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Ethanol stimulates endoplasmic reticulum inositol triphosphate and sigma receptors to promote withdrawal-associated loss of neuron specific nuclear protein/Fox-3

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Abstract

Background: Prior studies demonstrate that ethanol exposure induces the release of intracellular calcium (Ca^{2+}) in modulation of γ -Aminobutyric acid (GABA)-ergic tone and produces concomitant alterations in sigma (σ)-1 protein expression that may contribute to the development ethanol dependence. However, the influence of Ca^{2+} released from endoplasmic reticulum (ER)-bound inositol triphosphate (IP3) and σ -1 receptors in regulating hippocampal function has yet to be delineated.

Methods: Rat hippocampal explants were subjected to chronic intermittent ethanol exposure (CIE) with or without the addition of IP3 inhibitor xestospongine C (0–0.5 μ M) or σ -1 receptor antagonist BD-1047 (0–80 μ M). Hippocampal viability was assessed via immunohistochemical labeling of neuron specific nuclear protein (NeuN)/Fox-3 in CA1, CA3, and DG sub-regions.

Results: Exposure to CIE produced consistent and significant decreases of NeuN/Fox-3 in each primary cell layer of the hippocampal formation. Co-exposure to xestospongine reversed these effects in the CA1 sub-region, and significantly attenuated these effects in the CA3 and DG regions. Xestospongine application also significantly increased NeuN/Fox-3 immunofluorescence in ethanol-naïve hippocampi. Co-exposure to 20 μ M BD-1047 also reversed the loss of NeuN/Fox-3 during CIE exposure in each hippocampal cell layer, whereas exposure to 80 μ M BD-1047 did not alter NeuN-Fox-3 in ethanol-treated hippocampi. By contrast, 80 μ M BD-1047 application significantly increased NeuN/Fox-3 immunofluorescence in ethanol-naïve hippocampi in each sub-region.

Conclusions: These data suggest that ethanol stimulates ER IP3 and σ -1 receptors to promote hippocampal loss of NeuN/Fox-3 during CIE.

Keywords

ethanol withdrawal; neuron specific nuclear protein; calcium; inositol triphosphate; sigma-1

INTRODUCTION

Calcium (CA^{2+}) is a ubiquitous intracellular signal known to control a cadre of cellular processes. Internal CA^{2+} stores are held within membrane systems of the endoplasmic reticulum (ER) or sarcoplasmic reticulum of muscle cells, as well as mitochondria (Berridge, 1993). Release of intracellular CA^{2+} is controlled by various channels, such as the inositol-1,4,5-trisphosphate (IP3) receptor and ryanodine receptor families (Clapper et al., 1987). Ethanol application is known to produce concomitant alterations in ER-bound protein complexes (e.g., decreases in IP3 mRNA [Rodríguez et al., 1996]), ionotropic glutamatergic (e.g., inhibition of N-methyl-D-aspartate [NMDA] receptors [Loving et al., 1989]) and group-1 metabotropic glutamate-family proteins (i.e., decreases in mGlu1 mRNA [Simonyi et al., 1996]). These studies demonstrate that ethanol alters polyphosphoinositide signaling, but the functional effects of these neuroadaptive changes are not yet fully understood. For example, prior studies utilizing whole-cell voltage-clamp recordings demonstrate that presynaptic release of intracellular CA^{2+} is required for spontaneous release of γ -Aminobutyric acid (GABA) neurotransmission in cerebellar neurons following ethanol exposure (Kelm et al., 2007). Others demonstrate that application of pharmacologically relevant concentrations of ethanol (i.e., 25–50 mM) facilitates release of CA^{2+} from IP3-insensitive pools in brain microsomes (Daniell & Harris, 1989). Simultaneous application of ethanol and IP3, however, produced an additive release of intracellular CA^{2+} . Although these findings suggest that ethanol may stimulate other ER-bound proteins in addition to IP3 receptors, the etiology of these effects has yet to be delineated.

Located in close proximity to IP3 receptors are sigma (σ)-1 receptors, which are CA^{2+} -sensitive, ligand-regulated chaperones of the ER implicated in potentiation of prolonged IP3 receptor-mediated CA^{2+} signaling (Hayashi & Su, 2007). Sigma-1 receptors are highly concentrated in the hippocampus (Alonso et al., 2000) and therefore involved in mediation of synaptic plasticity that may contribute to the development of ethanol dependence. For example, a prior study found that chronic, intermittent ethanol (CIE) administration produced an upregulation of σ -1 receptor protein expression in the hippocampus (Sabeti, 2011). These increases in σ -1 polypeptide levels were attenuated by exposure to a σ -1 receptor antagonist (10 mg/kg BD-1047). In particular, prior studies demonstrate that σ -1 receptors mediate intrinsic plasticity in cornu ammonis (i.e., CA1) hippocampal neurons following exposure to CIE (Meunier et al., 2006). These effects contribute to the neurocognitive consequences produced by CIE (Meunier et al., 2006) and their inhibition reduced ethanol consumption in Sardinian alcohol-preferring rats (Sabino et al., 2009). Functional polymorphisms in the σ -1 receptor gene were also associated with a propensity for alcoholism in a case-controlled study (Miyatake et al., 2004) suggesting that σ -1 receptors may regulate development of dependence in the clinical population.

Collectively, these findings suggest that ethanol exposure may stimulate release of intracellular CA^{2+} that may contribute to the neurocognitive consequences of CIE. However, the role of CA^{2+} released from ER-bound IP3 and σ -1 receptors in regulating hippocampal viability has yet to be delineated. The purpose of the present studies, therefore, is to examine the effects of intracellular CA^{2+} release from IP3 and σ -1 receptor sensitive stores on

withdrawal-associated loss of neuron specific nuclear protein (NeuN)/Fox-3 in rat hippocampal explants.

MATERIALS AND METHODS

Organotypic Hippocampal Slice Culture Preparation

Whole brains from eight-day-old Sprague-Dawley rats (Harlan Laboratories; Indianapolis, IN) were aseptically collected, transferred to culture dishes containing frozen dissecting media composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), 10.60 μ M Amphotericin B solution (Sigma), and 50 μ M streptomycin/penicillin (Invitrogen), and bilateral hippocampi were removed and then carefully transferred to sterile plates containing chilled culture media composed of dissecting media, distilled water, 36 mM glucose (Fisher, Pittsburg, PA), 25% Hanks' Balanced Salt Solution (HBSS; Invitrogen), 25% (v/v) heat-inactivated horse serum (HIHS; Sigma), 0.05% Amphotericin B solution (Sigma), and 0.05% streptomycin/penicillin (Invitrogen). A stereoscopic microscope was used to detach excess hippocampal tissue prior to sectioning of unilateral hippocampi at 200 μ M using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Hippocampi were selected under the stereoscopic microscope for intact cell layers prior to careful placement onto Millicell-CM 0.4 μ M biopore membrane inserts that contained 1 mL of pre-incubated culture media, and were then placed in a sterile six-well culture plate. Each culture well plate generated eighteen intact hippocampal explants. Excess culture media was extracted off the top of each biopore membrane insert using the stereoscopic microscope. Explants were maintained in an incubator at 37°C with a gas composition of 5% CO₂/95% air for five days in order to allow tissue to adhere to the membrane inserts prior to the first media change (after Butler et al., 2010). Care of all animals was carried out in agreement with the University of Kentucky's Institutional Animal Care and Use Committee.

Ethanol Exposure and Withdrawal

The present study used an in vitro model of CIE that has been published previously (Reynolds et al., 2015A; Reynolds et al., 2015B) to delineate the influence of intracellular CA²⁺ from ER-bound receptors in promoting withdrawal-associated loss of hippocampal NeuN/Fox-3. In brief, male and female rat hippocampal explants were randomly transferred at 5 days in vitro to plates containing either 1 ml of the ethanol-naïve culture media (control) or ethanol-containing media (i.e., 0 and 50 mM) for five days with or without the addition of xestospongins C (0.5 μ M), a membrane-permeant inhibitor of IP₃-mediated CA²⁺ release or BD-1047 (20 and 80 μ M), a selective antagonist at the σ -1 receptor (Kim et al., 2010; Taylor & Tovey, 2010). At 11 days in vitro, explants were removed from their respective treatment groups and transferred to plates containing 1 ml of fresh ethanol-naïve culture media for a 24-hour ethanol withdrawal period. During each withdrawal period, tissue was drug naïve. This treatment regimen was repeated a total of three times. Xestospongins C was first dissolved in 100% dimethyl sulfoxide (DMSO; Fisher) to yield a final working concentration of 0.01% DMSO in the ethanol-naïve and ethanol-containing culture media (after Reynolds et al., 2015B). Concentrations of xestospongins C (i.e., 0–0.5 μ M) and BD-1047 (i.e., 0–80 μ M) were selected based on prior reports showing efficacy in reducing

withdrawal-associated neurotoxicity from other drugs of abuse, such as methamphetamine when applied acutely (Smith et al., 2008; Smith et al., 2010). The concentration of ethanol (i.e., 50 mM) was selected in the present report as it produces a consistent loss of NeuN in this model of CIE (Reynolds et al., 2015A; Reynolds et al., 2015B), as well as reflects binge-like ethanol consumption in the clinical population (Eckardt et al., 1998).

Immunohistochemistry

NeuN (Fox-3) is a protein found in nearly all post-mitotic neurons (Kim et al., 2009; Mullen et al., 1992), and was selected in the present study because it is a reliable marker of neuronal integrity using this model of CIE (Reynolds et al., 2015A; Reynolds et al., 2015B). Rat hippocampal explants were fixed by placing 1 ml of 10% formalin solution on the top and bottom of each culture plate well, incubated at room temperature for 30 minutes, washed twice with phosphate buffered saline (PBS), and then stored at 4°C until conducting immunohistochemistry. Immunohistochemical labeling with NeuN/Fox-3 was conducted by transferring formalin-fixed explants to a plate containing 1 ml of permeabilization (wash) buffer (200 ml PBS [Invitrogen], 200 μ L Triton X-100 [Sigma], 0.010 mg Bovine Serum [Sigma]). One ml of buffer was added to the top of each well, and explants were incubated at room temperature for 45 minutes in order to permeate cell membranes. Explants were then incubated at 4°C for 24 hours with the primary monoclonal antibody mouse anti-NeuN (1:200; Sigma). Explants were then washed with PBS and incubated for 24 hours with goat anti-mouse fluorescein isothiocyanate (FITC; 1:200; Sigma), and imaged with SPOT software 4.0.2 (advanced version) for Windows (W. Nuhsbahr Inc.; McHenry, IL, USA) through a 5x objective with a Leica DMIRB microscope (W. Nuhsbahr Inc.; McHenry, IL, USA) connected to a computer and captured with a SPOT 7.2 color mosaic camera (W. Nuhsbahr). A band-pass filter at 495 nm (520 nm emission) detected FITC immunofluorescence, allowing for NeuN/Fox-3 optical density determination after Butler et al., 2010.

Statistical analyses

Statistical analyses were conducted to delineate the influence of intracellular CA^{2+} release from ER-bound receptors in regulating hippocampal integrity following exposure to CIE. Studies included in the present report were conducted two times using two different rat litters. All data were converted to percent control and then combined for ease of interpretation. Data were analyzed using a two-factor analysis of variance (ANOVA) with media (i.e., ethanol-containing and ethanol-naïve) and drug (i.e., no drug and drug) as the factors for each hippocampal cell layer (i.e., CA1, CA3, and DG). Pairwise comparisons of means were conducted using Holm-Sidak's multiple comparisons test. Effects were considered significant at $p < 0.05$. For graphical representation and interpretation, data are presented as group mean $\pm$ the standard error of the mean (SEM).

RESULTS

Inhibitor of IP₃-mediated CA^{2+} release by Xestospongins C

In the pyramidal cell layer of the CA1, ANOVA analyses revealed significant main effects of media ($F[1,115] = 29.49$, $p = 0.0001$) and drug ($F[1,115] = 11.88$, $p < 0.0008$). CIE exposure

produced a 24% decrease in NeuN/Fox-3 protein immunofluorescence relative to ethanol-naïve tissue. Exposure to xestospongin C (0.5 μ M) attenuated the loss of NeuN/Fox-3 immunofluorescence produced by three cycles of CIE by 16% ($p=0.007$; Holm-Sidak's multiple comparisons) (Figure 1A). Application of xestospongin C resulted in significant increases of NeuN/Fox-3 immunofluorescence by 11% ($p=0.0009$; Holm-Sidak's multiple comparisons) in ethanol-naïve tissue (Figure 1A).

In the pyramidal cell layer of the CA3, ANOVA analyses revealed significant main effects of media ($F[1,115]=11.95$, $p<0.0001$) and drug ($F[1,115]=5.39$, $p=0.007$). In this sub-region, CIE exposure produced a 21% decrease in NeuN/Fox-3 protein immunofluorescence relative to ethanol-naïve tissue. Exposure to xestospongin C (0.5 μ M) attenuated the loss of NeuN/Fox-3 immunofluorescence produced by three cycles of CIE as expression of NeuN/Fox-3 detected in ethanol-treated hippocampi co-exposed to xestospongin C was not altered significantly as compared to ethanol and drug-naïve tissue ($p>0.05$; Holm-Sidak's multiple comparisons) (Figure 1B). In addition, application of xestospongin C resulted in significant increases of NeuN/Fox-3 immunofluorescence by 15% ($p=0.03$; Holm-Sidak's multiple comparisons) in ethanol-naïve tissue (Figure 1B).

A similar pattern was observed in the granule cell layer of the DG as ANOVA analyses revealed significant main effects of media ($F[1,115]=21.28$, $p<0.0001$) and drug ($F[1,115]=9.09$, $p=0.003$). CIE exposure produced a 15% decrease in NeuN/Fox-3 protein immunofluorescence relative to ethanol-naïve tissue in this sub-region. Exposure to xestospongin C (0.5 μ M) attenuated the loss of NeuN/Fox-3 immunofluorescence produced by three cycles of CIE in ethanol-treated hippocampi in a similar manner to the CA3 sub-region ($p>0.05$; Holm-Sidak's multiple comparisons) (Figure 1C). Application of xestospongin C resulted in significant increases of NeuN/Fox-3 immunofluorescence by 17% ($p=0.001$; Holm-Sidak's multiple comparisons) in ethanol-naïve tissue (Figure 1C).

σ -1 receptor antagonist BD-1047

In the pyramidal cell layer of the CA1, ANOVA analyses revealed a significant media-by-drug interaction ($F[2,66]=7.01$, $p=0.002$), a significant main effect of media ($F[2,66]=6.08$, $p=0.004$), and a significant main effect of drug ($F[2,66]=8.79$, $p=0.004$). CIE exposure produced a 26% decrease in NeuN/Fox-3 protein immunofluorescence relative to ethanol-naïve tissue. Exposure to the lower concentration of BD-1047 (20 μ M) reversed these effects by 58% ($p=0.003$; Holm-Sidak's multiple comparisons), whereas exposure to higher BD-1047 concentrations (i.e., 80 μ M) failed to restore levels of NeuN/Fox-3 protein immunofluorescence in ethanol-exposed hippocampi ($p>0.05$; Holm-Sidak's multiple comparisons) (Figure 2A). Application of BD-1047 at the lower concentration (i.e., 20 μ M) did not significantly alter levels of NeuN/Fox-3 protein immunofluorescence in ethanol-naïve tissue ($p>0.05$; Holm-Sidak's multiple comparisons) whereas exposure to higher concentrations of BD-1047 (i.e., 80 μ M) resulted in significant increases of NeuN/Fox-3 immunofluorescence by 36% ($p=0.0009$; Holm-Sidak's multiple comparisons) in ethanol-naïve tissue (Figure 2A).

In the pyramidal cell layer of the CA3, ANOVA analyses revealed a significant media-by-drug interaction ($F[2,66]=5.65$, $p=0.006$), a significant main effect of media ($F[2,66]=10.64$,

$p=0.002$), and a significant main effect of drug ($F[2,66]=10.09$, $p=0.002$). CIE exposure produced a 36% decrease in NeuN/Fox-3 protein immunofluorescence relative to ethanol-naïve tissue ($p=0.0009$; Holm-Sidak's multiple comparisons). Exposure to the lower concentration of BD-1047 (i.e., 20 μM) reversed these effects by 96% ($p=0.0004$; Holm-Sidak's multiple comparisons), whereas exposure to higher BD-1047 concentrations (i.e., 80 μM) failed to restore levels of NeuN/Fox-3 protein immunofluorescence in ethanol-exposed hippocampi ($p>0.05$; Holm-Sidak's multiple comparisons) (Figure 2B). Application of BD-1047 at the lower concentration (20 μM) did not significantly alter levels of NeuN/Fox-3 protein immunofluorescence in ethanol-naïve tissue ($p>0.05$; Holm-Sidak's multiple comparisons), whereas exposure to higher concentrations of BD-1047 (i.e., 80 μM) resulted in significant increases of NeuN/Fox-3 immunofluorescence by 36% ($p=0.0002$; Holm-Sidak's multiple comparisons) in ethanol-naïve tissue (Figure 2B).

A similar pattern was observed in the granule cell layer of the dentate gyrus as ANOVA analyses revealed a significant media-by-drug interaction ($F[2,66]=8.70$, $p=0.0008$), a significant main effect of media ($F[2,66]=18.75$, $p=0.0001$), and a significant main effect of drug ($F[2,66]=8.04$, $p=0.0008$). CIE exposure produced a 28% decrease in NeuN/Fox-3 protein immunofluorescence relative to ethanol-naïve tissue. Exposure to the lower concentration of BD-1047 (20 μM) reversed these effects by 58% ($p=0.003$; Holm-Sidak's multiple comparisons), whereas exposure to higher BD-1047 concentrations (i.e., 80 μM) failed to restore levels of NeuN/Fox-3 protein immunofluorescence in ethanol-exposed hippocampi ($p>0.05$; Holm-Sidak's multiple comparisons) (Figure 2C). Application of BD-1047 at the lower concentration (20 μM) did not alter levels of NeuN/Fox-3 protein immunofluorescence in ethanol-naïve tissue ($p>0.05$; Holm-Sidak's multiple comparisons), whereas exposure to higher concentrations of BD-1047 (i.e., 80 μM) resulted in significant increases of NeuN/Fox-3 immunofluorescence by 51% ($p=0.0001$; Holm-Sidak's multiple comparisons) in ethanol-naïve tissue (Figure 2C).

Depicted in Figure 3 are representative images of NeuN/Fox-3 immunofluorescence of ethanol and drug-naïve hippocampi (top left), ethanol-naïve hippocampi exposed to xestospongine C (0.5 μM) (top middle), ethanol-naïve hippocampi exposed to BD-1047 (20 μM) (top right), ethanol-exposed drug-naïve hippocampi (bottom left), hippocampi co-exposed to ethanol and xestospongine C (0.5 μM) (bottom middle), hippocampi co-exposed to ethanol and BD-1047 (20 μM) (bottom right).

DISCUSSION

The present study examined the influence of ethanol-induced activity of ER-bound receptors and presumptive intracellular CA^{2+} release on hippocampal viability, as reflected in NeuN immunoreactivity after Reynolds et al. 2015A. We first assessed the efficacy of xestospongine C, a macrocyclic bis-1-oxaquinolizidine and potent blocker of IP₃-mediated CA^{2+} release on the vesicles of the ER membrane (Gafni et al., 1997), to prevent ethanol withdrawal-associated loss of NeuN in hippocampi exposed to a CIE regimen. CIE produced significant decreases in NeuN/Fox-3 immunofluorescence in each of the primary cell layers of the hippocampal formation by 15–24%. These results are not unexpected and are consistent with the magnitude of effects typically observed following this CIE treatment regimen (Reynolds

et al., 2015A; Reynolds et al., 2015B). Although we have previously shown that the loss of NeuN using this model of CIE corresponds to loss of cellular marker thionine (Reynolds et al., 2015A), these effects could reflect either a decrease of NeuN protein expression or adverse effects on “physiological status” (Weyer & Schilling, 2003) rather than loss of neurons. However, prior studies suggest that NeuN loss correlates strongly with other measures of neuronal loss (Chen-Roetling et al., 2013; Liu et al., 2009). Liu et al. 2009, for example, demonstrate that NeuN is a more sensitive marker of neuronal loss than measuring infarct volume in a stroke model because the NeuN loss occurs before the obvious infarction. Nonetheless, co-application of ethanol and xestospongin C (0.5 μ M) for three consecutive cycles of CIE significantly attenuated levels of NeuN/Fox-3 immunofluorescence in each examined hippocampal sub-region, indicating that ethanol acutely induces activity of IP3-dependent signaling. Others have shown that release of intracellular CA^{2+} from IP3-sensitive stores plays a critical role in the alcohol-mediated death of cerebellar granule neurons (Kouzoukas et al., 2013). This is consistent with prior studies demonstrating that ethanol application engages IP3 receptors to release intracellular CA^{2+} in the hippocampus (Daniell & Harris; 1989) in a protein-kinase C (PKC)-dependent manner (Mironov & Hermann, 1996), and advances our understanding of the functional consequences of this release. These data suggest that ethanol may mobilize intracellular CA^{2+} via upstream activation of second messenger effectors, such as protein kinases, prior to withdrawal to promote the decreases of NeuN/Fox-3 observed in the present report. Given the notion that ethanol acutely inhibits NMDA receptor function (Lovinger et al., 1989) and CIE exposure produces decreased hippocampal viability via over-activation of NMDA receptors during period of withdrawal (Reynolds et al., 2015A), perhaps NMDA receptors may be activated down-stream of IP3 receptors prior to withdrawal to produce the loss of NeuN/Fox-3 observed in the present report. Additionally, the accumulation of CA^{2+} intracellularly may perturb neuronal integrity via myriad effects unrelated to glutamatergic tone, including mitochondrial swelling.

We further examined the influence of intracellular CA^{2+} release in mediating hippocampal function using the selective antagonist of the chaperone σ -1 protein BD-1047. Stimulation of these ER-bound, ligand-regulated receptors stabilizes IP3 receptors to prevent proteasome degradation (Hayashi & Su, 2007). Co-application of ethanol and BD-1047 (20 μ M) for three consecutive cycles of CIE reversed the loss of NeuN/Fox-3 immunofluorescence in the CA1, CA3, and DG sub-regions of the hippocampal formation. This ability of BD-1047 to mitigate the effects of CIE on NeuN loss is consistent with a prior study conducted by Sabeti (2011) suggesting that CIE exposure blunts intrinsic neuronal plasticity in a σ -1-dependent manner in the adolescent hippocampus. In general, it is unknown whether the ability of BD-1047 to restore hippocampal integrity in the present report is mediated via interactive effects of σ -1 chaperone proteins, NMDA receptors, or mGlu-containing proteins, which are known to be important in producing decreased hippocampal viability in this model (Reynolds et al., 2015B). In one study, application of BD-1047 restored activity-induced changes in synaptic strength (LTP) to basal levels in hippocampal slices withdrawn from a CIE treatment regimen in an NMDA-independent manner (Sabeti & Gruol, 2008). In another study, blocking intracellular CA^{2+} release increased GluN1 subunit expression in a similar manner to the observed ethanol-associated increases in GluN1 polypeptide levels

(Chen et al., 1999). Thus, the role of intracellular CA^{2+} function in modulating NMDA receptor function is not well understood.

Others have found that NMDA-dependent neuronal firing is mediated via σ -1 receptors of hippocampal pyramidal neurons (Monnet et al., 1990; Monnet et al 1992A) coupled to G-proteins (i.e., $G_{i/o}$ [Monnet et al 1992B]). In vivo administration of ifenprodil, a selective NMDA-receptor antagonist for the GluN2B complex and σ -1 receptor agonist (Hanner et al., 1996) has been shown to alleviate seizures during periods of ethanol withdrawal (Malinowska et al., 1999; for a review, see Debonnel & de Montigny, 1996). Consistent with this notion, we have previously found that group 1 G-coupled family-proteins (i.e., G_q) promote neuroadaptation to ethanol and withdrawal-associated hippocampal damage following CIE exposure in vitro and physical dependence in vivo (Reynolds et al., 2015B), suggesting a role for ethanol's activation of G_q -linked polyphosphoinositide signaling prior to CA^{2+} mobilization from internal stores. In addition, prior studies have demonstrated that BD-1047 administration restored cognitive impairments induced by blocking glutamatergic receptors (Maurice & Su, 2009) and chronic ethanol administration (Meunier et al., 2006), as well as attenuated ethanol-induced conditioned place preference (Maurice et al., 2003) and reduced ethanol consumption (Sabino et al., 2009). Therefore, the influence of IP3 and σ -1 receptors in regulating hippocampal integrity following CIE in the present report may have implications for the development of neurocognitive deficits associated with hippocampal integrity produced by CIE exposure (e.g., impaired cognitive flexibility [Bandanich et al., 2011] and working memory perturbations [Zhao et al., 2013]).

Interestingly, we found that the lower concentration of BD-1047 (20 μ M) reversed CIE-induced loss of NeuN in ethanol-treated hippocampi whereas the higher concentration (80 μ M) increased levels of NeuN immunofluorescence in ethanol-naïve hippocampi but failed to alter loss of NeuN in ethanol-treated hippocampi. Given that intracellular CA^{2+} enhances the sensitivity of ethanol on the GluN1-containing NMDA receptors (Mirshahi et al., 1998), the interaction observed in the present report could reflect inherent differences in NMDA receptor subunit stoichiometry produced by ethanol that confers sensitivity to agonist and/or antagonist application, such as fractional CA^{2+} permeability (Burnashev et al., 1995) though not yet confirmed. By contrast xestospongin application increased levels of NeuN in both ethanol-treated and ethanol-naïve hippocampi. The reasons for these effects are unknown, but likely reflect inherent characteristics of organotypic hippocampal slice culture preparations (i.e., ER stress) (for a review see Berridge et al., 2003; Verkhratsky, 2002), even in the absence of ethanol. For example, the preparation of hippocampal explants itself is known to produce basal neurotoxicity associated with influx of CA^{2+} (Mayer et al., 2002) likely through activation of glutamatergic mGlu5-containing proteins, NMDA receptors (Mayer et al., 2002; Driscoll et al., 1991), and extracellular glutamate accumulation (Driscoll et al., 1993). In addition, pharmacological blockade of intracellular CA^{2+} at the ER using antagonists at relatively low concentrations (e.g., 5-di(*tert*-butyl)-1,4-benzohydroquinone [BHQ]), modestly but significantly enhances survival by ~10% in primary cultures of cerebellar neurons (Mhyre et al., 2000), suggesting a neuroprotective role for blunting CA^{2+} mobilization in cell culture. The present findings taken together with prior studies, therefore, suggest that blocking intracellular CA^{2+} release at the ER may

protect hippocampal neurons from CA^{2+} -associated loss of NeuN due to preparation and maintenance (i.e., medium-change toxicity) of hippocampal explants.

Taken together, these findings suggest that ethanol mobilizes intracellular CA^{2+} via ER-bound IP3 and $\sigma-1$ receptors in possible modulation of glutamatergic tone to promote withdrawal-associated loss of NeuN/Fox-3. In addition, these ER-bound proteins are involved in regulation of hippocampal pyramidal and granule cell function and perhaps synaptic plasticity. These concomitant alterations in intracellular CA^{2+} signaling may contribute to the development ethanol dependence in vivo (Veatch & Becker, 2002; 2005) and in the clinical population (Ballenger & Post, 1978; Duka et al., 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Effects of IP3 inhibitor xestospongine C (0.5 μM) application on NeuN/Fox-3 immunofluorescence in ethanol (EtOH)-exposed and EtOH-naïve hippocampi within the pyramidal cell layer of the CA1 (A), CA3 (B), and the granule cell layer of the dentate gyrus (DG) (C). Data are presented as percent control of the mean SEM. *Statistical significance ($p < 0.05$) compared to EtOH- and -drug-naïve hippocampi; #statistical significance ($p < 0.05$) compared to EtOH-treated, but drug-naïve hippocampi. $N = 27$ for EtOH-treated hippocampi; $N = 29$ for hippocampi co-exposed to EtOH and xestospongine C; $N = 33$ for EtOH- and drug-naïve hippocampi; $N = 30$ for EtOH-naïve hippocampi exposed to xestospongine C.

Fig. 2.

Effects of r-1 chaperone protein antagonist BD-1047 (20 and 80 IM) application on NeuN/Fox-3 immunofluorescence in ethanol (EtOH) treated and EtOH naive within the CA1 (A), CA3 (B), and dentate gyrus (DG) (C). Data are presented as percent control of the mean SEM. *Statistical significance ($p < 0.05$) compared to EtOH- and drug-naive hippocampi; #statistical significance ($p < 0.05$) compared to EtOH-treated, but drug-naive hippocampi. N = 10 for EtOH-treated hippocampi; N = 14 for hippocampi co-exposed to EtOH and BD-1047 (20 IM); N = 11 for hippocampi co-exposed to EtOH and BD-1047 (80 IM); N = 11 for EtOH and drug-naive hippocampi; N = 13 for EtOH-naive hippocampi exposed to BD-1047 (20 IM); N = 13 for EtOH-naive hippocampi exposed to EtOH and BD-1047 (80 IM).

Fig. 3.

Representative images of NeuN/Fox-3 immunofluorescence of ethanol (EtOH)- and drug-naïve hippocampi (top left), EtOH-naïve hippocampi exposed to xestospongin C (0.5 IM) (top middle), EtOH-naïve hippocampi exposed to BD-1047 (20 IM) (top right), EtOH-treated, but drug-naïve hippocampi (bottom left), hippocampi co-exposed to EtOH and xestospongin C (0.5 IM) (bottom middle), and hippocampi coexposed to EtOH and BD-1047 (20 IM) (bottom right).