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 (45 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; Elsworth 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).
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% O2) 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 postero-lateral 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% extent of gross cerebral damage was not different among sub-

parent 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 postero-lateral 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; Direct). The infusion cannula (Brain Infusion Kit 3) was inserted using stereotoxic 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 stereotoxic 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):

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

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 stereotoxic surgery. Two weeks after chronic infusion, new neurons were evaluated in the SVZ and neostriatum of three
subjects from each group. Eight weeks after treatment, another matched groups of three mice each were also included to evaluate the long-term survival of newly generated striatal neurons. Briefly, the animals were sacrificed, given an intracardiac perfusion of 4% paraformaldehyde, and the brain tissues were harvested. These brain tissues were frozen and cryosectioned at 16-μm intervals, and immunohistochemical staining was performed on six sections over a range of 256 μm. Sections were stained with the cell proliferation marker BrdU and the neuronal marker βIII-tubulin (1:400, Covance, NJ, USA), and the double-labeled cells of BrdU/βIII-tubulin were assessed by confocal imaging. The area of the SVZ and neostriatum were obtained using the MetaMorph Imaging System (Molecular Device, Sunnyvale, CA, USA), and converted to the volume (area/16 μm) of the SVZ and neostriatum were obtained using the MetaMorph Imaging System (Molecular Device, Sunnyvale, CA, USA), and converted to the volume (area×16 μm); the number of newly generated neurons were quantified as the density (1/mm³). Then, total numbers of newly generated cells and new neurons were estimated by multiplying the densities of BrdU+ cells and BrdU/βIII-tubulin+ cells by total volume of the SVZ or striatum. Total volume was calculated from multiplying the summed areas of every cross-section of the SVZ or striatum by the section thickness (16 μm) in each sample. In addition, sections were co-stained with BrdU and the mature neuronal marker NeuN (1:400, Chemicon, Pemecula, CA, USA), and the double-labeled cells of BrdU+/NeuN+ in the striatum were also assessed so as to confirm the identity of mature neurons. They were also immunostained with BrdU and PSA-NCAM (1:400, Sigma-Aldrich), or doublecortin (DCX+; 1:400, Chemicon) to identify migrating neuroblast.

Infusion of a mitotic inhibitor Ara-C
Fifty seven mice were infused with 2% cytosine-b-d-arabinofuranoside (Ara-C; Sigma-Aldrich) or PBS into the ventricle (Alzet micro-osmotic pump model 1002; Durect) so as to inhibit mitogenic neurogenesis in response to BDNF and/or EGF. Mice, which had already received HI brain injury at 1 week of age, were randomly assigned to the one of the following treatment groups at 6 weeks of age: BDNF/EGF/Ara-C (n=15), BDNF/Ara-C (n=15), EGF/Ara-C (n=15), and PBS/Ara-C (n=12). Behavioral and immunohistochemical assessments of these mice were made as described above.

Statistical analysis
The effect of BDNF and/or EGF on the generation of new neurons and functional recovery after the intraventricular infusion was evaluated for each group. The numbers of BrdU+ or BrdU/βIII-tubulin+ cells (1/mm³) in the SVZ and neostriatum, and the results of behavioral tests were analyzed by using one-way ANOVA followed by post hoc Bonferroni t-test using SPSS (version 13.0). A statistically significant level was defined as P<0.05.

RESULTS

Immunohistochemistry
The ability of BDNF combined with EGF to induce neurogenesis in the SVZ and neostriatum was assessed immunohisto logically by counting the number of newly generated neurons co-labeled with BrdU and βIII-tubulin in 12 mice (three per group) with HI brain injury after 2 weeks of chronic infusion. BDNF was found to induce neurogenesis not only in the SVZ, but also in the neostriatum, a non-neurogenic area. Moreover, EGF potentiated striatal neurogenesis by expanding the mitotic pool of SVZ cells responsive to neuronal instruction by BDNF, increasing subependymal neurogenesis as a consequence (Fig. 2A–E). Namely, mice treated with a co-infusion of BDNF and EGF showed a significant induction of mitogenesis in the SVZ (196.8±43.4×10^3 BrdU+ cells/mm³) (one-way ANOVA, F=4.173, P=0.019, and post hoc t-tests of P=0.013) and neostriatum (1202.5±382.9 BrdU+ cells/mm³) (one-way ANOVA, F=4.104, P=0.02, and post hoc t-tests of P=0.019) of the ipsilateral hemisphere, compared with PBS controls (50.8±15.4×10^3 and 217.9±53.0, respectively). Furthermore, striatal neurogenesis in BDNF/EGF-treated mice (354.6±95.3 BrdU+/βIII-tubulin+ cells/mm³) was significantly increased relative to PBS controls (64.2±20.4) in the damaged striata (one-way ANOVA, F=3.288, P=0.042, and post hoc t-tests of P=0.033), although the contralateral hemisphere did not show a statistical difference among the groups (Table 1).

When total numbers of newly generated cells and new neurons were extrapolated by multiplying the densities of BrdU+ cells and BrdU+/βIII-tubulin+ cells by total volume of the SVZ or striatum, they exhibited same results as the densities themselves. Namely, BDNF/EGF-treated mice showed a significant mitogenesis in the SVZ (24.5±8.7×10^3 BrdU+ cells) (one-way ANOVA, F=3.522, P=0.034, and post hoc t-tests of P=0.027) and neostriatum (5.7±1.8×10^3 BrdU+ cells) (one-way ANOVA, F=4.526, P=0.014, and post hoc t-tests of P=0.015) of the ipsilateral hemisphere, compared with PBS controls (3.2±1.1×10^3 and 1.0±0.3×10^3, respectively). Furthermore, striatal neurogenesis in BDNF/EGF-treated mice (1.8±0.6×10^3 BrdU+/βIII-tubulin+ cells) was significantly increased relative to PBS controls (0.3±0.1×10^3) in the damaged striata (one-way ANOVA, F=3.683, P=0.029, and post hoc t-tests of P=0.027) (Table 1).

When a separate cohort of BDNF/EGF-treated mice (three per group) were assessed 8 weeks after chronic infusion, the number of new striatal neurons stained with BrdU+/βIII-tubulin+ (168.9±8.7) was substantially greater than in PBS controls (9.4±9.4) (one-way ANOVA, F=16.426, P<0.001, and post hoc t-tests of P<0.001). In addition, we found that the newly generated cells were expressed as migrating neuroblasts labeled with PSA-NCAM+ or DCX+ in the SVZ and the ventricular side of neostriatum (Fig. 2F, G). The new striatal neurons were also differentiated as mature neurons co-labeled with BrdU+/NeuN+ (Fig. 2H–J).

Rotarod performance
To provide a baseline with which to compare the behavioral effects of BDNF/EGF treatment, rotarod performance was assessed at a constant speed (48 rpm) and under accelerating conditions (4–80 rpm) prior to undergoing surgery for drug administration. In the pre-surgical evaluation, no between-group differences were seen. Improved performance in BDNF/EGF-treated mice relative to the other groups was evident after post-surgical 4 weeks, and a significant improvement in performance was seen by post-surgical 6 weeks at a constant speed of 48 rpm (F=3.548, P=0.012 by one-way ANOVA) (Fig. 3A), and at an accelerating speed (F=3.804, P=0.008 by one-way ANOVA) (Fig. 3B). When compared with the other groups, BDNF/EGF-treated mice exhibited significantly longer latency than PBS-treated group (post hoc t-tests of P=0.037
Fig. 2. Induction of striatal neurogenesis. Intraventricular infusion of BDNF and EGF dramatically enhanced neurogenesis in SVZ 2 wk after treatment (A), compared to mice treated with BDNF (B), EGF (C), PBS (D). The BDNF/EGF-treated mice exhibited newly generated striatal neurons stained with BrdU+/βIII-tubulin+ (E). In addition, they showed migrating neuroblasts labeled with PSA-NCAM+ (F) or DCX+ (G) in the SVZ. The new striatal neurons were also differentiated as mature neurons co-labeled with BrdU+/NeuN+ (H–J). Striatal neurogenesis maintained a substantial increment by 8 wk after treatment, compared with the other groups (K). Scale bars: 10 μm.
at a constant speed; \( P=0.042 \) at an accelerating speed, and untreated controls (post hoc \( t \)-tests of \( P=0.031 \) at a constant speed; \( P=0.010 \) at an accelerating speed).

Finally, 8 weeks after beginning treatment, their mean rotarod latency was shown to be significantly increased to 208.2±30.4 s at a constant speed (one-way ANOVA, \( F=3.484, P=0.013 \) (Fig. 3A), and to 222.9±18.7 s with an accelerating condition (one-way ANOVA, \( F=7.572, P<0.001 \) (Fig. 3B). In comparison with the other groups, BDNF/EGF-treated mice also showed a significant increment over PBS-treated group (88.4±21.3 s, \( P=0.029 \) at a constant speed; 120.7±17.4 s, \( P<0.001 \) at an accelerat-

![Table 1. Newly generated neurons in SVZ and neostriatum as a function of 2 wk-treatment in an animal model of neonatal HI brain injury](https://example.com/table1)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SVZ</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total volume (mm³)</td>
<td>BrdU × 10³ (cells/mm³)</td>
</tr>
<tr>
<td>Ipsilateral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/E</td>
<td>0.11±0.02</td>
<td>196.8±43.4*</td>
</tr>
<tr>
<td>BDNF</td>
<td>0.10±0.02</td>
<td>116.4±22.5</td>
</tr>
<tr>
<td>EGF</td>
<td>0.08±0.01</td>
<td>136.9±29.2</td>
</tr>
<tr>
<td>PBS</td>
<td>0.06±0.01</td>
<td>50.8±15.4</td>
</tr>
<tr>
<td>Contralateral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/E</td>
<td>0.09±0.01</td>
<td>109.2±33.0</td>
</tr>
<tr>
<td>BDNF</td>
<td>0.08±0.01</td>
<td>101.6±16.3</td>
</tr>
<tr>
<td>EGF</td>
<td>0.08±0.01</td>
<td>78.2±11.3</td>
</tr>
<tr>
<td>PBS</td>
<td>0.07±0.01</td>
<td>41.6±10.8</td>
</tr>
<tr>
<td>Ipsilateral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/E</td>
<td>4.7±0.3</td>
<td>1202.5±382.9*</td>
</tr>
<tr>
<td>BDNF</td>
<td>4.2±0.4</td>
<td>505.6±97.7</td>
</tr>
<tr>
<td>EGF</td>
<td>4.2±0.1</td>
<td>496.4±113.1</td>
</tr>
<tr>
<td>PBS</td>
<td>4.3±0.4</td>
<td>217.9±53.0</td>
</tr>
<tr>
<td>Contralateral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/E</td>
<td>5.4±0.2</td>
<td>420.2±97.3</td>
</tr>
<tr>
<td>BDNF</td>
<td>5.3±0.4</td>
<td>348.7±62.5</td>
</tr>
<tr>
<td>EGF</td>
<td>5.0±0.3</td>
<td>228.4±48.2</td>
</tr>
<tr>
<td>PBS</td>
<td>5.1±0.3</td>
<td>150.9±40.0</td>
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</table>

Values are mean±SE. HI, hypoxic-ischemic; SVZ, subventricular zone; B/E, BDNF/EGF.

* \( P<0.05 \) vs PBS controls, by one-way ANOVA with posthoc Bonferroni \( t \)-test.

Fig. 3. Rotarod performance. After intraventricular infusion of BDNF/EGF, mean rotarod latency was shown to be significantly increased at constant speed of 48 rpm post-treatment 6–8 wk, compared with those of PBS and untreated controls (A). Rotarod performance was also improved at accelerating speed (4–80 rpm) by 8 wk post-treatment, compared with mice treated with EGF and PBS as well as untreated controls (B).
ing speed), and untreated controls (87.8 ± 31.7 s, P = 0.028 at a constant speed; 115.4 ± 9.3 s, P < 0.001 at an accelerating speed). When compared to normal animals which did not receive HI brain injury were also assessed at 3 months of age, rotarod latency of BDNF/EGF-treated mice reached to a similar level of no HI group (237.3 ± 22.6 s at a constant speed; 252.0 ± 12.9 at an accelerating speed) (Table 2). Although a statistical difference from PBS-treated group and untreated controls was not shown, mice treated with BDNF alone exhibited a tendency for improved rotarod performance 4 weeks after surgery but not thereafter. Furthermore, mice treated with EGF alone showed a similar outcome to the controls, whose rotarod latency was unchanged after post-operative 2 weeks (Fig. 3A, B).

Forelimb-use asymmetry test

To evaluate the presence of asymmetry caused by unilateral HI brain injury, we used both the cylinder test and the ladder walking test. No differences between groups were not shown, mice treated with BDNF alone exhibited a tendency for improved rotarod performance 4 weeks after surgery but not thereafter. Furthermore, mice treated with EGF alone showed a similar outcome to the controls, whose rotarod latency was unchanged after post-operative 2 weeks (Fig. 3A, B).

Table 2. Rotarod performance and the ratio relative to animals with no HI injury 8 wk after treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Constant speed (48 rpm)</th>
<th>Accelerating speed (4–80 rpm)</th>
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<tr>
<td></td>
<td>Latency to fall (s)</td>
<td>Ratio of motor function (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HI injury</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/E</td>
<td>208.2 ± 30.4*†</td>
<td>87.8</td>
</tr>
<tr>
<td>BDNF</td>
<td>154.8 ± 39.3</td>
<td>65.2</td>
</tr>
<tr>
<td>EGF</td>
<td>106.6 ± 33.8</td>
<td>44.9</td>
</tr>
<tr>
<td>PBS</td>
<td>88.4 ± 21.3</td>
<td>37.2</td>
</tr>
<tr>
<td>Untreated</td>
<td>87.8 ± 32.9</td>
<td>37.0</td>
</tr>
<tr>
<td>No HI injury</td>
<td>237.3 ± 22.6*†</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>222.9 ± 19.4*†</td>
<td>88.5</td>
</tr>
<tr>
<td></td>
<td>167.6 ± 26.8</td>
<td>66.5</td>
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<td>134.1 ± 22.0</td>
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<td>120.7 ± 17.4</td>
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<tr>
<td></td>
<td>115.4 ± 6.9</td>
<td>45.8</td>
</tr>
<tr>
<td></td>
<td>252.0 ± 12.9*†</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are mean ± SE. HI, hypoxic-ischemic; B/E, BDNF/EGF. Ratio of motor function (%) is calculated from comparison with no HI injury group. Ratio of functional recovery (%) is calculated from comparison with untreated HI injury group.

* P < 0.05 vs untreated, † P < 0.05 vs PBS, ‡ P < 0.05 vs EGF, § P < 0.05 vs BDNF by one-way ANOVA with posthoc Bonferroni t-test.

In addition, the percentage of slips on transverse rungs of the ladder, compared to total steps with the hemiplegic forelimbs, were reduced sequentially in mice treated with EGF (6.8 ± 1.7%), BDNF (4.5 ± 1.1%), and BDNF/EGF (3.1 ± 1.1%), relative to PBS-treated mice (8.1 ± 1.4%) and untreated controls (8.5 ± 1.8%). Although a statistically significance was not seen, a strong trend of the reduction in the percentage of slips versus the controls was shown for BDNF/EGF-treated group (F = 2.320, P = 0.059 by one-way ANOVA) (Fig. 4B).

Ara-C mediated inhibition reverse functional recovery

In order to establish the relationship between induction of neurogenesis and behavioral benefits of the treatment, we...
assessed the effects of BDNF and EGF while simultaneously inhibiting mitogenesis and consequent neurogenesis. Subgroups of BDNF/EGF-, BDNF-, and EGF-treated mice with HI brain injury were co-infused chronically with a 2% solution of the mitotic inhibitor Ara-C, to block SVZ cell proliferation and the generation of new striatal neurons (Fig. 5A, B). First of all, we confirmed that all animals treated with Ara-C did not show any new neurons co-labeled with BrdU and βIII-tubulin in the neostriata. The concurrent suppression of induced neurogenesis in Ara-C-treated mice abolished the beneficial effect of BDNF/EGF on motor performance when evaluated relative to mice not given Ara-C, although there were no differences among the groups from the beginning. Namely, BDNF/EGF/Ara-C-treated mice exhibited the same rotarod latency (88.1±21.2 s) as the other groups, whereas rotarod latency in BDNF/EGF-treated mice not given Ara-C was increased significantly relative to PBS-treated and Ara-C-treated controls in the accelerating rotarod paradigm 8 weeks after surgery (one-way ANOVA, F=10.564, P<0.001, and post hoc t-tests of P<0.001) (Fig. 5C).

The co-infusion of Ara-C also reduced the beneficial effects of BDNF/EGF in the forelimb-use asymmetry cylinder test, with mice showing a percentage of hemiplegic limb contacts with the wall (36.9±5.6%) comparable to that in PBS- and Ara-C-treated controls. Thus, only BDNF/EGF-treated mice exhibited a tendency of higher contralateral forelimb use than the other groups (F=1.953, P=0.068 by one-way ANOVA) (Fig. 5D).

DISCUSSION

Since no definite treatment is available to CP resulting from HI exposure, different therapeutic strategies have continuously been investigated so far. Such strategies include transplantation of neural stem cells (Daadi et al., 2010) or adult stem/progenitor cells (Lee et al., 2010; Yasuhara et al., 2006) to the damaged regions for promoting cell replacement and axonal sprouting, as well as neuroprotective treatment for ameliorating brain damage by using growth factors (Gonzalez et al., 2009; Lin et al., 2005; Mizuno et al., 2008). However, in the present study, we addressed the role of striatal neurogenesis from endogenous stem/progenitor cells, resulting from administration of neurotrophic factors, and the contribution of new neurons to functional recovery, based on the observations that intraventricular administration of BDNF induced striatal neurogenesis in normal adult brain (Benraiss et al., 2001; Chmielnicki et al., 2004; Pencea et al., 2001) and Huntington’s disease (Cho et al., 2007), and perilesional neurogenesis in stroke model (Keiner et al., 2009).

Neuroprotection versus neurogenesis

In experimental studies using animal models of neonatal HI brain injury, Almli et al. have reported that intraventricular administration of BDNF, as a pre-treatment, reduced the deterioration of cognitive function after brain damage (Almli et al., 2000). Galvin and Oorschot showed that a 3-day treatment with BDNF and NT-3 protected approximately 40% neurons in the striatum (Galvin and Oorschot, 2003). Lin et al. stated that the administration of insulin-like growth factor-1 (IGF-1) promoted functional recovery by protecting oligodendrocytes (Lin et al., 2005). Katsuragi et al. have also reported a neuroprotective effect of cells secreting glial cell line-derived neurotrophic factor (GDNF), when transplanted prior to brain damage (Katsuragi et al., 2005). Additionally, the pharmacologic agents such as erythropoietin (Gonzalez et al., 2009; Mizuno et al., 2008), minocycline (Cai et al., 2006), and nitric oxide synthase inhibitor (Ji et al., 2009) attenuated HI injury in neonatal brain.
Likewise, the use of growth factors, neurotrophic factors, or other pharmacologic agents have been considered primarily for their neuroprotective potential targeting the survival of remaining neurons, rather than for the therapeutic benefits that they may offer as a result of their effects on endogenous neurogenesis and regeneration of the damaged area. On the other hand, the latter treatment strategy might be particularly suited to the adult period of cerebral palsy encountered frequently in clinics. Therefore, we studied the induction of neurogenesis and functional consequences in response to treatment with neurotrophic factor BDNF and mitogenic factor EGF at 6 weeks of adult age, 5 weeks after neonatal HI brain injury. This period seems to be a considerable lapse of time after neuroprotective treatment would be effective.

Optimization of neonatal hypoxic-ischemic brain injury

Based on the previous reports (Vannucci et al., 1999; Vannucci and Vannucci, 2005), hypoxic-ischemic brain damage was induced by the permanent unilateral carotid artery ligation on postnatal day 7 and the placement of mice in a hypoxic environment for approximately 90 min. To reduce the sampling bias of the results caused by the difference of cerebral damage, striatal volume, and subsequent hemiplegic paralysis of the individuals, the presence and the severity of brain damage were examined 1 week after the HI brain injury. By excluding animals with severe brain lesions and the largely destroyed neostriata at the time, subjects with matched striatal volumes could have been included in this study. Therefore, the striatal volumes were not different among the groups 8 weeks after treatment. In other words, functional recovery in BDNF/EGF-treated mice might be not derived from neuroprotective effect of attenuating the striatal volume loss but from striatal neurogenesis.

Experimental grouping in all experiments

When we clarified the sample size of each group as well as the total number of mice used for all experiments in this study, total 182 mice with mild to moderate HI brain injury were recruited. In the set of behavioral testing, 72 mice were grouped into mice treated with BDNF/EGF (n=20), BDNF (n=14), EGF (n=18), or PBS (n=20) before stereotaxic surgery at 6 weeks of age to investigate functional outcomes by continuous 2-week intraventricular infusion. Another 14 mice were also subjected to untreated HI controls, and 15 mice which did not receive HI brain injury were recruited as no HI group in order to estimate the extent of functional recovery in the treated mice. In the other set of immunohistochemistry, total 24 mice were recruited to evaluate newly generated neurons in the SVZ and neostriata at post-treatment 2 weeks (n=3 per group) or 8 weeks (n=3 per group). In additional set of the Ara-C experiment, total 57 mice with HI brain injury were infused with Ara-C concurrent with BDNF/EGF (n=15), BDNF (n=15), EGF (n=15), or PBS (n=12) to confirm that Ara-C mediated inhibition of mitogenic neurogenesis could reverse functional recovery.

Functional recovery by administration of BDNF and EGF

Performance was assessed in the rotarod test at both constant speed and accelerating speed to evaluate group differences in motor coordination and balance sensitively and reliably (Cho et al., 2007; Luessse et al., 2001). In addition, forelimb-use asymmetry test such as cylinder test was added to evaluate the hemiplegic asymmetry caused by unilateral cerebral lesions (MacLellan et al., 2006), and neonatal HI brain injury (Daadi et al., 2010; Lee et al., 2010). The behavioral results of our study showed that, after 8 weeks of treatment, mice treated with combination of BDNF and EGF had significantly improved rotarod performance both at constant speed and at accelerating speed. They also exhibited better contralateral forelimb use in the cylinder test as well as ladder walking test. These results suggest that intraventricular administration of BDNF and EGF promoted functional recovery consistently in various motor functions.

However, mice treated with BDNF alone, who had a modest striatal neurogenesis post-treatment 8 weeks, did not show a statistically significant increment in any behavioral testing, although they exhibited a strong tendency for improved rotarod performance. Furthermore, any behavioral performance in mice treated with EGF alone was not different than that in PBS-treated controls, suggesting that EGF itself had no neurogenic effect nor functional recovery. Rather than a direct benefit, EGF seems to expand the mitotic pool of SVZ cells responsive to striatal neurogenesis by BDNF, and thereafter to potentiate functional recovery, even though some authors have reported that EGF reduced neurogenesis after focal cerebral ischemia (Jin et al., 2004).

Estimation of the extent of functional recovery

When we calculated the ratio of motor function and functional recovery from the rotarod latency of no HI group which did not receive HI brain injury, BDNF/EGF-treated mice showed approximately 87.8% of motor function of no HI group at a constant speed, and 88.6% of motor function of no HI group at an accelerating speed 8 weeks after treatment. On the other hands, the mice treated with PBS did not show a difference with untreated controls (37.2% vs. 37.0% at a constant speed; 47.9% vs. 45.8% at an accelerating speed). We then estimated the extent of functional recovery by a subtraction from the ratio of motor function of untreated HI group, BDNF/EGF-treated mice exhibited 50.8% of functional improvement at a constant speed, and 42.7% of functional improvement at an accelerating speed (Table 2).

Functional recovery from striatal neurogenesis

The histological results of our study showed that the combination therapy of BDNF and EGF, after 2 weeks of chronic infusion, not only significantly induced neurogenesis in the SVZ, but also into the neostriatum which has been considered a non-neurogenic area. The survival of newly generated striatal neurons in BDNF/EGF group
(47.6%) was sustained relative to that in the other groups (BDNF, 29.9%; EGF, 12.4%; PBS, 14.6%) through post-operative 8 weeks when BDNF/EGF-treated mice showed a significant improvement on the above behavioral tests. Moreover, the newly generated cells, expressed as migrating neuroblasts in the SVZ, were recruited to differentiated functional neurons with proof of mature neuronal marker NeuN. It appears that new neurons are migratory, although this conclusion cannot be definitive in the absence of a time course analysis.

Taken together, BDNF/EGF-treated mice exhibited a significant enhancement in striatal neurogenesis at both 2 and 8 weeks of treatment, and the existence of migrating neuroblasts and mature neurons, suggesting that the newly generated cells might be recruited from the SVZ cell populations to the striatum as a mature neuronal identity, and survived for relatively longer period. The mitotic inhibitor Ara-C prevented the functional recovery induced by intraventricular co-infusion of BDNF and EGF, while suppressing mitogenic neurogenesis in the SVZ and blocking striatal neurogenesis. This suggests that an improvement in motor performance may be derived from the induction of striatal neurogenesis rather than from the neuroprotective effects of any agents, especially in the chronic stage of neonatal HI brain injury.

Limitation of this study

First, the quantitative histological data do still seem to present a shortcoming, although density and volume were used to estimate total cell number. It in itself was not stereological sampling, and was subject to potential sampling biases. Total number of newly generated striatal neurons might be overestimated from the striatal volume assessed by planimetry. We should conduct a rigorous stereological study as previously reported (Galvin and Oorschot, 2003).

Second, we could not prove neuronal differentiation in animals treated with BDNF/EGF or BDNF alone, whereas the treatments may serve to enhance proliferation, migration, and survival overall. The percentage of newly generated cells turning into neurons appears to be nearly identical among conditions, suggesting that there is no difference in the efficiency of neuronal differentiation between groups. Furthermore, the frequency at which newly generated cells differentiate into neurons in the PBS infused group seems to be higher than we hypothesized. We should repeat more samples and confirm whether the treatment has definite effect on neuronal differentiation or no effect on it.

Third, we used the anti-mitotic agent, Ara-C to inhibit neural stem/progenitor cell proliferation in SVZ, and the generation of new striatal neurons. However, HI brain injury can usually lead to a cascade of the proliferation of cells other than SVZ populations including endothelial cells for angiogenesis, microglia and macrophages to clear out debris, and astrocytes to block off the HI site and to return the region to homeostasis. For the future study, we must validate whether the Ara-C mediated reversal of functional recovery is the result of inhibiting striatal neurogenesis by evaluating endothelial, microglial, and astrocytic markers to provide information of the identity of the proliferating cells in the HI brain injury.

CONCLUSION

In conclusion, in an adult animal model of neonatal HI brain injury, intraventricular administration of BDNF and EGF in combination promoted functional recovery of motor behavior as well as induction of striatal neurogenesis. The treatment benefits might be derived from the addition of new neurons, since neither striatal neurogenesis nor motor performance improved in animals treated with BDNF or EGF alone, and concurrent infusion of Ara-C completely inhibited the neuronal regeneration and functional effects. These results suggest that this strategy could be applied to the treatment of CP, even in adults, and that it may also be relevant to the treatment of a variety of neurological diseases such as stroke, and other incurable diseases of the central nervous system.

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