Behavioural Brain Research 288 (2015) 1–10

Contents lists available at ScienceDirect

Behavioural Brain Research

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

Research report

Flupirtine attenuates chronic restraint stress-induced cognitive deficits and hippocampal apoptosis in male mice

Pengcheng Huang[a], Cai Li[a], Tianli Fu[a], Dan Zhao[a], Zhen Yi[a], Qing Lu[a,b,c], Lianjun Guo[a,b,c], Xulin Xu[a,b,c,*]

[a] Department of Pharmacology, School of Basic Medicine, Tongji medical college, Huazhong University of Science and Technology, Wuhan 430030, China
[b] The Key Laboratory for Drug Target Researches and Pharmacodynamic Evaluation of Hubei Province, Wuhan 430030, China
[c] The Institute of Brain Research, Huazhong University of Science and Technology, Wuhan, 430030, China

HIGHLIGHTS

- Flupirtine rescues spatial learning and memory impairments in stressed mice.
- Flupirtine alleviates neuronal apoptosis in the hippocampus CA1 of stressed mice.
- Flupirtine attenuates synaptic loss in the hippocampus CA1 of stressed mice.
- Flupirtine activates Akt/GSK-3β signaling pathway in the hippocampus of stressed mice.

ARTICLE INFO

Article history:
Received 25 November 2014
Received in revised form 1 April 2015
Accepted 4 April 2015
Available online 11 April 2015

Keywords:
Chronic restraint stress
Flupirtine
Cognitive impairment
Akt (protein kinase B)
Glycogen synthase kinase 3 beta

ABSTRACT

Chronic restraint stress (CRS) causes hippocampal neurodegeneration and hippocampus-dependent cognitive deficits. Flupirtine represents neuroprotective effects and we have previously shown that flupirtine can protect against memory impairment induced by acute stress. The present study aimed to investigate whether flupirtine could alleviate spatial learning and memory impairment and hippocampal apoptosis induced by CRS. CRS mice were restrained in well-ventilated Plexiglass tubes for 6 h daily beginning from 10:00 to 16:00 for 21 consecutive days. Mice were injected with flupirtine (10 mg/kg and 25 mg/kg) or vehicle (10% DMSO) 30 min before restraint stress for 21 days. After stressor cessation, the spatial learning and memory, dendritic spine density, injured neurons and the levels of Bcl-2, Bax, p-Akt, p-GSK-3β, p-Erk1/2 and synaptophysin of hippocampal tissues were examined. Our results showed that flupirtine significantly prevented spatial learning and memory impairment induced by CRS in the Morris water maze. In addition, flupirtine (10 mg/kg and 25 mg/kg) treatment alleviated neuronal apoptosis and the reduction of dendritic spine density and synaptophysin expression in the hippocampal CA1 region of CRS mice. Furthermore, flupirtine (10 mg/kg and 25 mg/kg) treatment significantly decreased the expression of Bax and increased the p-Akt and p-GSK-3β, and flupirtine (25 mg/kg) treatment up-regulated the p-Erk1/2 in the hippocampus of CRS mice. These results suggested that flupirtine exerted protective effects on the CRS-induced cognitive impairment and hippocampal neuronal apoptosis, which is possibly associated with the activation of Akt/GSK-3β and Erk1/2 signaling pathways.

1. Introduction

Substantial evidence implicates stress is an important factor in the vulnerability to depression and other behavioral disorders [1]. Chronic restraint stress (CRS) can exacerbate neurodegeneration and cognitive deficits [2–4]. Growing evidences have shown that chronic stress causes atrophy and functional impairment in several key brain areas such as the frontal cortex and hippocampus [5,6]. The hippocampus is a region that plays a crucial role in learning and memory and is an area also particularly susceptible to chronic stress [7,8]. Extensive researches have proved that CRS disrupts the hippocampus-dependent cognitive function [4,9,10].

Abbreviations: CRS, chronic restraint stress; Con, control; Flu, flupirtine; DMSO, dimethyl sulfoxide; NS, normal saline; PBS, phosphate-buffered saline; ANOVA, analysis of variance; Akt, protein kinase B; GSK-3β, glycogen synthase kinase 3 beta; Erk1/2, extracellular signal-regulated kinase 1/2; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

* Corresponding author at: Department of Pharmacology, School of Basic Medicine, Tongji medical college, Huazhong University of Science and Technology, 13 Hangkong Road, Wuhan 430030, China. Tel./fax: +86 27 83691762.
E-mail address: xulinxu@hust.edu.cn (X. Xu).

http://dx.doi.org/10.1016/j.bbr.2015.04.004
0166-4328/

Akt (Protein kinase B) has been thought to be involved in neuronal survival, and activation of the kinase confers neuroprotection in several models of apoptosis [11–13]. Glycogen synthase kinase-3β (GSK-3β), a ubiquitous cellular serine/threonine protein kinase, plays a role in various essential physiological processes in the mammalian brain, such as development, cell cycle, or apoptosis [14]. GSK-3β serving as an essential downstream effector of Akt, its activity is inhibited by Akt-mediated phosphorylation at serine 9 [15]. It has been shown that GSK-3β is activated in the hippocampus of chronic stressed animals [9,16], and inhibition of GSK-3β restores chronic stress-induced memory deficit [17] and neuronal apoptosis [18].

Flupirtine is clinically used as a non-opioid analgesic with muscle relaxant [19,20]. In addition to its well-characterized as a Kv7 channel activator, flupirtine also acts like an NMDA receptor antagonist and has $GABA_A$ receptor-agonistic properties [21,22]. Several studies have demonstrated that flupirtine has significant powerful anti-oxidative and anti-apoptosis effects either *in vitro* or *in vivo* [23–25]. Flupirtine alleviates neuronal degeneration and cognitive impairment induced by repetitive hyperthermic seizures [25]. However, the molecular mechanisms for neuroprotective effects of flupirtine have not yet been fully understood. Furthermore, we have previously shown that flupirtine can prevent impairment of acute stress on spatial memory retrieval via inactivation of GSK-3β [26]. In the present study, we investigated the effects and underlying mechanisms of flupirtine on cognitive deficits and hippocampal apoptosis induced by CRS.

2. Materials and methods

2.1. Animals

Adult male Kunming (KM) mice, weighing 20–25 g, were obtained from the Animal Center of Tongji Medical College. Five mice were kept in a cage and were allowed free access to water and food. The mice were maintained at a constant temperature of 23 ± 1 °C, humidity at 55 ± 5% and under a 12:12 light/dark cycle (lights on at 7:00 a.m). The mice were allowed to acclimatize for 7 days before experiments. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Huazhong University of Science and Technology in accordance with the NIH guidelines for the care and use of laboratory animals (approval number: S400/5/1/2011). All efforts were made to minimize animal suffering and number of animals necessary.

2.2. CRS model and animals treatment

Adult mice were subjected to CRS as previously described [27,28] with minor modifications. The mice were restrained for 6 h (from 10:00 to 16:00) daily in well-ventilated Plexiglass tubes (3.3 cm diameter, 10.5 cm length) for 21 consecutive days. During restraint stress, animals were not physically compressed, but food and water were deprived. The same handle was carried on the unstressed mice. Animals were divided into six groups randomly:

1. Control group: mice were treated with vehicle (normal saline containing 10% DMSO) for 21 days (Con, $n = 15$);
2. Flupirtine group: mice were injected with flupirtine (10 mg/kg or 25 mg/kg) for 21 days (Flu10, $n = 13$; Flu25, $n = 15$);
3. CRS group: mice were injected with vehicle 30 min before restraint stress for 21 days (CRS, $n = 16$);
4. Flupirtine treatment group: mice were treated with flupirtine (10 mg/kg or 25 mg/kg) 30 min before restraint stress daily for 21 days (CRS + Flu10, $n = 14$; CRS + Flu25, $n = 14$).

Flupirtine maleate (Targsense scientific Co. Ltd, Shanghai, China) was dissolved in normal saline containing 10% DMSO in concentrations of 1 mg/ml and 2.5 mg/ml. Flupirtine or vehicle was administered intraperitoneally (i.p.) in a volume of 10 ml/kg body weight 30 min prior to the onset of restraint stress.

2.3. Morris water maze

The spatial learning and memory performance was determined using the Morris water maze (MWM) test as previously described [29] with minor modifications. The Morris water maze consisted of a stainless-steel circular pool (90 cm diameter, 45 cm height) and a Plexiglass platform (15 cm diameter). The container was filled with water (23 ± 2 °C) that was dyed by using non-toxic paint. The Plexiglass platform was submerged approximately 2 cm below the surface of the water and placed in center of second quadrant during the training session. Mice were given four trials per day for four consecutive days after CRS. Mice were placed into the tank facing the wall of the pool, and were allowed to swim and find the hidden platform. The time to reach the platform (escape latency) was recorded in each trial. During each trial, each mouse was given 60 s to find the hidden platform. If the mice failed to find the platform within 60 s, they would be guided to find the platform and stayed on it for 20 s. On 5th day after stress, the platform was removed and mice were tested on a spatial probe trial for 60 s. The time of mice spent in the target quadrant was recorded. An automatic tracking system MT-200 (Chengdu instrument Co., Chengdu, Sichuan province, China) was used to monitor swimming activities. After the Morris water maze test, mice were sacrificed for further histological and biochemical analysis.

2.4. Hematoxylin and eosin staining

Mice ($n = 4$–5) were deeply anesthetized with 10% chloral hydrate (i.p., 0.35 ml/100 g) and perfused with normal saline solution, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). After perfusion, the brains tissues were removed carefully and kept in 4% paraformaldehyde at 4 °C overnight. After fixation and dehydration with gradient ethanol, the brain tissues were embedded in paraffin and sliced into in the coronal plane at 5 μm thickness using a section cutter (Leica, Germany). The sections (2 sections/mouse) were stained with hematoxylin and eosin (H&E). Morphology of hippocampus was observed by a light microscope (Olympus Corporation, Japan). Damaged neurons were identified by their acidophilic (eosinophilic) cytoplasm and pyknotic nuclei, which are suggestive of necrotic morphology [30,31]. Cell counting of injured cells was performed in the pyramidal layer of the hippocampus CA1 of the mice.

2.5. Golgi silver staining

Dendritic spine of pyramidal neurons in the hippocampus was observed by Golgi silver staining as previous study [32]. Mice ($n = 4$–5) were anesthetized with 10% chloral hydrate and perfused with normal saline solution. The brain tissues were removed and stored in Golgi-Cox solution for 14 days, then kept in 30% sucrose solution. Brain tissues were sectioned at 50 μm thickness in the coronal plane using a vibratome (Camden Instrument, MA752, Leicester, UK). Dendritic images were acquired and analyzed with a microscope of OLYMPUS BX51 and software of Image-Pro Express. The numbers of dendritic spine in the hippocampus CA1 neurons (50 μm segment, 70 dendritic segments on 35 neurons from each group) were counted by the observers who were blind to experimental conditions.