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Endogenous Neuronal Replacement in the Juvenile Brain Following Cerebral Ischemia

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Abstract—Replacement of dead neurons following ischemia, either via enhanced endogenous neurogenesis or stem cell therapy, has long been sought. Unfortunately, while various therapies that enhance neurogenesis or stem cell therapies have proven beneficial in animal models, they have all uniformly failed to truly replace dead neurons in the ischemic core to facilitate long-term recovery. Remarkably, we observe robust repopulation of medium-spiny neurons within the ischemic core of juvenile mice following experimental stroke. Despite extensive neuronal cell death in the injured striatum of both juveniles and adults at acute time points after ischemia (24 h and 7 d), mature newborn neurons replaced lost striatal neurons at 30 d post-ischemia. This neuronal repopulation was found only in juveniles, not adults, and importantly, was accompanied by enhanced post-ischemic behavioral recovery at 30 d. Ablation of neurogenesis using irradiation prevented neuronal replacement and functional recovery in MCAo-injured juvenile mice. In contrast, findings in adults were consistent with previous reports, that newborn neurons failed to mature and died, offering little therapeutic potential. These data provide support for neuronal replacement and consequent functional recovery following ischemic stroke and new targets in the development of novel therapies to treat stroke. Published by Elsevier Ltd on behalf of IBRO.

Key words: cerebral ischemia, endogenous recovery, neural stem cells, neurogenesis, neuron replacement.

INTRODUCTION

Approximately 800,000 Americans experience a new or recurrent stroke every year, and many survivors continue to live with permanent stroke-related disabilities, often leading to poor quality of life (Korda and Douglas, 1997; Mercier et al., 2001; Sun et al., 2014). Although stroke is one of the leading causes of death and disability worldwide, no successful long-term neuroprotective therapies have been found in clinical trials to date (Kidwell et al., 2001; Ginsberg, 2008; Minnerup et al., 2012), highlighting the need for novel therapeutic approaches. Neuronal replacement could result in direct recovery of function, since many post-ischemic impairments are due to neuronal damage or death. Neurogenesis (the birth of new neurons) is one emerging approach involving the generation of functionally integrated neurons from progenitor cells, and occurs throughout life in the brain of mammals, making it an appealing target for potential interventions to enhance post-stroke recovery.

Long-standing evidence indicates that cerebral ischemia initiates adult neurogenesis (Liu et al., 1998; Jin et al., 2001; Arvidsson et al., 2002; Nakatomi et al., 2002). Stroke-induced neurogenesis in adult mice involves vigorous proliferation and migration of neural progenitor cells, but most cells die within 4 weeks (Lichtenwalner and Parent, 2006; Liu et al., 2013; Tobin et al., 2014), unable to repair tissue and repopulate damaged areas (Zhao et al., 2008). The timeline for rapid proliferation is short-lived, peaking at 7 days post-injury (Lichtenwalner and Parent, 2006; Liu et al., 2013). Much research has focused on neonatal, perinatal, and adult rodents, yet few studies have assessed post-stroke neurogenesis in juveniles. Research centered on adult neurogenesis in rodent models of cerebral ischemia demonstrates little replacement of neurons lost following stroke-induced damage. Adult neurogenesis differs from developmental neurogenesis, where the brain is

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undergoing processes such as axon pathfinding, programmed cell death, and dendritic extension, which are limited in the mature neurons of the adult brain (Danzer, 2008). The juvenile brain is ideal for studying neurogenesis because 3–4-week-old mice have a fully developed brain that has reached neuronal maturity like adults, yet isn't vulnerable to the developmental processes found in the neonatal and perinatal brain. Differences between the juvenile and adult brain may shed light on potential interventions that could be used to enhance neurogenesis in adults, or help identify why newborn neurons do not survive in adults.

To test the hypothesis that neurogenesis and functional recovery may be enhanced in the juvenile brain, we compared an experimental ischemic stroke (Herson et al., 2013) model in juvenile and adult mice. We examined neurogenesis and neuronal replacement in the striatum with neurobehavioral assays of functional recovery and immunohistochemistry, including bromodeoxyuridine (BrdU) labeling and cell-type specific markers at 24 h, 7 d, and 30 d following 45-min transient middle cerebral artery occlusion (MCAo). The vast majority of neurons lost in the striatum following stroke are GABAergic medium-sized spiny neurons (MSN), which are the primary neuronal type (90–95%) in the region and are essential for motor function (Arlotta et al., 2008). To determine if striatal neurogenesis plays a role in post-stroke recovery of function, we tested an array of motor and locomotive functions at baseline, 7 d, and 30 d after cerebral ischemia.

Despite equivalent injury between age groups at acute time points (24 h and 7 d), we discovered a robust regenerative response in the juvenile brain at 30 d post-injury not found in adults. We found substantial neuronal replacement in areas of ischemic damage unique to the juvenile brain, along with improved functional outcomes on behavioral tests, revealing improved limb use and motor responses in MCAo-injured juvenile mice, but not adults.

EXPERIMENTAL PROCEDURES

Seventy-two male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) were randomly assigned to one of two groups for molecular experiments (MCAo Adult or MCAo Juvenile), one of four groups for behavior tests (MCAo Adult, Sham-operated Adult, MCAo Juvenile, or Sham-operated Juvenile), and the irradiation experiment included MCAo Juvenile + irradiation mice. Mice were single housed, in temperature- (23 ± 3 °C) and light (12:12 h, light:dark)-controlled rooms with ad libitum access to food and water. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory animals in biological research.

Middle cerebral artery occlusion (MCAo)

MCAo methods are as previously reported (Jia et al., 2011; Herson et al., 2013). Briefly, cerebral ischemia was induced under isoflurane anesthesia in juvenile (postnatal day 20–25, 10–15 g) and adult (8 weeks, 25–30 g) mice for 45 min with reversible MCAo via the intraluminal suture method. Minor variations were incorporated to accommodate the small size of P20-25 mice (a 6–0 nylon suture was heat-blunted and coated with silicone gel to obtain a smaller filament diameter of ~0.18 mm). The adequacy of MCAo was confirmed by laser Doppler flowmetry (>70% drop required for inclusion) measured over the ipsilateral parietal cortex in all mice.

Bromodeoxyuridine (BrdU) administration

Two injections of BrdU (50 mg/kg in 0.9% saline, i.p.; Sigma, St. Louis, MO, USA) were given at 24 h and 48 h after stroke, at peak expression times reported in the literature following stroke. A synthetic analog of thymidine, BrdU is commonly used in the detection of proliferating cells in living tissues.

Immunohistochemistry

Tissue collection, staining, and analyses were performed by a blinded investigator. Cellular proliferation and neurogenesis was assessed by BrdU co-localization with cell type-specific markers, since developing neurons express distinct markers during the maturation process. Immunofluorescence assays also included markers for GABAergic MSNs, the primary neuronal type in the striatum (90–95%), and for the remaining neuronal types (5–10%), cholinergic interneurons and GABAergic parvalbumin-immunoreactive interneurons (Chang and Kita, 1992; Arlotta et al., 2008). Staining of 50-μm sections consisted of phosphate-buffered saline washes (1× PBS, 3 × 5 min), 1-h incubation in blocking serum (5% normal donkey serum with 0.3% Triton X-100), overnight incubation at 4 °C in primary antibody, PBS washes (3 × 5 min), 1-h incubation in secondary antibody, PBS washes (3 × 5 min), Hoechst counterstain 5 min (1:10,000 in PBS), PBS washes (3 × 5 min), mount and coverslip with anti-fade mounting medium (Vectashield). For BrdU staining, sections were washed with 1× PBS (3 × 5 min), denatured (2 N HCl) for 20 min at 37 °C, neutralized with 0.1 M borate buffer (pH 8.5, 3 × 15 min), PBS washes (3 × 5 min), and finished using the protocol listed above. The following primary antibodies for cell-specific markers were used: rat anti-BrdU (1:300, Abcam), rabbit anti-doublecortin (DCX, 1:500, Abcam), mouse anti-NeuN (1:500, Millipore), rabbit anti-COUP-TF-interacting protein 2 (Ctip2, 1:300, Abcam), rat anti-Ctip2 (1:300, Abcam), rabbit anti-choline acetyltransferase (ChAT, 1:300, Millipore), mouse anti-parvalbumin (PV, 1:300, Sigma), goat anti-glial fibrillary acidic protein (GFAP, 1:500, Santa Cruz Biotech), and rabbit anti-oligodendrocyte transcription factor 2 (Olig2, 1:300, Millipore). The following secondary antibodies were used: Alexa Fluor 488, 594, or 647-conjugated IgG (1:500 or 1:600; Jackson Immuno) and Alexa Fluor 555 (1:500, Abcam). Cell death was assessed by a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, Cell Death Detection Kit, Roche) assay. Confocal microscopy was used to confirm co-localization of BrdU and cell type-specific markers using an Olympus FV1000 laser scanning confocal microscope and Olym-

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