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Research report

Activated microglia are implicated in cognitive deficits, neuronal death, and successful recovery following intermittent ethanol exposure

Ya-Nan Zhao, Fang Wang, Yan-Xia Fan, Guan-Fang Ping, Jing-Yu Yang, Chun-Fu Wu*

Department of Pharmacology, Shenyang Pharmaceutical University, 110016, Shenyang, PR China

HIGHLIGHTS

- Object recognition and spatial memory impairment were observed after intermittent ethanol binge.
- Long-term of neuronal death were detected during ethanol intoxication and abstinence.
- Microglia activation were remarkable during intoxication, while, microglia proliferation were obvious during withdrawal.
- microglia are involved in ethanol induced neurodegeneration and self-recovery after abstinence.

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ABSTRACT

Microglia function as the primary immune effector cells in the brain and play a pivotal role in the neuroinflammatory processes which are critical component of neurodegenerative diseases. Alcohol abuse has been considered as one of the common reasons for neurodegeneration although the causative factors are poorly understood. Here, we investigated whether activated microglia were implicated in neurodegeneration and cognitive dysfunctions in adult rats after intermittent alcohol abuse. Rats were given orally a priming dose of 5 g/kg ethanol and then 3 g/kg every 8 h for 4 days, followed by a 3-day ethanol-withdrawal period. These 4 days of ethanol treatments were repeated four times intermittently to simulate the binge drinking of human alcoholics. Neurodegeneration and microglial activation were detected by Fluoro-Jade B staining, Golgi staining, immunohistochemistry and ELISA, respectively, while cognitive function was assessed by Morris water maze and novel object recognition. The results showed that microglial activation and inflammatory cytokine expression were obvious in the parietal association cortex, entorhinal cortex and hippocampus accompanied by neurodegeneration following ethanol treatment. Moreover, learning and memory abilities also declined following ethanol treatments. However, the hypertrophied microglia disappeared accompanied by the decrease of inflammatory cytokines levels on day 4, and ramified microglial proliferated significantly on day 14 after ethanol withdrawal, along with a recovery from neuronal damage and cognitive impairment. Thus, the present study indicated that activated microglia might be involved in neurodegeneration and cognitive dysfunctions induced by intermittent ethanol exposure, and neurotrophic microglia appear to have a contribution to the recovery during abstinence.

1. Introduction

Ethanol abuse is one of the most costly health problems in the world [1]. Chronic excessive ethanol consumption commonly leads to structural and functional damage to the human brain, particularly in regions that are responsible for learning and memory [2–4]. Human epidemiological studies have demonstrated that alcoholics often suffer from cognitive deficits, including marked impairment in spatial learning and memory, as well as short term and declarative memory, which are associated with ethanol-induced impairment in the cortex and hippocampus [5–8]. Similarly, rats exposed to a single cycle of a binge alcohol paradigm have shown significant impairments in both spatial memory and non-spatial

Abbreviations: CD11b$^+$, CD11b-positive; CNS, central nervous system; DG, dentate gyrus; EC, entorhinal cortex; EtOH, ethanol-treated; Morris water maze, MWM; Novel object recognition, NOR; FJB, Fluoro-Jade B; FJB$^+$, FJB-positive; BrdU$^+$, BrdU-positive; PBS, phosphate buffered saline; ED-1$^+$, ED-1-positive; PtA, parietal association cortex.

* Corresponding authors at: Department of Pharmacology, Shenyang Pharmaceutical University, Box 31, 103 Wenhua Road, 110016, Shenyang, PR China.
Tel.: +86 24 23843567; fax: +86 24 23843567.
E-mail addresses: yangjingyu2006@gmail.com (J.-Y. Yang), wucf@syphu.edu.cn, chunfuw@gmail.com (C.-F. Wu).

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object recognition performance, paralleled with elevated levels of neurotoxicity in corticolimbic areas including the hippocampal dentate gyrus and the entorhinal cortex [9–12].

Microglia, the innate immune cells in the brain, have a beneficial healing effect as well as a toxic inflammatory effect in the central nervous system (CNS) [13,14]. Under normal conditions, microglia are typically found in a resting state as indicated by a ramified morphology releasing neurotrophic growth factors to support neurogenesis and increase neuronal survival [15,16]. In response to a multitude of CNS pathological conditions, microglia rapidly change to an activated state, characterized by a hypertrophied or bushy morphology, and they are toxic to neighboring neurons as observed in the neuroinflammation-associated pathogenesis of various CNS disorders [17–20].

In recent years, alcohol-induced neuroinflammation has been proposed as one of the alcoholism-induced neuropathological mechanisms since increased levels of microglial markers are observed in the brains of post-mortem human alcoholics [21]. Experimental evidence has demonstrated that ethanol induces microglial activation in vitro by stimulating the Toll-Like Receptor 4 response, and causes neuronal death when this microglia-conditioned medium is incubated with neuronal cells [22]. Corticolimbic brain damage in conjunction with microglial activation in the entorhinal cortex or hippocampus induced by binge ethanol exposure has similarly been found in rats [10,23]. However, the possible changes and potential roles of microglia in ethanol abuse have not been fully clarified.

Episodic alcohol intoxication, or intermittent binge-type drinking, such as alternating severe ethanol-intoxication and ethanol-withdrawal phases, is one of the drinking patterns of alcoholics [24]. Therefore, in the present experiment, following an intermittent ethanol binge, rats were used to simulate the alcoholism in human beings with binge drinking habits. How 'intermittent binge drinking' may affect the microglial response in the adult rat brain during ethanol intoxication as well as periods of abstinence, which was investigated for the first time. Meanwhile, neuronal activity and cognitive performance accompanied by microglia changes were examined in order to provide some clues about microglial functions in ethanol neuropathology.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats, initially weighing 270–300 g (age 8 weeks approximately), were supplied by the Experimental Animal Centre of Shenyang Pharmaceutical University. The animals were maintained under standard housing conditions (22 ± 2 °C, 50 ± 10% relative humidity, a 12 h light:dark cycle) and food and water were available ad libitum. All experiments were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and the experimental procedures were approved by the Local Committee on Animal Care and Use.

2.2. Intermittent ethanol exposure

Rats were subjected to intermittent ethanol exposure. Ethanol (25%, w/v) in a diluted nutritionally complete diet [50% (v/v) Vanilla Ensure (ABBOTT, Zwolle, Netherlands)] was administered intragastrically via an 18-gauge gavage needle. The ethanol-treated (EtOH) animals were given a priming dose of 5 g/kg ethanol (0.2 ml/10 g body weight), then an additional dose of 3 g/kg (0.12 ml/10 g body weight) every 8 h for 4 consecutive days at 7:00 AM, 3:00 PM, and 11:00 PM. After the 4-day ethanol exposure period, there was a 3-day ethanol withdrawal period. The total duration of the experiment was 25 days, including four cycles of ethanol intoxication and three cycles of ethanol withdrawal period. Control rats received a diet of 50% (v/v) Vanilla Ensure made isocaloric with dextrose, equal to the average of all the EtOH animals. All animals had free access to water throughout the ethanol treatment and free access to food and water during the ethanol withdrawal period. The doses, duration and the administration route of ethanol were based on previous studies [9,11,24,25].

The following experimental groups were used: one batch of animals [$n = 20$; 4 control animals; 16 EtOH animals] was used for histochemical analysis of Fluoro-Jade B (FJB) staining and immunohistochemistry (CD11b) at 1 h after the last dose of ethanol (D0), and withdrawal from ethanol for 4 days (D4), 7 days (D7) and 14 days (D14) following intermittent ethanol exposure. One batch [$n = 20$; 4 control animals; 16 EtOH animals] was used for immunohistochemistry (ED-1), immunofluorescence and Golgi straining. Another batch [$n = 40$; 8 control animals; 32 EtOH animals] was used for ELISA. Last batch ($n = 40$; 20 control animals; 20 EtOH animals) was used for behavioral assessment on D4, D7 and D14.

2.3. Blood ethanol determination

Blood samples were taken from the caudal vein 1 h after the last ethanol treatment and blood ethanol concentrations were measured by gas chromatography [26].

2.4. Bromo-deoxy-uridine (BrdU) incorporation

Rats were injected BrdU (50 mg/kg, dissolved in sterile saline, Sigma Chemical, Saint Louis, MO, USA) as previously described [27]. BrdU was given twice daily starting from D0 to D14. To examine a time course of changes in microglial proliferation after ethanol exposure, the EtOH groups were sacrificed at D0, D4, D7 or D14, respectively, and the control group was sacrificed at D14.

2.5. Behavioral analysis

2.5.1. Morris water maze (MWM)

Rats were tested in MWM to monitor their spatial learning and memory [25]. The water maze consisted of a pool that was 150 cm in diameter and 60 cm in height. It was painted black and filled with black nontoxic ink with the temperature at 22 ± 1 °C. The pool was divided into four quadrants named as northeast, southeast, southwest and northwest, respectively. A movable black circular platform (10 cm in diameter) was located in the center of quadrant and submerged 2 cm below the water surface. The water in the tank was stirred in between animal trials to disrupt odor trails. The room was furnished with several extramaze cues immobile throughout the entire experiment process. Task in the MWM comprised of two times-spatial reference memory tests and two times-reversal learning tests. The animal's movement was recorded and analyzed using a computerized video-tracking system (Ethovision® 8.0, Noldus Information Technology, Wageningen, Netherlands).

In the spatial reference memory test, from the fourth to sixth day or eleventh to thirteenth day post-ethanol treatments, rats were trained to find the hidden platform with the extramaze cues to examine acquisition of spatial reference memory. The platform was located in the southwest quadrant from the fourth to sixth day, or in the northwest from the eleventh to thirteenth day. Rats were trained twice a day for 3 consecutive days and each training consisted of four trials. In each trial, rats were placed into the pool, facing the wall at each of the four quadrant edges, pseudorandomly chosen across trials. If the rat found the platform within 60 s, it was allowed to stay in the platform for 10 s. If not, the experimenter guided the rat to the platform and allowed it to rest for 10 s. The rat was then returned to a holding cage for 60 s before the next trial. Two parameters were recorded: escape latency, swimming time to locate the hidden platform; path length, the distance of the swim path from the start location to the hidden platform.

In the reversal learning test, on D7 or D14 post-ethanol treatments separately, rats were assessed reversal learning ability. The platform was moved to the quadrant opposite that in which it had been placed during the prior reference memory task. Rats were tested once and each test consisted of four trials. The method used and parameters recorded were the same as reference memory task.

2.5.2. Novel object recognition (NOR)

The object recognition test was performed as previously described [12]. Briefly, rats were placed in a square box with an open top, painted black, 100 cm wide × 45 cm tall. The arena was dimly illuminated and surrounded by a black curtain to make environment uniform. The test consisted of ten consecutive 4 min sessions, each followed by resting in cages for 4 min. In session 1 (S1), the animal was placed in the empty open field. This first session served as a familiarization stage to prevent anxiety and to assess locomotor activity. In sessions 2–9 (S2–S9), the animal was exposed to a diagonal configuration of two objects 1 and 2 in the field and became habituated to this new environment. In session 10 (S10), object 1 was replaced by a novel object, object 3, to test recognition of the new object. All objects were located at the same distance from the wall of the field, and the field was cleaned with water and dried after every session. Each session was recorded by a video camera suspended above the field and interfaced with a computerized tracking system using Ethovision® 8.0 software (Noldus Information Technology, Wageningen, Netherlands). For each session, the duration of contacts was calculated. The duration of contacts in S10/S9 was used to measure the recognition memory. A contact was defined as any time the rat touched his nose or hands on the object to actively explore it, not just as a passing sniff as it passed by the object while walking around the field. The object recognition tests were performed on D4, D7 and D14 separately. For each test, different objects were used.