per se, may not play a critical role in the generation of alcohol withdrawal seizures.

Conclusions

We report in this study that alcohol withdrawal causes a downregulation of BK_{Ca} channels that outlast the finite period of enhanced susceptibility to audiogenic seizures following

alcohol withdrawal. Thus, downregulation of BK_{Ca} channels, per se, may not be fundamentally important for the generation of alcohol withdrawal seizures. Nevertheless, the imbalance between upregulation of Ca_v channels and downregulation of BK_{Ca} channels may provide a novel cellular mechanism for the occurrence of alcohol withdrawal seizures.

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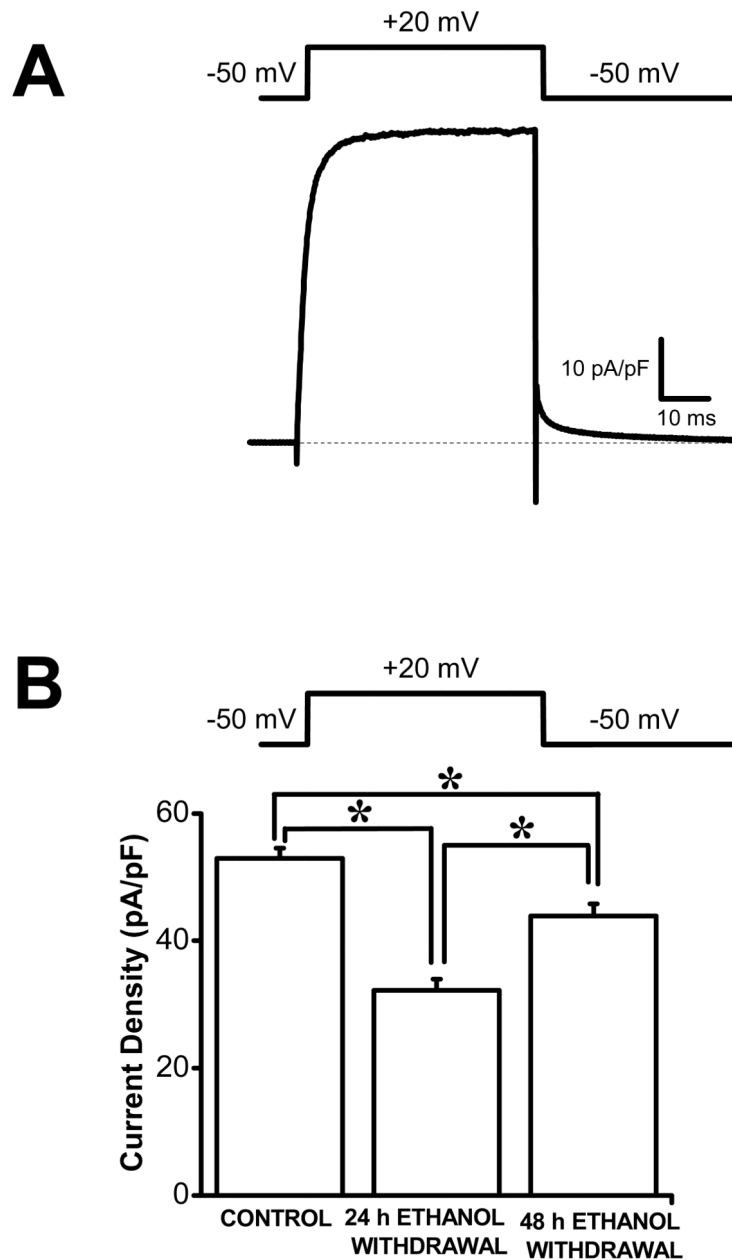


Figure 1. Downregulation total outward K^+ currents in IC neurons following ethanol withdrawal. **A.** Representative total outward K^+ currents in IC neuron obtained from naive SD rat. Whole cell outward K^+ currents were activated by voltage steps to +20 mV from the holding potential of -50 mV. **B.** The current density of total outward K^+ channels was significantly reduced in IC neurons of SD rats subjected to 24 h ($n=23$) and 48 h ($n=12$) ethanol withdrawal when the seizure susceptibility is present and absent, respectively, compared to controls ($n=23$). Additional decreases in current density were found in IC neurons of SD rats at 24 h ($n=23$) compared to 48 h ($n=12$) following ethanol withdrawal. $*P<0.05$ (two-way ANOVA). Data represents mean \pm S.E.M.

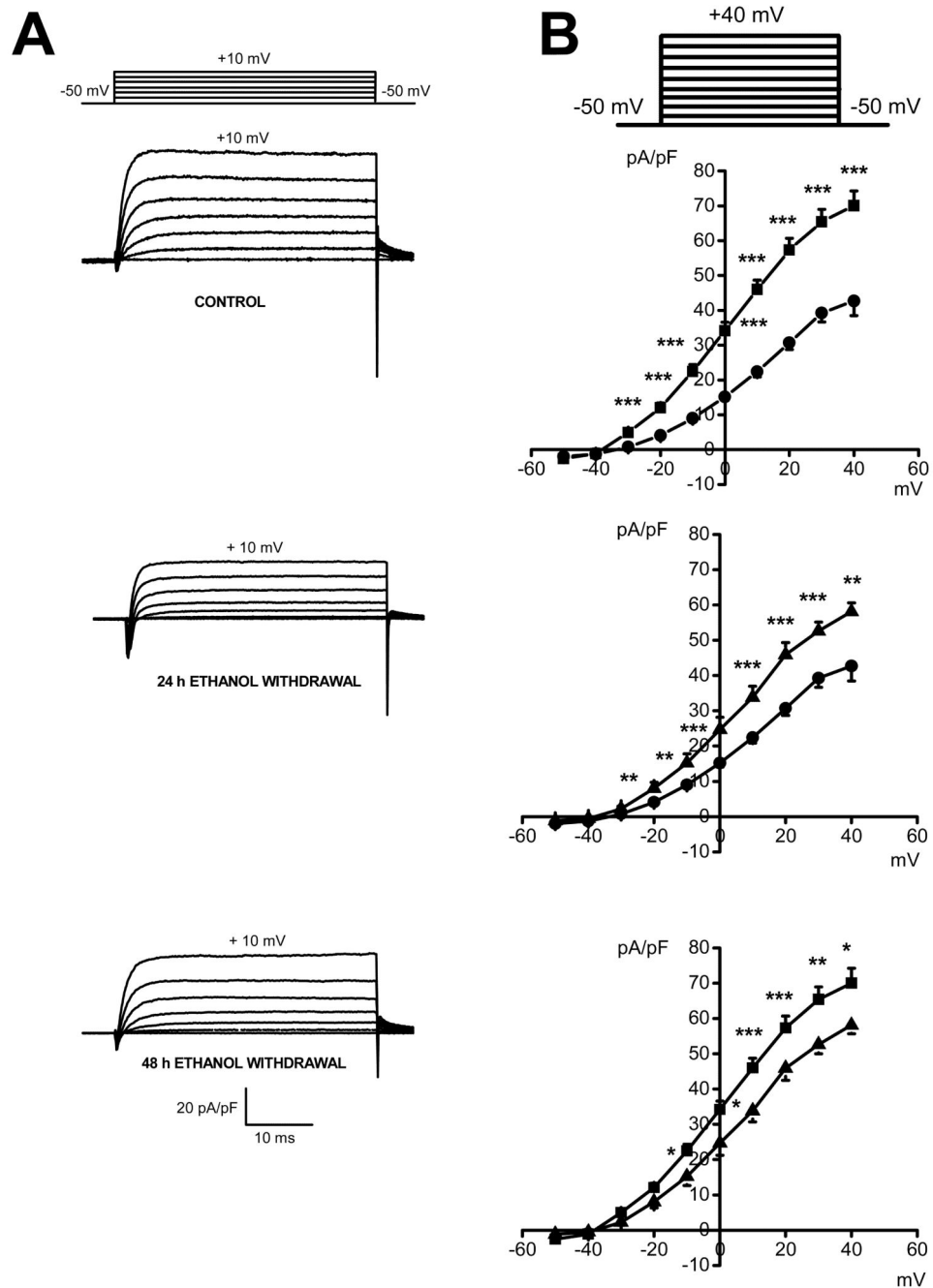
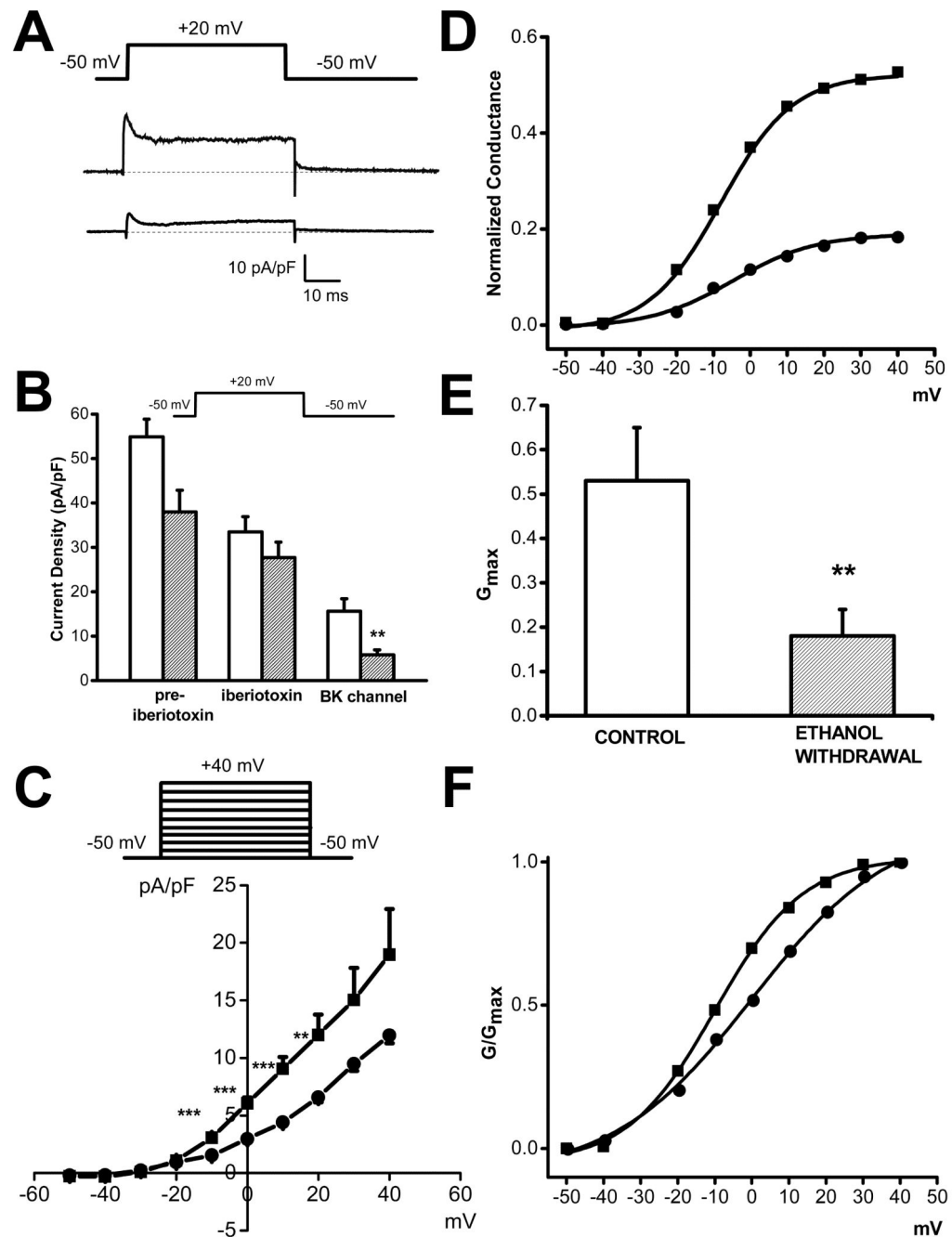


Figure 2. Voltage dependence of total outward K^+ currents in IC neurons following ethanol withdrawal. **A.** Representative whole cell outward K^+ current traces in IC neuron obtained in a control (upper traces) and rats subjected to 24 h (middle traces) and 48 h (lower traces) following ethanol withdrawal. In these examples, the peak current density was decreased by about 2-fold and 1.5-fold in IC neurons 24 h and 48 h following ethanol withdrawal, respectively, compared to controls. **B.** Voltage dependence of total outward K^+ currents in IC neurons obtained from controls (filled square, $n=12$), as well as 24 h (filled circle, $n=13$)

and 48 h (filled triangles, n=9) following ethanol withdrawal. The current density was significantly decreased 24 h following ethanol withdrawal (filled circles) at all voltages compared to the control group (filled squares; upper curves). Decreases in the current density was also found in IC neurons 24 h (filled circles) compared to 48 h (filled triangles) following ethanol withdrawal (middle curves). Quantification shows (lower curves) that the current density was significantly reduced at 48 h (filled triangles) following ethanol withdrawal, compared to controls (filled squares). Each point represents mean \pm S.E.M. * P <0.05, ** P <0.01, *** P <0.001 (one-way ANOVA).

**Figure 3.**

Downregulation of BK_{Ca} channel currents in IC neurons following ethanol withdrawal. **A**. Representative iberiotoxin-sensitive (BK_{Ca}) current traces in control (upper trace) and 24 h following ethanol withdrawal (lower trace). Outward K^+ currents were activated by +20 mV voltage steps from a holding potential of -50 mV using 2 mM Ca^{2+} as a charge carrier. **B**. Iberiotoxin-sensitive (BK_{Ca}) channel currents were significantly decreased at 24 h following ethanol withdrawal (filled bar graph, $n=8$) compared to controls (opened bar graph, $n=8$). **C**. Voltage dependence shows that iberiotoxin-sensitive currents were decreased in IC neurons

at 24 h following ethanol withdrawal (filled circle, n=6) compared to controls (filled square, n=6). **D.** Plots of normalized conductance as function of voltage revealed that ethanol withdrawal (24 h, filled circles, n=6) is associated with ~2.5 fold decreased conductance compared to controls (filled squares, n=6). **E.** The maximal conductance G_{\max} is markedly reduced in IC neurons following 24 h ethanol withdrawal (open bar, n=6) compared to controls (filled bar, n=6). **F.** Ethanol withdrawal (24 h, filled circles) did not significantly alter the voltage dependence of BK_{Ca} channel activation (G/G_{\max} curves fitted with a Boltzmann equation), as compared to controls (filled squares). Each point represents mean \pm S.E.M. ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA).

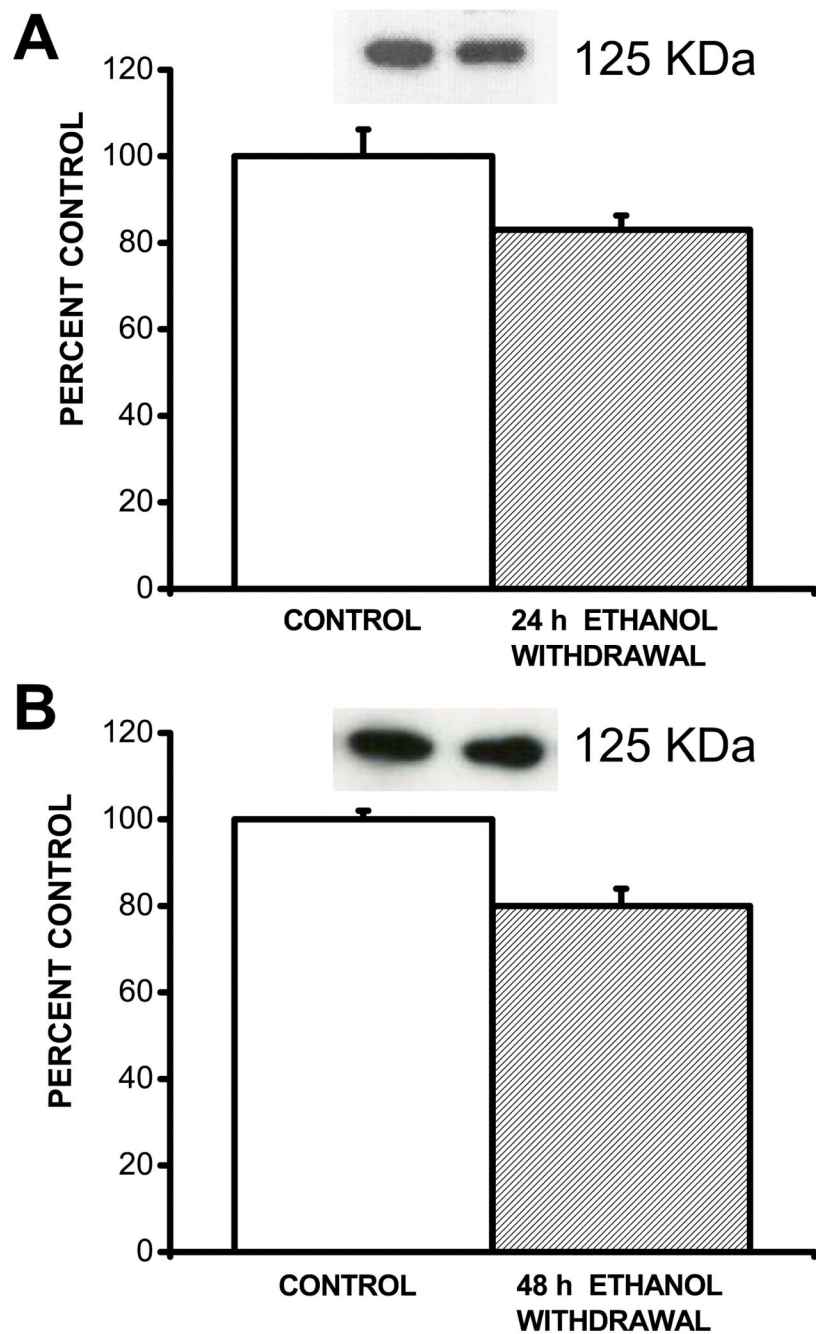


Figure 4. Downregulation of BK_{Ca} channel α -subunit in IC neurons following ethanol withdrawal. Representative immunoblots of BK_{Ca} channel α -subunit obtained from control IC neurons and following ethanol withdrawal are showed in insets. The density of the 125 kDa immunoreactive band was significantly reduced in IC neurons 24 h (filled bar, n=9, panel A) compared to controls (opened bar, n=9). Similarly, the density of the 125 kDa immunoreactive band was significantly reduced in IC neurons at 48 h (filled bar, n=8, panel

B) following ethanol withdrawal as compared to controls (opened bar, n=8). Data represents mean \pm S.E.M. * P <0.05 (two-way ANOVA).