Alcohol 49 (2015) 219–227

Contents lists available at ScienceDirect

Alcohol

journal homepage: http://www.alcoholjournal.org/

Ethanol withdrawal is required to produce persisting N-methyl-D-aspartate receptor-dependent hippocampal cytotoxicity during chronic intermittent ethanol exposure

Anna R. Reynolds [a,b,*], Jennifer N. Berry [a,b], Lynda Sharrett-Field [a,b], Mark A. Prendergast [a,b]

[a] University of Kentucky, Department of Psychology, 110 Kastle Hall, Lexington, KY 40506-0044, USA
[b] University of Kentucky, Spinal Cord and Brain Injury Research Center, 741 South Limestone St., Lexington, KY 40536-0509, USA

ARTICLE INFO

Article history:
Received 14 April 2014
Received in revised form
23 January 2015
Accepted 23 January 2015

Keywords:
Hippocampus
CIE
NMDA receptor
APV
NeuN
Thionine

ABSTRACT

Chronic intermittent ethanol consumption is associated with neurodegeneration and cognitive deficits in preclinical laboratory animals and in the clinical population. While previous work suggests a role for neuroadaptations in the N-methyl-D-aspartate (NMDA) receptor in the development of ethanol dependence and manifestation of withdrawal, the relative roles of ethanol exposure and ethanol withdrawal in producing these effects have not been fully characterized. To examine underlying cytotoxic mechanisms associated with chronic intermittent ethanol (CIE) exposure, organotypic hippocampal slices were exposed to 1–3 cycles of ethanol (50 mM) in cell culture medium for 5 days, followed by 24 h of ethanol withdrawal, in which a portion of slices were exposed to competitive NMDA receptor antagonist (2*R*)-amino-5-phosphonovaleric acid (APV; 40 μM). Cytotoxicity was assessed using immunohistochemical labeling of neuron-specific nuclear protein (NeuN; Fox-3), a marker of mature neurons, and thionine (2%) staining of Nissl bodies. Multiple cycles of CIE produced neurotoxicity, as reflected in persisting losses of neuron NeuN immunoreactivity and thionine staining in each of the primary cell layers of the hippocampal formation. Hippocampi aged *in vitro* were significantly more sensitive to the toxic effects of multiple cycles of CIE than were non-aged hippocampi. This effect was not demonstrated in slices exposed to continuous ethanol, in the absence of withdrawal, or to a single exposure/withdrawal regimen. Exposure to APV significantly attenuated the cytotoxicity observed in the primary cell layers of the hippocampus. The present findings suggest that ethanol withdrawal is required to produce NMDA receptor-dependent hippocampal cytotoxicity, particularly in the aging hippocampus *in vitro*.

Introduction

Prolonged alcohol dependence is known to produce neurodegeneration and cognitive decline that may be specifically associated with a common drinking pattern characterized by periods of heavy consumption followed by periods of abstinence (Mello & Mendelson, 1972; for review, see Duka et al., 2004). This intermittent pattern of intake is known to progressively increase the incidence of seizures during periods of withdrawal from alcohol (Ballenger & Post, 1978; Shaw, Waller, Latham, Dunn, & Thomson, 1998; Wojnar, Bizoń, & Wasilewski, 1999). Retrospective analyses of patient records have established a significant relationship between multiple prior withdrawals and seizures during acute withdrawal (Booth & Blow, 1993; Lechtenberg & Worner, 1991; Worner, 1996; see Duka et al., 2004 for a review). Brain volume abnormalities and cognitive decline are also thought to be expedited in dependent individuals who have experienced multiple seizures or detoxifications. For example, Sullivan, Marsh, Mathalon, Lim, and Pfefferbaum (1996) reported that temporal lobe white matter volume was inversely associated with prior alcohol withdrawal seizures. Duka, Townshend, Collier, and Stephens (2003) reported deficits in inhibitory control of prepotent motor responses in alcohol-dependent individuals that were statistically associated with a history of a greater number of prior detoxifications. Loeber et al. (2010) similarly reported that patients with a history of multiple prior detoxifications showed delays in their cognitive recovery at 3 months post-detoxification, when compared with those with fewer prior detoxifications.

Use of preclinical models of chronic intermittent ethanol (CIE) in rodents suggests that CIE exposure increases the rate, intensity, and duration of subsequent seizures (Stephens, Brown, Duka, & Ripley,

* Corresponding author. University of Kentucky, Department of Spinal Cord and Brain Injury Research Center, 741 South Limestone St., Lexington, KY 40536-0509, USA. Tel.: +1 859 323 0575; fax: +1 859 257 5737.
E-mail address: arreyn3@uky.edu (A.R. Reynolds).

http://dx.doi.org/10.1016/j.alcohol.2015.01.008
0741-8329/

2001; Veatch & Becker, 2002), decreases the development of subsequent long-term potentiation (Stephens et al., 2005), and produces neurodegeneration of the hippocampal formation (Collins, Zou, & Neafsey, 1998; Corso, Mostafa, Collins, & Neafsey, 1998; Zhao et al., 2013). For example, mice exposed to 3 cycles of vaporized ethanol for 16 h followed by 8 h of ethanol withdrawal demonstrated significant increases in handling-induced convulsions and electroencephalogram (EEG) activity (Veatch & Becker, 2005). As another example, Zhao et al. (2013) reported that a CIE model employing gavage ethanol administration produced neurodegeneration of the medial temporal lobe and working memory deficits in rats. *In vitro*, cultured cortical neurons were exposed to a CIE treatment regimen of 75 mM ethanol for 14 h followed by 10 h of withdrawal from ethanol, and repeated a total of 5 times and terminated by either a 2- or 5-day period of withdrawal (Qiang, Denny, & Ticku, 2007). Western blot and immunoblot analyses revealed that CIE produced selective increases in GluN1 and GluN2B subunit expression on the surface membrane. Collectively, these studies demonstrate that neuroadaptive changes in excitatory neurotransmission are produced by CIE, but the functional role of the NMDA receptor in mediating these neurodegenerative effects has yet to be established. The purpose of the present studies was, therefore, to examine the distinct roles of ethanol exposure and ethanol withdrawal, as well as to assess the influence of the NMDA receptor in promotion of hippocampal neurodegeneration produced by CIE in rat hippocampal explants.

Materials and methods

Preparation of organotypic hippocampal slice cultures

Whole brains from 8day-old Sprague–Dawley rats (Harlan Laboratories; Indianapolis, IN) were aseptically removed and transferred to culture dishes containing frozen dissecting medium composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), and 50 μM streptomycin/penicillin (Invitrogen). Bilateral hippocampi were then removed and transferred to plates containing culture medium composed of dissecting medium, distilled water, 36 mM glucose (Fisher, Pittsburgh, PA), 25% Hanks' Balanced Salt Solution (HBSS; Invitrogen), 25% (v/v) heat-inactivated horse serum (HIHS; Sigma), and 0.05% streptomycin/penicillin (Invitrogen). Excess hippocampal tissue was removed using a stereoscopic microscope, and unilateral hippocampi were sectioned at 200 μm using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Three to four intact hippocampal slices were plated onto Millicell-CM 0.4 μm biopore membrane inserts containing 1 mL of pre-incubated culture medium, with each plate containing 18–24 intact hippocampal slices. The tissue was maintained in an incubator at 37 °C with a gas composition of 5% CO_2/95% air for 5 days before any experiments were conducted. Care of all animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), as well as the University of Kentucky's Institutional Animal Care and Use Committee. All experiments included in the present report were replicated using at least two different rat litters.

CIE treatment regimen

The current studies examined the number of cycles of CIE required to produce cytotoxicity in hippocampal slice cultures. At 5 days *in vitro* (DIV), slices were assigned to one of the following treatment conditions: 1) 1 cycle of CIE, in which slices were exposed to medium with or without 50 mM ethanol for 5 DIV, followed by a 24 h withdrawal period; 2) 2 cycles of CIE, in which slices were exposed to medium with or without 50 mM ethanol for 5 DIV, followed by a 24 h withdrawal period, and repeated two times; 3) 3 cycles of CIE, in which slices were exposed to medium with or without 50 mM ethanol for 5 DIV, followed by a 24 h withdrawal period, repeated a total of three times; 4) continuous ethanol exposure, in which slices were maintained in medium with or without 50 mM ethanol for 18 consecutive days, in the absence of any withdrawal, and fixed (Fig. 1). An additional study assessed the effects of 3 cycles of CIE and protracted withdrawal for 7 days on neuron-specific nuclear protein (NeuN) immunofluorescence. Slices were exposed to medium with or without 50 mM ethanol for 5 DIV, followed by a 24 h withdrawal period, repeated a total of three times, followed by 7 days of withdrawal. This concentration of ethanol (50 mM) was selected in order to reflect binge alcohol use (Eckardt et al., 1998; Jones & Sternebring, 1992). For example, prior studies conducted in our laboratory demonstrate a decline in the actual ethanol concentration over the 5day exposure (i.e., from 198.39 mg/dL to 123.93 mg/dL) (Butler, Smith, Self, Braden, & Prendergast, 2008). During each 5 day exposure period, ethanol and control-treated slices were maintained inside Ziploc® bags filled with 5% CO_2/95% air and water bath solutions containing either distilled water (50 mL) for control plates or distilled water (50 mL) containing 50 mM ethanol for ethanol-treated plates, to minimize evaporation of ethanol described in prior reports (Prendergast et al., 2004). Slices were fixed by placing 1 mL of 10% formalin solution on the top and bottom of each well for 30 min. Slices were then washed twice with phosphate buffered saline (PBS) and stored at 4 °C until immunohistochemistry was initiated.

Effects of aging in vitro *on CIE-induced cytotoxicity*

Following the initial studies described above, a separate set of experiments was conducted in which tissue was maintained in culture for the same length of time as for 3 cycles of CIE, and fixed at the same time point. These studies were designed to account for potential effects of aging on CIE-induced loss of NeuN immunoreactivity and thionine staining. In these studies, hippocampal slices were prepared and maintained as described above. At 5 DIV, slices were randomly assigned to one of the following treatment conditions: 1) 1 cycle of CIE, in which slices were maintained in ethanol-free medium for 16 days (with a medium change every 5 days prior to ethanol treatment) and then exposed to medium with or without 50 mM ethanol for 5 DIV, followed by a 24 h withdrawal period; or 2) 2 cycles of CIE, in which slices were maintained in ethanol-free medium for 11 days (with medium changes every 5 days prior to ethanol treatment) and then exposed to medium with or without

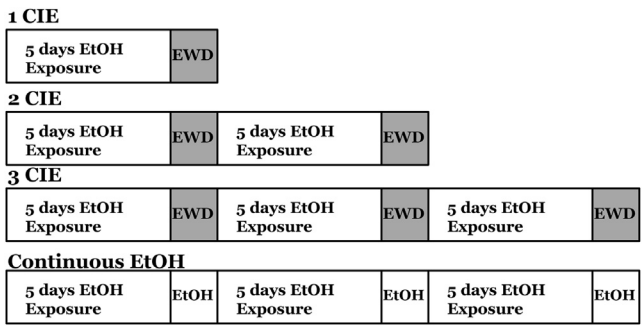

Fig. 1. Representative timelines of chronic, intermittent ethanol treatment of hippocampal slice cultures.