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Wheel running reduces ethanol seeking by increasing neuronal activation and reducing oligodendroglial/neuroinflammatory factors in the medial prefrontal cortex

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Abstract

The therapeutic effects of wheel running (WR) during abstinence on reinstatement of ethanol seeking behaviors in rats that self-administered ethanol only (ethanol drinking, ED) or ED with concurrent chronic intermittent ethanol vapor experience (CIE-ED) were investigated. Neuronal activation as well as oligodendroglial and neuroinflammatory factors were measured in the medial prefrontal cortex (mPFC) tissue to determine cellular correlates associated with enhanced ethanol seeking. CIE-ED rats demonstrated escalated and unregulated intake of ethanol and maintained higher drinking than ED rats during abstinence. CIE-ED rats were more resistant to extinction from ethanol self-administration, however, demonstrated similar ethanol seeking triggered by ethanol contextual cues compared to ED rats. Enhanced seeking was associated with reduced neuronal activation, and increased number of myelinating oligodendrocyte progenitors and PECAM-1 expression in the mPFC, indicating enhanced oligodendroglial and neuroinflammatory response during abstinence. WR during abstinence enhanced self-administration in ED rats, indicating a deprivation effect. WR reduced reinstatement of ethanol seeking in CIE-ED and ED rats, indicating protection against relapse. The reduced ethanol seeking was associated with enhanced neuronal activation, reduced number of myelinating oligodendrocyte progenitors, and reduced PECAM-1 expression. The current findings demonstrate a protective role of WR during abstinence in reducing ethanol seeking triggered by ethanol contextual cues and establish a role for oligodendroglia-neuroinflammatory response in ethanol seeking. Taken together, enhanced oligodendroglia-neuroinflammatory response during abstinence may contribute to brain trauma in chronic alcohol drinking subjects and be a risk factor for enhanced propensity for alcohol relapse.

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Keywords

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1.0 Introduction

A major factor contributing to the enduring nature of alcohol relapse is the persistence of subjective responses to contextual cues that were paired with alcohol consumption (O'Brien et al., 1998). These responses could be reduced by behavior-based approaches that have the potential to activate areas of the prefrontal cortex (PFC) associated with voluntary inhibitory control of motivational impulses (Phan et al., 2005, Diekhof and Gruber, 2010).

Growing evidence suggests that the rodent medial prefrontal cortex (mPFC) likely represents a functional homolog of the human medial and dorsolateral PFC (Vertes, 2006), and therefore rodent models of relapse (modeled as reinstatement of ethanol seeking) can be used to uncover neural correlates in the PFC that assist with enhanced propensity for relapse (Martin-Fardon and Weiss, 2013). For example, one such model, namely the extinction-reinstatement model elicits drug seeking in response to ethanol-associated environmental stimuli, such as ethanol context and cues after extinguishing responses to these environmental stimuli in a novel (nondrug-paired) context (Shaham et al., 2003). Using this model, it has been demonstrated that enhanced ethanol seeking triggered by ethanol cues, stress and ethanol itself is associated with alterations in neurotransmitter systems and neuronal activation in the extended amygdala, and pharmacological manipulations targeting the dysregulated neurotransmitter systems assisted with reducing propensity for relapse (Ciccocioppo et al., 2003, Zhao et al., 2006). However, neurobiological correlates in the mPFC in response to ethanol seeking triggered by ethanol context and contextual cues is unknown (Weiss et al., 2001), and could help determine risk factors in a brain region implicated in preoccupation/anticipation or craving stage of addiction (Koob and Volkow, 2010).

We have recently demonstrated that unregulated self-administration of ethanol in animals that experienced chronic intermittent ethanol vapor exposure (CIE-ED; a paradigm that produces alcohol dependence-like behavior) produces profound alterations in the birth and survival of oligodendroglial progenitors (OPCs) in the mPFC compared with regulated ethanol drinking (ED, a paradigm that maintains nondependent drinking; (Richardson et al., 2009, Kim et al., 2015, Somkuwar et al., 2015)). Particularly interesting is the long-lasting effect of withdrawal on OPCs in the mPFC, visualized as increases in proliferation and survival of progenitors in CIE-ED animals that also demonstrated enhanced drinking after prolonged abstinence, suggesting a permanent dysregulation in the oligodendroglial niche maintaining oligodendroglial homeostasis (Somkuwar et al., 2015). Protracted abstinence from CIE is also associated with increases in myelin associated proteins in the mPFC indicating additional compensatory changes in myelinating glia (Navarro and Mandyam, 2015). Such alterations in the expression of oligodendroglia and myelinating glia during withdrawal and abstinence in the mPFC may be regulated by neuroinflammatory response

during withdrawal, as interactions between oligodendroglia, myelin, endothelial cells and neuroinflammatory proteins have been demonstrated in models of brain injury, including, stroke and ischemia (Pham et al., 2012, Ortega et al., 2015). For example, proliferation and survival of OPCs is regulated by vascular endothelial cells, such that increase in endothelial response enhances OPC proliferation and survival (Arai and Lo, 2009). Notably, withdrawal from chronic ethanol experience enhances generation of inflammatory mediators such as cytokines in the cortex (Whitman et al., 2013, Harper et al., 2015) and increases in cytokines can upregulate expression of endothelial cell adhesion molecules in endothelial cells, that can in concert support leukocyte emigration via blood-brain barrier disruption (Woodfin et al., 2007, Larochelle et al., 2011). The recruitment of leucocytes by endothelial cell adhesion molecules is directly linked to more tissue damage and an increased release of inflammatory mediators, ultimately leading to uncontrolled inflammation (Privratsky et al., 2010).

In this context, platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) is known to produce neuroinflammation and subsequent blood brain barrier disruption, and accumulating evidence suggests that it could be used as a potential marker for neurological disorders (Losy et al., 1999, Zaremba and Losy, 2002, Hwang et al., 2005, Kalinowska and Losy, 2006, Woodfin et al., 2007, Privratsky et al., 2010, Cheung et al., 2015, Andrews et al., 2016). However, it is not known whether withdrawal from chronic ethanol experience regulates PECAM-1 and if the alterations in PECAM-1 correlate with withdrawal induced increases in oligodendroglia and myelinating glia.

Accumulating evidence demonstrates that behavioral therapies (e.g. physical activity) that augment cognitive flexibility and alter neuroinflammatory responses can be used to enhance recovery from brain injury in models of stroke and ischemia (Hu et al., 2010, Olver et al., 2015). Notably, clinical and preclinical studies also demonstrate an interaction between physical activity (via wheel running in rodents) and alcohol drinking behaviors. Specifically, aerobic exercise in humans and wheel running activity in animals increase drinking behaviors when access to both are not concurrent (Werme et al., 2002, Ozburn et al., 2008, French et al., 2009, Lisha et al., 2011, Lisha et al., 2013, Leasure et al., 2015), and lead to reward substitution when both are available concurrently (McMillan et al., 1995). However, it is not clear, at least in animal models of relapse, whether wheel running prevents ethanol seeking triggered by ethanol context and contextual cues after extinguishing ethanol responses in a novel context, and whether the behavioral outcomes correlate with running induced neuroadaptations (Deehan et al., 2011, Li et al., 2015).

The current study tested the hypothesis that wheel running during abstinence prevents ethanol seeking in CIE-ED and ED animals that demonstrate enhanced propensity for relapse. The study also tested the subhypothesis that the reduced ethanol seeking with wheel running is associated with running-induced decreases in oligdendroglia, myelinating glia and PECAM-1 and increases in neuronal activation in the mPFC.

2.0 Methods

2.1 Animals

Seventy adult male Wistar rats (Charles River) completed the study. All rats were 8 weeks old at the beginning of the study, and weighed approximately 220–250 g. The rats were maintained in reverse 12h light-12h dark cycle rooms and housed two/cage unless otherwise specified. Food and water were available *ad libitum*. All experimental procedures were carried out 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 were approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute.

2.2 Ethanol Self-Administration

The behavioral experiments conducted herein are presented as a detailed schematic in Figure 1a. Seventy adult male Wistar experimentally-naïve rats were given two 14-hour lever-responding training sessions in the operant conditioning boxes (Med Associates Inc, VT), on an fixed-ratio 1 schedule (FR1; one response resulted in one reinforce delivery), where one press on the available lever resulted in the delivery 0.1ml of water to a sipper cup mounted on the wall in between the two levers. The operant conditioning boxes were housed inside sound attenuating chambers. During these sessions, the house-light and white noise were turned off (Context A). Then, rats were trained to respond for 0.1ml of alcohol (10% v/v) over four daily 2-h FR1 sessions; all other conditions remained the same as before. Subsequently, the rats were trained to discriminate between two available levers to obtain on 0.1 ml ethanol (10% v/v) during daily 30-min FR1 sessions. During these sessions, active (right) lever responding resulted in the delivery of ethanol, while responding on the inactive (left) lever was recorded but had no programmed consequence. Each ethanol delivery followed by a 4-sec time-out during which responding on the active lever did not result in the delivery of ethanol. During this time-out period, the cue-light above the active lever remained on; thus the cue-light was paired with the delivery of ethanol. These 30-min discrimination training sessions continued till stable responding was obtained, where stable responding was defined as less than 10% variation in active lever responding for 3 consecutive 30-min FR1 sessions.

Subsequently, the rats were divided into two groups; one group received chronic intermittent ethanol vapor exposure (CIE; see procedure below) while the other group was exposed to air in their normal housing condition (did not experience ethanol vapors) for a duration of 7 weeks. Henceforth, these rats will be called CIE-ED (n=21) and ED (n=20) rats, respectively. All rats received two 30-min FR1 sessions per week (Tuesdays and Thursdays) during these 7 weeks. Responding was analyzed to determine escalation of self-administration compared to pre-vapor stable responding.

After 7 weeks of CIE, CIE-ED rats were withdrawn from ethanol vapors and both CIE-ED and ED rats were withdrawn from ethanol self-administration. Both CIE-ED and ED rats were divided into two groups and maintained as described for the remainder of the study. One group was maintained under standard housing conditions (referred as the sedentary

cohort, or as CIE-ED and ED, respectively). The second group of rats were housed in special cages equipped with running (see wheel running below) and referred to as CIE-ED-WR and ED-WR, respectively.

2.3 Chronic Intermittent Ethanol vapor exposure (CIE)

During CIE, rat cages were housed in specialized chambers and were exposed to alcohol vapors on a 14-h ON / 10-h OFF schedule. Alcohol (95% ethanol) from a large reservoir was delivered to a heated flask at a regulated flow rate using a peristaltic pump (model QG-6, FMI Laboratory, Fluid Metering). The drops of alcohol in the flask were immediately vaporized and carried to the vapor chambers containing the rat cages by controlled air flow (regulated by a pressure gauge). The air pressure and ethanol flow rates were optimized to obtain blood alcohol levels (BALs) between 125 and 250 mg/dl or 27.2 and 54.4 mM (Gilpin et al., 2008a); these BALs are 2–3 times the BAL observed in binge drinking, but not high enough to abolish righting reflex (Ernst et al., 1976, Courtney and Polich, 2009).

2.4 Tail bleeding for determination of BAL

For measuring BALs, tail bleeding was performed on the CIE-ED and ED rats, once a week (every Friday), between hours 13–14 of vapor exposure (Gilpin et al., 2008b). Rats were gently restrained while the tip of the tail was pricked with a clean needle. Tail blood (0.2 ml) was collected and centrifuged at 2000 rpm for 10 min. Plasma (5 μL) was used for measurement of blood alcohol levels (BALs) using an Analox AM1 analyzer (Analox Instruments USA Inc., MA). Single-point calibrations were performed for each set of samples with reagents provided by Analox Instruments (100 mg/dl). When plasma samples were outside the target range (125–250 mg/dl), vapor levels were adjusted accordingly.

2.5 Wheel running

Wheel running experiment was conducted according to our previously published method (Engelmann et al., 2014). Briefly, CIE-ED-WR and ED-WR rats were single housed in cages equipped with running wheels (Nalgene activity wheels, 34.5-cm diameter 9 9.7-cm wide). Age-matched ethanol naïve rats were also housed in identical cages with running wheels for the same duration and served as wheel running controls (WR; n=12). Each of the running wheels were equipped with magnetic switches that were connected to a computer console. Running activity was monitored for the entire duration as number of wheel revolutions collected in 10 min bins with VitalView Software (Minimitter Inc.). Additional set of ethanol naïve control rats were maintained under standard housing conditions for the same duration (ethanol naïve sedentary, n=12) alongside the sedentary CIE-ED and ED rats. These rats did not have access to locked running wheels in their home cages as rodents climb in locked running wheels, a form of physical activity (environmental enrichment) and a potential confound for a sedentary group. The rats were maintained under these housing conditions till they were euthanized.

2.6 Drinking during abstinence (DDA)

After 23 days of abstinence from CIE and ethanol self-administration, CIE-ED and ED rats, both with and without running wheel access, were given one 30 min FR1 session to lever

press for ethanol reinforcement (0.1 ml of 10% v/v ethanol) under cue-context conditions identical to that used for training and maintenance. Active and inactive lever responses were recorded.

2.7 Extinction

Following DDA, rats were subject to 6 daily 30-min extinction sessions under a different cue-context combination than that used for training and maintenance (Context B). Specifically, operant boxes different from those used for self-administration were used and the house-light and white noise were turned on, and no cue-lights were available following lever presses. Finally lever response did not result in the delivery of ethanol. Both lever responses were recorded.

2.8 Reinstatement

Following the 6th day of extinction, rats were subject to one session of cued-context reinstatement of ethanol seeking. Specifically, rats were introduced to operant chambers under conditions identical to training and maintenance (no house-light, no white noise; Context A). Active lever responses resulted in the presentation of the cue-light for 4 sec, but did not result in the delivery of ethanol. Both active and inactive lever responses were recorded.

2.9 5'- Bromo 2-deoxyuridine (BrdU) Labeling

Three days following removal from ethanol vapor, all rats, irrespective of treatment group, received an injection of BrdU (150 mg/kg, i.p.). The 3 day time-point was selected based on a previous publication that reported a burst in proliferation in the hippocampal SGZ using a comparable CIE model (Hansson et al., 2010). The single BrdU dose (150 mg/kg) was chosen to maximize labeling of proliferating cells by using a near-saturating concentration of BrdU (saturating concentration in rodents is ~200 mg/kg). Of note, while BrdU is used for experimentally labeling proliferating cells (Dayer et al., 2003, Mandyam et al., 2007a, Taupin, 2007), injections of BrdU are also indicated to have cytotoxic and teratologic effects (Kolb et al., 1999, Sekerkova et al., 2004, Ogawa et al., 2005, Kuwagata et al., 2007, Duque and Rakic, 2011, Rowell and Ragsdale, 2012), primarily because BrdU is a marker of DNA synthesis and not of cell division *per se* (Breunig et al., 2007). However, in adult rodent models BrdU cytotoxicity is typically evident at greater than 2 times the currently used dose ((Cameron and McKay, 2001, Eadie et al., 2005); for review, (Taupin, 2007)), suggesting that the BrdU dose used in the current study could be suitable to evaluate proliferation and survival of progenitors in adult rats.

Previous studies had revealed that 3-days after cessation of CIE and ethanol self-administration, cell proliferation is increased in the mPFC for CIE-ED rats compared to ED and age-matched ethanol and behaviorally naïve rats (Somkuwar et al., 2015). However, the effect of 3-days of running experience concurrently with cessation of CIE and ethanol self-administration on cell proliferation is not known. To measure cellular proliferation of newly born cells, two hours after the BrdU injection, a subset of CIE-ED-WR (n=4), ED-WR (n=6), and ethanol naïve WR (n=6) and sedentary (n=6) rats were sacrificed, and their mPFC sections were probed for BrdU. The remaining CIE-ED-WR (n=9), ED-WR (n=12)

and ethanol naïve control WR (n=6) and sedentary (n=6) rats were sacrificed 28 days after BrdU injection to measure the survival of newly born cells in the mPFC. All the CIE-ED (n=8) and ED (n=8) rats that did not have running wheel access during abstinence were also sacrificed 28 days after the BrdU injection. On the 28th day following BrdU administration, the responding for ethanol paired cue and context was evaluated using the 30-min self-administration reinstatement session. The rats were sacrificed 60–90 minutes after this session for measuring neuronal activation (cFos) and progenitor survival (BrdU).

2.10 Brain tissue collection

Rats were killed by rapid decapitation and the brains were isolated, and dissected along the midsagittal plane. The left hemisphere was snap frozen for Western blotting analysis (see below) and the right hemisphere was postfixed in 4% paraformaldehyde for immunohistochemistry. For tissue fixation, the hemispheres were incubated at room temperature for 36 hours and subsequently at 4 °C for 48 hours with fresh paraformaldehyde replacing the old solution every 12 hours. Finally, the hemispheres were transferred to sucrose solution (30% sucrose with 0.1% sodium azide) for cryoprotection and storage till tissue sectioning was conducted (Cohen et al., 2015). Subsequently, the tissue was sliced in 40μm sections along the coronal plane on a freezing microtome. Every ninth section through the PFC (+3.7 to +2.5 mm from bregma; 4 sections per rat) was mounted on Superfrost® Plus slides and dried overnight and used for BrdU analysis. Two sections through the PFC (+3.2 and +2.7 mm from bregma) were mounted as described before and processed for cFos analysis.

2.11 Quantitative Immunohistochemical Analysis for BrdU labelled cells

Quantitative immunohistochemical assay performed using a previously published optical fractionator method (Kim et al., 2015) using mouse monoclonal anti-BrdU (1:400; catalog # MCA2060, Serotec); the sections were counter-stained with Vector Fast Red (a nuclear stain). BrdU labelled cells were quantified in the mPFC with a Zeiss AxioImager Microscope equipped with Stereo Investigator 11.06 (MicroBrightField Bioscience, Williston, VT USA), a three-axis Mac 5000 motorized stage, a Zeiss digital MRc video camera, PCI color frame grabber, and computer work station. mPFC regions were contoured by referencing histological landmarks including corpus collosum, anterior commissure and rhinal fissure, using a 2.5x objective with a 10x eye piece and the above software (Paxinos and Watson, 1997). Cells were visually quantified within the contour using a 40x objective and a 10x eyepiece by an observer blind to the study using the following criteria - cells stained as dark brown to black, with the ability to focus the boundary of the cell within the mounted section thickness. A software generated 180 × 120 μm counting frame was systematically moved through the entire contoured area of the tissue to manually assess and count the BrdU-positive (BrdU+) cells. Mounted section thickness after immunohistochemistry was determined to be ~28 μm. The two-hour-old BrdU+ cells always appeared in clusters of irregularly shaped dark stained cells. The overlapping-pair arrangement and the number of cells in each cluster were confirmed by focusing on different layers of cells along the Z-axis. Twenty-eight-day old BrdU+ cells were predominantly distributed as single cells or pairs of oval to round, spotty or dark stained cells, rarely seen in clusters, and exhibited cell morphology different from the proliferating time point. Absolute

cell counting (complete counting of all immunoreactive cells in the contoured area) was performed in the mPFC; the data are presented as total number of cells per unit area (cells/mm^2 based on mounted section thickness) per animal.

2.12 Phenotypic Analysis of BrdU labelled cells

To determine the phenotype of twenty-eight day old BrdU+ cells, Superfrost slides with mPFC sections were immunoprobed for BrdU (sheep primary 1:100; CY3 donkey anti-sheep secondary 1:200), oligodendroglial marker Olig2 (rabbit primary: 1:100, FITC goat anti-rabbit secondary: 1:200 (Mandyam et al., 2007b, Kim et al., 2014)) and myelin marker myelin basic protein (MBP; mouse primary 1:250; CY5 donkey anti-mouse secondary 1:500). Confocal analysis of triple labeled cells was performed with a Zeiss Axiovert 100 M and LSM510 using a previously published method (Kim and Mandyam, 2014, Kim et al., 2014). Briefly, using a 60× oil immersion objective (equipped with a 10× eyepiece), immunoreactive cells were optically sectioned in the *z*-plane using multitrack scanning (section thickness of 0.45 μm). Colocalization of antibodies was assessed with the confocal system by analysis of adjacent *z*-sections (using gallery function and orthogonal function for equal penetration of the antibodies). The phenotypic distribution of the surviving BrdU+ cells was calculated as the percent of mPFC BrdU+ cells colabeled with Olig2 (BrdU +Olig2+) or Olig2 and MBP (BrdU+Olig2+MBP+) in each rat. A minimum of 15 BrdU+ cells were analyzed per animal from each of the rats.

2.13 Quantitative analysis of cFos labeled cells

The following primary antibody was used for cFos immunohistochemistry (IHC): (1:1000, catalog # sc-52, Santa Cruz Biotechnology (Recinto et al., 2012)). The sections were pretreated (Mandyam et al., 2004), blocked, and incubated with the primary antibody followed by biotin-tagged secondary antibody. Fos immunoreactive cells were examined and quantified with a Zeiss AxioImager Microscope as described previously. Live video images were used to draw contours delineating the subregions of the PFC (anterior cingulate, prelimbic and infralimbic cortices). The fields of the brain regions for quantification were traced separately at 25x magnification. A 150 × 150 μm frame was placed over the regions of interest using the StereoInvestigator stereology platform followed by analysis using the optical fractionator method. The frame was systematically moved over the tissue to cover the entire contoured area and the labeled cells in each subregion falling entirely within the borders of the contour were marked and analyzed. Immunoreactive cells were quantified (absolute cell counting in the area contoured for analysis) and were summed up for each PFC subregion. Data were then summed up for the entire PFC and are presented in Figure 2.

2.14 Western blotting

Western blot procedures optimized for measuring levels of both phosphoproteins and total proteins were employed (Graham et al., 2007, Kim et al., 2014, Galinato et al., 2015). Tissue punches from 500-um thick sections of mPFC were homogenized by sonication in ice-cold 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, St. Louis, MO), and protein concentration was determined using a detergent-compatible Lowry method (Bio-Rad, Hercules, CA). 20 μg protein samples subjected to gel electrophoresis and

transferred to PVDF membranes. The membranes were incubated with the appropriate primary/secondary antibody for detecting several oligovasculature markers (Supplementary content, Table S1). 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 ImageJ software. Blots were reprobed for β-Tubulin (1:8000, SCBT sc-53140) for normalization purposes. Protein expression is presented as percent of ethanol naïve sedentary control samples on the same blot to normalize for blot-to-blot variability.

2.15 Statistical analysis

Each parametric measure was compared between groups using appropriate statistical analyses detailed in supplementary content (Table S2). In rats without running wheel access, number of BrdU and cFos cells was correlated with the density of PECAM-1 using Pearson's product-moment correlation coefficient. Statistical significance was accepted at $p < 0.05$. Two-way ANOVA was conducted using GraphPad Prizm software, three-way ANOVA was conducted using SPSS version 20. Post-hoc tests were conducted to determine group differences and are indicated in Table S2.

3.0 Results

3.1 Escalation of Ethanol Drinking and Blood Alcohol Levels during CIE

Active lever responding for ethanol (10 % v/v; pre-vapor; $t_{(39)}=1.75$; p=0.090; Figure 1b), as well as inactive lever responding ($t_{(39)}=0.508$; p=0.61; Figure S1a) did not differ between the rats separated into the CIE-ED and ED groups prior to the onset of CIE. The amount of ethanol vapor experienced by each CIE-ED rat reached greater than 150 mg/dl of plasma; the BALs were significantly greater in CIE-ED compared to ED rats from week 1–7 of CIE ($F_{weekXtreatment}[6,49.2]=92.9$, $p<0.0001$; Figure S1b). Active lever responses for ethanol self-administration significantly increased from week 3–7 of CIE treatment in CIE-ED rats, but not in the ED rats, compared to the respective pre-vapor responding and ED rats ($F_{weekXtreatment}[6,234]=14.1$, $p<0.0001$; $F_{treatment}[1,39]=27.5$, $p<0.0001$; $F_{week}[6,234]=17.9$, $p<0.0001$; Figure 1b). After week 7, CIE-ED and ED rats were separated into CIE-ED and CIE-ED-WR, and ED and ED-WR groups and their active lever responding did not differ between the groups (last 3 days of self-administration; CIE-ED: $t_{(15)}=0.235$; p=0.82; ED: $t_{(19)}=1.91$; p=0.071; Figure 1c). Ethanol experience did not alter body weight (Figure S4).

3.2 Wheel Running during Abstinence

Running activity, measured as number of revolutions per day, did not differ between ED-WR, CIE-ED-WR and WR controls ($F_{dayXtreatment}[58,696]=0.818$, p=0.96; $F_{treatment}[2,24]=0.0237$, p=0.92; Figure S2).

3.3 Drinking during Abstinence

Following four weeks of abstinence, CIE-ED demonstrated higher drinking (higher active lever responses) compared to ED when rats were given access to ethanol, however, drinking was not different between CIE-ED-WR and ED-WR ($F_{sessionXtreatmentXwheel}[1,34]=0.267$,

p=0.61; $F_{sessionXtreatment}[1,34]=4.65$, p=0.038; $F_{sessionXwheel}[1,34]=4.24$, p=0.047; $F_{session}[1,34]=2.00$, p=0.17; Figure 1c). Importantly, drinking during abstinence was enhanced in ED-WR compared to ED (p=0.016), and ED-WR compared to responding prior to abstinence (p=0.0054). Inactive lever responses did not differ between CIE-ED and ED rats, and CIE-ED-WR and ED-WR rats (Figure S1d).

3.4 Extinction and Contextual Cued Reinstatement of Ethanol Seeking

Following drinking during abstinence, the rats were subjected to six sessions of extinction in a novel context (context B, different from self-administration context A). ED-WR, CIE-ED and CIE-ED-WR rats demonstrated enhanced seeking during extinction compared to ED rats ($F_{sessionXtreatmentXwheel}[5,160]=3.28$, p=0.046; $F_{sessionXwheel}[5,160]=1.75$, p=0.18; $F_{sessionXtreatment}[5,160]=2.15$, p=0.13; $F_{session}[5,160]=25.8$, p<0.0001; Figure 1e). CIE-ED and CIE-ED-WR rats extinguished operant responses without significant differences in latency to extinguish. In contrast, ED-WR rats demonstrated enhanced latency to extinguish lever responses compared to ED, CIE-ED and CIE-ED-WR rats (ps<0.05). Lever responses on the previously paired inactive lever (in context A) did not differ between groups ($F_{sessionXtreatmentXwheel}[5,165]=2.00$, p=0.12; $F_{sessionXwheel}[5,165]=0.445$, p=0.74; $F_{sessionXtreatment}[5,165]=0.544$, p=0.67; $F_{session}[5,165]=1.52$, p=0.21; Figure S1d). All rats extinguished prior to reinstatement testing.

Following extinction sessions, the rats were subject to contextual cued reinstatement (in context A where active lever responding with cues is indicative of delivery of alcohol). Reinstatement of ethanol seeking on the previously paired active lever was higher in ED and CIE-ED rats compared to ED-WR and CIE-ED-WR rats ($F_{sessionXtreatmentXwheel}[1,33]=3.16$, p=0.084; $F_{sessionXtreatment}[1,33]=2.28$, p=0.14; $F_{sessionXwheel}[1,33]=25.4$, p<0.0001; $F_{session}[1,33]=97.3$, p<0.0001; Figure 1f). In contrast, responding on the previously paired inactive lever was not altered in any of the groups ($F_{sessionXtreatmentXwheel}[1,33]=1.51$, p=0.24; $F_{sessionXtreatment}[1,33]=0.007$, p=0.93; $F_{sessionXwheel}[1,33]=0.30$, p=0.59; $F_{session}[1,33]=0.007$, p=0.93; Figure S1e).

3.5 Neuronal Activation in the Medial Prefrontal Cortex

Two-way ANOVA demonstrated significant group differences in neuronal activation in mPFC ($F_{treatmentXwheel}[2,43]=13.11$, p<0.0001; $F_{treatment}[2,43]=4.9$, p=0.01; $F_{wheel}[1,43]=44.33$, p<0.0001; Figure 2). Post hoc analysis revealed that the number of cFos cells were reduced in ED rats compared to sedentary controls (p=0.03), and access to running wheels did not alter the number of cFos cells in WR rats compared to sedentary rats. In contrast, ED-WR and CIE-ED-WR rats showed enhanced number of cFos cells following contextual cued reinstatement compared to WR controls, sedentary controls, ED and CIE-ED rats (ps<0.05).

3.6 Cell Survival and Phenotypic Analysis of Progenitors in the Medial Prefrontal Cortex

Newly born progenitors were labeled with BrdU 72h after CIE cessation and 2-h-old (Figure S3) and 28-day-old (Figure 3) BrdU cells were quantified. The number of 28-day-old BrdU+ cells were increased in ED and CIE-ED rats compared to ED-WR, CIE-ED-WR, sedentary and WR controls ($F_{treatmentXwheel}[2,43]=27.5$, p<0.0001; $F_{treatment}[2,43]=48.1$, p<0.0001;

F_{wheel}[1,43]=100, p<0.0001; Figure 3a–g). Post hoc analyses demonstrated higher number of BrdU cells in CIE-ED compared to ED (p=0.028); higher number of BrdU cells in ED compared to ED-WR, and CIE-ED compared to CIE-ED-WR (ps<0.01). Phenotypic analysis of 28-day-old OPCs revealed that a greater proportion of the BrdU+ cells expressed Olig2 (78–89%) compared with expression of Olig2 and MBP (0–15%) or neither of the markers (5–12%; $F_{phenotype}$[2,70]=523, p<0.0001; Figure 3n). Phenotypic distribution of BrdU+ cells did not significantly differ between treatment groups ($F_{phenotypeXtreatmentxwheel}$[4,70]=0.569, p=0.69; $F_{phenotypeXtreatment}$[5, 70]=1.15, 0.34; $F_{phenotypeXwheel}$[5, 70]=2.84, p=0.065).

3.7 Biochemical analysis of Oligodendroglia and myelinating glial Markers in the mPFC

Western blot analyses of proteins expressed during developmental stages of OPCs [NG2, PLP, CNPase, MBP, MOG, Olig2] were conducted on mPFC tissue lysates (Figure 4). Two-way ANOVA was performed for each protein indicated above and the detailed results are presented in Table S3. ED and CIE-ED had enhanced expression of NG2 and PLP in the oligodendroglial lineage compared to sedentary control as well as ED-WR and CIE-ED-WR rats (Figure 4b–c). CIE-ED had enhanced expression of MBP and MOG compared to sedentary control and CIE-ED-WR rats (Figure 4d–e). Overall, CIE-ED rats demonstrated an increase in expression of immature oligodendroglial and myelinating glial markers, whereas, ED rats demonstrated an increase in immature oligodendroglial markers (Figure 4f–g).

3.8 Expression of PECAM-1 in the mPFC

We used immunohistochemistry to determine qualitative changes in PECAM-1 levels in the mPFC, and staining demonstrated an increase in PECAM-1 expression in the entire mPFC in ED and CIE-ED rats compared to ethanol naïve controls, and WR groups (Figure 5a). Western blotting was performed to determine quantitative differences in PECAM-1.Two-way ANOVA of PECAM-1 demonstrated increased expression in ED and CIE-ED rats ($F_{treatment}$[2,47]=2.61, p=0.03; F_{wheel}[1,47]=6.0, p=0.01; Figure 5b–c), and post hoc analysis revealed that PECAM-1 expression was greater in CIE-ED and ED compared to sedentary controls, CIE-ED-WR and ED-WR rats (ps<0.05).

Linear regression analyses were also conducted between expression of PECAM-1, cFos, BrdU and all the oligodendroglial proteins in ED, CIE-ED, ED-WR and CIE-ED-WR. PECAM-1 expression in CIE-ED rats negatively correlated with cFos cells (r= −0.88, p=0.003) and positively correlated with the number of BrdU cells (r=0.73, p=0.03) (Figure 5d–e). No other correlations reached statistical significance.

4.0 Discussion

The first goal of the study was to identify differences in ethanol drinking behaviors and ethanol seeking behaviors during abstinence from chronic ethanol experience in ED and CIE-ED rats. The second goal was to determine whether wheel running during abstinence increases drinking behavior in ED and CIE-ED rats when ethanol was made available, and whether wheel running preserved extinction learning (a form of behavioral inhibition)

performed during abstinence to reduce reinstatement of ethanol seeking behaviors. Here, we report that access to ethanol after a period of abstinence increases drinking in CIE-ED rats compared with ED rats, and this difference in drinking behavior is abolished (via increased drinking in ED-WR and maintained higher drinking in CIE-ED-WR) in animals that had access to running wheels during abstinence. We also report that ED rats reinstate ethanol seeking to a similar degree compared with CIE-ED rats, despite the fact that ED rats demonstrated regulated ethanol intake and did not display elevated drinking when ethanol was made available during abstinence. Notably, wheel running during abstinence reduced reinstatement of ethanol seeking in ED-WR and CIE-ED-WR rats suggesting preserved behavioral inhibition. Additional biochemical and immunohistochemical studies were performed on postmortem tissue from all groups to determine neuroadaptations in the mPFC that were associated with ethanol seeking. Markers of oligodendroglia, myelinating glia and neuroinflammation were enhanced during abstinence, and these effects were more robust in CIE-ED rats compared with ED rats. The PECAM-1 responses correlated with reduced neuronal activation and enhanced oligodendroglial levels in the mPFC in CIE-ED rats. Wheel running during abstinence reduced the number of newly born oligodendroglia and levels of PECAM-1 in the mPFC, and these changes were associated with enhanced neuronal activation. Our data demonstrate an interplay between oligodendroglial and neuroinflammatory responses in the mPFC in regulating neuronal activation in a model of alcohol addiction.

The results confirm that CIE experience reliably increases drinking and produces unregulated and escalated ethanol intake in an operant paradigm compared to animals that experience drinking without CIE (O'Dell et al., 2004). Several preclinical studies show an interaction between wheel running and ethanol drinking, where concurrent access to running wheel and ethanol reduces ethanol drinking, suggesting reward substitution (McMillan et al., 1995, Ehringer et al., 2009, Hammer et al., 2010, Brager and Hammer, 2012, Darlington et al., 2014, Gallego et al., 2015). Contrary to expectations, however, introduction of running wheel access during abstinence from ethanol self-administration did not increase or decrease wheel running in CIE-ED-WR and ED-WR animals, suggesting a lack of reward substitution/withdrawal-induced hypolocomotion in wheel activity (Ozburn et al., 2008, Logan et al., 2012). It is also possible that a ceiling effect rendered further increase in wheel activity unachievable in this age group of rats studied, especially given that age is a factor regulating exercise output (Staples et al., 2015). Reintroduction of ethanol access after abstinence produced higher drinking in CIE-ED rats compared to ED rats, and wheel running during abstinence abolished the difference between the groups by enhancing drinking in ED-WR rats. The increased drinking during abstinence cannot be attributed to the nonspecific arousal effects of ethanol context and cues because responding at the inactive lever remained indistinguishable from those during maintenance of self-administration. More importantly, these findings support an ethanol deprivation effect in ED-WR rats (Werme et al., 2002, Ozburn et al., 2008), which was not observed in CIE-ED-WR rats, suggesting a ceiling effect in CIE-ED-WR rats. This finding is interesting because deprivation effect models certain human aspects of alcoholism, and is observed after several episodes of abstinence post heavy drinking (Heyser et al., 1997, Holly and Wittchen, 1998, Spanagel and Holter, 1999, Werme et al., 2002, Ozburn et al., 2008). Our findings show that

that wheel running during abstinence from moderate drinking potentiates deprivation effects, and these behavioral outcomes may enhance the propensity to consume excessive alcohol when alcohol is made available.

Although prior studies have investigated the interactions between running wheel and ethanol in the context of reward substitution and deprivation, no studies have examined the interaction of running wheel with ethanol seeking behaviors. Our findings reveal that CIE-ED animals exhibited greater resistance to extinction compared to ED animals. Wheel running during abstinence eliminated the difference by increasing seeking in ED-WR rats during extinction, suggesting that deprivation effects stimulated by wheel running continued into extinction sessions. However, all animals extinguished self-administration behavior by the last extinction session. Notably, CIE-ED rats and ED rats demonstrated similar ethanol-seeking behaviors triggered by ethanol contextual cues and wheel running prevented ethanol seeking in both groups to a similar extent. These findings support a protective effect of wheel running on propensity for contextual cued reinstatement of ethanol seeking. However, it is important to note that access to a running wheel is a form of environmental enrichment, and the change in seeking behaviors may be due to an enrichment-related effect. Thus, wheel running selectively facilitated inhibition of ethanol seeking, possibly by promoting ethanol-specific extinction learning mechanisms. Given that extinction training is being adopted in clinical behavioral therapy to promote recovery from relapse (Kiefer and Dinter, 2013), our findings suggest that physical exercise can enhance the beneficial effects of extinction training in alcoholic subjects, irrespective of previous intensity of alcohol drinking pattern.

With respect to wheel running-induced neuroadaptations in ethanol-experienced subjects, concurrent or delayed access to wheel running inhibits ethanol-induced reduction in hippocampal neurogenesis, and expression of hippocampal trophic factors, suggesting a neuroprotective effect (Crews et al., 2004, Maynard and Leasure, 2013, Gallego et al., 2015). This protection could assist with reduction in certain somatic signs of ethanol withdrawal in animals that experienced wheel running during or after ethanol exposure (Leasure and Nixon, 2010, Devaud et al., 2012, McCulley et al., 2012). However, other than the current study, no studies have examined the neuroadaptations by wheel running in the mPFC in ethanol-experienced subjects during protracted abstinence. Such studies could support the role of mPFC in inhibitory control of motivational impulses in reducing ethanol-seeking behaviors (Phan et al., 2005, Diekhof and Gruber, 2010, Chen et al., 2013, Mihindou et al., 2013). For example, studies employing the extinction-reinstatement model have demonstrated an increase in neuronal activation (via cFos labeling) in the mPFC in response to ethanol seeking triggered by ethanol itself and not footshock stress (Wedzony et al., 2003, Zhao et al., 2006, Dayas et al., 2007), suggesting that mPFC plays a role in incentive motivation in response to ethanol and does not regulate behavioral responses to stress. Notably, blood oxygen level-dependent (BOLD) functional magnetic resonance imaging (fMRI) studies in abstinent human subjects revealed that alcohol cues triggered enhanced brain activity in the PFC, suggesting that these responses could be interpreted as neuronal correlates of craving or incentive motivation (George et al., 2001, Karch et al., 2015, Kose et al., 2015). Our findings demonstrate that neuronal activity was reduced in the mPFC in ED and strong trend in CIE-ED rats that demonstrated enhanced ethanol seeking triggered by

ethanol contextual cues. These results are distinct from the findings reported in ED and CIE-ED rats when ethanol seeking was triggered by ethanol priming, suggesting recruitment of brain regions other than the mPFC in response to ethanol itself (Zhao et al., 2006, Dayas et al., 2007). It is also likely that reduced cFos expression in the mPFC in ED and CIE-ED rats is correlated with reduced behavioral inhibition in these animals as evidenced by enhanced ethanol seeking triggered by contextual cues (Rhodes and Killcross, 2004, Rhodes and Killcross, 2007, Chen et al., 2013, Mihindou et al., 2013). Wheel running enhanced cFos expression in the mPFC and reduced ethanol seeking triggered by contextual cues, suggesting that wheel running could be reversing ethanol-induced hypoactivity in the mPFC as seen with other drugs of abuse (Chen et al., 2013, Zlebnik et al., 2014). Supporting a previous finding, wheel running did not alter cFos expression in running controls in the mPFC, suggesting that cFos response to running experience *per se* is different in areas of the mPFC compared to other brain regions associated with reward and reinstatement (Zlebnik et al., 2014). Thus, wheel running might modulate inhibitory associations formed during extinction by contextual cues and promote ethanol-specific extinction learning mechanisms by activating distinct neuronal ensembles (Pfarr et al., 2015). These findings support the role of the mPFC in recovery and reinstatement of an extinguished conditioned response, and also implicate the mPFC in some aspects of the recall of extinction learning.

Other forms of plasticity in the mPFC may support the reduced neuronal activity (cFos) in response to ethanol seeking in ED and CIE-ED rats. For example, synaptic plasticity and neural connectivity in the brain are associated with cellular processes such as myelination of axons, elimination of and pruning of synapses and neuroimmune responses (Pascual et al., 2014, Jacobsen et al., 2016). Recent work in animals that experienced binge ethanol exposure demonstrate that ethanol-induced cytokine responses disrupt myelin associated proteins (Pascual et al., 2014), however, it is unclear how excessive cytokine production may alter myelin integrity. Work from research conducted in tissue inflammation and injury models demonstrates a relationship between cytokines and endothelial cell adhesion molecules, where PECAM-1 supports leukocyte emigration and enhances the release of proinflammatory factors (Privratsky et al., 2010). Notably, endothelial cells also regulate OPCs and myelinating glia (Arai and Lo, 2009). We therefore determined the alteration in the expression of PECAM-1, a marker for neuroinflammatory response and blood brain barrier disruption (Kalinowska and Losy, 2006, Woodfin et al., 2007). As predicted, both ED and CIE-ED rats had excessive expression of PECAM-1 in the mPFC. More importantly, higher levels of PECAM-1 predicted lower levels of cFos in CIE-ED rats, demonstrating a negative relationship between the two proteins. Wheel running completely abolished/prevented the increases in PECAM-1 expression, and these changes were associated with enhanced neuronal activity in the mPFC. This is an important finding because PECAM-1 is enhanced at the site of endothelial injury and is a marker for ischemic response and neuroinflammation (Rosenblum et al., 1994, Hwang et al., 2005, Kalinowska and Losy, 2006), effects that could be occurring in ethanol experienced subjects (Whitman et al., 2013, Harper et al., 2015). Therefore, it appears that wheel running provided neuroprotection by inhibiting expression of factors implicated in brain trauma.

It is demonstrated that PECAM-1 in the adult brain and periphery supports the proliferation and differentiation of cells and maintains an anti-apoptotic environment (Bergom et al.,

2005, Ohab and Carmichael, 2008). Given the exaggerated response in PECAM-1 expression in ED and CIE-ED rats, we examined the number and phenotype of newly born OPCs in the mPFC. We have recently demonstrated that CIE-ED rats have enhanced proliferation of OPCs during early withdrawal and enhanced survival of OPCs during protracted abstinence compared to ED rats, and these changes are associated with altered trophic factors regulating the gliogenic niche (Somkuwar et al., 2015). In the current report, we show that CIE-ED and ED rats exhibit enhanced survival of OPCs, and particularly enhanced myelinating OPCs and these changes were associated with enhanced ethanol seeking in both groups. More importantly, in CIE-ED rats, levels of PECAM-1 positively correlated with survival of newly generated OPCs (BrdU), indicating a strong relationship between neuroinflammatory response and oligodendroglial response during abstinence post chronic ethanol experience. These findings support previous observations of enhanced oligodendrogenesis in response to brain trauma (as seen in models of stroke and ischemia (Pham et al., 2012, Jing et al., 2015)), and indicate glial and inflammatory remodeling in response to brain injury generated by chronic ethanol experience (Tanaka et al., 2003, Jiang et al., 2011).

Notably, acute wheel running during withdrawal did not alter the proliferative burst in OPCs, however, continued running during abstinence reduced the survival of these progenitors. For example, running completely blocked the responses in newly born OPCs, and PECAM-1 and supported enhanced neuronal activation in the mPFC. These findings suggest a novel relationship between neuroinflammatory/oligodendroglial effects of wheel running in the mPFC and enhancement in inhibitory control and extinction learning mechanism to assist with reduced ethanol seeking behaviors. In sum, although correlational, these findings support the role of an oligodendroglial and neuroinflammatory response in the mPFC in modulating neuronal activity and reinstatement of ethanol seeking in the context of chronic ethanol intake.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Protracted abstinence enhances ethanol seeking triggered by contexual cues
- Enhanced ethanol seeking is associated with oligodendrogenesis and neuroinflammatory responses in the mPFC
- Wheel running during abstinence prevents ethanol seeking triggered by contexual cues
- Reduced seeking is associated with reduced oligodendrogenesis and neuroinflammatory responses in the mPFC

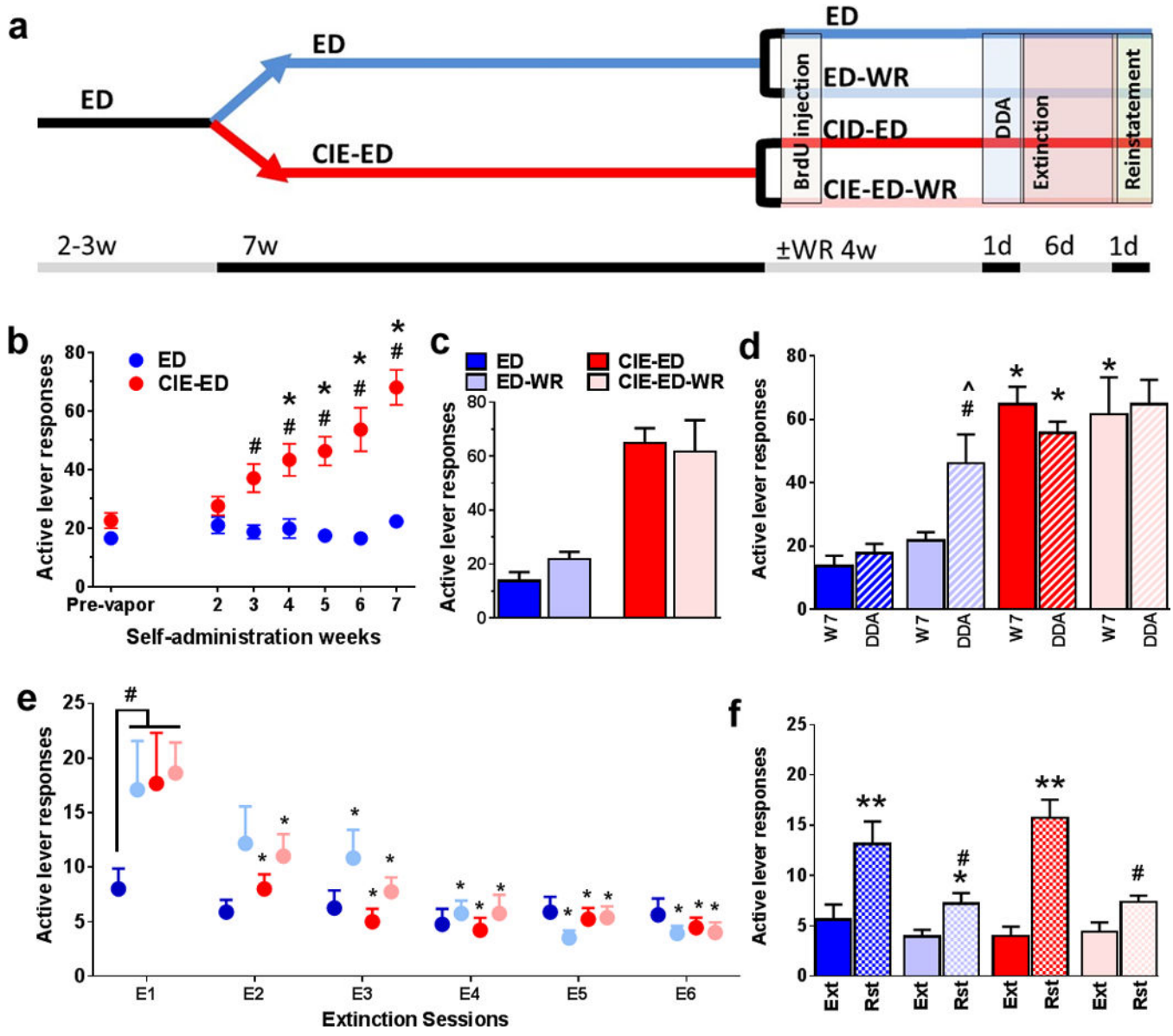

Figure 1.

(a) Schematic representation of behavioral paradigm for the entire study. ED animals are always indicated in shades of blue and ED-CIE animals are indicated in shades of red. Animals were either given access to running wheels during withdrawal and abstinence or no wheels in their home cage for the same duration of time indicated by ±WR (b) Ethanol self-administration indicated as active lever responses. (c) Active lever responses averaged from last three days of self-administration prior to separation into ED and ED-WR and CIE-ED and CIE-ED-WR groups. (d) Active lever responses from ED, ED-WR, CIE-ED, and CIE-ED-WR animals when they experience a single drinking session during abstinence. (e) Lever responses during extinction (in context B) on the previously paired active lever (in context A). (f) Reinstatement (active lever presses) triggered by ethanol contextual cues. Data are represented as mean ± S.E.M.; n=6–12 each group. #p<0.05 vs ED in (b), *p<0.05 vs. week 1 in (b). *p<0.05 compared to the respective ED groups; #p<0.05 compared to ED during

DDA, and ^p<0.05 vs. week 7 responses in ED-WR animals in (d). #p<0.05 indicates significant effect of wheel and CIE; *p<0.05 vs. day 1 extinction within each group in (e). *p<0.05, **p<0.01 vs. extinction and #p<0.05 versus sedentary groups in (f).

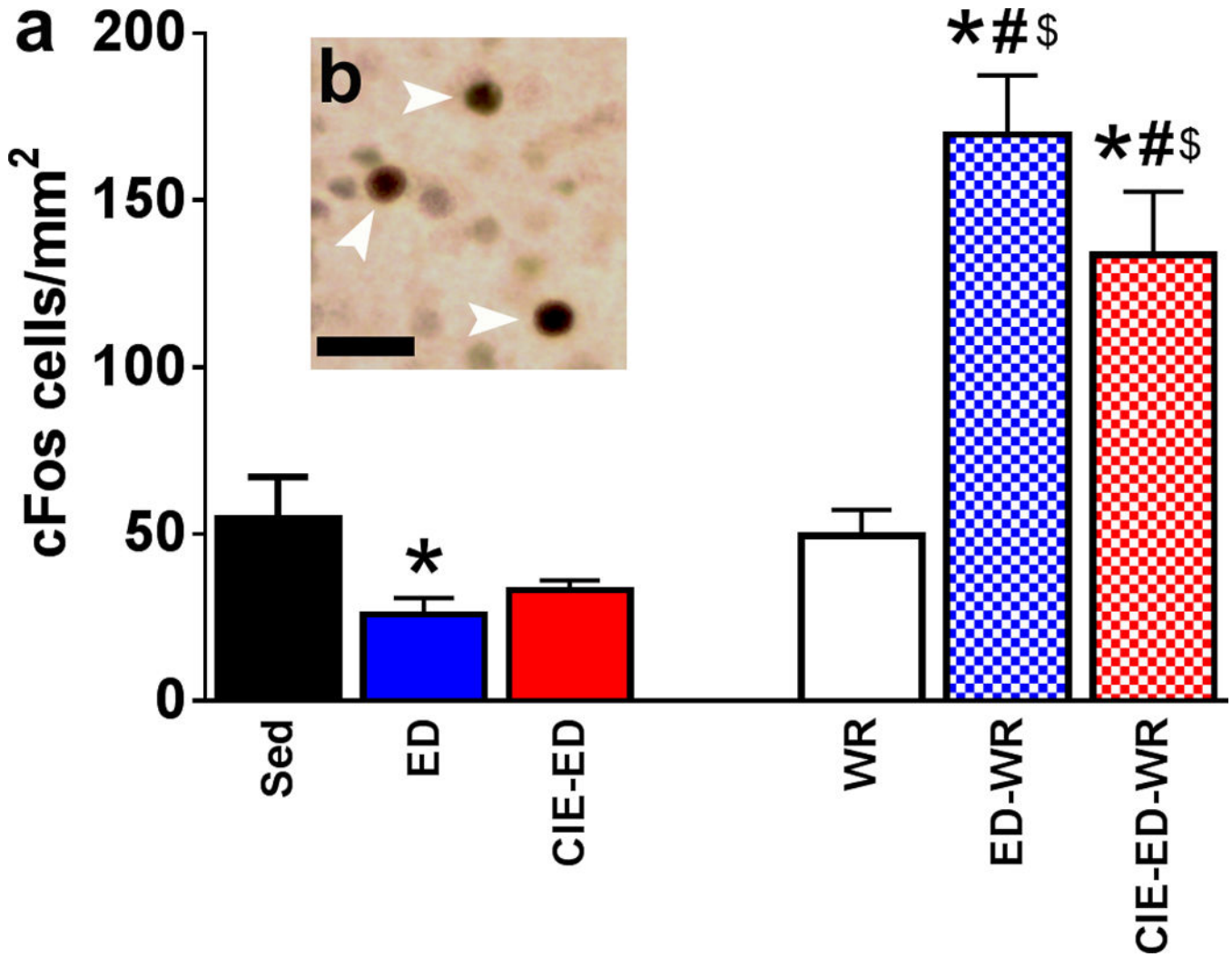

Figure 2.

Quantitative analysis (a) and qualitative representation (b) of cFos in all experimental groups. (a) Total number of cFos immunoreactive cells in the mPFC expressed as cells/mm2. (b) DAB stained cFos cells in the mPFC with arrowhead pointing to immunolabeled cells. Scale bar in (b) is 15 um. *p<0.05 vs. sedentary control; #p<0.05 vs. ED and CIE-ED groups respectively; $p<0.05 vs. WR. Data are represented as mean ± S.E.M.; n=6–12 each group.

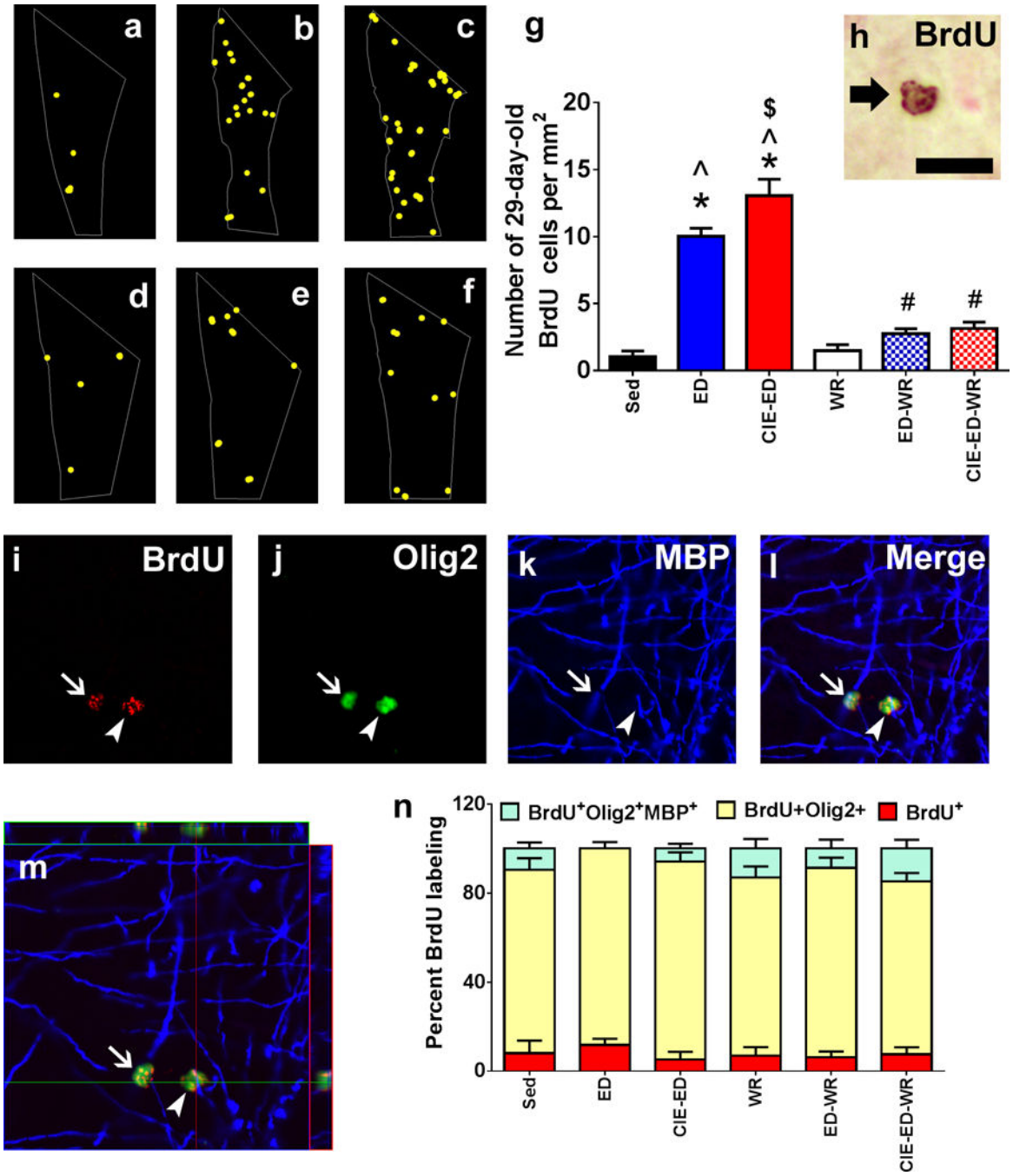

Figure 3.

Qualitative map (a–f) and quantitative analysis of BrdU labeled cells in the mPFC acquired via StereoInvestigator software. Map in (a) shows distribution of 28-day-old BrdU cells in sedentary control (a), ED (b), CIE-ED (c), WR (d), ED-WR (e) and CIE-ED-WR (f) animal. One representative section from a matched bregma was chosen to demonstrate cellular distribution in each group. BrdU cells are indicated as yellow dots. (g) Total number of BrdU cells per mm2 in the mPFC. (h) DAB labeled BrdU cells in the mPFC. BrdU cells always appeared in cluster of 2 or more cells overlapping in a counting frame. Arrow in (h)

points to two BrdU cells visualized as one on top of the other. (i–m) confocal triple labeling analysis of BrdU labeled cells. (i) BrdU, CY3; (j) Olig-2, FITC; (k) MBP, CY5, (l) merge. (m) Indicates an orthogonal section through the z-stack collected for each cell for qualitative analysis of the phenotype of the cells. Image is indicated in the x-z and y-z axis to demonstrate equal penetration of the all the three antibodies and fluorophores used for labeling. Arrow in (i–l) points to a BrdU/Olig2 double labeled cell placed on top of a myelin fiber, whereas arrowhead in (i–l) points to a BrdU/Olig2/MBP triple labeled cell. Scale bar indicated in (h) is 10 um for (h) and 20 um for (i–m). (n) Stacked bar graph representation of triple labeled population in each experimental group. *$p<0.05$ vs. sedentary control; ^$p<0.05$ vs. WR controls, \$$p<0.05$ vs. ED, #$p<0.05$ vs. respective non-wheel groups. Data are represented as mean ± S.E.M.; n=6–12 each group.

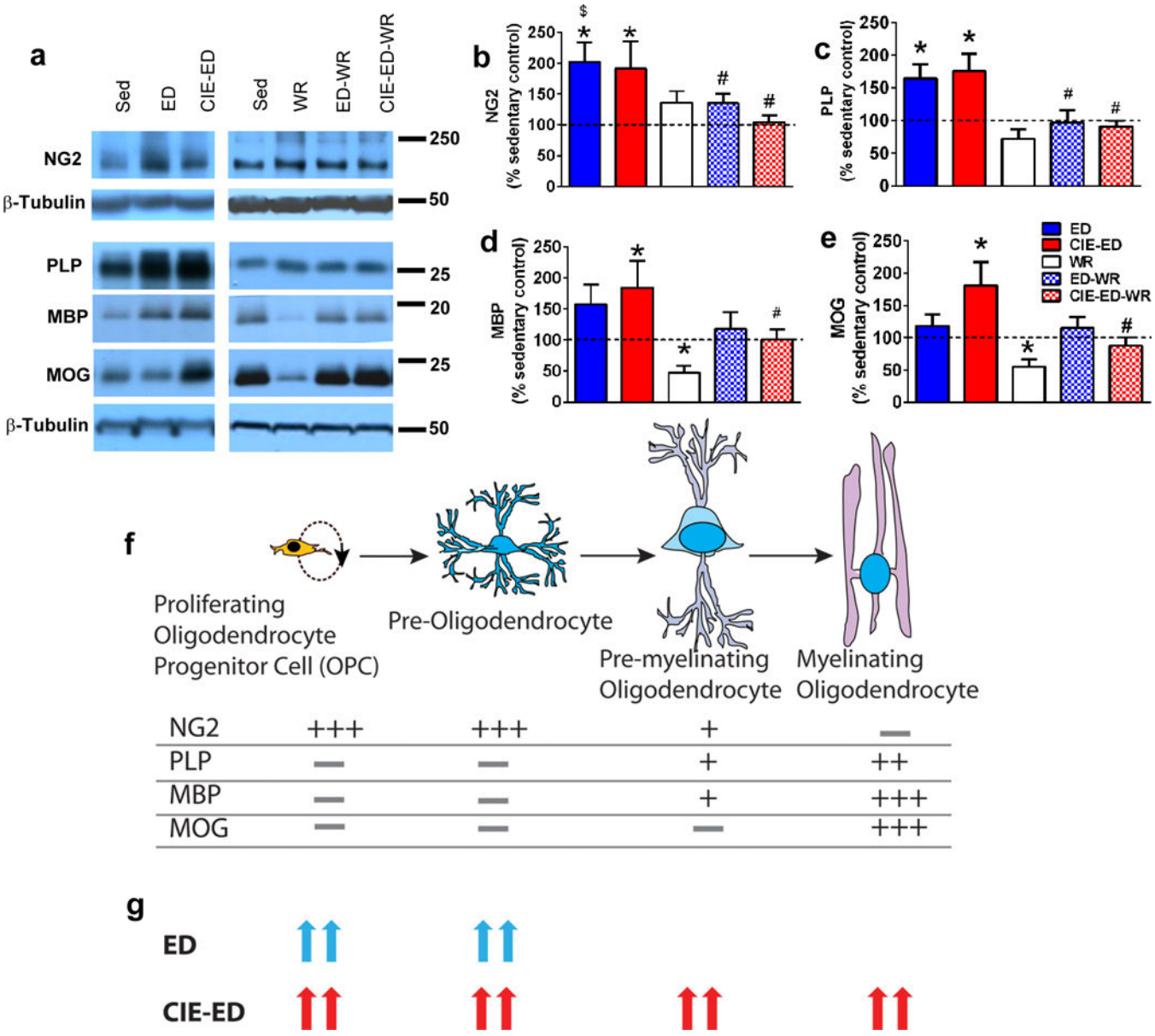

Figure 4.

(a) Representative immunoblot of NG2, PLP, MBP and MOG with corresponding blots for β-tubulin which was used as loading control. (b–e) Densitometric analysis of NG2 (b), PLP (c), MBP (d) and MOG (e) indicated as percent change from sedentary control. (f) Cartoon representation of different developmental stages of OPCs and corresponding levels of expression of proteins investigated in our study. The schematic is based on reviews from (Nishiyama et al., 2009, Barateiro and Fernandes, 2014). (g) Summary of the change in expression levels of OPC proteins tested in ED and CIE-ED animals compared to sedentary controls. Data are represented as mean ± S.E.M.; n=6–12 each group. *p<0.05 vs. sedentary control, $p<0.05 vs. WR, #p<0.05 vs. respective sedentary ethanol control groups.

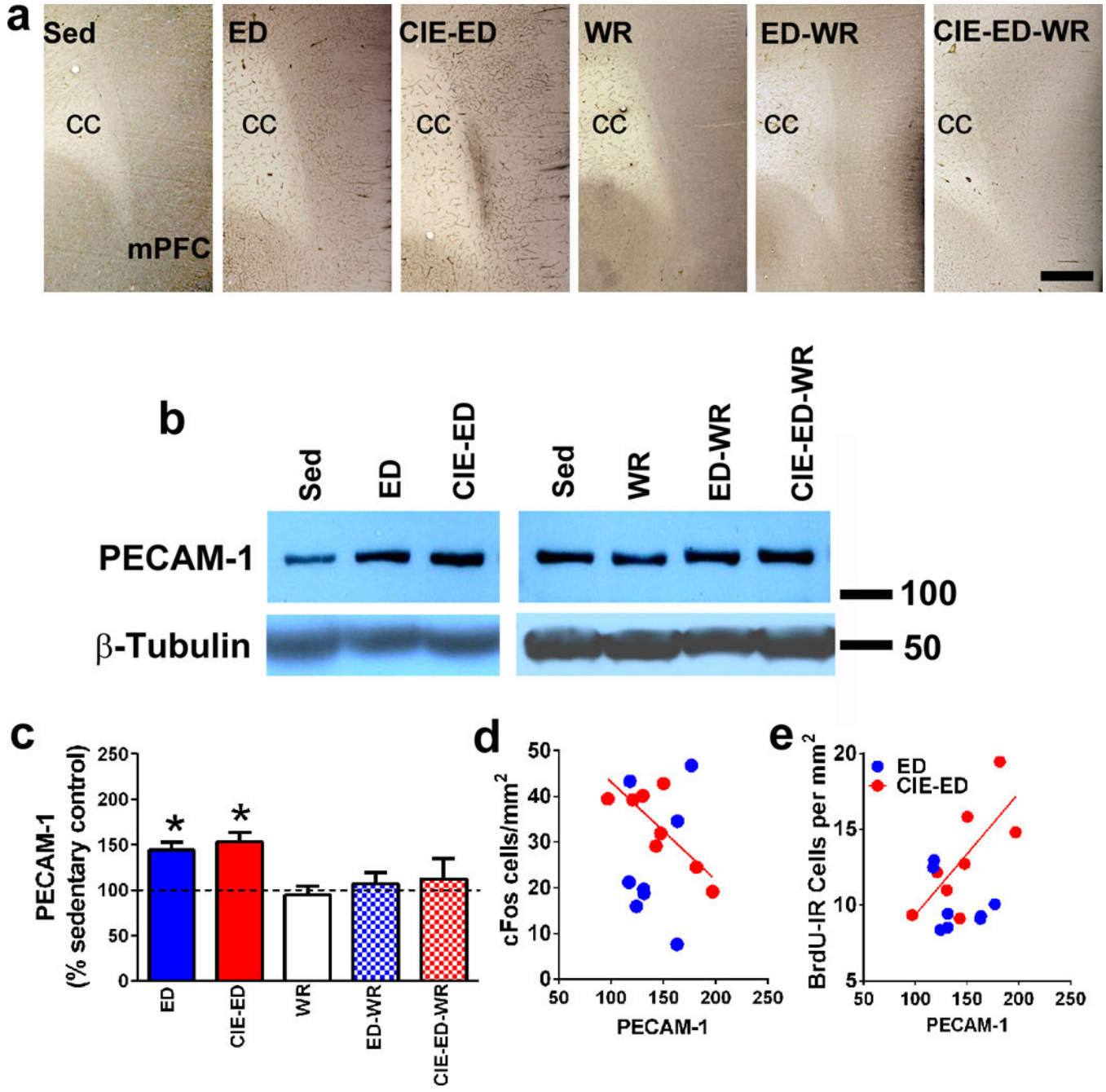

Figure 5.

(a) Qualitative images of the mPFC stained with PECAM-1 from sedentary, ED, CIE-ED, WR, ED-WR and CIE-ED-WR rats. Scale bar in CIE-ED-WR panel is 100um applies to all images in (a); cc is corpus callosum. (b) Representative immunoblot of PECAM-1 with corresponding blot for β-tubulin which was used as loading control. (c) Densitometric analysis of PECAM-1 indicated as percent change from sedentary control. *p<0.05 vs. sedentary control. Data are represented as mean ± S.E.M.; n=6–12 each group. (d–e)

PECAM-1 expression negatively correlates with the number of cFos cells (d) and positively correlates with the number of BrdU cells (e) in the mPFC in CIE-ED rats.