Effect of Brain-Derived Neurotrophic Factor Treatment and Forced Arm Use on Functional Motor Recovery After Small Cortical Ischemia

W.-R. Schäbitz, MD; C. Berger, MD; R. Kollmar, MD; M. Seitz, MS; E. Tanay, MS; M. Kiessling, MD; S. Schwab, MD; C. Sommer, MD

Background and Purpose—Both the administration of growth factors and physical therapy such as forced arm use (FAU) are promising approaches to enhance recovery after stroke. We explored the effects of these therapies on behavioral recovery and molecular markers of regeneration after experimental ischemia.

Methods—Rats were subjected to photothrombotic ischemia: sham (no ischemia), control (ischemia), brain-derived neurotrophic factor (BDNF; ischemia plus BDNF, 20 μg), and FAU (ischemia plus FAU, 1-sleeve plaster cast ipsilateral limb). Animals survived 1 or 6 weeks and underwent behavioral testing (Rotarod, beam balance, adhesive removal, plantar test, neuroscore). After the rats were killed, brain sections were immunostained for semiquantitative analysis of MAP1B, MAP2, synaptophysin, GFAP expression, and quantification of infarct volumes.

Results—Infarct volumes were not different between the groups 1 or 6 weeks after ischemia. BDNF-treated animals had better functional motor recovery (Rotarod, beam balance, neuroscore) compared with all other groups (P < 0.05). There was no significant adverse effect of early FAU treatment on motor recovery, although sensorimotor function (adhesive removal test) was impaired (P < 0.05). There were no differences between groups as measured by nociception of the left and right forepaw (plantar test). BDNF treatment transiently induced MAP1B expression in the ischemic border zone and synaptophysin expression within the contralateral cortex 6 weeks after ischemia (P < 0.05). Both BDNF and FAU reduced astrogliosis compared with controls (P < 0.05).

Conclusions—Postischemic intravenous BDNF treatment improves functional motor recovery after photothrombotic stroke and induces widespread neuronal remodeling. Early FAU treatment after stroke does not increase infarct size, impairs sensorimotor function, but leaves motor function unchanged. Postischemic astrogliosis was reduced by both treatments. (Stroke. 2004;35:992-997.)

Key Words: brain-derived neurotrophic factor ■ cerebral ischemia ■ forced arm use ■ photothrombosis ■ regeneration

A promising approach to increase recovery from behavioral dysfunction after brain injury is the administration of pharmacological agents. Growth factors may be ideal candidates because they control differentiation and growth in developmental processes and recovery and regeneration after traumatic brain lesions. There is a growing body of evidence that brain-derived neurotrophic factor (BDNF) might be particularly well suited because exogenous administration of BDNF exhibits potent protective effects after various types of ischemic lesions.1–4

Physical therapy is recognized as alternative approach to improve recovery after focal stroke.5 Immobilization of the unaffected arm, combined with physical therapy, called forced arm use (FAU), was shown to improve motor function of the impaired arm weeks after unilateral stroke.6–8 However, experimental studies indicated that early motor training or FAU of the impaired limb worsened functional outcome and exaggerated lesion size.9–13 These findings raise questions about early and intensive rehabilitation programs after stroke in humans.

In the present study, we further explored the effects of a drug treatment (BDNF) and physical therapy (FAU) on behavioral recovery and molecular markers of regeneration.

Materials and Methods

Experimental Groups

Experimental protocols were approved by the local ethics committee. Male Wistar rats (Charles River; 280 to 320 g) were randomly assigned to groups with end points at weeks 1 and 6: control (ischemia; n = 6 per group); BDNF (ischemia plus BDNF; n = 6 per

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Focal Cerebral Ischemia

Animals were anesthetized with an intramuscular injection of 100 mg/kg body weight ketamine hydrochloride (WDTr). Anesthesia was maintained with 50 mg/kg body weight. A PE-50 polyethylene tube was inserted into the right femoral artery for continuous monitoring of mean arterial blood pressure and blood gases. The right femoral vein was cannulated with a PE-50 tube for treatment infusion. During the experiment, rectal temperature was maintained and maintained at 37°C by a thermostatically controlled heating pad (Föhr Medical Instruments).

For illumination, a fiberoptic bundle with a 1.5-mm aperture was placed stereotaxically onto the skull 4 mm posterior to the bregma and 4 mm lateral from the midline (white-light beam; 150 W for 20 minutes). During the first 2 minutes of illumination, the dye rose bengal (0.133 mL/kg body weight, 10 mg/mL saline) was injected intravenously. Sham-operated animals underwent the same experimental procedures without infusion of rose bengal and illumination.

After surgery, catheters were removed, and the animals were allowed to recover from the anesthesia, given food and water ad libitum, and housed together (2 per cage).

Behavioral Testing

All animals were operated on and tested in parallel (1 animal per group at once). In all animals of the 6-week group, behavioral tests were performed before (baseline) and 3, 4, 5, and 6 weeks after ischemia by a blinded investigator (M.S.). For Rotarod tests, rats were placed on an accelerating Rotarod cylinder, and the time the animals remained on the Rotarod was measured.6,16 Speed was increased from 4 to 40 rpm within 5 minutes. The trial ended if the animal fell off the rungs or gripped the device and spun around or 2 consecutive revolutions without attempting to walk on the rungs. An arbitrary time limit of 500 seconds was set for the rats on the Rotarod cylinder in training and in the testing procedures.

The animals were trained 3 days before ischemia. The mean duration (seconds) on the device was recorded with 3 measurements 1 day before surgery. Motor test data are presented as percentage of mean duration (3 trials) on the Rotarod compared with the internal baseline control (before surgery).

The adhesive removal test was done both before and after ischemia.17 Initially, 2 pieces of adhesive-backed paper dots (113.1 mm²) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. The time to remove each stimulus from the forelimbs was recorded 5 trials per day for each forepaw. Individual trials were separated by 5 minutes. Before surgery, animals were trained for 3 days. Once the rats were able to remove the dots within 10 seconds, they were subjected to ischemia.

The changes in nociception were assessed by plantar test.19 Rats were placed in a clear plastic chamber (18 × 29 × 13 cm, 2.2-mm thickness). Acclimation of the animals to the environment occurred 20 minutes before baseline testing for 3 consecutive days with random activation of heating source to accommodate the animals to the pain stimulus. The heat source was then positioned under the glass floor beneath the right or left forepaw. Paw withdrawal automatically activated the timer with a latency of 0.1 second.

<table>
<thead>
<tr>
<th>TABLE 1. Neurological Score</th>
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<tbody>
<tr>
<td>Motor tests</td>
</tr>
<tr>
<td>Raising rat by tail</td>
</tr>
<tr>
<td>Flexion of forelimb</td>
</tr>
<tr>
<td>Flexion of hindlimb</td>
</tr>
<tr>
<td>Head moved &gt;10° to vertical axis within 30 s</td>
</tr>
<tr>
<td>Placing rat on floor (normal = 0; maximum = 3)</td>
</tr>
<tr>
<td>Normal walk</td>
</tr>
<tr>
<td>Inability to walk straight</td>
</tr>
<tr>
<td>Circling toward paretic side</td>
</tr>
<tr>
<td>Falls down to paretic side</td>
</tr>
<tr>
<td>Reflex absence and abnormal movements</td>
</tr>
<tr>
<td>Pinna reflex (head shake when auditory meatus is touched)</td>
</tr>
<tr>
<td>Corneal reflex (eye blink when cornea is lightly touched with cotton)</td>
</tr>
<tr>
<td>Startle reflex (motor response to a brief noise from snapping a clipboard paper)</td>
</tr>
<tr>
<td>Seizures, myoclonus, myodystony</td>
</tr>
<tr>
<td>Maximum points</td>
</tr>
</tbody>
</table>

Scores of 5 to 10 indicate severe injury; 4 to 6, moderate injury; and 1 to 3, mild injury.

Measurements were taken 5 times at 15-minute intervals and are given as averaged paw-withdrawal latency.

Beam balance performance of animals was assessed on a 6-point scale28: 0 = balances with steady posture; 1 = grasps side of beam; 2 = hugs beam and 1 limb, falls down from beam; 3 = hugs beam and 2 limbs, falls down from beam, or spins on beam >60 seconds; 4 = attempts to balance on beam but falls off >40 seconds; 5 = attempts to balance on beam but falls off >20 seconds; and 6 = falls off, no attempt to balance or hang onto beam <20 seconds.

Table 1 shows a modified neurological score as composite of motor, sensory, and reflex tests.17,18 Neurological function was graded on a scale of 0 (normal score) to 10 (maximal deficit score).

Immunohistochemistry

Immunohistochemistry was performed on coronal free-floating 50-μm Vibratome sections with the following antibodies: mouse anti-MAP1B (monoclonal, 1:8000; Chemicon), mouse anti-MAP2 (monoclonal, 1:4000; Chemicon), mouse anti-synaptophysin (monoclonal, 1:100; Dako), and rabbit anti-GFAP (polyclonal, 1:400; Dako). For semiquantitative analysis, optical densitometry was performed as previously described.29 Images were scanned with a DMC video camera (Polarmoid). The photothermotic lesion was selected on the monitor, and optical density was automatically determined in the immediate vicinity of the lesion (ischemic border zone), adjacent cortex, and corresponding contralateral cortex, respectively, with the AIS imaging research software (Imaging Research Inc.). Optical density of the corpus callosum was used as the reference value for background staining. For semiquantitative analysis of GFAP expression, the total area with reactive gliosis and consecutive increased optical density surrounding the ischemic lesion was automatically determined with the AIS software.

Data and Statistical Analyses

Infarct volume after 1 and 6 weeks was calculated from the equation for ellipsoids: 4/3πr1r2r3 (r = 1/2 length; r = 1/2 diameter; r = 1/2 height). Values are presented as mean ± SEM. After all data were acquired, the randomization code was broken. Statistical analysis was performed by use of Analyze-it. All data obtained at different time points were parametric and compared by use of 1-way analysis of variance (ANOVA), followed by the post hoc Fisher protected least significance difference test. An a error rate of 0.05 was taken as the criterion for significance.

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Results

Infarct Volumes and Functional Outcome

No differences were observed between groups for rectal temperature, pH, Pco2, Po2, mean arterial pressure, and mortality. There were no differences in weight gain over time between groups.

Infarct volumes after 1 week were 28.5 ± 2.3 mm3 (control), 31.7 ± 6.4 mm3 (BDNF), and 37.1 ± 3.6 mm3 (FAU) (P = NS); after 6 weeks, infarct volumes were 16.1 ± 4.3 mm3 (control), 10.1 ± 3.5 mm3 (BDNF), and 12.7 ± 3.5 mm3 (FAU) (P = NS).

BDNF-treated animals had a better motor recovery (Rotarod, beam balance, neuroscore) over time compared with controls and FAU-treated animals (P < 0.05; Figures 1 through 3). Interestingly, motor recovery was not impaired by FAU treatment compared with controls (Figures 1 through 3). FAU treatment impaired sensorimotor function of the forelimbs compared with controls, whereas BDNF had no effect (P < 0.05; Figure 4). There were no significant differences between all groups by measuring nociception of the left and right forepaw in the plantar test.

Immunohistochemistry

MAP1B

One week after photodethrombosis, a small rim of intact tissue close to the lesion (border zone) with increased MAP1B immunoreactivity (IR) was seen in all experimental groups that reached significance only in BDNF-treated rats compared with controls (Figure 1, available online at http://stroke.ahajournals.org). No significant changes were detect-

Figure 1. Rotarod test. Note the significant improvement in running function of BDNF-treated animals vs controls and FAU 5 and 6 weeks after ischemia (P < 0.05, ANOVA, Fisher’s test).

Figure 2. Beam-balance test. Note the significant improvement in beam balance of BDNF-treated animals vs controls and FAU 5 and 6 weeks after ischemia (P < 0.05, ANOVA, Fisher’s test).

Figure 3. Neurological score. Note the significant improvement in neurological outcome of BDNF-treated animals vs controls and FAU 5 and 6 weeks after ischemia (P < 0.05, ANOVA, Fisher’s test).

Figure 4. Adhesive removal test of right and left (paretic) forepaw. Note that the impairment in removing sticky paper dots of FAU-treated animals vs BDNF-treated animals and controls during experiment pronounced on the paretic limb (P < 0.05, ANOVA, Fisher’s test).
TABLE 2. Semiquantitative Analysis of MAP2 and Synaptophysin IR

<table>
<thead>
<tr>
<th></th>
<th>1 Week</th>
<th>6 Weeks</th>
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<tr>
<td><strong>MAP2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Border zone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100±24</td>
<td>100±15</td>
</tr>
<tr>
<td>BDNF</td>
<td>109±18</td>
<td>99±9</td>
</tr>
<tr>
<td>FAU</td>
<td>135±18</td>
<td>78±22</td>
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<tr>
<td>Adjacent cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100±27</td>
<td>100±12</td>
</tr>
<tr>
<td>BDNF</td>
<td>109±14</td>
<td>98±6</td>
</tr>
<tr>
<td>FAU</td>
<td>128±12</td>
<td>79±10</td>
</tr>
<tr>
<td>Contralateral cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100±11</td>
<td>100±13</td>
</tr>
<tr>
<td>BDNF</td>
<td>98±13</td>
<td>114±6</td>
</tr>
<tr>
<td>FAU</td>
<td>125±17</td>
<td>102±11</td>
</tr>
<tr>
<td><strong>Synaptophysin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Border zone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100±17</td>
<td>100±22</td>
</tr>
<tr>
<td>BDNF</td>
<td>123±19</td>
<td>135±21</td>
</tr>
<tr>
<td>FAU</td>
<td>116±11</td>
<td>124±15</td>
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<tr>
<td>Adjacent cortex</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>100±12</td>
<td>100±16</td>
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<tr>
<td>BDNF</td>
<td>109±16</td>
<td>131±21</td>
</tr>
<tr>
<td>FAU</td>
<td>110±7</td>
<td>129±16</td>
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<tr>
<td>Contralateral cortex</td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>100±14</td>
<td>100±21</td>
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<tr>
<td>BDNF</td>
<td>88±10</td>
<td>190±34</td>
</tr>
<tr>
<td>FAU</td>
<td>47±13</td>
<td>131±20</td>
</tr>
</tbody>
</table>

*P<0.05, ANOVA, Fisher’s test.

GFAP
At 1 week, a massive increase in GFAP-immunoreactive astrocytes was present within the cortex surrounding the ischemic lesion in all experimental groups. Increased astrogliosis was also present in the entire ipsilateral cortex, corpus callosum, and striatum. Within the contralateral hemisphere, scattered GFAP-positive astrocytes could be detected with identical distribution in control, BDNF, and FAU rats (Figure II, available online at http://stroke.ahajournals.org). After 6 weeks, GFAP-immunoreactive astrocytes were widely restricted to cortex and corpus callosum adjacent to the ischemic lesion. In FAU-treated and, more pronounced, in BDNF-treated rats, the area with astrogliosis around the lesion was significantly smaller compared with control animals (Figure II).

Discussion
To the best of our knowledge, the present study demonstrates for the first time that BDNF is not only a neuroprotectant but also a strong inducer of recovery after stroke.

In contrast to previous growth factor studies on recovery after stroke,22–24 we administered BDNF as an intravenous infusion. The dose was chosen below the reported neuroprotective dose of the substance (300 μg); indeed, 20 μg BDNF clearly improved functional outcome without affecting final infarct size. Such functional improvement could be caused by modulation of short- and long-term plasticity changes. As early as 24 hours after ischemia, the GABAergic system in the cortical peri-infarct zone is downregulated, leading to an imbalance between excitation and inhibition.25 This disinhibition can be prevented by exogenous BDNF (yet in a neuroprotective dose) after stroke.26 Long-term plastic changes include long-term potentiation, axonal regeneration, and sprouting and correlate with functional recovery after ischemic lesions.27,28 BDNF treatment was shown to increase long-term potentiation and cognitive function after transient forebrain ischemia.4 Similar to other drugs (amphetamines, basic fibrinogen growth factor), BDNF treatment may increase expression of markers for axonal sprouting and synaptogenesis such as MAP1/2 or synaptophysin.22,29 Although BDNF has recently been demonstrated to promote dendritic growth in vitro,30 our data with transient perilesional upregulation of the growth associated with MAP1B in the BDNF-treated group corroborate these findings in vivo. The specificity of this observation is underlined by the absence of significant changes in MAP2, which represents the propensity of this observation is underlined by the absence of significant changes in MAP2, which represents the growth in vitro,30 our data with transient perilesional upregulation of the growth associated with MAP1B in the BDNF-treated group corroborate these findings in vivo. The specificity of this observation is underlined by the absence of significant changes in MAP2, which represents the “late” MAP suggested to be involved in stabilization and maintenance of synaptic circuits in the mature brain.31 Interestingly, BDNF was also able to induce increased immunostaining of the presynaptic marker protein synaptophysin at 6 weeks in the contralateral hemisphere, indicating widespread remodeling processes. BDNF and, to a lesser extent, FAU treatment significantly reduced astrogliosis as measured by GFAP staining (Figure II). Activation of astrocytes is a common feature of neurological disorders, but whether beneficial or
adverse effects on neurons predominate is still unclear. Our results corroborate recent findings of Acarin and colleagues, demonstrating that inhibition of the glial response reduces neurotoxicity.

Physical therapy may be beneficial in improving sensorimotor function after focal lesions. Forced exercise on a running wheel early after focal cortical lesions improved functional outcome without changing lesion volume and improved functional outcome after early “adverse” moderate motor therapy of permanent middle cerebral artery occlusion. Furthermore, early and intensive learning after ischemia in a socially enriched environment can improve functional outcome but is accompanied by increased brain damage. As shown here, physical training modeled by FAU treatment for 14 days initiated 1 hour after photothrombotic stroke impaired neither functional motor outcome or infarct volume. Effects of FAU treatment on functional outcome are not completely understood. Although human studies indicate that FAU in the chronic phase after stroke significantly improves the function of the paretic arm, experimental studies are more controversial. FAU was reported to retard functional recovery and increase the original electrolytic lesion of forelimb sensorimotor cortex. No increase in cortical infarct volume but impaired functional recovery was reported after 45 minutes of FAU in distal middle cerebral artery occlusion, and no effect of FAU on lesion size or functional outcome was reported after transient suture occlusion. FAU was also reported to reduce damage and improve function in a rat model of Parkinson’s disease in which castrated animals were similar to sham rats in spontaneous limb use and apomorphine-induced rotation. All these findings point toward a lesion-dependent effect of FAU: Large lesions of the cortex such as electrolytic lesions or distal middle cerebral artery occlusions include larger penumbral areas with cells at risk where behavioral pressure may further increase energy failure and finally kill these cells. There is also evidence that FAU after large lesions involves glutamate because blockade of the N-methyl-D-aspartate receptor attenuated use-dependent exaggeration of the injury. Smaller or no lesions of the cortex such as transient suture occlusion, photothrombotic ischemia as shown in the present study, or a model of Parkinson’s disease have no or smaller penumbral areas with fewer cells at risk, so behavioral pressure could be compensated for by alive cells, which may then improve function through early training. In these milder pathophysiological conditions, excessive release of glutamate into the extracellular space may not be the key factor because FAU did not result in increases in glutamate levels when measured with in vivo microdialysis. Independent of lesion size, forced exercise such as FAU may have increased BDNF expression that could have supported recovery of function. This raises the question as to how far BDNF and FAU influence each other. Because of complex interactions, the effects of a combined therapy with BDNF and FAU are hard to predict and should be investigated in future experiments.

In conclusion, postischemic intravenous BDNF treatment clearly improved motor recovery without affecting the final infarct size and induced widespread neuronal remodeling. Early FAU treatment had no detrimental effect on infarct size, caused no deterioration of motor function, but impaired sensorimotor function.

Acknowledgments
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