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Withdrawal from chronic intermittent alcohol exposure increases dendritic spine density in the lateral orbitofrontal cortex of mice

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ABSTRACT

Alcohol use disorders (AUDs) are associated with functional and morphological changes in subfields of the prefrontal cortex. Clinical and preclinical evidence indicates that the orbitofrontal cortex (OFC) is critical for controlling impulsive behaviors, representing the value of a predicted outcome, and reversing learned associations. Individuals with AUDs often demonstrate deficits in OFC-dependent tasks, and rodent models of alcohol exposure show that OFC-dependent behaviors are impaired by chronic alcohol exposure. To explore the mechanisms that underlie these impairments, we examined dendritic spine density and morphology, and NMDA-type glutamate receptor expression in the lateral OFC of C57BL/6J mice following chronic intermittent ethanol (CIE) exposure. Western blot analysis demonstrated that NMDA receptors were not altered immediately following CIE exposure or after 7 days of withdrawal. Morphological analysis of basal dendrites of layer II/III pyramidal neurons revealed that dendritic spine density was also not affected immediately after CIE exposure. However, the total density of dendritic spines was significantly increased after a 7-day withdrawal from CIE exposure. The effect of withdrawal on spine density was mediated by an increase in the density of long, thin spines with no change in either stubby or mushroom spines. These data suggest that morphological neuroadaptations in lateral OFC neurons develop during alcohol withdrawal and occur in the absence of changes in the expression of NMDA-type glutamate receptors. The enhanced spine density that follows alcohol withdrawal may contribute to the impairments in OFC-dependent behaviors observed in CIE-treated mice.

Introduction

Cognitive and behavioral changes are characteristic symptoms of long-term alcohol (i.e., ethanol) consumption, and include increased anxiety and irritability, memory deficits, and impaired executive function (Abernathy, Chandler, & Woodward, 2010). The prefrontal cortex (PFC) and its subregions regulate executive function, and two of these regions, the medial PFC (mPFC) and the orbitofrontal cortex (OFC), mediate cognitive flexibility and decision making related to expected rewards, respectively (Fuster, 2008; Zald & Rauch, 2006). The OFC's role in representing the value of a given stimulus is particularly interesting because it not only integrates information from a variety of primary sensory modalities but also participates in abstract concepts such as monetary reward (O'Doherty, 2004; O'Doherty, Kringelbach, Rolls, Hornak, & Andrews, 2001). In addition, evidence suggests that the OFC specifically participates in coding the rewarding aspects rather than the direct sensory aspects of stimuli (O'Doherty et al., 2000). Not surprisingly, this region plays a critical role in regulating impulsive behaviors and reversal learning (Berlin, Rolls, & Kischka, 2004; Schoenbaum, Saddoris, & Stalnaker, 2007; Winstanley, Theobald, Cardinal, & Robbins, 2004). fMRI studies show that patients with alcohol use disorders (AUDs) have greater activation of the OFC when performing OFC-dependent tasks compared to healthy controls, suggesting that prolonged alcohol consumption impairs OFC function, thus forcing the region to work harder to execute the same task. Outside of the scanner, patients with AUDs also perform poorly on reversal-learning tasks (Fortier, Maksimovskiy, Venne, LaFleche, & McGlinchey, 2009; Fortier et al., 2008) in a manner similar to patients with OFC lesions (Hornak et al., 2004; Tsuchida, Doll, & Fellows, 2010).

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Consistent with deficits reported in individuals with AUDs, rodent models of alcohol exposure have been shown to induce OFC-dependent behavioral deficits. For example, a previous study reported that treatment of mice with chronic intermittent ethanol (CIE) exposure in vapor inhalation chambers impaired reversal learning in a naturalistic food foraging task (Badanich, Becker, & Woodward, 2011) that is sensitive to OFC dysfunction (Bissonette et al., 2008). CIE exposure also altered performance during reversal learning on a choice task in mice that is sensitive to OFC and dorsolateral striatum lesions (DePoy et al., 2013). Forced binge-like alcohol consumption in mice led to deficits in reversal learning in a Barnes maze without impairing the initial spatial learning portion of the task (Crews & Boettiger, 2009). Together, the results of these studies suggest that chronic alcohol exposure may disrupt selective regions of the frontal cortex, including the OFC. Recently, morphological adaptations in the PFC have been associated with alcohol exposure and subsequent behavioral deficits. In rodents, chronic alcohol exposure and withdrawal leads to changes in dendritic arborization and alterations in dendritic spine density (Holmes et al., 2012; Kim, Zamora-Martinez, Edwards, & Mandyam, 2014; Kroener et al., 2012). In the OFC, while recent studies have investigated the effects of alcohol on dendritic length (DePoy et al., 2013; Holmes et al., 2012), nothing is known about how CIE exposure affects spine density and morphology in this region.

In the present study, we determined the effects of CIE exposure and withdrawal on dendritic spine morphology in the lateral OFC of C57BL/6J mice. We also examined chronic alcohol-induced changes in NMDA receptor expression because studies have linked chronic alcohol exposure with altered NMDA receptors in multiple brain regions including the PFC (Holmes et al., 2012; Kroener et al., 2012; Lovinger & Roberto, 2013). The results from these studies indicate that neurons in the lateral OFC undergo withdrawal-dependent changes in spine morphology in a manner that is distinct from the medial PFC.

Materials and methods

Animals

Adult male C57BL/6J mice (25–30 g; Jackson Laboratories, Bar Harbor, ME) were individually housed under a 12-h light/dark cycle (lights on at 0200). Rodent chow (Harland Tekland, Madison, WI) and water were available *ad libitum*. Mice were maintained in an AAALAC-accredited facility with automated temperature, humidity, and light cycle control. These studies were approved by the Institutional Animal Care and Use Committee and conducted according to the requirements of the NIH Guide for the Care and Use of Laboratory Animals (2011).

CIE-exposure procedure

CIE exposure of mice was performed using vapor inhalation chambers as described in previous studies (Becker & Lopez, 2004; Lopez & Becker, 2005). Briefly, mice were exposed to alcohol vapor for 16 h per day followed by an 8-h withdrawal in their home cage. This exposure regimen was repeated for 4 consecutive days followed by a 3-day rest period before beginning the next cycle of alcohol vapor exposure. This exposure pattern was repeated for 4 weeks, and control mice were placed in air inhalation chambers following the same parameters. Alcohol levels in the chambers were monitored daily and adjusted as necessary to achieve stable BECs above 175 mg/dL (range: 180.8–243.7 mg/dL). BECs in mice were measured weekly by taking a blood sample from the retro-orbital sinus immediately after removal from the chamber. Prior to each chamber session, mice received intraperitoneal (IP) administration of 1.6 g/kg alcohol in combination with 1 mmol/kg pyrazole (an alcohol dehydrogenase inhibitor) in a final volume of 0.02 mL/g body weight to initiate intoxication. Control mice were similarly handled, but were dosed with pyrazole in saline prior to being placed in air chambers.

Subcellular fractionation and western blot analysis

Tissue punches were taken from the lateral OFC of control and CIE-exposed mice (n = 5–10/group) at 0 and 7-day withdrawal from CIE exposure. Triton X-100 insoluble fractions that are enriched in postsynaptic density proteins were prepared as previously described (Mulholland, Becker, Woodward, & Chandler, 2011). Briefly, a Dounce homogenate was prepared and centrifuged at 23,000×g for 30 min at 4 °C. The pellet containing membrane-associated proteins was resuspended with 0.5% Triton X-100 buffer. The suspension was rotated for 15 min at 4 °C and then centrifuged at 12,000×g for 20 min at 4 °C, generating detergent-soluble and detergent-insoluble fractions. The detergent-insoluble pellet was solubilized in 2% LDS, and western blots were performed on this fraction following previous methods (Badanich, Mulholland, Beckley, Trantham-Davidson, & Woodward, 2013). An aliquot of each sample was taken for determination of protein concentration by the bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL). The remaining samples were stored at −80 °C until immunoblot analysis.

Samples were diluted with NuPAGE 4X LDS sample loading buffer (Invitrogen Corp., Carlsbad, CA; pH 8.5) containing 50 mM dithiothreitol, and samples were denatured for 10 min at 70 °C. Five micrograms of each sample were separated using the Bis-Tris (375 mM resolving buffer and 125 mM stacking buffer, pH 6.4; 7.5% acrylamide) discontinuous buffer system with MOPS electrophoresis buffer (50 mM MOPS, 50 mM Tris, 0.1% SDS, 1 mM EDTA, pH 7.7). Protein was then transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA) using a semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). After transfer, blots were washed with phosphate-buffered saline containing 0.1% Tween 20 (PBST) and then blocked with PBST containing 5% nonfat dried milk (NFDM) for 1 h at room temperature with agitation. The membranes were then incubated overnight at 4 °C with primary antibodies diluted in PBST containing 0.5% NFDM and washed in PBST prior to 1-h incubation at room temperature with horseradish peroxidase conjugated secondary antibodies diluted 1:2000 in PBST. Membranes received a final wash in PBST and the antigen–antibody complex was detected by enhanced chemiluminescence using a ChemiDoc MP Imaging system (Bio-Rad Laboratories, Hercules, CA). The bands were quantified by mean optical density using computer-assisted densitometry with ImageJ v1.41 (National Institutes of Health, USA). Because the use of loading controls (e.g., actin, GAPDH) for normalization in western blot experiments are subject to quantitation errors (Aldridge, Podrebarac, Greenough, & Weiler, 2008; Dittmer & Dittmer, 2006), normalization to a total protein stain (i.e., amido black) was 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 response. GluN1 antibody was purchased from BD Pharmingen (1:4000; Catalog #556308; San Jose, CA). GluN1 C2′ antibody was purchased from Chemicon International (1:1000; Catalog #AB5050P; Billerica, MA), GluN2A antibody was purchased from EMD Millipore (1:2000; Catalog #07-732; Billerica, MA) and GluN2B antibody was purchased from the UC Davis/NIH NeuroMab Facility (1:2000; Catalog #75-097; Davis, CA). Phospho-GluN2B antibody was purchased from Cell Signaling Technology (1:1000; Catalog #4208; Danvers, MA).