Journal of Neuroimmunology 285 (2015) 169–179

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

# Chronic ethanol exposure combined with high fat diet up-regulates P2X7 receptors that parallels neuroinflammation and neuronal loss in C57BL/6J mice

Liana Asatryan [a,*], Sheraz Khoja [b], Kathleen E. Rodgers [a], Ronald L. Alkana [b], Hidekazu Tsukamoto [c], Daryl L. Davies [a]

[a] Titus Family Department of Clinical Pharmacy and Pharmaceutical Economics and PolicySchool of PharmacyUniversity of Southern California1985 Zonal Avenue, Los Angeles, CA, 90033, United States
[b] Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Avenue, Los Angeles, CA 90033, United States
[c] Southern California Research Center for Alcoholic Liver and Pancreatic Disease and Cirrhosis, Keck School of Medicine, University of Southern California, 1333 San Pablo Street, Los Angeles, CA 90033, United States

ARTICLE INFO

*Article history:*
Received 2 April 2015
Received in revised form 22 May 2015
Accepted 17 June 2015

*Keywords:*
Purinergic P2X7 receptors
Pro-inflammatory cytokines
Microglia
Astrocytes
Neuroinflammation and neuronal loss
Intragastric ethanol feeding
High fat diet

ABSTRACT

The present investigation tested the role of ATP-activated P2X7 receptors (P2X7Rs) in alcohol-induced brain damage using a model that combines intragastric (iG) ethanol feeding and high fat diet in C57BL/6J mice (*Hybrid*). The *Hybrid* paradigm caused increased levels of pro-inflammatory markers, changes in microglia and astrocytes, reduced levels of neuronal marker NeuN and increased P2X7R expression in ethanol-sensitive brain regions. Observed changes in P2X7R and NeuN expression were more pronounced in *Hybrid* paradigm with inclusion of additional weekly binges. In addition, high fat diet during *Hybrid* exposure aggravated the increase in P2X7R expression and activation of glial cells.



## 1. Introduction

Neurological and cognitive impairments leading to permanent brain damage are major consequences of chronic alcohol abuse (Vetreno et al., 2011). Studies indicate that 50% of detoxified alcoholics have measurable cognitive impairments and over 75% of chronic alcoholics have significant brain damage at autopsy (Vetreno et al., 2011). At the neuroanatomical level, adverse effects of chronic alcohol intake include reduction of brain volume due to white matter loss, thinning of the corpus callosum and atrophy of the mammillary bodies. Regional volume differences are, in part, due to neuronal loss and gliosis in the thalamus, forebrain, hippocampus and cerebellum (Obernier et al., 2002; Zahr et al., 2011).

Nutritional deficiencies are associated with severe conditions of alcohol related brain damage such as Wernicke encephalopathy–Korsakoff syndrome (Nardone et al., 2013). In contrast, increased caloric intake and obesity in the Western societies often accompany alcohol consumption and abuse. There is evidence that together, alcohol abuse and obesity, may lead to higher rates of liver damage compared to the effects of either factor alone (Deng et al., 2005; Xu et al., 2011). Moreover, alcohol abuse and obesity separately are among etiologies of neurocognitive deficiencies. However, little is known if there is a synergy between ethanol and obesity in causing brain damage.

Recent evidence regarding the molecular mechanisms of alcohol (ethanol)-induced brain damage suggests the involvement of ethanol-induced neuroinflammation (Crews and Nixon, 2009; Crews and Vetreno, 2014; He and Crews, 2008; Kelley and Dantzer, 2011). These studies demonstrated increases in the number of pro-inflammatory cytokines as well as oxidative stress markers in both animal models of

*Abbreviations:* CNS, central nervous system; Hipp, hippocampus; Striat, striatum; MidBr, midbrain; iG, intragastric; EtOH, ethanol; HCFD, high fat diet; ATP, adenosine triphosphate; NO, nitric oxide; iNOS, inducible NO synthase; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-10, interleukin 10; TNFα, tumor necrosis factor alpha; TGFβ, transforming growth factor beta; MCP-1, monocyte chemoattractant protein 1; CCL2 (same as MCP-1), chemokine CC ligand 2; TLR, toll-like receptor; P2X7R, purinergic P2X7 receptor; Iba1, ionized calcium binding adapter molecule 1; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclear antigen; HRP, horseradish peroxidase; RT-PCR, reverse transcription polymerase chain reaction.

* Corresponding author at: Titus Family Department of Clinical Pharmacy and Pharmaceutical Economics and Policy, School of Pharmacy, University of Southern California, 1985 Zonal Ave, PSC 508, Los Angeles, CA 90033, United States.
*E-mail address:* asatryan@usc.edu (L. Asatryan).

http://dx.doi.org/10.1016/j.jneuroim.2015.06.007
0165-5728/

chronic ethanol exposure as well as in post-mortem alcoholic human brain (Crews et al., 2006; Crews and Nixon, 2009; He and Crews, 2008; Qin et al., 2008). The important role of toll-like receptors (TLR4, TLR2) in ethanol-induced neuroinflammatory signaling cascades in glial cells and ethanol-related brain damage has been demonstrated (Alfonso-Loeches et al., 2010; Fernandez-Lizarbe et al., 2009). Moreover, the releases of pro- as well as anti-inflammatory cytokines were linked to long-term changes in ethanol-induced behaviors and neurodegeneration (Blednov et al., 2012; Crews et al., 2006; Crews and Nixon, 2009; Qin et al., 2008).

Neuroinflammation and related cognitive decline are also currently recognized as negative consequences of obesity (reviewed in Miller and Spencer, 2014). Elevated expression of pro-inflammatory cytokines and transcription factor NFkB, activation and infiltration of microglia have been found in hypothalamus of rats fed high fat diet (De Souza et al., 2005), in hippocampus of mice on 60% high fat diet for 20 weeks (Jeon et al., 2012) and of db/db mouse model of metabolic syndrome (Dinel et al., 2011). Neuroinflammation caused by high fat diet may, in part, be related to activation of central processes. As such, a role of central TLR4-signaling in pro-inflammatory cascades has been suggested (Milanski et al., 2009). Despite this knowledge, there remains a paucity of information regarding other receptor systems that are important for the development of ethanol- as well as obesity-induced neuroinflammation.

Purinergic P2X7 receptors (P2X7Rs) have recently become a focus of investigation in the areas of chronic inflammation, neurodegeneration, neuropsychiatric disorders (e.g., depression) and pain (reviewed in (Skaper et al., 2010)). P2X7Rs belong to the ATP-gated P2XR superfamily of ligand gated ion channels (P2X1–P2X7) (Khakh et al., 2001; North, 2002). In contrast to the other P2X subtypes, P2X7R subtype is unique in that it: 1) is activated at high, millimolar ATP concentrations such as released during a CNS insult; 2) is able to form a pore that allows passage of molecules of up to 900 Da (North, 2002) and 3) interacts with many intracellular adaptor and signaling proteins due to their unusual long C-terminus (Kim et al., 2001). Notably, P2X7Rs are widely expressed in neurons, astrocytes, microglia, oligodendrocytes and Schwann cells (Skaper et al., 2010; Takenouchi et al., 2010).

Signaling through P2X7Rs plays a vital role in the activation of neuroimmune cells (Monif et al., 2010). Most importantly, P2X7Rs were shown to modulate the release of IL-1β, a recognized mediator of neurodegeneration (Bernardino et al., 2008; Clark et al., 2010; Honore et al., 2009; Takenouchi et al., 2008). P2X7Rs are also mediators of TNFα and CC-chemokine ligand 3 secretion (Kataoka et al., 2009; Suzuki et al., 2004), production of superoxide (Parvathenani et al., 2003) and nitric oxide (NO) (Gendron et al., 2003) and matrix metalloproteinase 9 (Choi et al., 2010; Shin et al., 2010). The presence of P2X7Rs has been demonstrated within the β-amyloid plaques suggesting a role in the processes of neurodegeneration in Alzheimer's Disease (McLarnon et al., 2006; Parvathenani et al., 2003). P2X7Rs has been further implicated in neurodegenerative disorders through receptor up-regulation which is evident in epileptic brain, cerebral ischemia, amyotrophic lateral sclerosis, multiple sclerosis and chronic neuropathic pain (reviewed in Volonte et al., 2011). Involvement of P2X7Rs in obesity-induced renal inflammation and beta cell dysfunction has also been demonstrated (Glas et al., 2009; Solini et al., 2013). To date no information is available on the role of P2X7Rs in ethanol- and/or obesity-related brain damage.

To this end, the present study investigated the effects of concurrent administration of ethanol and high fat on the development of neuroinflammation and parallel changes in P2X7R expression. This was accomplished using a mouse model that combines an intragastric (iG) chronic ethanol exposure and high fat diet (*Hybrid*) in C57BL/6J mice. This model produces clinically relevant liver pathology of chronic alcoholic steatohepatitis with macrophage inflammation and features of alcoholic hepatitis (Lazaro et al., 2015).

## 2. Materials and methods

### 2.1. Materials

All reagents were of research grade unless otherwise indicated. Ethanol at 200 proof (Rossville Gold Shield) was used for intragastric feeding of mice.

### 2.2. Methods

#### 2.2.1. Animals, exposure to ethanol and diet

The experiments were performed on age matched, male C57BL/6J mice obtained from Jackson laboratories (Bar Harbor, ME). All animals were treated in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

For the majority of the studies, we used a model that combines exposure to ethanol (EtOH) and Western type high fat diet termed as *Hybrid* throughout the manuscript. This model was developed at the Southern California Center for Alcoholic Liver and Pancreatic Disease and Cirrhosis (Director Dr. Tsukamoto). Briefly, 8 weeks old C57BL/6J mice were started on ad libitum high fat solid diet designated as HCFD (HCFD pellets composed of 1% w/w cholesterol, 20%Cal lard, 17% corn oil:HCFD, Dyets Inc. #180724) for 2 weeks followed by a surgery for placement of iG catheters. After recovery from the surgery (~1 week), mice were divided into 3 groups. The first group denoted as HCFD-EtOH received continuous infusion of ethanol (~27 g/kg/day, 35%Cal) plus liquid high fat diet (corn oil, 25%Cal) via iG catheters at 60% of total required calories for the duration of 6 weeks. Second group, denoted as HCFD-EtOH+Binge, was similar to the first with additional exposure to a binge bolus dose (3.5–5 g/kg) which was administered once a week. This bolus dose was injected through the iG catheters during the dark cycle after the ethanol infusion was withdrawn for 5–6 h. The third control group denoted as HCFD-Glucose was infused with dextrose to account for the 35% of calories from EtOH. Mice in all 3 groups continued to consume solid HCFD for the remaining 40% calories throughout the duration of the experiment. In addition, for some experiments, a subset of EtOH + Binge mice were also fed ad libitum regular Chow at 40% of total calories (denoted respectively Chow-EtOH+Binge). The corresponding Glucose controls were fed ad libitum Chow and received dextrose (denoted as Chow-Glucose). Shortly after the exposures, the iG tubes were removed, mice anesthetized using xylene and ketamine, decapitated and brain tissues removed for further processing.

Blood ethanol concentrations (BECs) measured using ANALOX GM7 Analyzer (Analox Instruments USA, MA) achieved in both *Hybrid* models (HCFD-EtOH and HCFD-EtOH+Binge) were ~200–400 mg% or 50–100 mM. This approach normally causes significant liver fibrosis with neutrophil infiltration which is characteristic of alcoholic hepatitis (Deng et al., 2005; Xu et al., 2011).

#### 2.2.2. RT-PCR

Right brain hemispheres were snap frozen and kept in −80 °C till RNA isolation. RNA was isolated using RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA) after tissue homogenization with microbeads in the TissueLyser (Qiagen, Valencia, CA). cDNA was then synthesized from 1 μg RNA using High Capacity RNA-to-cDNA Kit (Life Technologies, Grand Island, NY). The real-time PCR was performed using ABI 7900 fast real-time system (Life Technologies). GAPDH was used as normalization control. The following sets of primers were used: IL-1β forward — 5′-TCGCTCAGGGTCACAAGAAA-3′, IL-1β reverse — 5′-CATCAGAGGCAAGGAGGAAAAC-3′; IL-6 forward — 5′-TCGGAGGCTTAATTACACATGTTC-3′, IL-6 reverse — 5′-CAAGTGCATCATCGTTGTTCATAC-3′; TNFα forward — 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, TNFα reverse — 5′-TGGGAGTAGACAAGGTACAACCC-3′; MCP-1 forward — 5′-CCACTCACCTGCTGCTACTCAT-3′, MCP-1 reverse — 5′-TGGTGATCCTCTTGTAGC