



VEGF regulates hippocampal neurogenesis and reverses cognitive deficits in immature rats after status epilepticus through the VEGF R2 signaling pathway

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ABSTRACT

Epilepsy is the most common chronic disease in children, who exhibit a higher risk for status epilepticus (SE) than adults. Hippocampal neurogenesis is altered by epilepsy, particularly in the immature brain, which may influence cognitive development. Vascular endothelial growth factor (VEGF) represents an attractive target to modulate brain function at the neurovascular interface and is a double-edged sword in seizures. We used the lithium-pilocarpine-induced epilepsy model in immature Sprague–Dawley rats to study the effects of VEGF on hippocampal neurogenesis in the acute phase and on long-term cognitive behaviors in immature rats following status epilepticus (SE). VEGF correlates with cell proliferation in the immature brain after SE. By preprocessing VEGF in the lateral ventricles prior to the induction of the SE model, we found that VEGF increased the proliferation of neural stem cells (NSCs) and promoted the migration of newly generated cells via the VEGF receptor 2 (VEGFR2) signaling pathway. VEGF also inhibited cell loss and reversed the cognitive deficits that accompany SE. Based on our results, VEGF positively contributes to the initial stages of neurogenesis and alleviates cognitive deficits following seizures; moreover, the VEGF/VEGFR2 signaling pathway may provide a novel treatment strategy for epilepsy.

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1. Introduction

Children are at a higher risk for seizures than adults. Status epilepticus (SE) is a common acute disorder in pediatric neurology. Frequent or prolonged seizures in the immature brain result in irreversible brain damage and long-lasting sequelae. These seizures may induce neuronal cell loss, interfere with developmental programs, lead to epilepsy and cognitive impairments, and may seriously influence children's quality of life [1–3]. Physicians must choose a reasonable treatment in time to reduce the damage following SE.

Hippocampal neurogenesis is altered by many neurological diseases, particularly in the immature brain, which may influence cognitive development [4–5]. Reports have described endogenous neural stem/progenitor cells (NSCs) in the hippocampus that undergo self-repair throughout epileptogenesis. As shown in our previous study,

endogenous NSCs are stimulated to undergo self-repair following SE. However, some of the newly proliferating cells underwent apoptosis. Cell proliferation and apoptosis are closely related to the surrounding environment post-SE [6]. Perhaps the newly proliferated cells lack the necessary survival mechanisms.

In the past few decades, there has been great interest in the factors that modulate the microenvironment, and these factors may play key roles in the proliferation, migration, differentiation, and survival of NSCs, as well as in the functions of neurotrophic factors following brain injury. These factors include brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and vascular endothelial growth factor (VEGF) [7]. However, the mechanisms that regulate endogenous stem cells are poorly understood.

Vascular endothelial growth factor is the most important factor in the microenvironment. VEGF (also known as VEGF-A) is a potent angiogenic and neurogenic growth factor and is one of the important factors in the NSC microenvironment. In the brain, VEGF receptors (VEGFRs) are localized to the vascular endothelium, glia, and neurons. To date, the following VEGF family members have been identified: VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF) [8–12]. VEGF has numerous roles in the diseased nervous system, including multiple direct beneficial effects on various types of neural cells. However,

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VEGF may be a double-edged sword because of its roles in inflammation and neuroprotection following SE [13,14].

Whether VEGF has opposite effects on the immature brain in epilepsy remains an open question. In this study, we first explored the spatiotemporal pattern of VEGF expression in the epileptic hippocampal formation of a rat model, particularly in the dentate gyrus (DG). Furthermore, we aimed to explore the role of VEGF preprocessing in regulating neurogenesis in the immature brain during the acute phase following SE. We also wanted to determine whether VEGF can reverse the cognitive deficits observed in the immature brain in the chronic phase following SE. Based on our results, therapies targeting the VEGF/VEGFR2 signaling pathway may be effective at enhancing hippocampal neurogenesis and alleviating cognitive impairments following SE.

2. Methods

2.1. Animals

All animal experiments were conducted in accordance with protocols approved by the Animal Care Committee of Chongqing Medical University, Chongqing, China. Animals were housed in a controlled environment (food and water available *ad libitum*, 21 ± 1 °C, humidity 60%, lights on from 7:00 AM–7:00 PM).

2.2. LiCl-pilocarpine-induced rat model of epilepsy

All subjects were 20-day-old male Sprague–Dawley rats weighing 40 to 50 g. Seizures were scored from stages 1–5, based on a modification of a previously published scale [15,16]. SE was defined as seizures with no intervening return to normal behavior for greater than 5 min. SE was induced by an intraperitoneal (i.p.) injection of pilocarpine (pilo, 30 mg/kg, Sigma) 18 h after the lithium chloride injection (127 mg/kg, i.p., Solarbio, Beijing, China). Atropine (Shuanghe, Beijing, China) was administered via an i.p. injection (1 mg/kg) 30 min prior to the pilo injection to reduce peripheral effects. Sixty minutes after SE, all rats received a single dose of diazepam (10 mg/kg, i.p.). Animals that did not reach SE were excluded from all subsequent analyses. Control animals received i.p. injections of the same volume of sterile saline. The rats were sacrificed by decapitation under deep anesthesia with sodium pentobarbital at various time points (12 h or 1, 3, 7, 14, 28 or 56 days after SE) to evaluate the dynamic changes in NSCs.

2.3. Bromodeoxyuridine labeling

The cell proliferation marker BrdU (Sigma, St. Louis, MO, USA) was dissolved in saline and was i.p. injected (50 mg/kg) 2 h after SE. BrdU was injected every 8 h for three times. The rats were sacrificed 24 h after the last BrdU injection.

2.4. VEGF and SU5416 administration

The rats were anesthetized via an i.p. injection of 10% chloral hydrate (3 ml/kg) and placed in a stereotactic frame. A Hamilton syringe was placed in the right lateral cerebral ventricle at predetermined coordinates (1.2 mm posterior and 1.2 mm lateral from lambda, and depth of 3.8 mm). The speed of administration was 0.5 μ l/min. Before drug administration, the validity of injection was determined by injecting a blue dye into a separate litter.

The subjects received a single intracerebroventricular injection of human recombinant VEGF165 (20 ng, 40 ng or 60 ng per rat, Peprotech, Rocky Hill, USA) 12 h before the induction of SE. Control animals received phosphate-buffered saline (PBS) with 0.4% bovine serum albumin (BSA). SU5416 (Selleck, USA), a VEGFR2 inhibitor, was dissolved in dimethylsulfoxide (DMSO) and was intracerebroventricularly injected at a dose of 5 mM per rat after the lithium injection.

2.5. VEGF enzyme-linked immunosorbent assay (ELISA)

Animals were sacrificed at various time points after SE to analyze the VEGF protein levels using an ELISA. The hippocampal tissue was collected and homogenized according to the instructions for the rat VEGF ELISA. The VEGF ELISA protocol was performed according to the instruction manual provided with the rat VEGF ELISA kit (Neobioscience, Shenzhen, China).

2.6. Nissl staining

Animals were euthanized with sodium pentobarbital 3 days after the induction of SE and transcardially perfused with heparinized saline (NS), followed by 4% paraformaldehyde in PBS. Brains were post-fixed in 4% paraformaldehyde for 48 h at 4 °C. Paraffin sections were cut coronally at a thickness of 5 μ m. The sections were stained with cresyl violet for the subjective evaluation of hippocampal damage.

2.7. Western blot analysis

The hippocampus of each rat was rapidly dissected and stored in liquid nitrogen immediately after dissection. A Bio-Rad protein assay kit (Bio-Rad Laboratories, USA) was used to determine the protein concentration. Samples containing 30 μ g of protein were loaded on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrophoretically separated. The proteins were then transferred to polyvinylidene difluoride membranes (0.22 μ m, Millipore Corp, Billerica, MA, USA). PBS containing 10% milk was used to block the membranes for 1 h. The membranes were incubated with the following specific primary antibodies overnight at 4 °C: anti-VEGFR2 (rabbit polyclonal antibody, Abcam, 1:1000) anti-caspase 3 (rabbit polyclonal antibody, Gene Tex, 1:1000), anti-phospho-AKT (rabbit polyclonal antibody, Cell Signaling Technology, 1:1500). Parallel western blots probed with an anti- β -actin antibody (1:500, Chemicon) were used to normalize the results. The intensities of the protein bands in the western blots were analyzed by densitometry using ACD see Pro2.5 and Image J software.

2.8. Immunofluorescence

Each rat brain was cut into consecutive frozen coronal sections at a thickness of 40 μ m and stored in cryoprotectant solution at 4 °C until immunofluorescence staining was performed. The following primary antibodies were used: anti-Ki67 (rabbit monoclonal antibody, Abcam, 1:50), anti-BrdU (mouse monoclonal antibody, Millipore, 1:100), anti-Dcx (rabbit polyclonal antibody, Abcam, 1:200), and anti-polysialic acid (PSA)-NCAM (mouse monoclonal antibody, Millipore, 1:80). All of the antibodies listed were incubated with the tissue overnight. All images were captured using a laser-scanning confocal microscope.

2.9. Morris water maze

On the first day, each animal was placed in a 150-cm diameter water maze for a 1-min adaptation period to avoid stress. For the acquisition trials, each animal was tested four times each day. For each trial, the animal was randomly placed at one of four starting positions (N, E, S, or W), with an inter-trial interval of 1 min. Each trial ended when the animal escaped onto a submerged, hidden goal platform for 3 s. Any animal that had not located the platform within 60 s was guided to the platform by hand and allowed to remain in place for 15 s. The mean latency to find the platform was calculated as the escape time. After 4 days of acquisition trials, the goal platform was removed for a spatial probe trial in which each animal was placed in the maze last 60s for 2 times, with an inter-trial interval of 30-min. The mean time spent in the target quadrant and the mean number of times the animals crossed the platform were recorded.

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