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TLR4 elimination prevents synaptic and myelin alterations and long-term cognitive dysfunctions in adolescent mice with intermittent ethanol treatment

Jorge Montesinos [a], María Pascual [a], Antoni Pla [a], Concepción Maldonado [b], Marta Rodríguez-Arias [b], Jose Miñarro [b], Consuelo Guerri [a,*]

[a] Department of Molecular and Cellular Pathology of Alcohol, Príncipe Felipe Research Center, C/Eduardo Primo Yúfera, 3, 46012 Valencia, Spain
[b] Department of Psychobiology, Facultad de Psicología, Universitat de Valencia, Avda. Blasco Ibáñez, 21, 46010 Valencia, Spain

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ABSTRACT

The adolescent brain undergoes important dynamic and plastic cell changes, including overproduction of axons and synapses, followed by rapid pruning along with ongoing axon myelination. These developmental changes make the adolescent brain particularly vulnerable to neurotoxic and behavioral effects of alcohol. Although the mechanisms of these effects are largely unknown, we demonstrated that ethanol by activating innate immune receptors toll-like receptor 4 (TLR4), induces neuroinflammation and brain damage in adult mice. The present study aims to evaluate whether intermittent ethanol treatment in adolescence promotes TLR4-dependent pro-inflammatory processes, leading to myelin and synaptic dysfunctions, and long-term cognitive impairments. Using wild-type (WT) and TLR4-deficient (TLR4-KO) adolescent mice treated intermittently with ethanol (3.0 g/kg) for 2 weeks, we show that binge-like ethanol treatment activates TLR4 signaling pathways (MAPK, NFκB) leading to the up-regulation of cytokines and pro-inflammatory mediators (COX-2, iNOS, HMGB1), impairing synaptic and myelin protein levels and causing ultrastructural alterations. These changes were associated with long-lasting cognitive dysfunctions in young adult mice, as demonstrated with the object recognition, passive avoidance and olfactory behavior tests. Notably, elimination of TLR4 receptors prevented neuroinflammation along with synaptic and myelin derangements, as well as long-term cognitive alterations. These results support the role of the neuroimmune response and TLR4 signaling in the neurotoxic and behavioral effects of ethanol in adolescence.

1. Introduction

Alcohol binge drinking is prevalent in adolescence, and a high percentage of European and USA teenagers have reported heavy episodic drinking, defined as consuming five or more drinks on one occasion in the past 2 weeks (Danielsson et al., 2012; Johnston et al., 2013). These high rates of heavy alcohol use are concerning since many studies have indicated that heavy alcohol consumption can impair neurocognitive development which affects several neuropsychological domains, including memory, executive functioning, visuospatial skills, and sustained attention (Brown et al., 2000; Giancola et al., 2001). Longitudinal studies have also shown adverse effects of adolescent drinking on the development of visuospatial processing, attention and working memory (Hanson et al., 2011; Squeglia et al., 2009; Tapert et al., 2002).

Adolescence is an important brain maturation period during which some brain regions undergo remodeling and functional changes, which increase neuronal connectivity and change synaptic plasticity (Alfonso-Loeches and Guerri, 2011; Toga et al., 2006). The prefrontal cortex (PFC), which coordinates higher-order cognitive processes and executive functions, is the last brain region to mature (Mills et al., 2014). Gray matter reduction begins primarily in early adolescence (≈12–14 years) in posterior brain regions and progresses to more anterior regions (Gogtay et al., 2004), such as the PFC, to continue into early adulthood (mid-20s) (Sowell et al., 2001). Reduction in gray matter, which is particularly evident in the PFC, is associated with the pruning of excess neurons (Paus, 2005), and with synapse stabilization. Concomitantly, the white

* Corresponding author at: Department of Molecular and Cellular Pathology of Alcohol, Centro de Investigación Príncipe Felipe, C/Eduardo Primo Yúfera 3, Valencia 46012, Spain. Tel.: +34 96 328 96 80; fax: +34 96 328 97 01.
E-mail address: cguerri@cipf.es (C. Guerri).

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matter volume and myelination of fiber tracts increases in the PFC during adolescence, event associated with enhanced neuronal conduction and communication (Barnea-Goraly et al., 2005; Giedd et al., 1999; Pfefferbaum, 2004). All these processes are an integral component of neurocognitive development and generate efficient information processing and improved cognition (Squeglia et al., 2013). These extensive developing changes in brain maturation might explain the adolescent brain's vulnerability to the deleterious effects of ethanol (Alfonso-Loeches and Guerri, 2011; Jacobus and Tapert, 2013). PFC-dependent behaviors have been described to be altered in human alcoholics (Fortier et al., 2009; Kamarajan et al., 2010) and also in PFC-damaged rodents (Bissonette et al., 2008), or after an acute ethanol challenge (Brown et al., 2007). Neuroimaging studies have also reported changes in microstructural and functional myelin integrity in different brain areas in adolescents with alcohol abuse (Bava et al., 2009a; Schweinsburg et al., 2010). These myelin alterations may be related with attention and spatial working memory deficits in human adolescents who participate in heavy alcohol abuse (Tapert et al., 1999, 2004).

PFC is one of the brain regions more affected by ethanol drinking during adolescence and can cause PFC-mediated control deficits in adulthood (Gass et al., 2014). The underlying mechanisms of neurotoxic and behavioral effects of ethanol in adolescence are presently unknown. Our previous studies indicated that by activating the innate immune system, particularly the toll-like receptor 4 (TLR4) signaling response, ethanol triggers the activation of transcription factors NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) and AP-1 (activating protein-1), which induce the production of cytokines and inflammatory mediators, that cause brain damage. Indeed, we have shown that by activating TLR4 responses in glial cells, chronic alcohol consumption induces neuroinflammation, gliosis, demyelination and brain damage (Alfonso-Loeches et al., 2010, 2012; Blanco et al., 2005; Fernandez-Lizarbe et al., 2009), and these effects have been associated with behavioral impairments (Pascual et al., 2011).

Our previous studies have also provided evidence that binge-like ethanol treatment in adolescent rats induces some inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), in the cerebral cortex and the hippocampus, causing neural death and cognitive alterations (Pascual et al., 2007). Neural death can, in turn, enhance neuroinflammation by the release of danger-associated molecular pattern (DAMP) molecules, such as high-mobility group box-1 (HMGB1), a nuclear protein which is passively released during stress, cell injury and necrosis, or is actively secreted as a cytokine (Andersson and Tracey, 2011). HMGB1 can bind and activate several receptors, including TLRs, receptors for advanced glycation end products (RAGE) and C-X-C chemokine receptor 4 (CXCR4) (Yang et al., 2013). Recent studies have demonstrated that ethanol treatment induces the release of HMGB1 in the brain and liver, which contributes to ethanol-induced pathology in both organs (Ge et al., 2014; Vetreno et al., 2013; Whitman et al., 2013).

Despite the involvement of TLR4 in adult neuroinflammation, whether TLR4 activation participates in the myelin and synaptic dysfunction, and long-term neuroinflammatory and cognitive behavioral impairments associated with binge drinking in adolescence is presently unknown. This study reveals, for the first time, the critical role of TLR4 receptors in neuroinflammation and brain damage induced by intermittent ethanol treatment in the PFC of adolescent wild-type (WT) mice since changes in neither the production of cytokines and inflammatory mediators nor in myelin and synaptic structures were observed in TLR4-deficient (TLR4-KO) mice treated with alcohol. Elimination of the TLR4 function also prevents ethanol-induced long-lasting cognitive alterations, which suggests the role of TLR4 signaling and neuroinflammation in ethanol-induced brain alterations in adolescence.

2. Materials and methods

2.1. Animals and treatments

Female C57BL/6 WT (Harlan Ibérica, Barcelona, Spain) and TLR4-KO knockout (KO) mice (C57BL/6 background, kindly provided by Dr. S. Akira, Osaka University, Suita, Japan) aged 30 days were used. All the animals were kept under controlled light and dark (12/12 h), temperature (23 °C), and humidity (60%) conditions. All the experimental procedures were carried out in accordance with the guidelines approved by the European Communities Council Directive (86/609/ECC) and by Spanish Royal Decree 1201/2005. The animal experiments were also approved by the *Ethical Committee of Animal Experimentation of the* Príncipe Felipe Research Center (Valencia, Spain).

For the binge ethanol treatment, WT and TLR4-KO mice were housed (4 animals/cage) and maintained with water and solid diet *ad libitum*. Morning doses (9–10 a.m.) of either saline or 25% (v/v) ethanol (3 g/kg) in isotonic saline were administered intraperitoneally to 30-day-old mice on 2 consecutive days with 2-day gaps without injections for 2 weeks (PND 30 to PND 43), as previously described (Pascual et al., 2007). Then, some mice were maintained without alcohol treatment until postnatal day (PND) 65. A single dose of ethanol to adolescent mice resulted in a peak of BECs of 178.23 ± 16.75 mg/dL at 30 min post-injection. Some animals were sacrificed by decapitation 24 h after the last (8th) ethanol or saline administration (PND 44, short-term ethanol effects) or after 3 weeks upon ethanol or saline administration (PND 65, long-term ethanol effects). Brains from adolescent (PND 44) and young adult mice were collected, and the PFC were dissected and stored at −80 °C until use. In addition, some animals were anesthetized, perfused with paraformaldehyde (PF)/glutaraldehyde and used for the electron microscopy analysis. Behavioral studies were conducted 3 weeks after the last dose of ethanol or saline treatment (long-term effects).

2.2. Western blot analysis

The Western blot technique was performed in the PFC tissue lysates, as described elsewhere (Fernandez-Lizarbe et al., 2009). To analyze the HMGB1 levels, nuclear and cytosolic fractions were isolated following the procedure of (Ishida et al., 2002). The primary antibodies used were: CNPase (2′,3′-cyclic-nucleotide 3′-phosphodiesterase), PLP (proteolipid protein), MBP (myelin basic protein), MAG (myelin-associated glycoprotein), MOG (myelin oligodendrocyte glycoprotein) and HMGB1 (Abcam, Cambridge, UK); NG2 (neuron/glial antigen 2), p-ERK (extracellular signal-regulated kinase), p-JNK (c-Jun N-terminal kinase), p-p38 and p-p65 (Cell Signaling Technology, Leiden, The Netherlands); iNOS (BD Transduction Laboratories, California, USA) and COX-2 (Cayman Chemical, Michigan, USA); synapsin IIa, syntaxin 4, SNAP-25 (synaptosomal-associated protein-25) and synaptotagmin (BD Transduction Laboratories). Membranes were washed, incubated with the corresponding HRP-conjugated secondary antibodies and developed using the ECL system (ECL Plus; Thermo Scientific, Illinois, USA). All the membranes were stripped and incubated with the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibodies (Chemicon, California, USA), Lamin A/C (BD Transduction Laboratories), NeuN (neuronal nuclei protein) (Chemicon, California, USA) or the corresponding total form of each phosphorylated protein (ERK, JNK, p38 and p65; Santa Cruz Biotechnology) as loading controls. Band intensity was quantified with the ImageJ 1.44p analysis software (National Institutes of Health, USA). The densitometry analysis is shown in arbitrary units normalized to the respective loading control.