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Alcohol withdrawal upregulates mRNA encoding for $Ca_V2.1$-α1 subunit in the rat inferior colliculus

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ABSTRACT

We previously reported increased current density through P-type voltage-gated Ca^{2+} channels in inferior colliculus (IC) neurons during alcohol withdrawal. However, the molecular correlate of this increased P-type channel current is currently unknown. Here, we probe changes in mRNA and protein expression of the pore-forming $Ca_V2.1$-α1 (P/Q-type) subunits in IC neurons during the course of alcohol withdrawal-induced seizures (AWSs). Rats received three daily doses of ethanol or the vehicle every 8 h for 4 consecutive days. The IC was dissected at various time intervals following alcohol withdrawal, and the mRNA and protein levels of the $Ca_V2.1$-α1 subunits were measured. In separate experiments, rats were tested for acoustically evoked seizure susceptibility 3, 24, and 48 h after alcohol withdrawal. AWSs were observed 24 h after withdrawal; no seizures were observed at 3 or 48 h or in the control-treated rats. Compared to control-treated rats, the mRNA levels of the $Ca_V2.1$-α1 subunit were increased 1.9-fold and 2.1-fold at 3 and 24 h, respectively; change in mRNA expression was nonsignificant at 48 h following alcohol withdrawal. Western blot analyses revealed that protein levels of the $Ca_V2.1$-α1 subunits were not altered in IC neurons following alcohol withdrawal. We conclude that expression of the *Cacna1a* mRNA increased before the onset of AWS susceptibility, suggesting that altered $Ca_V2.1$ channel expression may play a role in AWS pathogenesis.

Introduction

Generalized tonic-clonic seizures (GTCSs) are one of the most severe withdrawal symptoms following the cessation of chronic alcohol intake (Alldredge & Lowenstein, 1993; Hillbom, Pieninkeroinen, & Leone, 2003; Pilke, Partinen, & Kovanen, 1984). A robust rat model of the human alcohol withdrawal-induced generalized tonic-clonic seizures has been developed to identify their underlying mechanisms. In this model, the inferior colliculus (IC) is critically involved in the initiation of acoustically evoked reflex seizures (i.e., audiogenic seizures) during alcohol withdrawal (i.e., alcohol withdrawal-induced seizures or AWSs) (Chakravarty & Faingold, 1998; Eckardt et al., 1986; Faingold & Riaz, 1995; Frye, McCown, & Breese, 1983; McCown & Breese, 1990, 1993). Electrophysiology studies revealed elevated excitability of IC neurons following alcohol withdrawal (Evans, Li, & Faingold, 2000; Faingold, Li, & Evans, 2000; N'Gouemo, Caspary, & Faingold, 1996). Interestingly, increased firing of IC neurons was observed both prior to and during AWSs (Chakravarty & Faingold, 1998; Faingold & Riaz, 1995). These findings support the importance of IC neurons in the networks that underlie AWSs (Faingold, N'Gouemo, & Riaz, 1998). The mechanisms underlying alcohol withdrawal-induced IC neuronal hyperexcitability are not fully understood.

Altered voltage-gated Ca^{2+} (Ca_V) channels play an important role in the mechanisms that underlie AWSs (Whittington, Lambert, & Little, 1993). We previously reported that the increase in total current density through Ca_V channels observed 3 h (i.e., when no AWS susceptibility is observed) and 24 h after alcohol withdrawal (i.e., when AWS susceptibility peaks) in IC neurons returns to control levels 48 h later, when the animal is no longer susceptible to AWSs (N'Gouemo, 2015). Interestingly, changes in Ca_V current density follow a parallel time course with the occurrence of AWS

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susceptibility (Faingold, 2008; N'Gouemo & Morad, 2003; N'Gouemo, 2015). Because alcohol withdrawal preferentially affects P-type $Ca_V2.1$ channels (N'Gouemo & Morad, 2003), we examined the extent to which mRNA and protein expression of pore-forming $Ca_V2.1$-α1 (P/Q-type) subunit are altered in IC neurons following alcohol withdrawal, and whether these changes are correlated with the development of AWS susceptibility.

Materials and methods

Animals

Male Sprague–Dawley rats (250–300 g; Taconic, Germantown, MD, USA) were used for these experiments. The animals were housed in a temperature- and humidity-controlled room on a 12-h/12-h 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 (National Research Council (U.S.), 2011).

Ethanol administration

Ethanol (prepared from a 95% stock solution) was administered by oral gavage as a 30% (v/v) solution in ISOMIL Infant Formula Concentrate (the ISOMIL formula was diluted 1:1 with water). Ethanol was administered three times per day (at 8-h intervals) for 4 days, and the level of intoxication was evaluated using a standard behavior-rating scale (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., an accentuated staggering gait and considerable elevation of the pelvis) but not severe (i.e., lethargy without pelvic or abdominal elevation) degree of intoxication (Faingold, 2008); the total amount of ethanol administered was 2–12 g/kg/day. On the fourth day, ethanol treatment was terminated after the second dose. The behavioral signs of ethanol intoxication and subsequent doses of ethanol were determined based on a well-described intoxication scale (Faingold, 2008; Majchrowicz, 1975): 0, Neutrality or absence of signs of intoxication or withdrawal (5 g/kg); **1, Sedation** (4 g/kg); **2, Ataxia 1**, characterized by the lowest degree of gait impairment (4 g/kg); **3, Ataxia 2**, corresponding to an intermediate degree of gait impairment (3 g/kg); **4, Ataxia 3**, characterized by a marked level of intoxication or recovery of the righting reflex (2 g/kg); **5, Loss of righting reflex** (1 g/kg); and **6, Coma** or absence of movements, eye closure, and absence of the eye blink reflex (0 g/kg). The behavioral signs of ethanol withdrawal include hyperactivity, tremors, tail spasticity, spontaneous seizures (myoclonus and forelimb clonic seizures), and acoustically evoked startle responses and seizures (Faingold, 2008; N'Gouemo, Yasuda, & Morad, 2006). The control-treated animals were maintained under similar conditions but received three times daily the ISOMIL formula alone (without ethanol).

Seizure testing

To determine the animals' susceptibility to develop acoustically evoked AWSs, each control-treated (n = 10) and ethanol-treated (n = 10) rat was selected at random and tested repetitively for seizures during the course of ethanol withdrawal: 2–3 h (the 3-h group), 23–24 h (the 24-h group), and 47–48 h (the 48-h group) after the last dose of ethanol. Animals were placed into a Plexiglass chamber located in a sound attenuating cubic equipped with a ventilation fan, light, sound generator, and video monitoring system (Med Associates, St. Albans, VT, USA). To induce seizures, an acoustic stimulus that consisted of pure tones at 100–110-dB sound pressure level was presented until wild running seizures (WRSs) was elicited or for 60 s if no seizure activity was observed. Convulsive seizure behavior was classified into four stages based on the scale for acoustically evoked seizures (Jobe, Mishra, & Dailey, 1992): stage 0, no seizures in response to acoustic stimuli; stage 1, one episode of WRSs; stage 2, two episodes of WRSs; stage 3, one episode of WRSs and/or jumping followed by bouncing GTCSs (or clonus) involving forelimbs and hindlimbs; stage 4, two episodes of WRS followed by clonus; stage 5, one episode of WRSs and clonus followed by tonic forelimb extension and clonus of the hindlimbs. Animals that did not exhibit a seizure during the first trial were tested again 2 h later using mixed sounds delivered via an electrical bell, based on the fact that seizure threshold varies between animals. The animals used in the molecular studies were not subjected to acoustically evoked seizure testing, as evoked seizures can induce a long-lasting increase in extracellular GABA levels (Ueda & Tsuru, 1995). Such GABA release and various degrees of seizure severity and duration may alter $Ca_V2.1$ channels expression.

Blood ethanol concentrations

In a separate set of experiments, blood ethanol concentration (BEC) was measured in the control group (n = 4) and in the ethanol-treated group at 3 h (n = 4), 24 h (n = 4), and 48 h (n = 4) during ethanol withdrawal. The rats were anesthetized (50 mg/kg Nembutal; intraperitoneally [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, London, UK).

Western blot analysis

At 3, 24, and 48 h 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% IGEPAL (Sigma-Aldrich, St. Louis, MO, USA), 10% glycerol, 1-mM EDTA, and 1-mM Na_3VO_4. The homogenates were cleared by centrifugation (13,800 × *g*, 4 °C, and 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 (BCA) assay and a Bio-Rad Model 680 spectrophotometer (Bio-Rad, Hercules, CA, USA). 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, Lincoln, NE, USA) for 1 h and then probed overnight at 4 °C with primary rabbit antibodies against the α1A subunit ($Ca_V2.1$-α1) (1:200; Alomone Lab, Jerusalem, Israel). The membranes were also incubated with anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) antibody (1:10,000; Thermo Fisher Scientific, Waltham, MA) 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 h 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 $Ca_V2.1$-α1 subunits in the IC, another set of rats (n = 8 per group) were subjected to the