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Involvement of TLR4 in the long-term epigenetic changes, rewarding and anxiety effects induced by intermittent ethanol treatment in adolescence

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ABSTRACT

Studies in humans and experimental animals have demonstrated the vulnerability of the adolescent brain to actions of ethanol and the long-term consequences of binge drinking, including the behavioral and cognitive deficits that result from alcohol neurotoxicity, and increased risk to alcohol abuse and dependence. Although the mechanisms that participate in these effects are largely unknown, we have shown that ethanol by activating innate immune receptors, toll-like receptor 4 (TLR4), induces neuroinflammation, impairs myelin proteins and causes cognitive dysfunctions in adolescent mice. Since neuroimmune signaling is also involved in alcohol abuse, the aim of this study was to assess whether ethanol treatment in adolescence promotes the long-term synaptic and molecular events associated with alcohol abuse and addiction. Using wild-type (WT) and TLR4-deficient (TLR4-KO) adolescent mice treated intermittently with ethanol (3 g/kg) for 2 weeks, we showed that binge-like ethanol treatment in adolescent mice promotes short- and long-term alterations in synaptic plasticity and epigenetic changes in the promoter region of bdnf and fosb, which increased their expression in the mPFC of young adult animals. These molecular events were associated with long-term rewarding and anxiogenic-related behavioral effects, along with increased alcohol preference. Our results further showed the participation of neuroimmune system activation and the TLR4 signaling response since deficient mice in TLR4 (TLR4-KO) are protected against molecular and behavioral alterations of ethanol in the adolescent brain. Our results highlight a new role of the neuroimmune function and open up new avenues to develop pharmacological treatments that can normalize the immune signaling responsible for long-term effects in adolescence, including alcohol abuse and related disorders.

1. Introduction

Adolescence is a developmental stage during which the brain undergoes remodeling and functional changes in synaptic plasticity, as well as neuronal connectivity in different regions, including the cortical and subcortical structures which undergo modifications in white- and gray-matter densities (Gogtay et al., 2004; Sowell et al., 2001). Adolescence is also a time when alcohol use and abuse are initiated, and adolescent binge drinking impacts the developing brain, particularly the prefrontal cortex (PFC), causing cognitive dysfunction and impairing attentional functioning (Koskinen et al., 2011), visuospatial ability (Giancola et al., 1998; Tapert et al., 2002) and executive control (White et al., 2011).

Adolescence binge drinking has also been linked to greater risk-taking and novelty-seeking behavior, and to higher prevalence of drug abuse and risk of relapse (Blakemore, 2008). Both prospective and retrospective human studies have revealed that alcohol use onset is a reliable predictor of later problematic use and dependence on alcohol and other drugs (DeWit et al., 2000; Grant and Dawson, 1997; Hawkins et al., 1997; Labouvie et al., 1997). Stress is also a consistent predictor of increased alcohol use and alcohol-related disorders, and stressors in adolescence may be particularly detrimental (Casement et al., 2015; Green et al., 2010; Lloyd and Turner, 2008; McLaughlin, 2010).

Although the molecular mechanisms of alcohol actions in the adolescent brain are not well understood, we have demonstrated that by activating the innate immune receptor TLR4 signaling in glial cells, ethanol triggers signaling pathways, which induce the release of inflammatory mediators and consequent brain damage (Alfonso-Loeches et al., 2010). Thus, using adolescent rats (Pascual et al., 2007) and mice (Montesinos et al., 2015), we

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demonstrated that intermittent binge-like alcohol treatment triggers pro-inflammatory cytokines and mediators (iNOS and COX-2) in the brain, which causes inflammatory damage in the PFC and impairs synaptic and myelin structures (Montesinos et al., 2015). Binge ethanol exposure during rodent adolescence also leads to the persistent loss of neurogenesis in the hippocampus (Vetreno and Crews, 2015). All these events have been associated with long-lasting cognitive dysfunctions (Montesinos et al., 2015; Pascual et al., 2007; Vetreno and Crews, 2015) and anxiety-like behavior (Vetreno and Crews, 2015; Vetreno et al., 2015) in young adult mice treated with alcohol in adolescence. The role of neuroinflammation and the TLR4 response in the actions of ethanol on the adolescent brain has been supported by data which show that anti-inflammatory compounds (Pascual et al., 2007) or the genetic elimination of TLR4 receptors prevents neuroinflammation, along with synaptic and myelin derangements, long-term cognitive alterations (Montesinos et al., 2015; Pascual et al., 2007), as well as motor impairment (Wu et al., 2012). However, whether inflammation and up-regulation of brain cytokines are also involved in some long-term behavioral effects as anxiety disorders, addiction and alcohol preference remains unknown.

TLRs are key regulators of immune activation in the CNS in response to pathogens and damage, and they initiate the innate and adaptive immunity during infection (Iwasaki and Medzhitov, 2004). Nevertheless, ethanol can activate the TLR4 response triggering cytokines, chemokines and pro-inflammatory signaling which modulate some behavioral effects of alcohol, such as the regulation of voluntary ethanol intake in rodent models (see rev. Blednov et al., 2011; Robinson et al., 2014). Ethanol intake also activates the immune system in humans since changes in the expression of immune-related genes have been reported in human alcoholic brains (Lewohl et al., 2000). Recent studies have also shown the involvement of neuroimmune signaling in the regulation of neuroplasticity, and in learning and memory processes (Williamson and Bilbo, 2013; Yirmiya and Goshen, 2011).

Increasing evidence suggests a role of epigenetic mechanisms in the long-lasting behavioral effects induced by alcohol consumption, including alcohol preference and addiction-related behavioral abnormalities as anxiety disorders (Nestler, 2014; Pandey et al., 2015; Sakharkar et al., 2014). Epigenetic mechanisms can affect the chromatin structure by regulating the expression of the genes involved in addiction in specific brain regions, including the ventral tegmental area and nucleus accumbens (Renthal and Nestler, 2008). For instance, changes in histone acetylation in the promoter region of cFos, Cdk5 and FosB have been associated with ethanol-induced place conditioning in adult rats treated with ethanol in adolescence (Pascual et al., 2012). One major encephalic structure involved in the expression of emotional states and anxiety-like behaviors is the prelimbic medial prefrontal cortex (mPFC) (Saitoh et al., 2014; Vialou et al., 2014). This brain area has bidirectional connections with a wide range of neuromodulatory systems (e.g. dorsal raphe, ventral tegmental area, locus coeruleus) and plays an important role in adaptive responses to rewarding and stressful events (Euston et al., 2012). Recent studies have suggested that repeated binge and withdrawal episodes in young adults may sensitize the mPFC to additional dysfunction thus facilitating the transition to alcohol dependence (George et al., 2012). However, the role of mPFC in the long-term synaptic remodeling and behavioral changes induced by binge-like ethanol treatment in adolescence is uncertain.

Here we report that intermittent binge-like ethanol treatment in adolescence induces long-term aberrant synaptic remodeling, increases histone acetylation at the promotor region of the bdnf and fosb genes and up-regulates their expression in the mPFC of young adult animals. These events were associated with long-term rewarding and anxiogenic-related behavior effects, along with alcohol preference, in young adult mice treated with alcohol in adolescence. Our findings also provide evidence for the participation of the neuroimmune system and TLR4 signaling response in the neurochemical and behavioral dysfunctions caused by alcohol abuse in adolescence since TLR4-deficient mice (TLR4-KO) are protected against the neurochemical and behavioral actions of ethanol on the adolescent brain.

2. Materials and methods

2.1. Animals and treatments

Female C57BL/6 WT (Harlan Ibérica, Barcelona, Spain) and TLR4 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). Female mice were used because previous studies showed more ethanol-induced inflammatory damage in female than in male mice (Alfonso-Loeches et al., 2013), and also to correlate the present results with previous findings on adolescent female mice exposed to binge-like ethanol treatment (Montesinos et al., 2015).

For the intermittent ethanol treatment, WT and TLR4-KO mice were housed (4 animals/cage) and maintained with water and solid diet *ad libitum*. Morning doses 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 with no injections, for 2 weeks (PND30 to PND43), as previously described (Pascual et al., 2007). 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 (PND44, 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 mPFC were dissected and stored at −80 °C until use. Behavioral tests were performed with young adult mice (PND 65).

2.2. Western blot analysis

The Western blot technique was performed in the mPFC tissue lysates, as described elsewhere (Fernandez-Lizarbe et al., 2009). The primary antibodies used were: acetyl H3 lysine 9 (Ac-H3 K9) FosB, ΔFosB, p-CREB, NF-κB p-p65, tri-methyl H3 lysine 4 (3me-H3 K4) (Cell Signaling Technology, Leiden, The Netherlands); acetyl histone 4 (H4) lysine 5 (Ac-H4 K5), acetyl H4 lysine 12 (Ac-H4 K12), cyclin-dependent kinase-5 (Cdk5), major histocompatibility complex class II (MHCII), cluster of differentiation molecule 11b (CD11b) (Abcam, Cambridge, UK), brain-derived neurotrophic factor (BDNF) (Santa Cruz Biotechnology, Madrid, Spain), GluR1 and NR1 (Millipore Iberica, Madrid, Spain). Membranes were washed, incubated with the corresponding HRP-conjugated secondary antibodies and developed with the ECL system (ECL Plus; Thermo Fisher Scientific, Illinois, USA). All the membranes were stripped and incubated with GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (Chemicon, California, USA), postsynaptic density protein 95 (PSD95), total H3 (T-H3), total H4 (T-H4) (Abcam) or the