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Neuroplasticity of A-type potassium channel complexes induced by chronic alcohol exposure enhances dendritic calcium transients in hippocampus

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Abstract

Rationale—Chronic alcohol-induced cognitive impairments and maladaptive plasticity of glutamatergic synapses are well-documented. However, it is unknown if prolonged alcohol exposure affects dendritic signaling that may underlie hippocampal dysfunction in alcoholics. Back-propagation of action potentials (bAPs) into apical dendrites of hippocampal neurons provides distance-dependent signals that modulate dendritic and synaptic plasticity. The amplitude of bAPs decreases with distance from the soma that is thought to reflect an increase in the density of Kv4.2 channels toward distal dendrites.

Objective—The aim of this study was to quantify changes in hippocampal Kv4.2 channel function and expression using electrophysiology, Ca²⁺ imaging, and western blot analyses in a well-characterized in-vitro model of chronic alcohol exposure.

Results—Chronic alcohol exposure significantly decreased expression of Kv4.2 channels and KChIP3 in hippocampus. This reduction was associated with an attenuation of macroscopic A-type K⁺ currents in CA1 neurons. Chronic alcohol exposure increased bAP-evoked Ca²⁺ transients in the distal apical dendrites of CA1 pyramidal neurons. The enhanced bAP-evoked Ca²⁺ transients induced by chronic alcohol exposure were not related to alteration of synaptic targeting of NMDA receptors or morphological adaptations in apical dendritic arborization.

Conclusions—These data suggest that chronic alcohol-induced decreases in Kv4.2 channel function possibly mediated by a down-regulation of KChIP3, drive the elevated bAP-associated Ca²⁺ transients in distal apical dendrites. Alcohol-induced enhancement of bAPs may affect metaplasticity and signal integration in apical dendrites of hippocampal neurons leading to alterations in hippocampal function.

Keywords

back-propagating action potentials; CA1 pyramidal neurons; chronic alcohol; dendritic calcium transients; KChIP3; Kv4.2 channels

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Introduction

It is well established that the hippocampus is a critical brain structure in learning and memory, and is increasingly being shown to function as a central hub (gate) to modulate information flow both within and between multiple disparate brain structures such as the prefrontal cortex (PFC) and amygdala (Maren et al. 2013). Hippocampal dysfunction may not only affect declarative memory but also impact many complex behaviors, affective disorders and addiction. Addiction to alcohol (ethanol) and other drugs induces plastic events and leads to lasting impairments in cognitive function that contribute to high relapse rates and poor treatment outcomes (Gould 2010; Kroener et al. 2012; Lovinger and Roberto 2013; Spiga et al. 2008). This signifies an essential need for a better understanding of the effects of chronic alcohol exposure on neuroadaptations within the hippocampus that may lead to the development of novel pharmacotherapeutic strategies to treat alcohol-related cognitive deficits.

Prolonged alcohol exposure engages neural mechanisms responsible for experience-dependent synaptic plasticity, and remodels glutamatergic synapses and alters glutamate signaling in key brain regions within the addiction circuitry (Mulholland and Chandler 2007). For example, chronic intermittent ethanol (CIE) exposure by vapor inhalation leads to enhanced NMDA receptor expression and long-term potentiation (LTP) in hippocampus (Nelson et al. 2005; Sabeti 2011; Sabeti and Gruol 2008), and produces a metaplastic shift from long-term depression to LTP in nucleus accumbens medium spiny neurons (Jeanes et al. 2011). Moreover, we have recently demonstrated that CIE exposure enhanced spike timing-dependent synaptic plasticity (STDP) in the PFC (Kroener et al. 2012). This aberrant plasticity was associated with poor performance on a PFC-dependent attentional set-shifting task. The induction of STDP requires the precise timing of presynaptic glutamate release and back-propagating action potentials (bAP) into the dendrite (Caporale and Dan 2008). While it is unknown if chronic alcohol exposure influences the mechanisms that control bAPs, these bAPs can activate voltage-gated Ca^{2+} channels and facilitate Mg^{2+} unblock of NMDA receptors leading to enhanced Ca^{2+} signaling and the induction of LTP (Caporale and Dan 2008; Colbert 2001).

Voltage-dependent potassium (Kv) channels composed of Kv4.2 α subunits underlie the transient A-type K^{+} current (I_A) in hippocampal dendrites where they shape postsynaptic responses, constrain coincidence detection, and control the amplitude of bAPs (Chen et al. 2006; Hoffman et al. 1997). Kv4.2 mRNA is targeted to dendritic compartments where its translation is coupled to changes in NMDA receptor activity (Jo and Kim 2011), and emerging evidence suggests that there is a functional coupling between Kv4.2 channels and NMDA receptors that may be critical for homeostatic plasticity (Jo and Kim 2011; Jung et al. 2008; Kaufmann et al. 2012). Of particular interest is recent evidence demonstrating an important role of Kv4.2 channels in both synaptic plasticity and cognition (Jung et al. 2008; Lockridge and Yuan 2011; Lugo et al. 2012; Truchet et al. 2012). Therefore, it is possible that neuroadaptations in Kv4.2 channels may be an important factor underlying altered synaptic plasticity and poor cognitive performance associated with prolonged alcohol

consumption. The present study was designed to investigate alterations in Kv4.2 channel expression and function in the hippocampus in response to chronic alcohol exposure.

Experimental procedures

All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committees of the Medical University of South Carolina and The University of Texas at Dallas.

Preparation and treatment of hippocampal cultures

Organotypic hippocampal slice cultures (OHSCs) were prepared and treated with chronic alcohol exposure following routine procedures (Mulholland et al. 2011). Briefly, four- to five-day-old Sprague-Dawley rat pups were rapidly decapitated and whole brains were aseptically removed and placed in chilled (4°C) dissection medium, consisting of Basal Medium Eagle (with Earle's salts) plus 25 mM HEPES, 2 mM GlutaMAX, and 100 µg/ml streptomycin (pH 7.2). Bilateral hippocampi were removed and placed in chilled culture medium comprised of dissection medium plus 36 mM glucose, 25% (v/v) Earle's balanced salt solution, and 25% PPHS. Each hippocampus was coronally sectioned at 400 µm using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK), yielding approximately 13 slices (26 per animal), and placed into fresh chilled culture medium. Four slices were transferred onto Millicell-CM 0.4-µm biopore membrane inserts and placed in 35-mm six-well culture plates in pre-incubated culture medium. Cultures were placed in a 37°C incubator with an atmosphere of 7.5% CO₂/92.5% air for 10 days ensuring complete development of intrinsic network pathways prior to the start of 7–9 day alcohol exposure (25 – 75 mM). Media was changed to one containing a reduced amount of platelet-poor horse serum (5%) after 4 days in-vitro and was replaced every 4 days. Each set of cultured slices was prepared from 6–8 rats pups and each treatment group within an experiment contained slices from at least 2 separate rat pups. To avoid litter effects, all experiments were completed with 4–7 replicates. At the completion of treatment, OHSCs were then prepared for western blots, electrophysiology, or calcium imaging.

Western blot analysis

Standard procedures were used for preparation of crude membrane and bis(sulfosuccinimidyl)suberate cross-linked fractions (Mulholland et al. 2011). Crude membrane fractions were prepared from organotypic hippocampal slices cultures treated with chronic alcohol. There were 4–7 replicates/experiment and each treatment group within an individual experiment contained 8 slices from 2 or more different rat pups. In brief, OHSCs were transferred into ice-cold homogenization buffer (50 mM Tris-HCl, 50 mM NaCl, 10 mM EGTA, 5 mM EDTA; 2 mM Na⁺ pyrophosphate, 1 mM activated Na⁺ orthovanadate, 1 mM Na⁺ fluoride, pH 7.5, containing Complete Protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The tissue was then probe sonicated and centrifuged at 23,100 × g for 30 min at 4°C. The resulting supernatant was removed and the pellet was solubilized in 2% lithium dodecyl sulfate (LDS). An aliquot was taken for determination of protein concentration by the bicinchoninic acid assay (Pierce

Biotechnology, Inc., Rockford, IL). The remaining pellet was stored at -80°C until western blot analysis. Because the use of loading controls (e.g., actin, GAPDH) for normalization in western blot experiments are subject to error in quantitation (Aldridge et al. 2008; Dittmer and Dittmer 2006), they were not used in these studies. Before each study, a series of western blots was performed using different titrations of sample and antibody to establish the linear range for each protein.

Methods for BS³ cross-linking of surface proteins followed procedures described previously (Mulholland et al. 2011). Slices were washed in ice-cold incubation buffer containing (in mM): NaCl (140), KCl (5.4), CaCl₂ (1.8), glucose (15), HEPES (25), and MgCl₂ (2), and were placed in a 1 mg/ml solution of BS³ in incubation buffer for 30 min at 4°C . The slices were then washed $3\times$ for 5 min in incubation buffer containing 20 mM Tris. The Tris wash buffer was completely removed and the slices were solubilized into 2% LDS and stored at -80°C until analysis. Primary antibodies used in these studies were Kv4.2 (1:1000; NeuroMab, Antibodies, Inc. & UC Davis, Davis, CA), GluN1 (1:4000; BD Pharmingen, Franklin Lakes, NJ), GluN2B (1:1000; NeuroMab, Antibodies, Inc. & UC Davis, Davis, CA), KChIP3 (1:1,000; NeuroMab), and Kv1.4 (1:1000; NeuroMab). Specificity of Kv4.2, GluN2B, KChIP3, and Kv1.4 primary antibodies have been confirmed in knockout mice (Alexander et al. 2009; Badanich et al. 2011; Deng et al. 2011; Menegola and Trimmer 2006). The membranes were then washed in PBST prior to 1 h incubation at room temperature with horseradish peroxidase conjugated goat anti-mouse secondary antibody diluted 1:2000 in PBST containing 0.5% NFDM. The antigen-antibody complex was detected by enhanced chemiluminescence using a ChemiDoc MP Imaging system (Bio-Rad Laboratories, Hercules, CA). The band corresponding to the appropriate molecular weight was quantified by mean optical density using computer-assisted densitometry with ImageJ v1.41 (NIH, USA).

Electrophysiology

Slices for the electrophysiology and imaging experiments were maintained in culture media containing alcohol until just before recording and imaging. Voltage-clamp recordings of macroscopic A-type K⁺ currents (I_A) in CA1 pyramidal neurons from OHSC were carried out at room temperature ($22-24^{\circ}\text{C}$) using an Axopatch 700B amplifier (Molecular Devices Corporation, Union City, CA) immediately following chronic alcohol treatment as previously described (Chen et al. 2006). A Zeiss AxioExaminer D1 microscope fitted with a $40\times$ water immersion objective and Dodt contrast was used to view CA1 neurons. Slice cultures were bathed with an extracellular recording solution designed to isolate whole-cell voltage-dependent I_A (osmolarity ~ 320 mOsm, saturated with 95% O₂-5% CO₂) containing the following (in mM): NaCl (125), KCl (2.5), MgCl₂ (1.3), glucose (25), HEPES (10), and tetrodotoxin (0.001). Ca²⁺ was excluded from the recording buffer and 2 mM MnCl₂ was added to block Ca²⁺ currents and Ca²⁺-activated K⁺ channels (Chen et al. 2006; Kass and Tsien 1975). Patch pipettes (2–3 M Ω resistance) were pulled from thin wall borosilicate glass (1.17 mm ID; Warner Instruments, Hamden, CT, USA) and filled with internal solution (pH 7.3 using KOH, osmolarity ~ 300 mOsm) containing (in mM): K-methylsulfate (120), KCl (20), NaCl (8), Mg-ATP (4), EGTA (0.2), HEPES (10), phosphocreatine (14), and GTP (0.3). Currents were acquired at 10 kHz using an ITC-18 interface and collected on

a PC running Axograph X software (Axograph Scientific, New South Wales, Australia). Neurons were held at -80 mV and depolarizing voltage steps (100 ms, from -80 to $+20$ mV) were applied with 100 ms between the two steps. Peak amplitude of the I_A that occurred after each depolarizing pulse was measured, averaged from 3 sweeps, and normalized to cell capacitance values. Steady-state current density was measured by dividing average current of the last 50 ms of the first step by the cell capacitance value. Access resistance (<30 M Ω) was monitored throughout, and a $<15\%$ change was deemed acceptable in all recordings.

Current-clamp recordings and fluorescence Ca^{2+} imaging

Recordings of membrane properties and action potential (AP) characteristics of CA1 neurons, as well as fluorescence imaging of calcium transients in the apical dendrites of these cells were performed in current-clamp mode. Therefore, individual OHSCs were transferred from their incubation media to a recording chamber where they were superfused at a rate of 2 ml/min with bubbled ACSF (95% O_2 -5% CO_2) and were maintained at room temperature (22–24°C). The recording ACSF was composed of (in mM): NaCl (126), KCl (2.5), Na_2HPO_4 (1.2), Na_2HCO_3 (25), glucose (10), CaCl_2 (2), and MgCl_2 (1). Using infrared DIC video microscopy we obtained whole-cell current-clamp recordings from visually-identified pyramidal neurons in the CA1 region of the hippocampus. Recording micropipettes pulled from borosilicate glass had an open tip resistance of 2–3 M Ω when filled with the following solution (in mM): K-gluconate (125), KCl (20), HEPES (10), phosphocreatine (10), ATP- Mg^{2+} (4), Na-GTP (0.3), Oregon Green 488 BAPTA-1 (0.1), and Alexa 594 (0.04), pH adjusted to 7.2–7.3 using KOH. Recordings were performed using a MultiClamp 700B amplifier. Signals were low-pass filtered at 3 kHz, digitized at 10 kHz, and stored on a PC for off-line analysis. Data acquisition and analysis were performed using Axograph X. The apparent input resistance (R_{in}) was measured from the average ($n > 10$) of the responses to small hyperpolarizing current pulses (-30 pA; 100-ms duration) preceding individual sweeps. The rheobase current and the basic properties of the first action potential (latency, voltage threshold, half-width) were analyzed from series of hyperpolarizing and depolarizing current steps (-100 pA to 460 pA, 20 pA steps, 1 sec duration). The action potential threshold was defined as the initial point of rapid voltage deflection, and the amplitude of the action potential and the fast postspike afterhyperpolarization (AHP) were measured from threshold to the positive and negative peak, respectively. Half-width of the action potential was the duration of the action potential at half-amplitude.

Changes in intracellular Ca^{2+} concentration were imaged using a confocal microscope [(LSM 510, Zeiss, Oberkochen, Germany) or (FV1000, Olympus Optical, Tokyo, Japan)]. Neurons were loaded via the recording pipette with the Ca^{2+} -sensitive fluorescent dye Oregon Green BAPTA-1 (OGB-1) (100 μM , Invitrogen, Carlsbad, CA) added to the pipette recording solution, and cells were held at -70 mV and were filled for at least 30 min before recording. OGB-1 was excited using the 488 line of an argon laser and emitted fluorescence was detected via a dichroic mirror (510 nm) and long-pass filter (505 nm). Changes in fluorescence following bAPs were measured with line scans at 0.3–1 kHz across apical dendrites. A minimum of three repetitions was acquired for each condition and averaged for statistical comparison. A maximum of two recording sites from each neuron were used for analysis. A second dye of a different wavelength (Alexa Fluor 594; 40 μM) was also

included in the internal recording solution to aid reconstruction of the cell's morphology in high-resolution z-stacked images at the end of the experiments. Alexa 594 was excited at 594 nm via a HeNe laser and emission was long-pass filtered at 560 nm. Raw data were background subtracted, and changes in Ca^{2+} influx in response to bAPs were calculated as the change in fluorescence relative to the resting fluorescence ($F/F = (F - F_{\text{basal}})/F_{\text{basal}}$, where F is the fluorescence at any time point, and F_{basal} is the baseline fluorescence averaged across the whole imaging duration. Analysis used Zeiss LSM (V3.2) or Olympus Fluoview imaging software, respectively. Because chronic alcohol exposure is known to increase NMDA receptor function and NMDA receptors have been shown to contribute to bAP-evoked Ca^{2+} transients, we also examined the effects of NMDA receptor blockade in these slices. Therefore, baseline recordings were acquired before and after bath application of the NMDA receptor antagonist CPP (10 μM).

Morphological analysis

Z-stacked confocal images of CA1 neurons filled with Alexa 594 during whole cell recordings were collapsed to generate images of the entire apical dendritic arbor. Apical dendrite complexity was analyzed using Imaris XT 3D imaging software (Bitplane, Zurich, Switzerland; version 6.4.2). The filament module of Imaris XT was utilized to determine dendritic arborization and length of apical dendrites based on branch order.

Statistical analyses

All data are expressed as means $\pm$ SEM, and a p value of 0.05 was used to determine significance. Western blot data from the 4–7 replicate experiments were analyzed, and each cell from the electrophysiology and morphology experiments were included in the analysis. Western blot and electrophysiology data were analyzed by two-tailed t-tests, and one- or two-way ANOVAs. Data collected from morphological studies were analyzed by two-tailed t-tests, two-way ANOVA, or χ^2 test for trends. Two-tailed t-tests, one-way ANOVAs and χ^2 test for trends were analyzed with Prism 6.02 (GraphPad Software, Inc.), and two-way ANOVA were analyzed using SigmaPlot (version 11.0, Sysstat Software, Inc.). Student-Newman-Keuls (SNK) post-hoc tests were used, when appropriate.

Results

Chronic alcohol and surface expression of Kv4.2 channels

Alterations in Kv4.2 channel expression and function were determined after chronic alcohol exposure using a culture model suited to study homeostatic and dendritic plasticity (Holopainen 2005). OHSCs were exposed to chronic alcohol (25 – 75 mM, 7–9 days) and a crude membrane fraction was prepared at the end of the exposure regimen. Studies on individuals admitted to the emergency room or detoxification units have reported blood alcohol concentrations (BALs) of 270 mg% to over 700 mg% in alcoholics (Cartledge and Redmond 1990; Lindblad and Olsson 1976; Teplin et al. 1989; Urso et al. 1981). The concentration of alcohol used in this study was selected to match BALs reported in this heavy drinking population. Alcohol treatment produced a concentration-dependent reduction in expression of Kv4.2 channels (Fig. 1A). To determine if chronic alcohol exposure reduced surface Kv4.2 channels, control and alcohol-exposed hippocampal cultures were

treated with a BS₃ crosslinking reagent at the end of the exposure period to separate plasma from intracellular membrane-bound Kv4.2 channels. Chronic alcohol exposure significantly reduced Kv4.2 channels in the total, but not intracellular fraction (Fig. 1B), suggesting a significant decrease in the surface pool of Kv4.2 channels. Along with Kv4.2, the Kv1.4 subunit contributes to macroscopic I_A in CA1 pyramidal neurons (Coetzee et al. 1999). Chronic alcohol did not alter protein expression levels of Kv1.4 channels in OHSCs (Fig. 1C). Interestingly, modulating functional Kv4.2 channels in hippocampal neurons has been reported to bi-directionally regulate expression of GluN1 and GluN2B, but not GluN2A subunits of the NMDA receptor (Jung et al. 2008). Consistent with this, we observed that chronic alcohol exposure elevated expression levels of GluN1 and GluN2B subunits of the NMDA receptor, but had no effect on GluN2A (Fig. 1D). Surface expression of functional Kv4.2 channels is modulated, in part, by Kv channel-interacting proteins (KChIPs) (Covarrubias et al. 2008), and KChIP3 also interacts with the C-terminal region of GluN1 to inhibit the surface expression of NMDA receptors (Zhang et al. 2010). Similar to our findings with Kv4.2 channels, chronic alcohol exposure of OHSCs produced a concentration-dependent reduction in expression levels of KChIP3 (Fig. 1E).

Chronic alcohol, AP properties, and A-type currents

In addition to a known role in regulating the amplitude of bAPs in hippocampal neurons, Kv4.2 channels can influence membrane excitability and AP properties (Kim et al. 2005). To complement the observation of changes in protein expression and confirm there was a functional reduction in surface Kv4.2 channels, the effects of chronic alcohol exposure on basic membrane properties, AP characteristics, and I_A density were examined using patch-clamp electrophysiology. Basic membrane properties and AP characteristics of control and chronic alcohol-treated CA1 neurons are shown in Table 1 and Fig. 2. Chronic alcohol exposure did not affect resting membrane potential, input resistance, rheobase, AP threshold or half-width, or latency to first AP. However, there was a significant reduction in the amplitude of the AHP in the chronic alcohol-exposed slice cultures (Fig. 3B,C).

The next set of studies was designed to directly measure changes in the contribution of K⁺ currents that contribute to I_A . The Kv1- and Kv4-mediated components of I_A can be distinguished by their different time constants of recovery from inactivation: recovery of Kv1 channels is > 1 sec, whereas Kv4 channel recovery from inactivation is <100 ms (Castellino et al. 1995; Jerng et al. 2004; Johnston et al. 2000). Taking advantage of these differences in the kinetics of recovery, we used a two-step protocol to examine whether chronic alcohol exposure (75 mM) altered the relative contribution of Kv4 and Kv1 channels to I_A density in CA1 pyramidal neurons. Neurons were held at -80 mV and then depolarized to +20 mV twice with 100 ms separating each voltage step (Fig. 3A). Because of the longer recovery from inactivation for Kv1, the second transient may represent Kv4-mediated currents. The contribution of Kv1 channels to the overall I_A can be determined by subtracting the second from the first transient. A significant reduction in I_A density by chronic alcohol was observed at both transients (Fig. 3B). Chronic alcohol exposure did not significantly alter Kv1-mediated currents in CA1 pyramidal neurons (Fig. 3C). The density of outward steady-state K⁺ currents were not different between control and chronic alcohol-treated CA1 neurons (control: 22.39 ± 6.48 pA/pF; chronic alcohol: 18.82 ± 3.62 pA/pF; t-

test, $p = 0.66$, $n = 8/\text{group}$). Together, these data are consistent with our observation that in-vitro chronic alcohol exposure reduces the membrane surface expression of Kv4.2, and further demonstrates that this reduction is reflected as a selective decrease in the Kv4-mediated component of I_A in CA1 pyramidal neurons.

Chronic alcohol exposure enhances bAP-evoked Ca^{2+} transients

In hippocampal CA1 pyramidal neurons, there is a distance-dependent increase in the density of I_A along the apical dendrite that has been shown to play a critical role in limiting the amplitude of bAPs into the dendrite (Hoffman et al. 1997). Furthermore, this distance-dependent increase in the density of the I_A along the dendrite is thought to reflect the progressive increase in the density of Kv4.2 channels relative to the uniform distribution of Na^+ channels (Bernard and Johnston 2003; Hoffman et al. 1997; Johnston et al. 2000; Kerti et al. 2012; Magee 1998; Yuan et al. 2002). Our observation that chronic alcohol exposure reduces Kv4.2 surface expression and I_A density suggests that bAP-evoked Ca^{2+} transients might be increased at distal sites of the apical dendrites. To examine this possibility, bAP-evoked Ca^{2+} transients were recorded in the apical dendrites from control and chronic alcohol-treated slices using an internal solution containing the Ca^{2+} indicator OGB-1 (100 μM) and Alexa-594 (40 μM) (Fig. 4A). High-resolution Z-stacked images of Alexa-594 obtained at the end of the recordings were used for subsequent analysis of morphological changes in dendritic arborization (Fig. 4A and 5A). As hypothesized, chronic treatment with alcohol significantly increased Ca^{2+} transients induced by a single bAP in apical dendrites of CA1 neurons (Fig. 4B). The difference between the intercepts of the regression lines for bAP-evoked Ca^{2+} transients in control and chronic alcohol-treated neurons was highly significant ($F(1,35) = 26.082$, $p < 0.0001$). As expected, chronic alcohol exposure significantly increased averaged magnitude of the bAP-evoked Ca^{2+} transients by 162% above control transient values (Fig. 4C).

Chronic alcohol exposure is well-documented to increase NMDA receptor expression, and NMDA receptor activation can impact bAP-evoked Ca^{2+} transients (Wu et al. 2012). Thus, it is possible that an increase in NMDA receptors in the distal dendrites could have contributed to the increase in bAP-evoked Ca^{2+} transients following chronic alcohol exposure. To test this hypothesis, bAP-evoked Ca^{2+} transients were recorded in CA1 neurons in control and chronic alcohol-exposed slice cultures before and after bath application of the NMDA receptor antagonist CPP (10 μM). Compared to baseline, bath application of CPP significantly reduced bAP-evoked Ca^{2+} transients in control and chronic alcohol-exposed CA1 pyramidal neurons (Fig. 4D). However, the magnitude of the reduction in Ca^{2+} transients by CPP did not differ significantly between the two treatment groups (t-test, $p = 0.12$, $n = 11\text{--}14/\text{group}$). Thus, up-regulation of NMDA receptors did not appear to substantially contribute to the enhanced Ca^{2+} signal in CA1 neurons from alcohol-exposed slices, again consistent with the increase resulting from reduced dendritic Kv4.2 channels.

Chronic alcohol does not affect apical dendrite length

Prolonged alcohol consumption has been reported to produce morphological alterations in the apical dendrites of CA1 pyramidal neurons (Mitra and Mukherjee 2001), which could

impact Ca^{2+} signaling in hippocampal dendrites. Thus, we examined alcohol-induced morphological adaptations in the apical dendrites using images collected from neurons filled with Alexa 594 during recordings of Ca^{2+} transients (Fig. 5A). Chronic alcohol did not alter the total length of the dendritic tree (Fig. 5B), the length of the main apical dendrite (Fig. 5B), the total number of branches (Fig. 5C), or the length of 2nd, 3rd, or 4th order branches (Fig. 5D). However, there was a lack of higher-order oblique branches (5th and 6th order) in the stratum radiatum layer (Fig. 5E). This suggests that chronic alcohol exposure has a subtle effect on pruning of the arborization of higher-order oblique dendrites in CA1 pyramidal neurons.

Discussion

The major finding of these studies is that chronic alcohol exposure reduces Kv4.2 channel function and surface expression and alters Kv4.2 channel complexes in the hippocampus. The down-regulation of Kv4.2 channels by chronic alcohol exposure was associated with a decrease in KChIP3 expression and elevated bAP-evoked Ca^{2+} transients in distal apical dendrites of CA1 pyramidal neurons. The increase in Ca^{2+} transients did not appear to be influenced by chronic alcohol-induced up-regulation of NMDA receptors, compensatory changes in Kv1.4-mediated I_A , or gross morphological changes in the apical dendritic arborization of CA1 pyramidal neurons. Taken together, these data suggest that chronic alcohol exposure reduces surface expression and function of Kv4.2 channels.

Kv4.2 channels are targeted to the apical dendrites and their density increases with distance from the soma (Hoffman et al. 1997; Kerti et al. 2012). These observations lead us to hypothesize that the reduced function and surface expression of Kv4.2 channels in response to chronic alcohol exposure may lead to changes in the amplitude of bAPs in the dendrite. In support of this, we observed a significant increase in bAP-evoked Ca^{2+} transients in the distal dendrites of CA1 pyramidal neurons that were exposed to chronic alcohol. In addition to Kv4.2 channels, extrasynaptic but not synaptic NMDA receptors have been reported to contribute to a portion of bAP-evoked Ca^{2+} transients in CA1 apical dendrites (Wu et al. 2012). An increase in NMDA receptors by chronic alcohol was also observed, and previous evidence suggests that this increase is due to synaptic targeting of NMDA receptors (Carpenter-Hyland et al. 2004; Clapp et al. 2010). As expected, blocking NMDA receptors slightly reduced the Ca^{2+} transients in apical dendrites. However, the magnitude of the reduction in the Ca^{2+} transients by NMDA receptor blockade was similar between control and alcohol-treated neurons. This lack of effect is consistent with the suggestion that expression of extrasynaptic NMDA receptors is not affected by chronic alcohol exposure. Together, these data provide evidence that it is the alcohol-induced down-regulation of Kv4.2 channels, and not the up-regulation of synaptic NMDA receptors that facilitates enhanced bAP propagation in distal dendrites. These data provide the first evidence that chronic alcohol exposure produces neuroadaptations in mechanisms that regulate bAPs in CA1 pyramidal neurons.

In congruence with our observation of a down-regulation of surface Kv4.2 channels, chronic alcohol exposure also decreased whole-cell I_A recorded from CA1 pyramidal neurons. In hippocampus, Kv4.2 and Kv1.4 α subunits are the predominant subunits that mediate

macroscopic I_A (Coetzee et al. 1999). Using a depolarization protocol that can separate Kv4- and Kv1-mediated I_A (Chen et al. 2006), we demonstrated that chronic alcohol selectively reduced Kv4-mediated currents without a significant effect on Kv1-mediated currents. Even though space-clamp is a known issue when trying to accurately record distal dendritic I_A at the soma, chronic alcohol exposure reduced surface expression of Kv4.2 channels and elevated bAP-evoked Ca^{2+} transients in distal apical dendrites. Interestingly, previous evidence suggests that there is a compensatory increase in Kv1 currents and an apparent coordinated decrease in KChIP3 expression in mice with a genetic deletion of Kv4.2 channels (Chen et al. 2006). In our studies, chronic alcohol did not produce a compensatory change in Kv1 function to counteract the reduction in Kv4.2. Together, these data suggest that Kv4.2 channel expression and function are reduced by chronic alcohol exposure. Further work is necessary to determine if this important component of regulating signal processing in hippocampus is a critical factor underlying altered synaptic plasticity and poor cognitive performance associated with prolonged alcohol consumption.

The induction of STDP requires the precise timing of presynaptic glutamate release and bAPs into the dendrite (Caporale and Dan 2008), and previous evidence suggests that Kv4.2 channels can modulate STDP in CA1 neurons (Watanabe et al. 2002). Knock-down of Kv4.2 channels enhanced the induction of LTP (Chen et al. 2006) and produced spatial learning deficits on the Morris water maze (Lockridge and Yuan 2011; Lugo et al. 2012), while pharmacological blockade of Kv4.2 channels impaired reference memory on a radial arm maze task (Truchet et al. 2012). KChIP3 knockout mice exhibit reduced I_A density recorded from CA1 pyramidal neurons, and facilitated LTP and learning on the NOR task (Fontan-Lozano et al. 2011). Lockridge and colleagues hypothesized that these memory deficits in Kv4.2 knockout mice might be attributable to an aberrant form of enhanced synaptic plasticity and altered signaling events in CA1 pyramidal neurons (Lockridge and Yuan 2011). Our data showing an alcohol-induced reduction in KChIP3 and Kv4.2 channels are consistent with this hypothesis. Perhaps not surprising, KChIP3 has also been implicated in regulation of synaptic plasticity and hippocampal-dependent learning (Alexander et al. 2009; Lilliehook et al. 2003). Interestingly, emerging evidence shows that chronic alcohol exposure leads to aberrant forms of enhanced synaptic plasticity (Jeanes et al. 2011; Kroener et al. 2012). It is possible that chronic alcohol-induced down-regulation of Kv4.2 channel complexes may be a critical factor underlying altered synaptic plasticity and poor cognitive performance associated with prolonged alcohol consumption.

An additional interesting finding in the present study is the observation that chronic alcohol exposure reduces KChIP3 expression. KChIP3 binding to the functional expression regulating N-terminal (FERN) domain of the α subunit of Kv4.2 channels promotes forward trafficking from the *trans* Golgi Network (TGN) to the plasma membrane (Kunjilwar et al. 2013). The FERN domain is thought to contain an endoplasmic reticulum (ER) retention signal, and this domain acts as a dominant negative to prevent trafficking from the TGN to plasma membranes when it is not bound with KChIP3. Thus, as presented in Fig. 6, we propose that the reduction in KChIP3 expression by chronic alcohol leads to decreased binding of KChIP3 to the FERN domain of Kv4.2 channels and attenuation of its trafficking to the plasma membrane. Along with its role in stabilization of surface Kv4.2 channels in

pyramidal neurons (Chen et al. 2006; Nerbonne et al. 2008), KChIP3 also acts as a Ca^{2+} -sensitive modulator for negative feedback regulation of NMDA receptors. Elevated Ca^{2+} levels enhance the association of KChIP3 and GluN1, whereas chelation of Ca^{2+} reduces KChIP3 and GluN1 binding (Zhang et al. 2010). Acute alcohol is known to inhibit NMDA receptor function (Lovinger and Roberto 2013), and the synaptic targeting of NMDA receptors is thought to be activity-dependent (Carpenter-Hyland et al. 2004). It is possible that inhibition of Ca^{2+} entry through NMDA receptors by alcohol leads to a decrease in KChIP3-NMDA receptor coupling. Although the exact mechanism for how KChIP3 influences surface expression of NMDA receptors is unknown, it has been suggested that KChIP3 binding to the C0 cassette of the GluN1 subunit may act as a signal for receptor endocytosis (Zhang et al. 2010). Thus, reduced coupling of KChIP3-GluN1 and KChIP3-Kv4.2 by chronic alcohol may account for a decrease in NMDA receptor endocytosis and targeting of Kv4.2 channels to the plasma membrane (Fig. 6).

In the present study, we measured expression levels of Kv4.2 channel complexes and NMDA receptors after chronic alcohol exposure in the absence of any withdrawal period. Consistent with previous findings in the hippocampus (Carpenter-Hyland and Chandler 2006; Carpenter-Hyland et al. 2004; Clapp et al. 2010; Hendricson et al. 2007; Mulholland et al. 2011), we found that chronic alcohol exposure of organotypic cultures increases GluN1 and GluN2B, but not GluN2A protein levels. Chronic alcohol-induced increases in NMDA receptor expression and function have also been reported in the frontal cortex, nucleus accumbens (NAc), basolateral amygdala, and bed nucleus of the stria terminalis (Clapp et al. 2010; Kash et al. 2009; Kroener et al. 2012; Lack et al. 2007; Obara et al. 2009; Qiang et al. 2007; Zhou et al. 2007). However, recent evidence suggests that this is not a global effect of chronic alcohol on the brain with some studies reporting no changes or decreases in synaptic NMDA receptor expression and function in the NAc and frontal cortex (Abraham et al. 2013; Holmes et al. 2012; McGuier et al. In Press; Obara et al. 2009). While the reasons for the apparent discrepancies are unclear, the studies showing decreases in NMDA receptors were performed 3 to 60 days after withdrawal from chronic alcohol, suggesting that heightened glutamatergic neurotransmission during withdrawal may influence synaptic NMDA receptor function and expression. Indeed, Clapp et al (2010) reported that the synaptic clustering of NMDA receptors produced by chronic alcohol exposure of hippocampal neurons is rapidly reversed by acute withdrawal. We hypothesize that up- or down-regulation of NMDA receptor function by chronic alcohol and withdrawal are driven by homeostatic mechanisms that govern neuronal excitability processes that attempt to restore normal signaling.

In summary, these findings demonstrate mechanistic neuroadaptations in Kv4.2 channels and KChIP3 induced by chronic alcohol exposure that could influence an important component of dendritic signaling that is critical for plasticity and learning events. While these homeostatic changes in response to chronic alcohol exposure likely function to reestablish the normal balance between excitatory and inhibitory neurotransmission in the hippocampus, these changes may also engage aberrant plasticity processes that contribute to hippocampal dysfunction in individuals with alcohol use disorders.

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Fig. 1.

Reduction in Kv4.2 channel expression by in-vitro chronic alcohol exposure. **(A)** Concentration-response curve for Kv4.2 channel expression levels in organotypic hippocampal slice cultures exposed to chronic alcohol (one-way ANOVA, $F(3,15) = 9.534$, $p = 0.004$, *SNK post-hoc, $*p < .05$, $**p < .01$; $n = 4$ replicate experiments). **(B)** Chronic alcohol (75 mM) reduced expression of Kv4.2 channels in the total, but not intracellular (IC) fraction (two-way ANOVA; $F(1,16) = 5.296$, $p = 0.035$, SNK post-hoc, $*p < 0.05$, $n = 5$ replicate experiments). **(C)** Chronic alcohol exposure did not alter Kv1.4 channel expression levels (t-test, $t(11) = 0.241$, $p = 0.814$, $n = 6-7$ replicate experiments). **(D)** Chronic alcohol exposure increased expression of GluN1 (t-test, $t(8) = 2.73$, $*p < 0.05$, $n = 6/\text{group}$) and GluN2B (t-test, $t(10) = 2.306$, $*p < 0.05$, $n = 5$ replicate experiments), but not GluN2A (t-test, $t(10) = 0.2377$, $p = 0.817$, $n = 6$ replicate experiments) subunits of the NMDA receptor. **(E)** Similar to the effects of chronic alcohol exposure on Kv4.2 channel expression, chronic alcohol exposure significantly reduced expression of KChIP3 (one-way ANOVA, $p = 0.033$, SNK post-hoc, $*p < 0.05$, $n = 4-5$ replicate experiments).

Fig. 2.

In-vitro alcohol (75 mM) exposure significantly reduces the afterhyperpolarization (AHP) in CA1 pyramidal neurons. **(A)** Representative traces of evoked action potentials recorded from control and chronic alcohol-treated CA1 pyramidal neurons. **(B,C)** The amplitude of the AHP was significantly reduced by chronic alcohol exposure (t-test, $t(32) = 2.253$, $*p < 0.05$, $n = 16-18$ cells/group).

Fig. 3.

Prolonged exposure to 75 mM alcohol selectively reduces I_A density mediated by Kv4 channels in CA1 neurons. **(A,B)** A two-pulse protocol revealed a significant reduction in the transient I_A density in chronic alcohol-treated CA1 pyramidal neurons (two-way ANOVA, $F(1,24) = 8.073$, $p < 0.009$, SNK post hoc test, $*p = .009$, $n = 8$ cells/group). **(C)** Chronic alcohol exposure does not alter I_A mediated by Kv1 channels (t-test, $t(12) = 0.696$, $p = 0.50$, $n = 8$ cells/group).

Fig. 4.

Enhanced bAP-induced Ca^{2+} transients in distal dendrites of CA1 apical dendrites following chronic alcohol (75 mM) exposure. **(A)** Representative image of a somatic AP (top, left) and the resulting Ca^{2+} transient (bottom, left) observed in the apical dendrite of a control CA1 pyramidal neuron. Corresponding images of the OGB-1 (top, middle) signal and Alexa-594 filled neuron (right) demonstrating the location of the line scan that was used to record the Ca^{2+} transient. **(B)** Ca^{2+} transient in distal apical dendrites evoked by bAPs in control and chronic alcohol treated CA1 pyramidal neurons as a function of distance from the soma. **(C)** Chronic alcohol exposure significantly increased averaged bAP-evoked Ca^{2+} transients in distal dendrites (t-test, $t(36) = 4.562$, $*p < 0.0001$, $n = 16-22/\text{group}$). **(D)** The NMDA receptor antagonist CPP significantly reduced bAP-evoked Ca^{2+} transients in distal dendrites of control and chronic alcohol treated CA1 pyramidal neurons (two-way repeated-measures ANOVA, $F(1,25) = 13.137$, $p = .001$, SNK post-hoc, $***p < .001$, $n = 12-15/\text{group}$).

Fig. 5.

Chronic alcohol (75 mM) exposure does not change the length of apical dendrites in CA1 pyramidal neurons. **(A)** Representative line drawings of apical dendrites in control and chronic alcohol treated neurons. Chronic alcohol exposure did not affect the **(B)** total length of the apical dendrites (t-test, $t(15) = 0.333$, $p > 0.05$; $n = 8-9$ neurons/group), **(B)** the length of the main apical dendrite (t-test, $t(15) = 2.008$, $p > 0.05$), or **(C)** the average number of branch points (t-test, $t(15) = 0.303$, $p > 0.05$). **(D)** Chronic alcohol exposure did not significantly affect the length of dendritic branches when classified by branch order (two-way ANOVA, $F(1,46) = 2.094$, $p > 0.05$). **(E)** Chronic alcohol exposure produced a reduction in the number of higher-order (5th and 6th order) dendrites ($\chi^2 = 4.02$, $df = 1$, $*p < 0.05$; $n = 17$).

Fig. 6.

Schematic representation showing the bidirectional modulation of Kv4.2 channels and NMDA receptors by KChIP3 after chronic alcohol exposure in hippocampus. *Left panel*, In the basal state, KChIP3 binding to the functional expression regulating N-terminal (FERN) domain of the α subunit of Kv4.2 channels promotes forward trafficking from the *trans* Golgi Network (TGN) to the plasma membrane. KChIP3 also binds to the intracellular C0 domain of the GluN1 subunit of NMDA. KChIP3 binding to the C0 domain is thought to promote NMDA receptor endocytosis. *Right panel*, Chronic alcohol exposure reduces KChIP3 and surface Kv4.2 channel expression and increases synaptic NMDA receptors. We propose that the reduction in KChIP3 expression leads to reduced binding of KChIP3 to the FERN domain of Kv4.2 channels in the TGN. The FERN domain is thought to contain an ER retention signal. When KChIP3 is not bound to this domain it acts as a dominant negative to prevent Kv4.2 channel trafficking from the TGN to the plasma membrane, and inhibition of Ca^{2+} entry through NMDA receptors by alcohol may lead to a decrease in KChIP3-mediated endocytosis of synaptic NMDA receptors.

Table 1

Membrane and action potential characteristics of control and chronic ethanol (75 mM) treated CA1 pyramidal neurons.

	<i>N</i>	Input resistance (M Ω)	Rheobase (pA)	Resting Membrane Potential (mV)	AP threshold (mV)	AP half-width (ms)	Latency to first AP (ms)
CTRL	13–16	157.93 $\pm$ 11.00	197.0 $\pm$ 12.33	-68.3 $\pm$ 1.4	-41.45 $\pm$ 0.85	1.55 $\pm$ 0.09	91.78 $\pm$ 21.60
Chronic EtOH	12–18	135.82 $\pm$ 11.11	212.35 $\pm$ 13.45	-66.7 $\pm$ 1.8	-43.48 $\pm$ 0.89	1.53 $\pm$ 0.11	73.43 $\pm$ 19.85
t-test		t(32) = 1.408, p=0.17	t(32) = 0.833, p=0.41	t(23) = 0.708, p=0.49	t(32) = 1.639, p=0.11	t(32) = 0.023, p=0.98	t(32) = 0.627, p=0.91