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UCH-L1 inhibition aggravates mossy fiber sprouting in the pentylenetetrazole kindling model

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ABSTRACT

Mossy fiber sprouting (MFS) is a pathological phenomenon that is commonly observed in epilepsy, and plentiful data reveal that abnormal phosphorylated modification of tau protein plays a critical role in MSF by the regulation of microtubule dynamics and axonal transport. Ubiquitin C-terminal hydrolase L1 (UCH-L1), a proteasomal deubiquitinating enzyme, has been proved to be associated with tau aggregation through mediating degradation of ubiquitinated and hyperphosphorylated tau. Thus, this study aimed to determine the expression of UCH-L1 in the rat hippocampus during the pentylenetetrazole (PTZ)-induced process and to demonstrate the possible correlation with MFS in epileptogenesis. Seizures were established by intraperitoneal injection of PTZ and LDN-57444 was used to inhibit the hydrolase activity of UCH-L1. We used western blot, immunofluorescence, immunoprecipitation, and timm staining to detect phosphorylated modification of tau and MSF. The results presented that LDN-57444 induced the deteriorated severity of seizures, increased phosphorylation of tau and increased distribution of Timm granules in both the supragranular region of the dentate gyrus (DG) and the stratum pyramidale of CA3 subfield. Our results suggest that UCH-L1 may be associated with hippocampal MSF followed the epileptogenesis through mediating phosphorylation of tau. UCH-L1 may be a potential and novel therapeutic target to limit epileptogenesis.

1. Introduction

Epilepsy is one of the most common neurological disorders characterized by neuronal hyperexcitability and suddenly synchronized discharges that can manifest as seizures. In about 30%–40% of patients with epilepsy are refractory to current anti-epileptic drugs, resulting in serious cognitive impairment, sudden unexpected death in epilepsy, accidents, suicide, vascular disease, pneumonia et al [1]. It has been widely ascertained that mossy fiber sprouting (MFS) contributes to morphological synaptic changes and hyper-excitable neuronal transmission, which involves in the development of epilepsy-epileptogenesis [2]. MSF is a phenomenon that the mossy fibers branch out of the dentate hilus and abnormally innervate the dentate inner molecular layer of dentate gyrus (DG) subfield and the stratum pyramidale of CA3 subfield [3]. MSF has been described in almost all epileptic models and has been consistently identified in humans with condition [4]. Sutula et al., for example, found that MSF is positively correlate with seizure frequency and severity in the pilocarpine-induced epilepsy model [5]. However, the mechanisms underlying MFS involved in epileptogenesis are quite complex and remains unclear.

Tau protein is a microtubule-associated protein which is almost exclusively found in axon, although dendrites, oligodendrocytes, and astrocytes can also present low levels of tau protein [6,7]. It promotes microtubule assembly and stabilizes the microtubule network, thereby regulating neural polarization, axonal transport, axonogenesis, neuronal signal, synaptical plasticity, and neuronal survival [8,9]. Those functions are mediated by post-translational

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modifications of tau protein and phosphorylation of tau is the most common and crucial to induce loss of its function by preventing its interaction with microtubules [9]. The abnormal microtubules caused by accumulation of hyperphosphorylated tau plays an important role in the MSF in epilepsy [10,11]. Although various kinases participate in mediating phosphorylation of tau, it has been suggested that other non-kinases are also related with phosphorylation of tau.

Ubiquitin C-terminal hydrolase L1 (UCH-L1) is an important component of the ubiquitin-proteasome system and is abundantly expressed in the brain where it can make up to 5% of total neuronal protein [12]. UCH-L1 is absolutely required for the maintenance of axonal integrity and its dysfunction is implicated in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [12]. Since UCH-L1 has both hydrolase and ligase activity, it mediates the degradation of post-translational modifications of tau. Xie et al. suggested that UCH-L1 inhibition could suppress tau aggresome through decreasing degradation of ubiquitinated and hyperphosphorylated tau [13,14]. Thus, UCH-L1 may also function in epileptogenesis, particularly in MFS by a mechanism involving tau phosphorylation. In this study, we used LDN-57444 to inhibit hydrolase activity of UCH-L1 in pentylenetetrazole (PTZ)-induced epilepsy, and then explored the correlation between UCH-L1 and tau and the involvement of UCH-L1 in epileptogenesis.

2. Materials and methods

2.1. Experimental animals

A total of 100 male Sprague–Dawley rats (8 weeks old) weighing 200–250 g were obtained from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). The rats were raised in a temperature-controlled room ($22 \pm 2\,^{\circ}C$) with a 12 h light/12 h dark cycle as well as free access to food and water. All animal procedures were approved by the Commission of Chongqing Medical University for the Ethics of Experiments on Animals in accordance to international standards. Animal care and sacrifice were conducted according to methods approved by the Chongqing Medical University Animal Experimentation Committee.

2.2. PTZ kindling and drug treatments

Animals were intraperitoneally injected with convulsive doses of PTZ (Sigma-Aldrich, USA) 35 mg/kg in saline every second day for 29 days (15 injections). After each injection, rats' behavior was immediately monitored for 1 h according to Racin's Scale [15]: grade 0, no change in behavior; grade 1, chewing; grade 2, gazing and head nodding; grade 3, unilateral forelimb clonus, twitching, and scratching; grade 4, rearing with bilateral forelimb clonus; grade 5, widespread muscle spasms, rearing with bilateral forelimb clonus and falling back. The rats that showed seizure activity with a score of at least a 4 for three consecutive days were considered successfully kindled. Only the successfully kindled rats were used as the PTZ group. The rats in control group received equal amount of saline instead of PTZ. UCH-L1 inhibitor LDN-57444 (0.4 mg/kg, San Diego, USA) was intraperitoneally injection 1 h before the PTZ injection each day, and these rats were selected as the PTZ + LDN-57444 group [16]. Saline (1 ml/kg) was intraperitoneally injection 1 h before the PTZ injection each day, and these rats were selected as the PTZ + saline group.

2.3. Tissue processing

After 24 h of last PTZ injection, rats were deeply anesthetized by intraperitoneal injection of 3.5% chloral hydrate (10 ml/kg). Hippocampus was separated and stored at − 80 °C for western blotting and co-immunoprecipitation (Co-IP). For immunofluorescence, the rat brains were removed after perfusion with saline and 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS) by cardiac puncture via the left ventricle, and then underwent 24-h dehydration using 10%, 20% and 30% sucrose respectively. After dehydration, rat brains were sectioned at 10 μm and stored at − 80 °C. For timm staining, The rats were anesthetized with chloral hydrate and intracardially perfused with 0.9% saline followed by 0.375% sodium sulfide and 4% PFA in PBS. The brains were removed carefully post-fixed 20 h and then equilibrated sequentially with 30% sucrose at 4 °C for 72 h. Brains were sectioned into 30 μm -thick coronal sections and processed for Timm staining.

2.4. Western blotting and Co-IP

To extract proteins, hippocampus of rats was homogenized in RIPA lysis buffer (Beyotime, Haimen, China) containing protease and phosphatase inhibitor mixture and cleared of debris by centrifugation at 12000 rpm, 4 °C for 30 min. After measuring protein concentrations by bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Haimen, China), harvested proteins were mixed with 5 × sodium dodecylsulfate (SDS) loading buffer and boiled for 5 min. Electrophoresis and electrotransfer were performed according to previous studies [17–19]. The primary antibodies included rabbit anti-UCH-L1 (1:1000, Proteintech, China), rabbit anti-PHF-1 (a tau phosphorylated sites at Ser 396/404; 1:2000, Abcam, UK), rabbit anti-p-tau-S396 (1:1000, Abcam, UK), rabbit anti-tau (1:1000, Abcam, UK) and rabbit anti-GAPDH (1:3000, Proteintech, China). Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000, Proteintech, China) was used as secondary antibodies. Immunoreactivity was visualized were by an enhanced chemiluminescence (ECL) kit (Beyotime, China). The density of the bands were analyzed by and quantified by the Fusion-FX7 system (Vilber Lourmat, France) and The mean optic density (OD) was normalized by GAPDH or tau. Co-IP was performed using Protein A/G agarose (Beyotime, China) following the manufacturer's instructions and normal rabbit anti-IgG (Abcam, UK) was used as control antibody. Harvested samples containing input (protein pre-treated with nothing), IgG (protein pre-treated with A/G agarose and rabbit anti-IgG), anti-UCH-L1 (protein pre-treated with A/G agarose and rabbit anti-UCH-L1) and anti-tau (protein pre-treated with A/G agarose and rabbit anti-tau) were analyzed with Western blot.

2.5. Immunofluorescence

Immunofluorescence was conducted to detect the location of UCH-L1 and co-location between UCH-L1 and tau in according to previous studies [17–19]. Primary antibodies containing rabbit anti-UCH-L1 (1; 50, Proteintech, China), mouse anti-GFAP (1:50, Boster, China), mouse anti-NeuN (1:100, Millipore, USA) and mouse anti-Tau (1:50, Abcam, UK) were used. Secondary antibodies containing goat anti-rabbit FITC (1:50, Proteintech, Wuhan, China) and goat anti-mouse Alex Flour 555 (1:50, Beyotime, Haimen, China) were used. After staining the nucleus by DAPI, images were captured using confocal laser scanning microscope ($A1^{+}R$, Nikon, Tokyo, Japan).

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