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Mild hypothermia inhibits the Notch 3 and Notch 4 activation and seizure after stroke in the rat model

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ABSTRACT

Ischemic brain injury is an important cause for seizure. Mild hypothermia of the brain or the whole body is an effective way to remit the post-stroke seizure. Our previous study revealed an implication of Notch 1 and 2 in the post-stroke seizure. This study further investigated the involvement of Notch 3 and 4 in post-stroke seizure and the effect of mild hypothermia on these two factors. A global cerebral ischemia (GCI) model was conducted in Sprague Dawley rats. Seizure activity was evaluated by the frequency of seizure attacks, seizure severity scores, and seizure discharges. Seizures were frequently occurred in the first and the second 24 h after GCI, however active whole-body cooling (mild hypothermia) and DAPT (Notch inhibitor) injection into the hippocampus, alone or in combination, alleviated seizure activity after GCI. Immunohistochemistry and Western blot assays revealed the up-regulation of Notch intracellular domain (NICD) 3 and 4 in the cerebral cortex and hippocampus following GCI, but mild hypothermia and DAPT inhibited the up-regulation of NICD 3 and 4. NF-κB, PPARα, PPARγ, cyclin D1, Sox2 and Pax6 are associated with the pathogenesis of diverse type of seizures. GCI induced NF-κB, cyclin D1, and Pax6 activity, but suppressed PPARγ. These effects of GCI were abolished by both mild hypothermia and DAPT treatment. This indicated the implication of Notch signaling in the effects of GCI. Collectively, mild hypothermia inhibits Notch 3 and Notch 4 activation and seizure after stroke in the rat model. This study adds to the further understanding of the pathogenesis of post-stroke seizures and the protective mechanism of mild hypothermia.

1. Introduction

Stroke is a leading cause for seizures although the underlying mechanisms remain to be determined [1–3]. Post-stroke seizures are characterized by recurrent unprovoked seizures with hyperexcitability of neurons predominantly in cerebral cortex and hippocampus. This type of seizure not only causes a poor life quality, but also increases mortality of the patients [1–3]. Clinical evidence shows that seizures are associated with increased fatality and mortality at the first year after stroke, even in patients with a less severe stroke [1]. Stroke-induced seizures are often refractory to conventional antiepileptic drugs, but mild hypothermia (32–35 °C) of the brain or the whole body is effective against seizures in neonates with perinatal hypoxic ischemic encephalopathy [4]. Therapeutic hypothermia selectively cooling the head or whole body prevents the neonatal brain from cerebral ischemia injury and improves following neurodevelopment of infants, which are associated to relatively lower seizure incidence after cerebral ischemia [4–6].

Increasing evidence supports that abnormal activation of Notch signaling is an important cause of the occurrence and development of seizures [7]. Mammalian Notch is known as transmembrane receptors containing four receptor isoforms Notch 1–4 [8,9]. Notch activation is dependant on combination with its mammalian ligands Delta and Serrate and subsequent enzymatic cleavages to generate the Notch intracellular domain (NICD). NICD with transcriptional activity mediates a highly conserved signaling cascade [8,9]. It has known that Notch signaling pathway participates in various physiological functions of brain [10], however aberrant activation of Notch signaling causes the occurrence of seizures in an epilepsy model [7]. In addition, our recent study revealed that Notch 1 and 2 are activated in the cerebral cortex and hippocampus after cerebral ischemia and associated to the post-stroke seizures[11]. There is also evidence implying the implication of Notch 3 and 4 in the pathogenesis of seizures. Previous study found that Notch 3 mutation causes the inherited subcortical infarcts and leukoencephalopathy (CADASIL) [11,12]. Patients with CADASIL have complications, including seizures, epilepsy, coronary artery disease, and abdominal aorta aneurysm [12,13]. A genetic association study using transgenic mice shows that up-regulation of active Notch 4

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provokes arteriovenous malformations. Arteriovenous malformations can lead to neurological impairments and the occurrence of seizures, headaches, and haemorrhage [14].

This study was aimed to investigate the implication of Notch 3 and 4 in the pathogenesis of post-stroke seizures and the influence of mild hypothermia to Notch 3 and 4 expression following stroke. The results were supposed to add to the further understanding of the pathogenesis of post-stroke seizures and the protective mechanism of mild hypothermia.

2. Materials and methods

2.1. Animals

The animal study was approved by the Animal Care and Use Committee of the Central South University. Seventy male Sprague Dawley (SD) rats (8–10 week old, Central South University, Changsha, China) were kept under environmentally controlled conditions (12 h light/dark cycles, 20–23 °C and 50% relative humidity). Food and water were provided *ad libitum*. All animal procedures were carried out in accordance with National Institutes of Health Guidelines for the care and use of animals. Rats with severe epilepsy were treated with clinically appropriate anticonvulsants. Experiments were completely randomized and were analyzed by personnel kept blind to treatment and time point.

2.2. Global cerebral ischemia (GCI)

Global cerebral ischemia was performed using a 'L' shape stick according to method described in previous study [15]. Briefly, animals were anesthetized with 2% isoflurane and subsequently placed in a supine position with four extremities fastened to the table. The 'L' shape stick was inserted into the mediastinum at the level of the second intercostal segment and the distal end of the stick was twisted 45° to be positioned under the bundle of major cardiac blood vessels. To interrupt blood flowing to brain, the stick was lifted up with finger pressure from outside of the chest. The compression for 2–3 min could result in cardiac arrest. At this time, rats were immediately worn a rodent respirator to help to recover heart beating and blood supply to brain. Rats (n = 9) in the sham group were subjected to the same procedure without compression of the carotid arteries.

2.3. Experimental design

Rats with convulsive seizures (n = 3 each experimental run) after GCI were randomly selected and humanely euthanized at indicated time points (3 h, 6 h, 12 h, 24 h, 48 h, 3 d and 7 d). In addition, a group of rats after GCI were randomly divided into 4 subgroups: GCI group (no further treatment, n = 18), mild hypothermia group (n = 24), DAPT group (n = 24) and mild hypothermia + DAPT group (n = 30). Rats in last 3 groups were subjected to treatment with mild hypothermia and DAPT injection, alone or in combination. Protein levels of NICD 3 and 4 in specific regions of the brain were tested using a western blot and immunohistochemistry assays.

2.4. Mild hypothermia

Active whole-body cooling was performed via a blanket cooling device (Cincinnati Subzero Blanketrol III, Cincinnati, Ohio., USA) immediately after the GCI. Core temperatures of rats as measured by a rectal probe were maintained at 33.5 °C for 30 min. At the end of the cooling period, the animal was transferred to a heating pad and allowed to warm up to normal body temperature. Thereafter, the animal was released into the normal housing cage.

2.5. DAPT injection

DAPT injection was following the method described by Sha et al. (7). DAPT (D5942, Sigma, St. Louis, MO, USA) was dissolved in 0.5 μl PBS (pH7.4) for a concentration of 50 μM. PBS containing 0.5% DMSO was used as the vehicle. The DAPT solution or vehicle was unilaterally injected into the hippocampus (0.5 μl/2 min) with a needle protruding about 2.3 mm from the guide cannula, after mice regained consciousness following GCI injury (20–30 min) and the treatment was continued once daily until rats were slaughtered.

2.6. Evaluation of seizure severity

To detect seizures following MCAO, animals were placed under continuous visual surveillance by experimenters with concurrent EEG recordings. The appearance of seizures were exemplified by rapid running, jumping, barrel rolling (≥3 turns), falling (loss of righting reflex) with tonic limb flexion, and repetitive tail erection. The seizure severity was evaluated by scoring method [16]: 0 = normal behavior; 1 = immobility; 2 = spasm, tremble, or twitch; 3 = tail extension; 4 = forelimb clonus; 5 = generalized clonic activity; 6 = jumping or running seizures; 7 = full tonic extension and 8 = death. The seizure severity scores were given by at least two neurologists blinded to treatments and to time post-injury.

EEG recordings was performed in free-moving animals using an amplitude-integrated EEG monitor as previously described [17]. Rat restrainer was used when rat exhibited vigorous convulsive behavior such as jumping and rapid running. Video-EEG monitoring was connected to a custom-built digital video-EEG monitoring system (model 1800, AM Systems, Carlsborg, WA, USA). Electrodes were implanted bilaterally into the hippocampal CA1 (bregma −2.3 mm, lateral 2.0 mm and depth 2.0 mm) and parietal cortex (bregma −0.6 mm, lateral 1.5 mm and depth 1 mm). Signals were collected in a frequency bandwidth of 0.1–1000 Hz, amplified 1000 times and then digitized at ≥5 KHz (Digidata 1300, Molecular Devices; Sunnyvale, CA, USA). Data were analyzed using pClamp software (version 10; Molecular Devices).

2.7. Western blot assay

After rats were anesthetized with 5% isoflurane, the cerebral cortex and hippocampus were harvested and stored in −80 °C until further use. Homogenized tissue samples were suspended in a cell lysis buffer containing 10% protease/phosphatase inhibitor cocktail. The extracted protein were run on 4–20% gradient SDS gels (Invitrogen) and subsequently transferred onto nitrocellulose membranes. Nitrocellulose membranes were blocked with non-fat milk for 2 h, followed by overnight incubation in primary antibodies against NICD3 (1:500 dilution; 55114-1-AP, Proteintech Inc., Chicago, USA), NICD4 (1:500 dilution; sc-5594, SANTA, Santa Cruz, CA, USA), p-p65(S536) (1:500 dilution; bs-0982R, BIOSS, Boston, WWLP, USA), PPARα (1:500 dilution; sc-9000, SANTA), PPARγ (1:500 dilution; sc-7196, SANTA), Cyclin D1 (1:500 dilution; #2926, CST, Shanghai, China), SOX-2 (1:400 dilution; sc-20088, SANTA), PAX6 (1:1000 dilution; ab5790, Abcam, Cambridge, UK) and GAPDH (SC-365062, SANTA), at 4 °C. Nitrocellulose membranes were incubated in horseradish peroxidase-conjugated secondary antibodies that target mouse IgG (ab97040, Abcam) or rabbit IgG (A0545, Sigma, St Louis, MO, USA) at a 1:2,000 dilution for 2 h at room temperature. Reactive proteins were detected using Enhanced Chemiluminscent and SuperSignal™ Chemiluminescent substrates (Pierce, Rockford, IL, USA).

2.8. Immunohistochemistry

The cerebral cortex and hippocampus tissues were fixed in 10% formalin with 20% sucrose overnight at 4 °C and subsequently rinsed several times with PBS (pH 7.4.). Tissue sections were blocked with PBS