Neuroscience 226 (2012) 475–488

ADOLESCENT BINGE DRINKING INCREASES EXPRESSION OF THE DANGER SIGNAL RECEPTOR AGONIST HMGB1 AND TOLL-LIKE RECEPTORS IN THE ADULT PREFRONTAL CORTEX

R. P. VETRENO * AND F. T. CREWS

The Bowles Center for Alcohol Studies, Department of Psychiatry, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

Abstract—Adolescence is a critical developmental stage of life during which the prefrontal cortex (PFC) matures, and binge drinking and alcohol abuse are common. Recent studies have found that ethanol increases neuroinflammation via upregulated high-mobility group box 1 (HMGB1) signaling through Toll-like receptors (TLRs). HMGB1/TLR 'danger signaling' induces multiple brain innate immune genes that could alter brain function. To determine whether adolescent binge drinking persistently increases innate immune gene expression in the PFC, rats (P25–P55) were exposed to adolescent intermittent ethanol (AIE [5.0 g/kg, 2-day on/2-day off schedule]). On P56, HMGB1/TLR danger signaling was assessed using immunohistochemistry (i.e., +immunoreactivity [+IR]). In a separate group of subjects, spatial and reversal learning on the Barnes maze was assessed in early adulthood (P64–P75), and HMGB1/TLR danger signaling was measured using immunohistochemistry for +IR and RT-PCR for mRNA in adulthood (P80). Immunohistochemical assessment at P56 and 24 days later at P80 revealed increased frontal cortical HMGB1, TLR4, and TLR3 in the AIE-treated rats. Adolescent intermittent ethanol treatment did not alter adult spatial learning on the Barnes maze, but did cause reversal learning deficits and increased perseverative behavior. Barnes maze deficits correlated with the expression of danger signal receptors in the PFC. Taken together, these findings provide evidence that adolescent binge drinking leads to persistent upregulation of innate immune danger signaling in the adult PFC that correlates with adult neurocognitive dysfunction.

Key words: ethanol, reversal learning, neuroimmune, innate immunity, Barnes maze.

INTRODUCTION

Chronic alcohol exposure in adulthood activates microglia and astrocytes (Qin and Crews, 2012), and increases the expression of cytokines, chemokines, oxidases, and other innate immune genes in mouse brain (Qin et al., 2008; Crews et al., 2011; Qin and Crews, 2012). These findings in animal models mimic changes in postmortem human alcoholic brain (He and Crews, 2008; Qin and Crews, 2012). In humans, initiation of alcohol use typically occurs during adolescence. Alcohol consumption during adolescence is highly prevalent as 8% of 8th grade, 16% of 10th grade, and 25% of 12th grade adolescent individuals reported heavy episodic drinking (i.e., < 5 consecutive alcohol drinks per episode) over the past 2 weeks (Johnston et al., 2009). This heavy drinking pattern persists into college as 44% of students report binge drinking every 2 weeks and 19% report > 3 binge drinking episodes per week (Wechsler et al., 1995; O'Malley et al., 1998). Heavy drinking among adolescent males was found to increase impulsivity the following year in those individuals predisposed to adolescent-typical impulsivity (White et al., 2011). Furthermore, early onset of alcohol use (< 13 years of age) was associated with increased drinking frequency and physical violence (Gruber et al., 1996), which is consistent with diminished impulse inhibition. In addition, adolescents and adults with alcohol use disorders evidence deficits in executive functioning (Brown et al., 2000; Tapert and Brown, 2000; Hanson et al., 2011). The frontal cortex continues to develop throughout adolescence and is uniquely sensitive to ethanol neurotoxicity (Crews et al., 2000) and studies in adults find neuroimmune activation contributes to ethanol neurotoxicity. These findings prompted investigation into the impact of models of underage binge drinking on adult prefrontal cortex (PFC) expression of HMGB1/TLR danger signaling molecules, neuroimmune activation, and cognition.

Activation of the innate immune system persists for long periods of time in the adult brain (Qin et al., 2007, 2008). However, the long-term effect of neuroimmune activation in the adolescent brain has not been studied. The high incidence of underage drinking increases the importance of understanding whether there are long-term persistent changes in the brain. We hypothesized that adolescent intermittent ethanol (AIE) exposure would persistently alter adult prefrontal cortical structure and function by elevating neuroinflammatory HMGB1/TLR

*Corresponding author. Address: The Bowles Center for Alcohol Studies, The University of North Carolina at Chapel Hill, Department of Psychiatry, CB #7178, 1021 Thurston-Bowles Building, Chapel Hill, NC 27599, USA.
E-mail address: rvetreno@email.unc.edu (R. P. Vetreno).

Abbreviations: AIE, adolescent intermittent ethanol; BEC, blood ethanol content; FC2, frontal cortex area 2; HMGB1, high-mobility group box 1; IHC, immunohistochemistry; IL, infralimbic cortex; +IR, immunoreactivity; LPS, lipopolysaccharide; OFC, orbitofrontal cortex; PFC, prefrontal cortex; PrL, prelimbic cortex; TLR, Toll-like receptor.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.08.046

danger signal expression. Twenty-four hours after AIE treatment concluded, HMGB1/TLR + IR was assessed using immunohistochemistry. In early adulthood (P80), we assessed + IR using immunohistochemistry and mRNA expression of danger signal receptors (i.e., TLR4, TLR3, TLR2) as well as the danger signaling agonist HMGB1 25 days after the conclusion of ethanol treatment. Furthermore, we assessed spatial and reversal learning on the Barnes maze to evaluate cognition. We found that AIE treatment upregulated the expression of TLR4, TLR3, and HMGB1 in the adolescent PFC that persisted into adulthood, and impaired reversal learning while increasing perseveration. Persistent upregulation of frontal cortical danger signaling molecules correlated with reversal learning deficits. Together, these findings support the hypothesis that early onset of alcohol exposure persistently activates innate immune danger signaling in the brain, which might contribute to adult neurocognitive dysfunction.

EXPERIMENTAL PROCEDURES

Animals

Time-mated young pregnant female Wistar rats (embryonic day 17), ordered from Harlan Sprague–Dawley (Indianapolis, IN), were allowed to acclimate to the animal facilities prior to birthing. On postnatal day (P)1 (24 h after birth), litters were culled to 10 pups per dam. Litters were housed on a 12/12 h light–dark cycle (light onset at 700 h) in a temperature (20 °C) and humidity-controlled vivarium, and provided *ad libitum* access to food and water. All experimental procedures were approved and conducted according to the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Adolescent intermittent ethanol treatment paradigm

On P21, male subjects were weaned and randomly assigned to one of the following treatment conditions: (i) adolescent intermittent ethanol (AIE, $n = 16$) or (ii) water-treated control (CON, $n = 16$). Beginning on P25, animals in the AIE condition received intragastric (i.g.) administration of ethanol (5.0 g/kg, 20% ethanol w/v) on a 2-day on/2-day off schedule until P55. Animals in the CON condition received comparable volumes of H_2O on a 2-day on/2-day off schedule for the same duration. As seen in Fig. 1, the body weights of AIE-treated animals did not differ significantly from the CON animals ($p > 0.35$). Tail blood was collected to assess blood ethanol content (BEC) one hr after ethanol administration on P38 and P54 as depicted in Fig. 1. Tail BECs were calculated using a GM7 Analyzer (Analox; London, UK). Mean BEC levels (±SEM) on P38 were 134 ± 8 mg/dL and at P54 were 165 ± 17 mg/dL. These BEC levels following i.g. ethanol treatment are consistent with previous studies using i.g. ethanol treatment of adolescent mice (Crews et al., 2006b).

Barnes maze spatial and reversal learning assessment

The Barnes maze was used to assess spatial learning and memory as well as the rats' ability to reverse a previously learned response. The Barnes maze (121 cm in diameter) was constructed of gray PVC and situated 100 cm from the floor. It contained 20 holes (10 cm in diameter) that were evenly spaced along the perimeter of the maze (see Cheng et al., 2006). A hidden box was situated directly beneath one of the holes to serve as an escape. The testing room had numerous visual cues, and the maze was brightly illuminated with spotlights to provide incentive for the rat to enter the hidden escape box. An automated tracking system (Ethovision XT 8.0, Noldus Ethovision; Leesburg, VA) was used to measure the distance traveled (cm), latency to enter goal box (s), velocity (cm/s), error duration (s), and frequency. The criterion for acquisition of learning was a 15-s average latency of all the rats in each treatment group to enter the hidden escape box. Between each trial, the maze and escape box was thoroughly cleansed (Roccal-D Plus, Fisher Scientific; Pittsburgh, PA) to remove all olfactory cues.

Spatial learning. Spatial learning commenced on P64 with each subject semi-randomly assigned a specific escape hole (1–20) beneath which the box was situated. The assigned escape hole was maintained for each animal for the duration of spatial learning. Before the start of each trial, the animal was placed in an opaque start cylinder that prevented the subject from determining its spatial location. The cylinder containing the rat was then placed on the Barnes maze in one of four possible randomly ordered start locations. Following a 10-s interval, the cylinder was lifted and the animal was allotted 5 min to locate and enter the escape box. Rats were given one trial per day for 6 days until the criterion of acquisition of learning was achieved.

Reversal learning. The animals were assessed for their ability to ''unlearn'' the previous location of the escape box and to learn a new location of the escape box. Reversal learning training began on P70, and the escape hole used during the spatial learning component was rotated 180°. The rat was released from the start cylinder identically to the spatial learning component. Rats were given one trial per day for 6 days until the criterion of learning was achieved. In addition to the measures collected, time and entries into the spatial goal quadrant were collected as a measure of perseveration. Perseveration was defined as repetitive entry and/or increased time spent in the previous spatial goal quadrant during reversal learning.

Probe trial. Five days after the completion of reversal learning, the rat was returned to the Barnes maze on P79 for a 2-min trial in which the escape box was not available. Duration and number of entries into the newly learned quadrant was collected.

Perfusion, brain tissue preparation, and immunohistochemistry

On P56 and P80, rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4.0% paraformaldehyde in PBS. Brains were extracted and post-fixed in 4.0% paraformaldehyde/PBS solution for 24 h followed by 4 days of fixation in a 30% sucrose solution. Brain tissue was sectioned coronally (40 μm) on a sliding microtome (MICROM HM450; ThermoScientific, Austin, TX). Sections were sequentially collected into well plates, and stored at −20 °C in a cryoprotectant solution consisting of 30% glycol and 30% ethylene glycol for immunohistochemistry (IHC). Free-floating sections were washed in 0.1 M PBS, incubated in 0.3% H_2O_2, and blocked with normal serum (goat or rabbit; MP Biomedicals, Solon, OH). Sections were incubated in primary antibody (Abcam, Cambridge, MA: rabbit anti-TLR4 [1:200], rabbit anti-HMGB1 [1:500]; Santa Cruz Biotechnology, Santa Cruz, CA: goat anti-TLR3 [1:100], goat anti-TLR2 [1:100]) for