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FORCED LIMB-USE ENHANCED NEUROGENESIS AND BEHAVIORAL RECOVERY AFTER STROKE IN THE AGED RATS

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Abstract—Constraint-induced movement therapy (CIMT) after stroke enhances not only functional reorganization but also structural plasticity of the brain in the adult rats. We examined whether forced limb-use which mimicked CIMT could influence ischemia-induced neurogenesis, apoptosis and behavioral recovery in the aged rats. Aged rats were divided into a sham group, an ischemia group, and an ischemia group with forced limb-use. Focal cerebral ischemia was induced by injection of endothelin-1. Forced limb-use began on post-stroke day 7 by fitting a plaster cast around the unimpaired upper limbs of rats for 3 weeks. Behavioral recovery was evaluated by tapered/ledged beam-walking test on postoperative day 32. The expression of doublecortin, neuronal nuclei, glial fibrillary acidic protein and Iba-1 were measured by single or double immunohistochemistry, and apoptosis was measured by TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay. The production of neuroblasts in the subventricular zone (SVZ) was significantly increased after stroke. Forced limb-use enhanced the proliferation of newborn neurons in the SVZ, as well as increased the long-term survival of newborn neurons. Furthermore, forced limb-use suppressed apoptosis and improved the motor functions after stroke in the aged rats. Forced limb-use exerted few effects on inflammation. Neither the number nor dendritic complexity of newborn granule cells in the hippocampus was affected by forced limb-use. Forced limb-use is effective in enhancing neurogenesis and behavioral recovery after stroke even in the aged rats.

Key words: aging, behavioral recovery, forced limb-use, neurogenesis, stroke.

INTRODUCTION

Despite extensive research, current effective treatments for stroke are limited. Constraint-induced movement therapy (CIMT) is a method of neurorehabilitation which encourages use of the impaired limb by restraint of the unaffected limb in daily life. CIMT, which has been extensively used for stroke rehabilitation, may induce not only functional reorganization but also structural plasticity (Gauthier et al., 2008; Maier et al., 2008; Kononen et al., 2012; Sterling et al., 2013; Zhao et al., 2013).

Stroke is strongly associated with advanced age and is principally a disease of the elderly population (Barnett, 2002; Kelly-Hayes et al., 2003; Donnan et al., 2008). Both experimental and human studies suggest that old age is associated with poor outcome and impaired functional recovery after stroke (Badan et al., 2003; Brown et al., 2003; Kelly-Hayes et al., 2003; Rosen et al., 2005). Various physiological and neurochemical changes during aging may alter the neurobiological response to brain insults (Peters, 2006; Popa-Wagner et al., 2011; Popa-Wagner et al., 2012). Therefore, the experimental data in aged animals might be more appropriate from a translational standpoint. However, studies addressing the effect of aging on CIMT-induced structural plasticity remain limited (Maier et al., 2008).

In the present study, we tested the hypothesis that forced limb-use in aged rats which mimicked CIMT in stroke patients could enhance neurogenesis and in turn promote behavioral recovery after focal cerebral ischemia.

EXPERIMENTAL PROCEDURES

Animals

Aged (18–20 months) male Sprague–Dawley rats (600–800 g) used in the study were purchased from Liaoning Changsheng Biological Technology Company Limited.

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Abbreviations: BrdU, 5-bromo-2-deoxyuridine; CIMT, constraint-induced movement therapy; DCX, doublecortin; DG, dentate gyrus; ET-1, endothelin-1; GFAP, glial fibrillary acidic protein; HPF, high-power field; NeuN, neuronal nuclei; SGZ, subgranular zone; SVZ, subventricular zone; TUNEL, TdT-mediated dUTP-biotin nick-end labeling.

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The rats were randomly assigned to three groups: sham-operated rats ($n = 6$, sham), rats subjected to cerebral ischemia ($n = 8$, ischemia), and rats with cerebral ischemia treated with forced limb-use ($n = 8$, forced limb-use). All experiments with respect to the operators responsible for surgical procedures and outcome assessments were performed in a double-blinded fashion. All rats were kept in standardized cages ($54.5 * 39.5 * 20.0\ cm^3$, 3–4 animals per cage) with a 12-h light/dark cycle and the rats had free access to food and water. Anesthesia was induced using a mixture of 3% isoflurane in 30% oxygen and 70% nitrous oxide and animals were maintained with 1.5% isoflurane for the surgeries. Buprenorphine (0.03 mg/kg, ip) was administered for postoperative pain relief. All efforts were made to ensure animal welfare and to reduce the number of animals used. All work involving rats was approved by the Institutional Animal Care and Use Committee of China Medical University [No.: SCXK (Liao) 2008-0005].

Endothelin-1 (ET-1) stroke model

To induce cortical ischemia, the vasoconstrictive peptide ET-1 (Sigma, St. Louis, MO, USA) was injected at the following three coordinates: (1) AP + 3.5 mm ML + 2.8 mm DV − 1.0 mm; (2) AP + 2 mm ML + 2.8 mm DV − 1.0 mm; and (3) AP + 0.5 mm ML + 2.8 mm DV − 1.0 mm according to the rat brain atlas by Paxinos and Watson (Soleman et al., 2010). ET-1 was injected at 0.5 μl/min (0.5 μg/μl) by an infusion pump, and the needle was left in place for three minutes before being slowly withdrawn over the course of one minute to avoid aspirating the ET-1 back through the needle track (Lowrance et al., 2013). The volume of each injection was 1.5 μl. Sham-operated animals received the same surgery except saline was injected instead of ET-1. Rats that failed to show unilateral lesions performed in the hemisphere contralateral to the dominant forelimb or died after ET-1 lesions were excluded in the further study.

Forced limb-use

Seven days after ischemia, forced limb-use was started by fitting a plaster cast around the unimpaired upper limb of the rats as described previously (Muller et al., 2008). The plaster applied over the soft cotton gauze allowed considerable mobility of the upper limb in the cast, but not large movements (Ishida et al., 2011). After 3 weeks, the cast was removed before behavioral testing (Fig. 1).

5-Bromo-2-deoxyuridine (BrdU) labeling

To label newly generated cells, all rats received twice daily intraperitoneal injections of BrdU (100 mg/kg, Sigma–Aldrich, St. Louis, MO, USA) during postoperative days 5–6 (Fig. 1) (Inta et al., 2013).

Tapered/ledged beam-walking test

To evaluate changes in forelimb functions, six rats of each group were tested using a tapered/ledged beam (Zhao et al., 2005; Zhao et al., 2013). The rats were pre-trained for 3 days to readily undergo beam-traversing task before ischemia induction and tested on postoperative day 32. Performance in the beam walking test was videotaped and later analyzed by calculating the slip ratio of the impaired (contralateral to lesion) forelimb (number of slips/number of total steps) (Fig. 1).

Tissue preparation

After follow-up on day 33, the rats were transcardially perfused, and then the brains were dissected and postfixed. A series of contiguous 40-μm-thick sections were cut on a cryotome (Thermo Electron, Waltham, MA, USA) from forebrain blocks for immunostaining.

Measurement of infarct volume

For assessment of infarct volume, coronal sections (40 μm) were picked up from +4.5 to −2.5 mm from the bregma at 1-mm intervals. Sections were mounted on slides, air dried, and stained with cresyl violet (Sigma, St. Louis, MO, USA) (Popp et al., 2009). The contralateral and ipsilateral hemisphere areas were measured using NIH ImageJ. The intact area in the ipsilateral (injured) hemisphere was subtracted from the area of the contralateral hemisphere in each section and the areas were multiplied by the distance between sections to obtain the total infarct volumes.

Immunohistochemistry

Sections (40 μm) were processed for immunohistochemistry as previously described (Jessberger et al., 2007). BrdU staining was preceded by DNA denaturation and incorporated BrdU was detected using sheep anti-BrdU (1:500, Abcam, Cambridge, MA, USA) (Eriksson et al., 1998). The following antibodies for phenotyping were applied in combination with anti-BrdU: guinea-pig anti-doublecortin (DCX) (1:800, Millipore, Billerica, MA, USA), mouse anti-neuronal nuclei (NeuN) Alexa

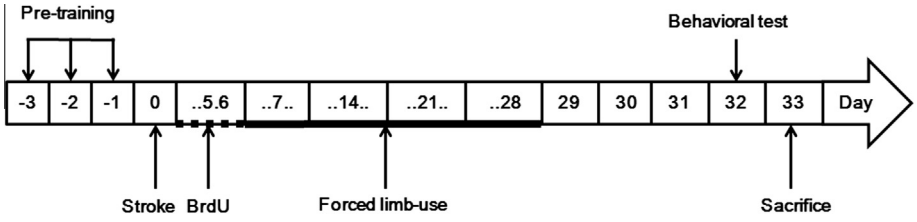

Fig. 1. Study design. The arrows indicate the timing of pre-training, induction of stroke, BrdU labeling, forced limb-use treatment, behavioral testing and sacrifice.