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# Broad-spectrum protein kinase inhibition by the staurosporine analog KT-5720 reverses ethanol withdrawal-associated loss of NeuN/Fox-3

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ABSTRACT

Chronic, intermittent ethanol (CIE) exposure is known to produce neuroadaptive alterations in excitatory neurotransmission that contribute to the development of dependence. Although activation of protein kinases (e.g., cyclic AMP [cAMP]-dependent protein kinase) is implicated in the synaptic trafficking of these receptors following CIE exposure, the functional consequences of these effects are yet to be fully understood. The present study sought to delineate the influence of protein kinase in regulating cytotoxicity following CIE exposure, as well as to examine the relative roles of ethanol exposure and ethanol withdrawal (EWD) in promoting these effects. Rat hippocampal explants were exposed to a developmental model of CIE with or without co-application of broad-spectrum protein kinase inhibitor KT-5720 (1 μM) either during ethanol exposure or EWD. Hippocampal cytotoxicity was assessed via immunofluorescence (IF) of neuron-specific nuclear protein (NeuN) with thionine staining of Nissl bodies to confirm IF findings. Concomitant application of ethanol and KT-5720 restored the loss of NeuN/Fox-3 IF in pyramidal CA1 and granule DG cell layers produced by CIE, but there was no restoration in CA3. Application of KT-5720 during EWD failed to significantly alter levels of NeuN IF, implying that ethanol exposure activates protein kinases that, in part, mediate the effects of EWD. KT-5720 application during EWD also restored thionine staining in CA1, suggesting kinase regulation of both neurons and non-neuronal cells. These data demonstrate that CIE exposure alters protein kinase activity to promote ethanol withdrawal-associated loss of NeuN/Fox-3 and highlight the influence of kinase signaling on distinct cell types in the developing hippocampus.



## Introduction

Patterns of binge-like ethanol consumption and multiple detoxifications (i.e., ethanol withdrawal [EWD]) predict poorer neurologic outcomes. These effects include physical manifestations of EWD (e.g., Veatch & Becker, 2005), neurocognitive perturbations (e.g., Zhao et al., 2013), and hippocampal neurodegeneration (e.g., Corso, Mostafa, Collins, & Neafsey, 1998) in adult rodents. These effects are associated with neuroadaptive changes in excitatory neurotransmission (e.g., Christian, Alexander, Diaz, & McCool, 2013; Nelson et al., 2005; Veatch & Becker, 2005). As an example, a prior study utilizing electrophysiological techniques demonstrated that EWD from CIE produced increased presynaptic glutamate function in the adult rat basolateral amygdala (Christian et al., 2013). Other electrophysiological studies have found amplified *N*-methyl-D-aspartate (NMDA)-receptor-mediated responses in the pyramidal CA1 cell layer of the hippocampal formation following exposure to CIE, relative to age-matched controls (Nelson et al., 2005). In addition, enhanced mGlu-1 and NMDA GluN signaling within the central nucleus of the amygdala (Cozzoli et al., 2014), as well as increased expression of group 1 mGlu-family and NMDA GluN2 proteins (Cozzoli et al., 2009), are observed in adult C57BL/6J mice subjected to binge-like ethanol administration. These behavioral and neurobiological data suggest that the behavioral effects of EWD are associated with alterations in excitatory neurotransmission.

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Western blot and immunoblot analyses revealed that CIE produced selective increases in GluN1 and GluN2B subunit expression on the surface membrane in fetal cultured cortical neurons (Qiang, Denny, & Ticku, 2007). Exposure to KT-5720 (i.e., 1 μM), an inhibitor of cyclic AMP-dependent protein kinase (PKA), and other similar protein kinases (e.g., mitogen-activated protein kinases [MAPK]), prevented increases in GluN1 and partially prevented increases in GluN2B expression in the developing cortex (Qiang et al., 2007). Another study demonstrated that ethanol exposure promotes trafficking of NMDA receptors in developing hippocampal neurons via activity-dependent processes (e.g., protein kinases) (Carpenter-Hyland, Woodward, & Chandler, 2004). Within the nucleus accumbens, activation of the cAMP-dependent protein kinase, PKA, confers the sensitivity of NMDA receptors following ethanol application in modulation of dopaminergic tone in periadolescent (i.e., 3–4 weeks old) rats (Maldve et al., 2002; see Lovinger, 2002 for a brief review). In addition, protein kinases (e.g., MAPK and extracellular signal-regulated kinases [ERK]) are known to phosphorylate group 1 metabotropic glutamate receptors (for a review, see Mao & Wang, 2016). A recent study conducted in our laboratory demonstrated that group 1 metabotropic glutamate (mGlu)-family proteins contribute to cytotoxicity in a developmental model of CIE (Reynolds, Williams, Saunders, & Prendergast, 2015). Taken together, these findings suggest that protein kinase activation might regulate neuroadaptive alterations in glutamatergic neurotransmission observed following CIE, particularly in the developing central nervous system (CNS). However, the functional role of protein kinase activity in promoting hippocampal cytotoxicity following CIE exposure is not clearly understood. Further, the relative roles of ethanol exposure and EWD in activating these intracellular signals to promote the cytotoxic effects of CIE have not been delineated. In the present report, we examined the functional effects of broad-spectrum protein kinase inhibition by the broad-spectrum staurosporine analog KT-5720 on the cytotoxic effects of ethanol in a developmental model of CIE.

## Methods

### *Organotypic hippocampal slice culture preparation*

Whole brains were aseptically removed from 8-day-old Sprague–Dawley rats (Harlan Laboratories; Indianapolis, IN) and transferred to sterile culture dishes containing frozen dissecting medium (Minimum Essential Medium [MEM; Invitrogen, Carlsbad, CA], 25 mM HEPES [Sigma, St. Louis, MO], 10.60 μM Amphotericin B solution [Sigma], and 50 μM streptomycin/penicillin [Invitrogen]). Bilateral hippocampi were extracted and carefully transferred to sterile plates containing chilled culture medium (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], 0.05% Amphotericin B solution [Sigma], and 0.05% streptomycin/penicillin [Invitrogen]). Excess hippocampal tissue was carefully 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). Hippocampi with cell layers intact (i.e., CA1, CA3, and DG) were selected under the stereoscopic microscope and then carefully plated using transfer pipettes onto Millicell-CM 0.4-μM biopore membrane inserts placed in a sterile 6-well culture that contained 1 mL of pre-incubated culture medium per well. Each culture well plate generated 18–24 hippocampi. Excess culture medium was extracted off the top of each biopore membrane insert and hippocampi were maintained in a water-jacketed incubator at 37 °C with a gas composition of 5% $CO_2$/95% air for 5 days prior to experimental manipulation for adequate membrane adherence (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.

### *Chronic, intermittent ethanol (CIE) regimen*

An *in vitro* model of CIE that has been published previously (Reynolds, Saunders, & Prendergast, 2016) was used to assess the functional role of cAMP-dependent protein kinase activation in promoting the cytotoxic effects of CIE. At 5 days *in vitro*, hippocampi were randomly transferred to plates containing either 1 mL of the ethanol-naïve culture medium (control) or ethanol-containing medium (i.e., 50 mM) for 5 days with or without the addition of KT-5720 (1 μM), a broad-spectrum protein kinase inhibitor (Bain et al., 2007). During each 5-day exposure period, ethanol and control-treated hippocampi 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 ethanol (50 mM) for ethanol-treated plates, so as to maintain ethanol at 50 mM. At 11 days *in vitro*, hippocampi were removed from culture plates and transferred to new plates containing 1 mL of fresh control culture media for a 24-h EWD period with or without the addition of KT-5720 (1 μM). This treatment regimen was repeated a total of three times (see Fig. 1). It is worthwhile to note that KT-5720 was applied either concomitantly with ethanol or during EWD for each of the three cycles of CIE at the same time points in ethanol- and control-treated slices. The concentration of ethanol (i.e., 50 mM) was selected based on prior reports demonstrating ubiquitous decreases of NeuN IR and thionine staining of Nissl bodies in this model of CIE (Reynolds, Berry, Sharrett-Field, & Prendergast, 2015). This concentration has also been shown to reflect patterns of binge drinking (Eckardt et al., 1998). The concentration of KT-5720 (i.e., 1 μM) was selected based on a prior report by Ticku and colleagues showing efficacy for protein kinase inhibition (Qiang et al., 2007). KT-5720 was first dissolved in 100% dimethyl sulfoxide (DMSO; Fisher) to yield a final working concentration of 0.01% DMSO in control and ethanol-treated culture medium.

### *Immunohistochemistry*

Following the CIE treatment regimen described above, hippocampi were fixed for immunohistochemical procedures by pipetting 1 mL of 10% formalin solution on the top and bottom of each culture plate well, with incubation for 30 min at room temperature. Wells were then washed twice with phosphate-buffered saline (PBS) and then stored at 4 °C until immunohistochemistry was performed. NeuN (Fox-3) is a protein located in nearly all post-mitotic neurons (Kim, Adelstein, & Kawamoto, 2009), and is a reliable marker of neuronal integrity (Butler et al., 2010; Reynolds et al., 2016). Immunohistochemistry was performed by transferring hippocampi to plates containing 1 mL of permeabilization (wash) buffer (200 mL PBS [Invitrogen], 200 μL Triton X-100 [Sigma], 0.010 mg bovine serum [Sigma]) on the bottom of each well. One mL of buffer was then added to the top of each well for a 45-min incubation period at room temperature to permeate cell membranes. Hippocampi were then transferred to plates containing 1× PBS on the bottom of each well, and primary monoclonal antibody mouse anti-NeuN (1:200; Sigma) was carefully pipetted on the top of each well as hippocampi were incubated at 4 °C for 24 h. Hippocampi were washed twice with 1× PBS and then incubated for 24 h with goat anti-mouse fluorescein isothiocyanate (FITC; 1:200; Sigma). Hippocampi were washed twice with 1× PBS. NeuN IF was visualized using SPOT software 4.0.2 (advanced