Neuroscience 169 (2010) 259–268

INDUCTION OF STRIATAL NEUROGENESIS ENHANCES FUNCTIONAL RECOVERY IN AN ADULT ANIMAL MODEL OF NEONATAL HYPOXIC-ISCHEMIC BRAIN INJURY

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Abstract—While intraventricular administration of epidermal growth factor (EGF) expands the proliferation of neural stem/progenitor cells in the subventricular zone (SVZ), overexpression of brain-derived neurotrophic factor (BDNF) is particularly effective in enhancing striatal neurogenesis. We assessed the induction of striatal neurogenesis and consequent functional recovery after chronic infusion of BDNF and EGF in an adult animal model of neonatal hypoxic-ischemic (HI) brain injury. Permanent brain damage was induced in CD-1® (ICR) mice (P7) by applying the ligation of unilateral carotid artery and hypoxic condition. At 6 weeks of age, the mice were randomly assigned to groups receiving a continuous 2-week infusion of one of the following treatments into the ventricle: BDNF, EGF, BDNF/EGF, or phosphate buffered saline (PBS). Two weeks after treatment, immunohistochemical analysis revealed an increase in the number of BrdU$^+$ cells in the SVZ and striata of BDNF/EGF-treated mice. The number of new neurons co-stained with BrdU and βIII-tubulin was also significantly increased in the neostriata of BDNF/EGF-treated mice, compared with PBS group. In addition, the newly generated cells were expressed as migrating neuroblasts labeled with PSA-NCAM or doublecortin in the SVZ and the ventricular side of neostriata. The new striatal neurons were also differentiated as mature neurons co-labeled with BrdU$^+$/NeuN$^+$. When evaluated post-surgical 8 weeks, BDNF/EGF-treated mice exhibited significantly longer rotarod latencies at constant speed (48 rpm) and under accelerating condition (4–80 rpm), relative to PBS and untreated controls. In the forelimb-use asymmetry test, BDNF/EGF-treated mice showed significant improvement in the use of the contralateral forelimb. In contrast, this BDNF/EGF-associated functional recovery was abolished in mice receiving a co-infusion of 2% cytosine-b-D-arabinofuranoside (Ara-C), a mitotic inhibitor. Induction of striatal neurogenesis by the intraventricular administration of BDNF and EGF promoted functional recovery in an adult animal model of neonatal HI brain injury. The effect of Ara-C to completely block functional recovery indicates that the effect may be the result of newly generated neurons. Therefore, this treatment may offer a promising strategy for the restoration of motor function for adults with cerebral palsy (CP). Published by Elsevier Ltd on behalf of IBRO.

Key words: brain-derived neurotrophic factor, epidermal growth factor, intraventricular infusion, neurogenesis, cerebral palsy.

It has been reported that neural stem cells and progenitors are present in the subventricular zone (SVZ) of the adult mammalian brain (Goldman, 1998). These cells can be proliferated by the administration of growth factors such as epidermal growth factor (EGF) (Sugiura et al., 2005), fibroblast growth factor (FGF) (Wada et al., 2003; Matsuoka et al., 2003; Ellsworth et al., 2003) or their combination (Baldauf and Reymann, 2005; Tureyen et al., 2005) into the ventricle after focal cerebral ischemia. Additionally, newborn neurons are mobilized to migrate not only toward the olfactory bulb along the rostral migratory stream, but neuronal progenitor cells are also recruited into a non-neurogenic neostriatum in response to the subependymal overexpression of brain-derived neurotrophic factor (BDNF) (Pencea et al., 2001; Benraiss et al., 2004; Chmielnicki et al., 2004; Cho et al., 2007). Likewise, adult neurogenesis persists in specific brain regions throughout lifetime and can be enhanced from endogenous progenitor cells residing in the SVZ by growth factors or neurotrophic factors, suggesting this strategy will be able to treat the damaged brain. In other words, if the differentiation of newly generated neurons could be directed toward specific functional brain regions, it may be possible to target the recovery of specific functions in the treatment of incurable neurological diseases.

Cerebral palsy (CP) caused by neonatal hypoxic-ischemic (HI) brain injury is the representative neurological disease. The incidence is approximately two per 1000 children, and among them, a majority exhibit neurodevelopmental impairment (Vannucci et al., 1999; Yager, 2004).

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Abbreviations: AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care; ANOVA, analysis of variance; Ara-C, cytosine-b-D-arabinofuranoside; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2-deoxyuridine; CP, cerebral palsy; DCX, doublecortin; EGF, epidermal growth factor; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; HI, hypoxic-ischemic; IGF-1, insulin-like growth factor-1; IRB, Institutional Review Board; PBS, phosphate buffered saline; SVZ, subventricular zone.

0306-4522/10 $ - see front matter. Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2010.04.038

In the experimental studies, voluntary running exercise (Praag et al., 1999a, b; Yasuhara et al., 2007) and enriched environment (Kempermann et al., 1997; Komitova et al., 2005; Olson et al., 2006) increased endogenous neurogenesis in the SVZ and hippocampus. However, because of the prevailing view that the regeneration of the damaged brain is extremely limited especially in the chronic stage, the clinical treatment of these conditions is usually focused on supportive care such as the prevention of complications or a modest reduction of abnormal movement patterns and spasticity rather than functional recovery derived from neurorestoration.

Therefore, in this study, the continuous intraventricular administration of low dose BDNF and EGF was used to determine if the induction of striatal neurogenesis could promote functional recovery in adult mice after neonatal HI brain injury, an animal model of adults with CP.

EXPERIMENTAL PROCEDURES

Neonatal hypoxic-ischemic brain injury

All animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), using a 12 h light/dark cycle according to animal protection regulations. The experimental procedure was approved by the Institutional Review Board (IRB). Permanent ischemic brain damage was induced in 7-day-old CD-1® (ICR) mice by unilateral carotid artery ligation (right side), and hypoxic brain injury (8% O_2 for 90 min) was also induced as described previously (Yager, 2004; Vannucci et al., 1999; Vannucci and Vannucci, 2005; Ong et al., 2005). Body temperature was maintained constantly at 37 °C within the hypoxic chamber. One week after HI brain injury, a scalp incision was made in order to identify the brain lesion in the posterolateral area of the right hemisphere. The presence and the extent of brain injury of all subjects were assessed through the semi-transparent skull. Animals with severe brain lesions of more than 50% of the unilateral hemisphere were excluded to eliminate the sampling error inherent with the condition due to the volumetric changes in neostriata derived from severe brain damage. The extent of gross cerebral damage was not different among subjects, showing ipsilateral lesion in the posterolateral hemisphere (Fig. 1A).

Experimental groups

For behavioral testing, at 6 weeks of age, 72 mice with HI brain injury were randomly assigned to receive a continuous 2-week intraventricular infusion of one of the following four treatments: a combination of BDNF and EGF (BDNF/EGF) (n=20); BDNF (n=14); EGF (n=18); or phosphate buffered saline (PBS) (n=20). BDNF and EGF were each infused at a concentration of 1 μg/mL using an Alzet micro-osmotic pump (model 1002; 0.25 μl/h infusion rate, 100 μl volume; Durect). The infusion cannula (Brain Infusion Kit 3) was inserted using stereotaxic coordinates (AP −0.5 mm from Bregma; ML −0.7 mm from Bregma; DV −2.0 mm from dura) to the lateral ventricle (Fig. 1B), and the osmotic pump connected to this was inserted to the dorsal s.c. tissue. Another cohort of 14 mice were recruited as untreated controls which did not receive stereotaxic surgery after HI brain injury, and 15 mice which did not receive HI brain injury were also recruited as no HI group to provide a more comprehensible understanding of the extent of recovery in the subjects. In the other set of immunohistochemistry, 2 weeks after chronic infusion, newborn neurons were evaluated in the SVZ and neostriatum of the subjects (n=3 per group). Eight weeks after treatment, another matched groups (n=3 each) were also included to evaluate the long-term survival of the new striatal neurons. The schematic timeline of this experiment from birth to 14 weeks of age was provided in Fig. 1C.

Behavioral assessments

Rotarod performance. A rotarod test was used to assess motor coordination and balance. All animals received a pre-operative performance evaluation at 5–6 weeks of age. Rotarod tests were then performed at 2-week intervals until post-surgical 8 weeks, using constant speed (48 rpm) and accelerating speed (4–80 rpm) paradigms. The latency of mice falling from the rod was measured twice at each test, and individual tests were terminated at a maximum latency of 300 s.

Forelimb-use asymmetry test. To evaluate functional asymmetry resulting from unilateral brain lesion and consequent hemiplegia, the cylinder test and the ladder walking test were performed 8 weeks after treatment. In the cylinder test, the numbers of time each forelimb contacted the cylinder wall while the mouse was rearing straight was evaluated over 10 min. The percentage of contacts with the hemiplegic forelimb use was evaluated by the following formula (MacLellan et al., 2006):

$$\frac{[\#\text{ of contacts with contralateral limb} + 1/2(\#\text{ contacts with both limbs})]\times 100(\%)}{\#\text{ contacts with ipsilateral limb} + \#\text{ contacts with contralateral limb} + \#\text{ contacts with both limbs}}$$

In the ladder walking test, the mice were required to walk three to four times over a distance of 1 m on a horizontal ladder with metal rungs randomly located at diverse distances. The numbers of slips with each forelimb from the transverse rungs was measured by videotape analysis.

Immunohistochemistry

Mice were given an i.p. injection of 5-bromo-2-deoxyuridine (BrdU; 50 mg/kg, Sigma-Aldrich) once per day for 12 days, beginning 1 day after stereotaxic surgery. Two weeks after chronic infusion, new neurons were evaluated in the SVZ and neostriatum of three

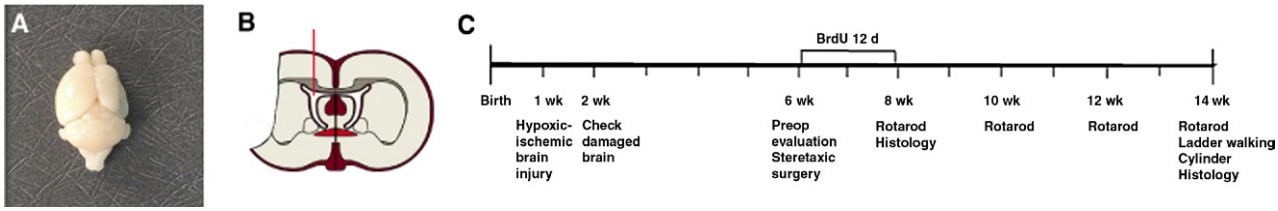

Fig. 1. Animal model and experimental design. Neonatal hypoxic-ischemic brain injury was induced by unilateral carotid artery ligation, and the mice were monitored during hypoxic condition (8% O_2), maintained within a hypoxic chamber for 90 min. The damaged brain showed ipsilateral lesion in the posterolateral hemisphere (A). At 6 wk of age, intraventricular infusion of BDNF and/or EGF was performed as above schematic (B). Experimental schedule from birth to 14 wk of age (C).