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Chronic ethanol intake induces partial microglial activation that is not reversed by long-term ethanol withdrawal in the rat hippocampal formation

Catarina Cruz[a,b], Manuela Meireles[b,c], Susana M. Silva[a,b,*]

[a] Unit of Anatomy, Department of Biomedicine, Faculty of Medicine, University of Porto, Alameda Professor Hernâni Monteiro, 4200-319, Porto, Portugal
[b] Center for Health Tecnology and Services Research (CINTESIS), Rua Doutor Plácido da Costa, 4200-450, Porto, Portugal
[c] School of Health, University of Algarve, Rua Eng. José Campos Coroa, 8000-510, Faro, Portugal

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ABSTRACT

Neuroinflammation has been implicated in the pathogenesis of several disorders. Activation of microglia leads to the release of pro-inflammatory mediators and microglial-mediated neuroinflammation has been proposed as one of the alcohol-induced neuropathological mechanisms. The present study aimed to examine the effect of chronic ethanol exposure and long-term withdrawal on microglial activation and neuroinflammation in the hippocampal formation. Male rats were submitted to 6 months of ethanol treatment followed by a 2-month withdrawal period. Stereological methods were applied to estimate the total number of microglia and activated microglia detected by CD11b immunohistochemistry in the hippocampal formation. The expression levels of the pro-inflammatory cytokines TNF-α, COX-2 and IL-15 were measured by qRT-PCR. Alcohol consumption was associated with an increase in the total number of activated microglia but morphological assessment indicated that microglia did not exhibit a full activation phenotype. These data were supported by functional evidence since chronic alcohol consumption produced no changes in the expression of TNF-α or COX-2. The levels of IL-15 a cytokine whose expression is increased upon activation of both astrocytes and microglia, was induced by chronic alcohol treatment. Importantly, the partial activation of microglia induced by ethanol was not reversed by long-term withdrawal. This study suggests that chronic alcohol exposure induces a microglial phenotype consistent with partial activation without significant increase in classical cytokine markers of neuroinflammation in the hippocampal formation. Furthermore, long-term cessation of alcohol intake is not sufficient to alter the microglial partial activation phenotype induced by ethanol.

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1. Introduction

Chronic excessive alcohol intake in humans leads to significant brain structural changes, including a decrease in neuronal density in the cerebellum, diencephalon and cerebral cortex, particularly in the hippocampal formation, and impaired synaptic ultrastructure (Harper and Matsumoto, 2005; Sullivan, 2003). In animal models, chronic alcohol exposure also results in structural and functional changes in multiple brain regions (Crews and Nixon, 2009; Madeira and Paula-Barbosa, 1999). In previous studies from our lab, we have shown that the consumption of a 20% aqueous ethanol solution over 6 months leads to a variety of morphological and neurochemical changes in the hippocampal formation, including neuronal loss (Lukoyanov et al., 1999; Paula-Barbosa et al., 1993), structural alterations at the dendritic and synaptic level (Cadete-Leite et al., 1989a, 1989b) and an increase of oxidative stress (Assuncao et al., 2007). These changes are not restricted to the hippocampal formation, since in the hypothalamic supraoptic, paraventricular and suprachiasmatic nuclei, prolonged ethanol exposure results in loss of neurons (Madeira et al., 1993; Silva et al., 2002a) and/or reduction in neuronal synthesis and expression of several peptides (Madeira et al., 1997; Silva and Madeira, 2012; Silva et al., 2002b, 2009). Using the same experimental model of alcohol consumption, which consistently increased blood alcohol concentrations to similar levels that, in addition, did not differ from those determined in the present study, we have also demonstrated

* Corresponding author: Unit of Anatomy, Department of Biomedicine, Faculty of Medicine, University of Porto, Alameda Professor Hernâni Monteiro, 4200-319, Porto, Portugal.
E-mail address: ssilva@med.up.pt (S.M. Silva).

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that the neurodegenerative changes induced by chronic ethanol intake are not reversed upon withdrawal in several regions of the brain (Cadete-Leite et al., 2003; Madeira et al., 1997; Pereira et al., 2014; Silva and Madeira, 2012; Silva et al., 2009) including the hippocampal formation (Lukoyanov et al., 1999; Paula-Barbosa et al., 1991, 1993; Pereira et al., 2016). Consistent with these data, reference memory is significantly impaired in ethanol abstinent rats (Lukoyanov et al., 1999; Munro et al., 2000).

Microglia are the resident immune cells of the central nervous system (CNS) and, in non-pathologic conditions, remain in a quiescent state. In response to a multitude of pathological stimuli, microglia rapidly become activated. Although different terms have been used to classify activated microglia, it has long been recognized that microglia exhibit a graded response, both morphologically and in terms of cytokine expression profile (Marshall et al., 2013). Raivich has defined a resting stage (stage 0), in which microglia display a highly ramified morphology and four different stages of activation. In stage 1, the lowest stage of activation, microglia have a less ramified appearance and express transforming growth factor-beta (TGF-β). Further development of the microglial response (stages 2 and 3a) is characterised by a reduction in ramification, with microglia adopting an amoeboid morphology and expression of tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β) and interferon gamma (IFNγ) (Raivich et al., 1999). Recent studies have suggested that microglia-mediated neuroinflammation is one of the mechanisms by which alcohol induces brain damage (Qin et al., 2008; Valles et al., 2004; Yang et al., 2014). In acute, sub-chronic and intermittent ethanol exposure animal models, there is a significant increase in the number of activated microglia (He and Crews, 2008; McClain et al., 2011; Ward et al., 2009) and in the levels of pro-inflammatory cytokines (Qin et al., 2008). Interestingly, following 5,5 (Riikonen et al., 2002) or 10 months (Dlugos and Pentney, 2001) of ethanol exposure, the morphology and the density of microglia were unchanged. However, in other models of chronic exposure, there is up-regulation of IL-1β, cyclooxigenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) expression (Pascual et al., 2015; Valles et al., 2004).

There is evidence that ethanol withdrawal after acute intermittent or chronic exposure may prolong or even exacerbate the pro-inflammatory state (Freeman et al., 2012). After twenty-eight days of withdrawal from a 4 days alcohol binge exposure, the number of microglial cells returned to control levels, although the immunoreactivity levels of a microglial marker remained high in all areas of the hippocampal formation except the dentate gyrus (DG) (Marshall et al., 2013). Acute alcohol withdrawal maintains a sustained elevation of the levels of several cytokines (TNF-α, IL-1β, IL-17) and chemokines and induces the expression of IFN-γ in the striatum of chronically exposed mice (Pascual et al., 2015). In other studies, the levels of some cytokines and neuroinflammatory response markers were significantly elevated 24 h or 48 h after withdrawal following chronic ethanol exposure (Freeman et al., 2012; Whitman et al., 2013).

Considering the pivotal role of the hippocampal formation in cognitive functions, the aim of this study was to assess the effects of long-term ethanol consumption and withdrawal on microglia activation and hippocampal neuroinflammation. We estimated the total numbers of microglia and activated microglia in the DG, and in the CA2/3 and CA1 subfields of the dorsal hippocampal formation after 6 months of ethanol exposure and a 2-month long withdrawal period. We also evaluated whether the increase in the number of activated microglia following ethanol exposure and withdrawal is associated with changes of the expression levels of key pro-inflammatory mediators.

2. Materials and methods

2.1. Animals and treatments

A total of 30 (n = 10/group) male Wistar rats was used in this study. Six-week old animals were obtained from the Institute for Molecular and Cell Biology (Porto, Portugal) and housed three to four per cage in a temperature (22 °C) and humidity (50%) controlled room under 12:12 h light/dark cycles with free access to solid food (4RF21/C Mucedola, Milan, Italy) and water for 2 weeks. At 2 months of age, rats were randomly assigned either to the ethanol-treated (ET) group (n = 20) or to the control (C) group (n = 10). Rats of the ET group had free access to a 20% aqueous ethanol (Merck KGaA) solution whereas rats of the C group had free access to tap water. Rats initially received *ad libitum* a 5% (v/v) ethanol solution for one day and subsequently the ethanol concentration was raised 1% per day up to 20% (v/v) during 2 weeks. After 6 months of treatment, half of the ET rats were sacrificed and the remaining were switched to water for 2 months as the withdrawal (W) group. In a 2-week period, ethanol concentrations were progressively reduced by 1% a day. All groups received beverages and food *ad libitum* throughout the experimental period. The amount of solid and fluid intake was calculated daily and rats were weighed weekly. The handling and care of the animals were conducted according to European Communities Council guidelines in animal research (2010/63/EU) and Portuguese Act 129/92 and approved by the animal welfare ethics review board (ORBEA) of the Faculty of Medicine, University of Porto. All efforts were made to minimise the number of animals used and their suffering.

2.2. Blood ethanol levels

Blood samples were collected monthly at two time points: 2 h after light and dark onset. Samples were stored at 4 °C for 3 h until complete clot formation and centrifuged twice at 2000 rpm for 10 min. Serum was collected and stored undiluted at −80 °C until further analysis. Serum ethanol concentrations were determined with a commercial colorimetric assay (K620-100; BioVision, USA) with a detection limit of 0.4 ppm.

2.3. Tissue preparations

At the end of the treatments, animals were anaesthetised with an intraperitoneal injection of sodium pentobarbital (80 mg/kg body weight). Half of the animals from each group (n = 5) were subjected to transcardiac perfusion with 250 mL of 0.1 M phosphate buffer pH 7.6, followed by 400 mL of a fixative solution containing 4% paraformaldehyde in phosphate buffer. Brains were removed from the skulls, coded for blind processing, post-fixed for 1 h in the fixative solution and transferred to 10% sucrose in phosphate buffered saline (0.1 M, pH 7.2) (PBS) where they were stored overnight at 4 °C. Subsequently, brains were mounted on a vibrating microtome, serially sectioned in the coronal plane at a nominal thickness of 40 μm (Leica VT1000S; Germany) and collected in PBS. Sections were stored in a cryoprotectant solution at −20 °C until processing for immunohistochemistry. The remaining animals (n = 5/group) were transcardially perfused with a saline solution. The brains removed from the skulls and the hippocampal formations were dissected, quickly frozen in liquid nitrogen and stored at −80 °C until use for RNA isolation and quantitative real-time polymerase chain reaction.

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