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Research report

Enriched housing promotes post-stroke neurogenesis through calpain 1-STAT3/HIF-1α/VEGF signaling

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ABSTRACT

Enriched environment (EE) has been shown to promote neurogenesis and functional recovery after ischemic stroke. However, the underlying molecular mechanisms are not fully understood. In this study, C57BL/6 mice underwent middle cerebral artery occlusion (60 min) followed by reperfusion, after which mice were housed in either standard environment (SE) or EE and allowed to survive for 3, 4, 6 or 10 weeks. Ipsilateral subventricular zone (SVZ) or striatum cells were dissociated from ischemic hemispheric brains of enriched mice at 14 days post-ischemia (dpi) and cultured *in vitro*. Our data showed that post-ischemic EE inhibited calpain 1 activity, and increased the expression of phosphorylated signal transducer and activator of transcription 3 (p-STAT3) in the ischemic hemisphere of enriched mice at 21 dpi. Calpain 1-specific inhibitor PD151746 further increased p-STAT3 expression and augmented the promoting effects of EE on post-stroke SVZ neural precursor cells (NPCs) proliferation and functional recovery. EE also increased the expression of hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) in the ischemic hemisphere at 21 dpi. Inhibition of the JAK/STAT3 pathway with AG490 decreased the expression of HIF-1α and VEGF. Furthermore, inhibition of HIF-1α with 2-methoxyestradiol robustly abolished EE-induced elevation of VEGF l expression. Furthermore, VEGF-A promoted the production and secretion of high mobility group box-1 protein (HMGB1) from reactive astrocytes *in vitro*. Culture supernatant from reactive astrocytes treated with VEGF-A promoted the proliferation and differentiation of NPCs. Glycyrrhizin reversed the promoting effects of EE on post-stroke neurorepair and functional recovery *in vivo*. Taken together, our data indicate that EE promotes post-stroke functional recovery through the inhibition of calpain 1 activity, and subsequent STAT3-HIF-1α-VEGF-mediated neurogenesis.

1. Introduction

Stroke is a major cause of death and disability worldwide because of the brain's limited capacity for neural repair and spontaneous recovery (Donnan et al., 2008; Go et al., 2014). An enriched environment (EE), including social interactions, voluntary and varied physical activity, introduction of novel objects, has been a classic paradigm for studying the effects of a complex combination of physical, cognitive, and social stimulation in rodents. EE is shown to be able to increase neurogenesis in the adult subventricular zone (SVZ) and angiogenesis (Komitova et al., 2005; Yu et al., 2014; Zhang et al., 2016a,b), ultimately promotes spontaneous recovery in animals after ischemic stroke (Madinier et al., 2014; Yu et al., 2014; Zhang et al., 2016a,b). However, the underlying molecular mechanisms are not fully understood.

Calpain activity is shown to be modulated during neural differentiation of rat pheochromocytoma cells (Pinter et al., 1994; Vaisid et al., 2005). As the most abundant calpain molecules in the brain (Liu et al., 2008), calpain 1 maintains stemness and represses neural differentiation of NPCs, while calpain 2 acts as potential modulator of gliogenesis *in vitro* (Santos et al., 2012).Furthermore, one previous study suggests an essential role of calpain 1 in astrocytic IL-17A-mediated neurogenesis after ischemic stroke (Zhang et al., 2016a,b). Based on these findings, we aim to explore the potential role of calpain 1 in EE-induced neurogenesis in the later phases of stroke recovery.

Signal transducer and activator of transcription-3 (STAT3) is a member of the STAT protein family of transcription factors, which

Abbreviations: EE, enriched environment; SE, standard environment; SVZ, subventricular zone; Dpi, day(s) post-ischemia; STAT3, signal transducer and activator of transcription 3; NPCs, neural precursor cells; HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular endothelial growth factor; HMGB1, high mobility group box-1 protein; MCAO, middle cerebral artery occlusion; 2ME2, 2-methoxyestradiol; BrdU, 5-Bromo-2′-deoxyuridine; EBST, elevated body swing test; LPS, lipopolysaccharide; GFAP, glial fibrillary acidic protein; SBDP145, aII-spectrin breakdown products of 145 kDa

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coordinate and integrate signals from extracellular stimuli and play an important role in the growth and differentiation of a variety of cell types (Horvath, 2000; Reich and Liu, 2006). Upon phosphorylation, STAT3 dimerizes and translocates to the nucleus, where it serves as a dynamic, sensitive molecular on-off switch for transcription of target genes (Reich and Liu, 2006). STAT3 inhibition mediates ciliary neurotrophic factor (CNTF) repression, whereas endogenous CNTF stimulates normal neuroblast formation from the SVZ, thus promotes neurogenesis in the adult central nervous system (CNS) (Keasey et al., 2013). STAT3 is shown to be needed for motor neuron differentiation in the developing CNS (Lee et al., 2013). STAT3 is also activated in response to injurious stimuli and may play an important role in neuronal survival and regeneration (Dziennis and Alkayed, 2008). Evidence has shown that cerebral ischemia promotes activation and nuclear translocation of STAT3 and STAT3-dependent transcription of target genes, including hypoxia-inducible factor 1-alpha (HIF-1α) (Jung et al., 2005; Sehara et al., 2013). STAT3 is a potential modulator of HIF-1α-mediated vascular endothelial growth factor (VEGF) expression (Jung et al., 2005). HIF-1α is a classic activator of VEGF production. HIF-1α/VEGF pathway plays an important role in the neurorepair and functional recovery following experimental stroke (Khan et al., 2015). Thus, we speculate that STAT3-mediatedh HIF-1α/VEGF signaling could be involved in EE-mediated neurorepair and functional recovery after ischemic stroke.

In the present study, we sought to examine the role of calpain 1 and STAT3/HIF-1α/VEGF signaling pathway in EE-mediated post-ischemic neurogenesis and functional recovery in a mouse middle cerebral artery occlusion (MCAO) model. The intrinsic relationship between calpain 1 and STAT3/HIF-1α/VEGF signaling in EE-mediated neurorepair is our second goal.

2. Materials and methods

2.1. Animals, surgery, and housing conditions

Male C57BL/6 mice (8–10 weeks old, 23–25 g) were purchased from Beijing Vital River Laboratory Animal Technology Company. Mice used for all experiments were housed under specific pathogen-free conditions at Animal Laboratory Center of Tongji Medical College. All experiments with mice were performed in accordance with protocols approved by the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. Mice were anaesthetized i.p. with ketamine (100 mg/kg) and xylazine (8 mg/kg). Focal cerebral ischemia was induced by MCAO with a 6–0 silicone-coated nylon monofilament for 1 h, to block the origin of the MCA (Zhang et al., 2014a,b). Sham-operated rats were manipulated in the same way, but the MCA was not occluded.

Standard-housed controls were housed for the same duration in a standard cage (270 × 225 × 140 mm^3) (3–4 mice/cage). The EE mice were introduced in EE 2 days after surgery. The EE mice were housed in a spacious cage (860 × 760 × 310 mm^3) containing novel objects such as tunnels, shelters, toys, and running wheels for voluntary exercise (12–15 mice/cage).

2.2. In vivo drug treatments

Treatment groups were assigned in a randomized and blinded manner (Fig. 1). Mice intracerebroventricular (i.c.v.) injection was performed under anesthesia using a stereotaxic instrument (RWD Life Science Co., Ltd.) with a sterile 26-G Hamilton microsyringe (80330; Hamilton Company, Reno, NV). PD151746 was dissolved in 1% DMSO at 0.8 mg/mL. Calpain 1 inhibitor PD151746 (0.2 mg/kg) was injected to EE mice i.c.v. daily for 2 week, starting at 7 days post-ischemia (dpi). Anti-VEGF neutralizing antibody (1 μg/μl; NeoMarkers, Fremont, CA) or IgG isotype control was injected to EE mice i.c.v. daily for 2 weeks, starting at 7 dpi. JAK-STAT3 signaling pathway inhibitor AG490 (Calbiochem, San Diego, CA) or HIF-1α inhibitor 2-methoxyestradiol (2ME2; Sigma-Aldrich, St Louis, MO) was dissolved in 1% DMSO at 0.5 mg/mL and injected to EE mice (5 mg/kg) i.p. daily for 2 weeks, starting at 7 dpi. 10 mg/mouse glycyrrhizin (TCI, Shanghai, China) or normal saline was administrated intraperitoneally daily for 2 weeks, starting at 7 dpi.

2.3. 5-Bromo-2′-deoxyuridine (BrdU) labeling

Mice were treated twice with BrdU (dissolved at 10 mg/mL in normal saline, 50 mg/kg per injection; Sigma, St. Louis, MO, USA) with an 8-h interval between injections at 20 dpi; on the following day, the animals were humanely killed to analyze BrdU labeling of dividing cells.

2.4. Functional assays

The elevated body swing test (EBST) was performed to evaluate the symmetry of motor behavior at 3, 4, 6 and 10 weeks after MCAO (Sun et al., 2012). The mice were examined for lateral movements/turning when their bodies were suspended 100 mm above the testing table by lifting their tails. A swing was recorded when mice moved their head away from the vertical axis (angle > 10°) in three sets of 10 trials, performed over 5 min. Results are expressed as the ratio of total number of contralateral swings.

The rotarod test provided an index of forelimb and hindlimb motor coordination and balance (Sun et al., 2012). Mice were trained daily on an accelerating (5–40 rpm) rotating rod for 3 days before MCAO; only those mice able to remain on the rod for 20 s at 40 rpm were subjected to MCAO. Test sessions consisting of three trials at 40 rpm were carried out just before MCAO, and at 3, 4, 6 and 10 weeks after MCAO. The final score was expressed as the mean time that a mouse was able to remain on the rod over three trials.

The pole test was used to assess forelimb strength, ability to grasp and balance performed in a blinded fashion at 3, 4, 6 and 10 weeks after MCAO (Gertz et al., 2012). Mice were placed head upward near the top of a vertical steel pole (600 mm high with rough surface). Both times taken to orientate the body completely downwards and to reach the floor with all four paws were recorded.

2.5. In vitro lipopolysaccharide (LPS) stimulation and treatments in the astrocyte culture

Cells were dissociated from ischemic hemispheric brains at 14 dpi and cultured *in vitro* as described in one previous study (Lin et al., 2016). Purified astrocytes were treated with LPS (100 ng/mL; Sigma, St. Louis, MO, USA) in the presence or absence of recombinant mouse VEGF-A (0.1 ng/mL; Chemicon International, Inc.; Temeca, CA) dissolved in artificial cerebrospinal fluid (aCSF) or aCSF. At 16 h after treatment, cells were collected by either direct cell lysis for western blotting analysis or by fixation using 4% paraformaldehyde for histological analysis. The supernatant of cell culture was collected for western blotting analysis, or added to the medium of cultured neural precursor cells (NPCs) with or without the addition of glycyrrhizin (500 μmol/L; TCI, Shanghai, China).

2.6. Assessment of NPCs proliferation and differentiation

Insulin-transferrin-selenium (ITS), and poly-L-ornithine were purchased from Sigma Chemicals. Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were purchased from PeproTech. Penicillin plus streptomycin was from Beijing Solarbio Science & Technology. Heparin was purchased from StemCell Technologies. B27 supplement, laminin, and FBS were purchased from Invitrogen/Gibco. DMEM/F-12 medium was from Thermo Scientific HyClone.

Ipsilateral SVZ cells were dissociated from ischemic hemispheric