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Protracted abstinence from chronic ethanol exposure alters the structure of neurons and expression of oligodendrocytes and myelin in the medial prefrontal cortex

Alvaro I. Navarro and Chitra D. Mandyam

Committee on the Neurobiology of Addictive Disorders, The Scripps Research Institute, La Jolla, CA, USA

Abstract

In rodents, chronic intermittent ethanol vapor exposure (CIE) produces alcohol dependence, alters the structure and activity of pyramidal neurons and decreases the number of oligodendroglial progenitors in the medial prefrontal cortex (mPFC). In this study, adult Wistar rats were exposed to seven weeks of CIE and were withdrawn from CIE for 21 days (protracted abstinence; PA) and tissue enriched in the mPFC was processed for Western blot analysis and Golgi-Cox staining to investigate the long-lasting effects of CIE on structure of mPFC neurons and levels of myelin associated proteins. PA increased dendritic arborization within apical dendrites of pyramidal neurons and these changes occurred concurrently with hypophosphorylation of the NMDA receptor 2B (NR2B) at Tyr-1472. PA increased myelin basic protein (MBP) levels that occurred concurrently with hypophosphorylation of the premyelinating oligodendrocyte bHLH transcription factor Olig2 in the mPFC. Given that PA is associated with increased sensitivity to stress and hypothalamic-pituitary-adrenal (HPA) axis dysregulation, and stress alters oligodendrocyte expression as a function of glucocorticoid receptor (GR) activation, the levels of total GR and phosphorylated GR were also evaluated. PA produces hypophosphorylation of the GR at Ser-232 without affecting expression of total protein. These findings demonstrate persistent and compensatory effects of ethanol in the mPFC long after cessation of CIE, including enhanced myelin production and impaired GR function. Collectively, these results suggest a novel relationship between oligodendrocytes and GR in the mPFC, in which stress may alter frontal cortex function in alcohol dependent subjects by promoting hypermyelination, thereby altering the cellular composition and white matter structure in the mPFC.

Keywords

Ethanol vapors; pyramidal neurons; NMDA; glucocorticoid receptor; phosphorylation; Olig2

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Correspondence: Chitra D. Mandyam, Ph.D., Committee on the Neurobiology of Addictive Disorders, The Scripps Research Institute, 10550 North Torrey Pines Road, SP30-2400, La Jolla, CA 92037 USA; Tel: (858) 784-9039; Fax: (858) 784-2086; cmandyam@scripps.edu.

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Introduction

In humans, alcohol use disorder (AUD) deleteriously alters the architectural structure of and function dependent on the frontal cortex, and these deficits persist during protracted abstinence (Mancall, 1961; Goldstein and Shelly, 1971; Harper et al., 1987; Parsons and Nixon, 1993; Nixon and Bowlby, 1996; Sullivan et al., 2000; Vetreno et al., 2013; Le Berre et al., 2014). This dysfunction is associated with the loss of white matter, or myelin (Carlen and Wilkinson, 1987; Lewohl et al., 2005), as well as gray matter (Jernigan et al., 1991; Fein et al., 2002), suggesting that alterations caused by chronic alcohol exposure in the glial and neuronal contents of the frontal cortex are partly responsible for the behavioral impairments observed in alcoholics months after abstaining from alcohol (Seo et al., 2013).

Comparable to human alcoholics, alcohol rats made dependent via chronic intermittent ethanol vapor exposure (CIE) exhibit compulsive alcohol consumption and anxiety (Roberts et al., 2000; Overstreet et al., 2002), and are therefore comparable models for investigating long lasting neurobiological alterations associated with AUD. A neurobiological substrate that is affected by ethanol exposure is the glutamatergic N-methyl-D-aspartate receptor (NMDAR), which is a critical component of neuronal development and synaptic plasticity (Hoffman, 2003). Prior observations suggest ethanol exposure may regulate NMDAR function by altering the phosphorylation state of the NR2B subunits via the activation of kinases or phosphatases (Suvarna et al., 2005; Wang et al., 2007; Holmes et al., 2012; Kroener et al., 2012). For example, chronic inhibition of NMDAR function via recurring chronic ethanol exposure has been found to induce an upregulation of the NR2B subunit in cortical neurons (Follesa and Ticku, 1995; Nagy et al., 2003; Sircar and Sircar, 2006; Kroener et al., 2012), suggesting a homeostatic-type mechanism. In addition, the alterations produced by ethanol on the function of pyramidal neurons have been found to be associated with increased dendritic arborization and spine density of pyramidal neurons in the medial prefrontal cortex (mPFC), suggesting that structural and functional plasticity of pyramidal neurons are associated with altered NR2B function during chronic ethanol exposure (Holmes et al., 2012; Kroener et al., 2012). Our recent findings in adult rats experiencing CIE for seven weeks without an abstinence period extended these prior studies and demonstrated that CIE enhanced components of neuron structure within the apical and basal dendrites of pyramidal neurons in the mPFC and these alterations occurred concurrently with enhanced expression of NR2B during CIE (Kim et al., 2014). However, whether the alterations in structure of mPFC neurons (dendritic arborization and spine density) and expression of NR2B in the mPFC persist into prolonged abstinence after cessation of CIE is unknown. Therefore, this study used a similar paradigm of alcohol exposure as reported in Kim *et al* 2014 in a separate cohort of adult rats and tested the hypothesis that CIE alters the structure of pyramidal neurons and function of NR2B in the mPFC, an effect that would persist into prolonged abstinence.

Oligodendrogenesis, or generation of premyelinating glial cells from progenitor cells occurs in the adult brain (Emery, 2010), however, the functional significance of oligodendrogenesis is unknown (Mandyam and Koob, 2012; Nave and Ehrenreich, 2014). In the mPFC, progenitor cells generate premyelinating oligodendrocytes (Mandyam et al., 2007; Kim et al., 2014), which could generate myelin (Rivers et al., 2008; Kang et al., 2010) to affect

neuronal plasticity. In the context of AUD, we have previously reported that CIE in rats reduced proliferation, differentiation and survival of premyelinating oligodendrocytes in the mPFC, and these alterations in oligodendrocyte progenitors are associated with reduced myelin basic protein expression during CIE (Richardson et al., 2009; Kim et al., 2014). However, whether the alterations in the expression of proteins linked to oligodendrogenesis and myelin persist into prolonged abstinence from chronic ethanol exposure is unknown. Therefore, this study also tested the hypothesis that chronic ethanol exposure alters the expression of premyelinating oligodendrocytes and myelin, an effect that would persist into prolonged abstinence.

Materials and Methods

Animals

Adult male Wistar rats (Charles River), weighing 250–300 g and 8 weeks old at the beginning of the experiments, were housed in groups of 2–3 per cage in a temperature-controlled (22°C) vivarium on a 12 h/12 h light/dark cycle (lights on at 8:00 P.M.) with *ad libitum* access to food and water. All procedures were performed during the dark phase of the light/dark cycle. Twenty-one rats started and completed the study. Experimental procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Chronic ethanol exposure in alcohol vapor chambers

Vapors were delivered on a 14 h on/10 h off schedule for 7 weeks. This schedule of exposure has been shown to induce physical dependence. The flow rate was set to deliver vapors that result in blood alcohol levels (BALs) between 125 and 250 mg% (Figure 1) or 27.2 and 54.4 mM. In this model, rats exhibit somatic withdrawal signs and negative emotional symptoms reflected by anxiety-like responses, hyperalgesia, and elevated brain reward thresholds (Schulteis et al., 1995; Roberts et al., 2000; Valdez et al., 2002; Rimondini et al., 2003; O'Dell et al., 2004; Zhao et al., 2007; Richardson et al., 2008; Sommer et al., 2008; Edwards et al., 2012; Vendruscolo et al., 2012). Control rats were not exposed to ethanol vapor.

Measurement of blood alcohol levels

Blood sampling (tail bleedings) was performed immediately after daily bouts of alcohol vapor exposure in dependent animals twice during the first week of vapor exposure and once during each subsequent week of vapor exposure (Figure 1a). Plasma (5 µL) was used for measurement of blood alcohol levels using an Analox AM 1 analyzer (Analox Instruments). Single-point calibrations were done for each set of samples with reagents provided by Analox Instruments (25–400 mg% or 5.4–87.0 mM). When blood samples were outside the target range (150–250 mg%), vapor levels were adjusted.

Golgi-Cox staining and analysis

To determine the effect of prolonged abstinence from alcohol on cortical pyramidal neuronal structure, a group of vapor exposed rats ($n = 9$) were killed by rapid decapitation under isofluorane anesthesia 20 d after the last vapor exposure (CIE-protracted abstinence (CIE-PA)). Air exposed rats were used as controls ($n = 6$). Golgi-Cox staining was performed as previously reported (Kim et al., 2014). Brains were coded before sectioning to ensure that experimenters were blind to treatments.

For each animal, the apical and basal trees of 4 pyramidal neurons in the layer 2/3 of the medial prefrontal cortex (3.7 to 2.0 mm from bregma; infralimbic and prelimbic cortices were combined) were traced, and morphological measurements were analyzed as previously reported (Figure 2a–d; (Kim et al., 2014)). For each reconstructed neuron, an estimate of dendritic complexity was obtained using the Sholl ring method. A 3D Sholl analysis was performed in which concentric spheres of increasing radius (in 20 μm increments) were layered around the cell body until dendrites were completely encompassed (Figure 2d). The number of dendritic intersections at each increment was counted, and results were expressed as total intersections and the number of intersections per radial distance from the soma.

For all neurons, spine density was measured at 100 $\times$ magnification with an oil immersion lens (equipped with a 10x eye piece) on the same dendritic branches used for Sholl analysis. Dendritic spine density was measured on five dendritic segments from both the basal and apical tree. For the basal tree, spines were counted along four 10 μm segments of 2–5 $^\circ$ order branches located at least 50 μm away from the cell body. For the apical tree, spines were counted along one 30 μm segment of primary apical branches located at least 80 μm away from the cell body. Dendritic spines were classified into 2 categories (mushroom or nonmushroom) based on their length and neck and head width. Stringent criteria were used for the categorization of spine heads into mushroom and nonmushroom type spines and were based on several seminal reports (Peters and Kaiserman-Abramof, 1970; Harris et al., 1992). The total number of dendritic spines visible along both sides of the segment was counted and expressed as number of spines per 10 microns of dendrite.

Western blotting

Procedures optimized for measuring levels of both phosphoproteins and total proteins were employed (Kim et al., 2014). Tissue punches from 500 μm thick PFC sections from CIE-PA rats ($n = 9$) and controls ($n = 6$), were homogenized on ice by sonication in buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1% SDS, with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III diluted 1:100; Sigma), heated at 100 $^\circ\text{C}$ for five minutes, and stored at -80°C until determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad). Samples were mixed (1:1) with a Laemmli or Tricine sample buffer containing β -mercaptoethanol. Each sample containing protein from one animal was run (30 μg per lane) on an 8–12% SDS-PAGE gel (Bio-Rad) and transferred to polyvinylidene fluoride membranes (PVDF pore size 0.2 μm). Blots were blocked with 2.5–5% milk (w/v) in TBST (25 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for 16–20 h at 4 $^\circ\text{C}$ and were incubated with the primary antibody for 16–20 h at 4 $^\circ\text{C}$: antibody to NR2B (1:200, Santa Cruz cat. no. sc-9057, predicted molecular

weight 178 kDa, observed band ~180 kDa), antibody to pNR2B Tyr-1472 (1:200, Cell Signaling cat. no. 4208S, predicted molecular weight 190 kDa, observed band ~190 kDa), antibody to PSD-95 (1:1000, Millipore, cat. no. 04-1066, predicted band size 95 kDa, observed band ~95 kDa), antibody to oligodendrocyte lineage transcription factor 2 (Olig2; 1:10000, predicted molecular weight 37 kDa (Ligon et al., 2006), observed band ~37 kDa), antibody to phosphorylated Olig2 (pOlig2) Ser-10, 13, 14 (1:500, predicted molecular weight 35 kDa, observed band ~35 kDa (Sun et al., 2011)), antibody to myelin basic protein (MBP; 1:500, Abcam, cat. no. ab40390, predicted band size 18–23 kDa, observed band ~20 kDa), antibody to GR (1:500, Cell Signaling Technology, cat. no. 12041S, predicted band size 91–95 kDa, observed band ~93 kDa), antibody to pGR-Ser-211 (a human analogue of the rat Ser-232; (Chen et al., 2008)); 1:500, Cell Signaling Technology, cat. no. 4161S, predicted band size 95 kDa, observed band ~98 kDa). β -tubulin (1:8,000, Santa cruz cat. no. sc-5274, predicted band size 50 kDa, observed band ~50 kDa) was used as a loading control. Blots were then washed three times for 15 min in TBST, and then incubated for 1 h at room temperature (24 °C), appropriately with either horseradish peroxide–conjugated goat antibody to rabbit (1:2000, BioRad) or horseradish peroxide–conjugated goat antibody to mouse IgG1 (1:2000, BioRad) in TBST. After another three washes for 15 min with TBST, immunoreactivity was detected using SuperSignal West Dura chemiluminescence detection reagent (Thermo Scientific) and collected using HyBlot CL Autoradiography film (Denville Scientific) and a Kodak film processor. Net intensity values were determined using the Image Studio Lite software (LI-COR Biosciences). For normalization purposes, following chemiluminescence detection, blots were either stripped for 20 minutes at room temperature (Restore, Thermo Scientific) and reprobed for total protein levels of β -Tubulin (for NR2B, Olig2, MBP) or blots were incubated with 0.125% coomassie stain for 5 minutes and washed three times for 5–10 minutes in destain solution (GR).

GR and pGR analysis was also conducted on tissue lysates from controls and CIE rats euthanized 3h after the last vapor exposure. Data from these animals and BALs for these animals have been published elsewhere (Kim et al., 2014). This experiment was performed to determine the changes in levels of GR and pGR during CIE.

Statistical analysis

For analysis of dendritic arborization and dendritic spine density, the tracing data from the neurons per animal were averaged (4 neurons per animal, 6–9 animals per group) and were subjected to statistical analysis. The effects of type of dendrite (apical vs. basal) or group (control vs. CIE-PA), and distance from the soma on dendritic length were analyzed using two-way ANOVA, with repeated measures for distance from the soma, followed by Fisher's LSD post hoc tests. Differences in density of proteins were also analyzed by Student's *t* tests or one-way ANOVA where appropriate. Data are expressed as mean $\pm$ SEM. Values of *p* < 0.05 were considered statistically significant. Graphs were generated using GraphPad Prism 5.0 software.

Results

Chronic inhalation of intermittent ethanol vapors produces steady blood alcohol levels and CIE does not affect body weight gain in adult Wistar rats

Measurements of animal body weights (Figure 1c) were not significantly different between control and CIE-PA groups. All animal body weights increased an average of 250 g over the entire experimental time for both groups. Measurements of the experimenter-adjusted BALs (Figure 1d) were maintained at a target BAL, between 150 and 250 mg%, during all 7 weeks of the CIE treatment period.

CIE-PA alters the structure of pyramidal neurons in the mPFC

Apical and basal trees of mPFC pyramidal neurons were traced separately for each pyramidal neuron (Figure 2a–d), and morphological measurements were analyzed per animal per group using 3D sholl analysis (Figure 2d). Two-way ANOVA revealed a significant main effect of distance from soma in apical and basal dendrites in control animals ($F_{(18, 190)} = 108.2$, $p < 0.0001$). Further analysis indicated a greater number of intersections at distances 10–90 μm from the soma in apical dendrites (Figure 2e; $p < 0.001$), and at distances 150–170 μm from the soma in both apical and basal dendrites (Figure 2e–f; $p < 0.05$), indicating an increase in dendritic arborization closer to the soma. CIE-PA increased the number of dendritic intersections in apical dendrites, without affecting the number of dendritic intersections in basal dendrites (Figure 2e–f). Two-way ANOVA revealed a significant interaction (ethanol treatment $\times$ distance from soma; $F_{(15, 195)} = 1.8$, $p = 0.03$), and a significant main effect of distance from soma ($F_{(15, 195)} = 36.6$, $p < 0.0001$). Further analysis showed a significant difference between CIE-PA and control at 70 μm from the soma ($p < 0.01$). CIE-PA did not alter the combined lengths of apical or basal dendritic trees, or the soma-tip distance of apical or basal dendritic trees (data not shown). Two-way ANOVA demonstrated a significant effect of dendrite type on total number of spines ($F_{(1, 13)} = 6.2$, $p = 0.02$), but did not detect a significant interaction. CIE-PA did not alter total dendritic spine density (mushroom vs. nonmushroom type spine density was also unaltered, data not shown).

CIE-PA produces hypophosphorylation of NMDAR subunit NR2B at Tyr-1472 without altering total levels of NR2B in the mPFC

To investigate the effects of CIE-PA on the total levels and phosphorylation of NR2B subunits in the mPFC, Western blot analyses were performed. CIE-PA significantly decreased the levels of phosphorylation of NR2B at Tyr-1472 (Figure 3a–b; $p = 0.0257$), and did not alter total NR2B subunit levels. CIE-PA did not alter the levels of PSD-95 (data not shown).

CIE-PA increases MBP levels and produces hypophosphorylation of the bHLH transcription factor Olig2 in the mPFC

We next examined the effects of CIE-PA on expression of total Olig2, the phosphorylation of Olig2, and total MBP in the mPFC. CIE-PA did not alter expression of total Olig2,

whereas it increased expression of MBP levels (Figure 4a–b; $p = 0.005$), both associated with the hypophosphorylation of Olig2 at Ser-10, 13, 14 (Figure 4a–b; $p = 0.024$).

CIE-PA produces hypophosphorylation of GR at Ser-232 without affecting total GR levels in the mPFC

We also examined the effects of CIE and CIE-PA on expression of total GR and the phosphorylation of GR at Ser-232 in the mPFC. CIE did not alter the expression of total GR or phosphorylation of GR. CIE-PA did not alter expression of total GR, whereas CIE-PA reduced expression of pGR levels compared with ethanol naïve controls (Figure 5a–b; ($F_{(2, 32)} = 6.8$, $p = 0.003$).

Discussion

We have recently demonstrated that chronic ethanol exposure produces alterations in the structure of pyramidal neurons in the mPFC and these changes are associated with enhanced expression of NR2B during CIE (Kim et al., 2014). Therefore, the first goal of the present study was to determine whether the effects of CIE observed in the structure of the pyramidal neurons and expression of NR2B during alcohol exposure were long-lasting. We have also demonstrated that chronic ethanol exposure reduces the expression of premyelinating oligodendrocytes and myelin associated proteins in the mPFC during CIE (Kim et al., 2014). Accordingly, the second goal of the study was to determine whether protracted abstinence from CIE produces compensatory effects in the expression of premyelinating oligodendrocytes and myelin basic protein in the mPFC. Consistent with our hypothesis, withdrawal and protracted abstinence from CIE enhanced dendritic arborization within apical dendrites of pyramidal neurons in the mPFC without affecting dendritic arborization within the basal dendrites or dendritic spine densities within apical and basal dendrites. The changes in neuronal structure occurred concurrently with dephosphorylation of Tyr-1472 of the NR2B subunit of the NMDARs, demonstrating changes in plasticity proteins associated with neuronal signaling. Protracted abstinence from CIE produced dephosphorylation of the triple Ser motif of Olig2 without altering expression of total Olig2, and these changes were associated with enhanced expression of MBP. These long-lasting effects of chronic ethanol exposure on mPFC pyramidal neurons and myelin following withdrawal and protracted abstinence demonstrate the detrimental neurobiological alterations contributing to the neurotoxicity of chronic alcohol exposure.

Dendritic arborization and dendritic spine density are elements of neuron structure and their plasticity is regulated by both cell intrinsic and extrinsic signals, and these elements of neuron structure undergo significant modifications during excitotoxicity (Smart and Halpain, 2000; Cline, 2001). For example, chronic ethanol exposure increases dendritic arborization and dendritic spine density of mPFC neurons, indicating dendritic remodeling during CIE (Holmes et al., 2012; Kroener et al., 2012; Kim et al., 2014). Although alterations in neuron structure are not required for alterations in neuron function (Lang et al., 2004), changes in dendritic structure of mPFC neurons have been correlated with changes in functional plasticity of mPFC neurons during alcohol exposure (Holmes et al., 2012; Kroener et al., 2012). This association between alterations in structure and function of

mPFC neurons has been extended into abstinence, as a seven week withdrawal post CIE did not abolish the structural and functional changes seen during alcohol exposure (Kroener et al., 2012). Our findings extend this study, and demonstrate that some structural alterations produced during alcohol exposure (increases in dendritic arborization within apical dendrites 50–70 μm from the soma; (Kim et al., 2014)) persist into protracted abstinence from CIE (current findings). This finding is important because CIE induces emotional memory deficits dependent on the mPFC which persist into abstinence (George et al., 2012; Holmes et al., 2012), and these behavioral impairments could be attributable to the maladaptive plasticity of pyramidal neurons. For example, the apical dendrites of the pyramidal neurons in the mPFC receive afferent inputs from the basolateral nucleus of the amygdala which make connections at approximately 50–70 μm from the soma (Hoover and Vertes, 2007; Gabbott et al., 2012). Therefore, it is tempting to speculate that a hypertrophy of these dendrites during protracted abstinence may suggest increased synaptic transmission from the amygdala, thus promoting emotional memory deficits and behaviors like the anxiety and compulsive behavior exhibited by alcohol dependent individuals after several weeks of abstinence (Overstreet et al., 2002; Seo et al., 2013).

The glutamatergic NMDAR is a heterotetrameric protein complex that mediates excitatory neurotransmission and plays a critical role in synaptic plasticity. NMDARs in the mPFC undergo significant regulation under ethanol exposure and withdrawal (Hu and Ticku, 1995; Chandler, 2003; Qiang et al., 2007; Kim et al., 2014). Specifically, acute ethanol exposure inhibits NMDAR function (Tu et al., 2007; Weitlauf and Woodward, 2008), while CIE-mediated chronic ethanol exposure increases NMDAR expression and function (Holmes et al., 2012; Kroener et al., 2012; Kim et al., 2014). A week of withdrawal from CIE abolishes CIE-induced enhanced expression of NR2B subunits of the NMDARs without affecting CIE-induced alterations in structure and function of pyramidal neurons in the mPFC (Kroener et al., 2012). The mechanism(s) driving alterations to NMDARs observed during withdrawal and protracted abstinence are not clear, however, functional changes to NMDARs have been associated with the phosphorylation and dephosphorylation of NR2B subunits (Ali and Salter, 2001; Nakazawa et al., 2001). For example, the NR2B subunit contains three tyrosine phosphorylation sites at the C-terminus; the Fyn-mediated Tyr-1472 being the major site of phosphorylation (Nakazawa et al., 2001). This site is located within a tyrosine-based motif (YEKL) that directly binds to the adaptor protein-2, a protein complex that links internalized proteins to clathrin (Slepnev and De Camilli, 2000; Roche et al., 2001; Lavezzari et al., 2003). The phosphorylation of Tyr-1472 by Fyn kinase disrupts the interaction between NMDARs and the adaptor protein-2 complex, ultimately preventing the clathrin-mediated endocytotic pathway and stabilizing the receptor (Lavezzari et al., 2003; Prybylowski et al., 2005); whereas, dephosphorylation of NMDARs at Tyr-1472 promotes endocytosis of NMDARs (Snyder et al., 2005; Venkitaramani et al., 2011). The present findings demonstrate that withdrawal and protracted abstinence produces dephosphorylation of the NR2B subunit at Tyr-1472. Inhibition of Tyr-1472 phosphorylation during protracted abstinence would suggest a protective mechanism against glutamate excitotoxicity that occurs during chronic ethanol exposure by allowing NMDA receptors to be internalized to offset the enhanced expression of the receptor during ethanol exposure (Kroener et al., 2012; Kim et al., 2014).

Olig2, a basic-helix-loop-helix transcription factor with significant roles in gliogenesis, is expressed in premyelinating oligodendrocyte progenitors and in differentiated myelinating oligodendrocytes, where it appears to have ongoing biological function (Rivers et al., 2008). During development and adulthood, Olig2 function is highly modulated by the phosphorylation of a conserved triple serine motif (Ser-10, 13, and 14) within the N-terminal domain (Sun et al., 2011). Specifically, phosphorylation of the triple serine motif decreases as progenitors mature into differentiated myelinating oligodendrocytes (Sun et al., 2011; Meijer et al., 2014). This indicates there is a positive correlation between dephosphorylation of Olig2 and an increased differentiation of Olig2 progenitors into differentiated myelinating oligodendrocytes and myelin. In the present study, protracted abstinence produced dephosphorylation of Olig2 and increased the levels of MBP. Since expression of MBP does not determine myelin gain-of-function or myelin activity, a minor limitation of the current study is the lack of such measures. Nevertheless, the dramatic increase in myelin associated protein could be pathological or compensatory and could be associated with altered structure of mPFC pyramidal neurons.

In the context of the above hypothesis, decreases in myelin and white matter have been documented with increased stress reactivity in aging, cognitive impairment during aging, and mental health disorders such as depression and post-traumatic stress disorder which are evident in alcohol dependent subjects (Regenold et al., 2007; Fields, 2008; Jackowski et al., 2008; Ziegler et al., 2010). Concurrently, increases in myelin and white matter have been associated with cognitive decline and suicidality, and these alterations are hypothesized to occur as consequence of high stress responsiveness (Lyons et al., 2004; Ehrlich et al., 2005; Morisaki et al., 2010). These findings suggest that stress induces alterations of the oligodendrocyte/myelin:neuron ratio in the adult brain which could affect cognition due to oligodendrocytic roles in synapse formation (Fawcett et al., 1989; Bandtlow et al., 1990). Notably, recent studies in the hippocampus indicate that exposure to the glucocorticoid stress hormone, corticosterone, induces a transcriptional program that promotes an increase in oligodendrogenesis, which in turn enhances levels of myelin (Chetty et al., 2014). In addition, there is emerging evidence that oligodendrocyte progenitor cells express glucocorticoid receptors, and corticosteroids regulate myelin expression in the central nervous system via regulation of the differentiation of oligodendrocyte progenitor cells (Chari, 2014; Jenkins et al., 2014). Therefore, the increases in myelin expression during protracted abstinence could be associated with changes in corticosterone or GR itself. For example, it has been demonstrated that CIE attenuates basal corticosterone levels by altering the function of the hypothalamic-pituitary-adrenal axis, and produces a blunted (reduced) corticosterone response during abstinence (Zorrilla et al., 2001; Richardson et al., 2008). Notably, blockade of GRs during withdrawal and abstinence reduces the negative affect symptoms associated with alcohol dependence (Vendruscolo et al., 2012). Therefore, the crude increase in myelin basic protein levels during protracted abstinence may be attributed to altered GR expression and function during withdrawal and protracted abstinence. For example, phosphorylation of the N-terminal site Ser-232 on the GR has been demonstrated to affect GR function and play a role in transcriptional activation via its interaction with coactivator proteins (Ismaili and Garabedian, 2004; Chen et al., 2006; Galliher-Beckley and Cidlowski, 2009). Analysis of phosphorylated and total GR levels demonstrates that

protracted abstinence produces dephosphorylation of GRs at Ser-232 without affecting total GR levels in the mPFC. These findings suggest that during protracted abstinence hypothalamic-pituitary-adrenal axis dysregulation via blunted corticosterone levels leads to reduced phosphorylation of GRs, thereby reducing GR function. However, more mechanistic studies are required to directly relate corticosterone levels to functional GR changes during protracted abstinence in the mPFC. Nevertheless, the present findings suggest that the hypermyelination in the mPFC during protracted abstinence could be resulting from allostatic-like changes in the extrahypothalamic GR system during chronic ethanol exposure (Vendruscolo et al., 2012).

Collectively, this study proposes that protracted abstinence produces long-lasting alterations in the structure of mPFC pyramidal neurons, with accompanying abnormalities in myelinating oligodendrogenesis and MBP. The finding that mPFC pyramidal neuron structure and myelin expression are affected long after cessation of chronic ethanol exposure indicates that chronic alcohol abuse may increase the risk of cognitive and emotional behaviors dependent on the frontal cortex long after cessation of excessive alcohol consumption.

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Highlights

- Chronic ethanol produces persistent changes in the structure of mPFC neurons
- Protracted abstinence enhances dendritic arborization within apical dendrites
- Protracted abstinence produces hypermyelination in the mPFC
- Protracted abstinence produces hypophosphorylation of GR at Ser-232

Figure 1.

(a) Experimental timeline for ethanol vapor exposure and withdrawal from ethanol vapors. Red arrow indicates when BALs were measured. Animals experienced CIE for 7 weeks. A cohort of CIE animals were euthanized 3h after the last vapor exposure and the findings from these animals are recently published in Kim *et al* 2014 (Kim *et al.*, 2014). Another cohort of CIE animals were euthanized 3 weeks after abstinence (CIE-PA) and the findings from these animals are reported in the current study. (b) Schematic of a coronal section of an adult rat brain indicating the region examined for Golgi-Cox analysis (shaded quadrilateral in light gray); and Western blotting analysis (dark gray circles indicating the region of tissue punches). (c) Animal body weights, expressed in grams, before, during the course of the 7 week CIE period, and after protracted abstinence. (d) Blood alcohol levels (BALs), expressed in mg%, over the seven week CIE period. $n = 12$ in controls (6 for Golgi-Cox and 6 for Western blotting analysis), and $n = 9$ in protracted abstinence group (left hemisphere for Golgi-Cox and right hemisphere for Western blotting analysis). CIE-PA, Chronic intermittent ethanol vapor exposure-protracted abstinence.

Figure 2.

(a) A representative example of a Golgi-Cox stained pyramidal neuron in the mPFC, indicating apical (arrow) and basal (filled arrowhead) dendrites, and dendritic spines on apical and basal dendrites (open arrowhead); (b–c) Representative neuron tracings from (b) one control and (c) one CIE-PA animal. (d) Sholl ring analysis at 20 μm ring distance with a starting distance of 10 μm of the neuron indicated in (c); Scale bar in (a) is 25 μm. (e–f) Structural analysis of mPFC pyramidal neurons, data of apical (e) and basal (f) dendrites illustrated as the number of intersections of the dendrites, (g) Spine density analysis of pyramidal neurons; Total number of dendritic spines per 10 μm of the dendrite from distal portions of the dendrite relative to the cell body. $n = 6$ animals in control and $n = 9$ animals in CIE-PA. Neuron data is from 24 neurons for control and 34 for CIE-PA. $**p < 0.01$ vs. control in (e).

Figure 3.

(a–b) CIE-PA decreases the phosphorylation of NR2B (pNR2B) at Tyr-1472 in the mPFC without affecting the levels of total NR2B. (a) Representative immunoblots of pNR2B vs. total NR2B compared with b-Tubulin. (b) Densitometric analysis of proteins in CIE-PA animals expressed as percent change compared with controls. $n = 6$ animals in control and $n = 9$ animals in CIE-PA. * $p < 0.05$ compared with controls by unpaired t test.

Figure 4. (a–b) CIE-PA decreases the phosphorylation of bHLH transcription factor Olig2 (pOlig2) at Ser-10, 13, 14 without affecting the levels of total Olig 2. CIE-PA also increases MBP levels. (a) Representative immunoblots of pOlig2, total Olig2, MBP, and corresponding blots for b-Tubulin. (b) Densitometric analysis of proteins in CIE-PA animals expressed as percent change compared with controls. $n = 6$ animals for control and $n = 9$ animals for CIE-PA. * $p < 0.05$ and ** $p < 0.01$ compared with controls by unpaired t test.

Figure 5.

(a, c) CIE does not alter phosphorylation of GR at Ser-232 and does not affect the levels of total GR. (b, d) CIE-PA decreases the phosphorylation of GR at Ser-232 without affecting the levels of total GR. (a, b) Representative immunoblots of pGR, total GR, and corresponding blot for coomassie blue from one control and CIE and one control and CIE-PA rat. (c, d) Densitometric analysis of proteins in CIE and CIE-PA animals expressed as percent change compared with controls. $n = 12$ animals in controls and $n = 12$ animals in CIE group. $n = 6$ animals in control and $n = 9$ animals in CIE-PA group. * $p < 0.05$ vs. control by unpaired t test.