Brain-Derived Neurotrophic Factor Contributes to Recovery of Skilled Reaching After Focal Ischemia in Rats

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Background and Purpose—Brain-derived neurotrophic factor (BDNF) is involved in neuronal survival, synaptic plasticity, learning and memory, and neuroplasticity. Further, exogenous treatment with BDNF or exposing animals to enrichment and exercise regimens, which also increase BDNF, enhances behavioral recovery after brain injury. Thus, the beneficial effects of rehabilitation in promoting recovery after stroke may also depend on BDNF. We tested this hypothesis by evaluating the contribution of BDNF to motor skill relearning after endothelin-1–induced middle cerebral artery occlusion in rats.

Methods—Antisense BDNF oligonucleotide, which blocks the expression of BDNF (or saline vehicle) was infused into the contralateral lateral ventricle for 28 days after ischemia. Animals received either a graduated rehabilitation program, including running exercise and skilled reaching training, which simulates clinical practice, or no rehabilitation. Functional recovery was assessed with a battery of tests that measured skilled reaching, forelimb use asymmetry, and foraging ability.

Results—Rehabilitation significantly improved skilled reaching ability in the staircase task. Antisense BDNF oligonucleotide effectively blocked BDNF mRNA, and negated the beneficial effects of rehabilitation on recovery of skilled reaching. Importantly, antisense BDNF oligonucleotide did not affect reaching with the unaffected limb, body weight, infarct size, or foraging ability, indicating the treatment was specific to relearning of motor skill after ischemia.

Conclusions—This study is the first to identify a critical role for BDNF in rehabilitation-induced recovery after stroke, and our results suggest that new treatments to enhance BDNF would constitute a promising therapy for promoting recovery of function after stroke. (Stroke. 2009;40:1490-1495.)

Key Words: hemiplegia ■ neurotrophins ■ plasticity ■ rehabilitation ■ stroke

Stroke is the leading cause of adult disability in developed countries, and most stroke survivors live with residual impairments that diminish independence and quality of life. After stroke, rehabilitation is used to promote relearning of original movements as well as learning compensatory movement patterns. The specific brain structures and mechanisms of recovery involved in motor learning (and relearning) after brain injury have not been identified. However, research in the past 10 years has identified brain-derived neurotrophic factor (BDNF) as playing an important role in various forms of neuroplasticity in both the intact and the damaged brain. BDNF is one of a family of neurotrophins that influences neuronal proliferation, survival, and differentiation as a result of binding to its tyrosine kinase receptor and subsequent downstream activation of several signal transduction pathways. Both BDNF and tyrosine kinase receptor are widely distributed throughout the brain, with highest expression in the hippocampus. BDNF is stored and released from glutamate neurons in a use-dependent fashion and has been implicated in long-term potentiation, learning, memory formation, depression, and recovery from brain injury. Administering BDNF in the acute postischemic period reduces cell death and infarct volume, whereas delayed treatment facilitates motor recovery in rats. Interestingly, exercise also upregulates BDNF and improves behavioral outcome in rodent stroke models. After injury, rehabilitation and motor learning aid in reorganizing cortical maps, which is thought to be one of the main effectors of recovery after stroke. Motor learning increases BDNF levels in the cortex, which may contribute to cortical map reorganization, increased synaptogenesis, enhanced dendritic spine formation and branching, and other forms of neuronal plasticity implicated in recovery after stroke. Furthermore, pharmacologically enhancing downstream BDNF signaling pathways/targets such as cAMP response-element-binding protein facilitates cortical reorganization and functional recovery after focal ischemia in rats.

Alternatively, inhibiting BDNF appears to have the potential to attenuate recovery processes. Recent findings show...
that healthy individuals with a val66met polymorphism in the BDNF gene show reduced ability to change cortical maps in response to motor training.19 In addition, blocking BDNF decreases cAMP response-element–binding protein and synapsin 1, which are regulated by BDNF, and this impairs learning and memory.20,21 Similarly, BDNF-mutant mice exhibit impaired long-term potentiation and are unable to learn.22,23 Together, these findings provide support for the role of BDNF in motor map reorganization and learning and memory, processes that are likely important for relearning motor skills after stroke.

Most of the data implicating BDNF in recovery processes are correlational in nature. Thus, we decided to directly test the hypothesized role of BDNF in rehabilitation-induced functional recovery after stroke. In the present study, we infused antisense BDNF oligonucleotide (or vehicle) into the contralateral lateral ventricle to block the expression of BDNF mRNA after focal ischemia. Rats were then exposed to either a social housing condition or a challenging graduated rehabilitation program that effectively promotes recovery of skilled reaching.13

Subjects and Methods

Subjects
Thirty-two male Sprague-Dawley rats (Charles River Laboratories, Montreal, Canada) weighing 230 to 275 grams at the time of surgery were used in this study. Animals were housed in pairs in Plexiglas cages on a 12-hour reverse light/dark cycle with water and food ad libitum. All procedures were performed during the animals’ dark phase. Efforts were made to minimize suffering and reduce the number of animals used (ie, group sizes determined from experience with ischemia model and behavioral tests). Experimental protocols were performed in accordance with Canadian Council on Animal Care guidelines and approved by the Memorial University Animal Care Committee. Figure 1 illustrates the timeline of experimental procedures.

Behavioral Training

Staircase Task
Animals were trained to reach for food pellets in the staircase test of independent forelimb reaching ability24 over 2 weeks (2 trials per day; 15 minutes per trial). Rats were food deprived to ~90% of free feeding weight during training and placed in the staircase apparatus (length, 300 mm; width, 68 mm; height, 120 mm) with 7 stairs descending on each side. Each stair was baited with 3 food pellets (45 mg; Noyes Precision Pellets, Research Diets), and staircases were positioned so pellets on the left stairs could only be retrieved with the left paw, and on the right stairs with the right paw. Further, pellets could not be retrieved from the bottom of the apparatus once they had been displaced from the stairs. Baseline (prestroke) reaching performance was determined from the last 2 trials.

Forelimb Use Asymmetry (Cylinder) Task
Two days before surgery, rats were placed in a Plexiglas cylinder (diameter, 200 mm; height, 350 mm) on a glass tabletop and video-recorded from below (via an angled mirror) for 4 minutes. Spontaneous forelimb use during wall exploration was analyzed by recording the number of ipsilateral, contralateral, and bilateral (simultaneous) contacts used for body support against the cylinder wall. The percent use of the affected (contralateral) limb was expressed as25:

\[ \text{(number of contacts with contralateral limb)} \times 100 \]

(ipsilateral limb contacts + contralateral limb contacts)

Focal Ischemia
Rats were anesthetized with isoflurane (3% induction, 1.5% maintenance in 30% O2, and 70% N2O) and placed in a stereotaxic frame. After a midline scalp incision, a hole was drilled at anterior-posterior +0.9 mm, medial-lateral 5.2 mm, relative to Bregma contralateral to the preferred paw as determined in the staircase task. To produce focal ischemia, the vasoconstrictive peptide endothelin-1 (1200 pmol in 3 μL sterile water; Calbiochem) was injected adjacent to the middle cerebral artery (dorsal-ventral −8.7 mm) over 13 minutes.16 After infusion, the needle was kept in position for an additional 5 minutes to minimize backflow. The wound was sutured closed and topical anesthetic and antibiotic ointments were applied. Body temperature was maintained between 36.5°C and 37.5°C during surgery with a self-regulating heating blanket (Harvard Apparatus).

Treatment Condition Assignment
Three days after ischemia, animals were tested in the staircase and cylinder tasks and stratified according to level of impairment (mild, 60%–80% baseline performance; moderate, 26%–59%; severe, <25%). Rats were further randomized into treatment condition on day 4, whereby half of the animals received antisense BDNF oligonucleotide to block BDNF levels (block), whereas the others received vehicle infusion (vehicle). The rats were then further divided (day 5) into groups that received either rehabilitation (rehab) or the standard housing condition (no rehab).

Osmotic Mini-Pump Implantation
Four days after ischemia, rats were anesthetized as described and placed in a stereotaxic frame. A hole was drilled at anterior-posterior −8.0 mm, medial-lateral 1.5 mm from Bregma, contralateral to ischemic hemisphere. A cannula was inserted into the lateral cerebral ventricle at a depth of 4.0 mm and secured with dental cement. Osmotic mini-pumps (Model 2004; Alzet) were filled with either antisense BDNF oligonucleotide (sequence 5’-TCT GCC CTT TTT GGT-3’; IDT) or saline vehicle, attached to the cannula via polyvinylchloride tubing (~150 mm length; Brain Infusion Kit 2; Alzet), and placed subcutaneously between the scapulae. Antisense oligonucleotide dissolved in sterile saline (100 nm/200 μL) or saline vehicle was released at a rate of 0.25 μL per hour for 28 days. Pumps, cannulae, and tubing were assembled under sterile conditions and were weighed both before and after filling to ensure the pumps were accurately filled.

Rehabilitation Protocols
One week before focal ischemia, animals were oriented to both the motorized running wheel and the rehabilitation reaching apparatus twice daily for 2 days. Rats ran for 10 minutes at a speed of 5.5 meters per minute in the running wheel (diameter, 360 mm; width, 130 mm) and received 30 minutes of reach training in the reaching apparatus (tray height, 60 mm).

Rehabilitation (or control condition) began 5 days after ischemia (Figure 2) and was graduated, and became more challenging over time. The total duration of rehabilitation (running and reaching rehabilitation) progressed from 30 minutes per day on day 1 to 120 minutes per day by day 27. The motorized running wheel was initially set for a slow pace (5.5 m/min) and short duration (15 min).
Three days after the last rehabilitation session (Tissue Processing and Injury Assessment normal latencies to explore and obtain 10 food pellets scattered administered at 4 weeks to evaluate whether rats demonstrated ischemia in the staircase and cylinder tasks. A foraging test was daily and given an amount of food pellets equivalent to that of rats progressing to 60 min/session). For the last 14 days, a plastic insert was placed in the reaching apparatus immediately after running exercise (wheel exercise (A) followed by skilled reaching (B). A tray filled with food pellets was placed at successively greater heights, (60 mm–140 mm) and durations (15 min maximum speed of 9.5 meters per minute over 60 minutes. Rats were placed in the reaching apparatus immediately after running exercise and were required to reach for food pellets (45 mg; Noyes) at successively greater heights, and a plastic insert was used to prevent rats from reaching with their less affected forelimb.

Running speed and duration progressively increased each week to a maximum speed of 9.5 meters per minute over 60 minutes. Rats were placed in the reaching apparatus immediately after running exercise and were required to reach for food pellets (45 mg; Noyes) at successively greater heights (60 mm–140 mm) and durations (15 min progressing to 60 min/session). For the last 14 days, a plastic insert was placed into the tray to prevent reaching with the less affected forelimb. Rats that did not receive rehabilitation were handled daily and given an amount of food pellets equivalent to that of rats in the rehabilitation condition.

Behavioral Testing
Functional outcome was assessed 2, 3, and 4 weeks after focal ischemia in the staircase and cylinder tasks. A foraging test was administered at 4 weeks to evaluate whether rats demonstrated normal latencies to explore and obtain 10 food pellets scattered evenly over the floor of a clean animal cage. The time required to find and eat all pellets was recorded.21,26

Tissue Processing and Injury Assessment
Three days after the last rehabilitation session (~4.5 weeks after ischemia), rats were deeply anesthetized with sodium pentobarbital (65 mg/kg, intraperitoneally) and transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in phosphate-buffered saline. The integrity of the pump, cannulae, and tubes was examined. Pumps were removed and the remaining liquid was withdrawn to ensure that the appropriate volume had been released. Brains were removed from the skull, fixed in 4% paraformaldehyde in phosphate-buffered saline for 90 minutes at 4°C, and then transferred to 20% sucrose at 4°C. Forty-µm-thick coronal sections were cut with a cryostat and every eighth section (spaced 320 µm apart) was stained with cresyl violet to assess tissue loss. To obtain the area of the injured tissue in the cortex and striatum, the area of the intact cortex and striatum in the injured hemisphere was subtracted from the area of healthy tissue in the contralateral hemisphere using NIH Image software. The total volume of injury (mm³) was calculated for the cortex and striatum separately by multiplying the area recorded from each slice by the distance between the initial and last slices.13 The remaining sections were stored in cryoprotectant at −20°C.

Total RNA Extraction and cDNA Synthesis
Thirty-six 40-µm sections (taken every 320 µm) from each ischemic animal were used for total RNA isolation. Lipids were removed using a chloroform extraction and total RNA was extracted using the High-Pure RNA Paraffin Kit (Roche) according to the manufacturer’s protocol. Total RNA concentration for each sample was measured with a spectrophotometer at 260 nm (1 optical density unit=40 µg/mL) and stored at −80°C. cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems) with random primers. Total RNA (4 µg) was used to synthesize cDNA for BDNF assays.

Relative Quantification of Target Gene Expression
Quantitative polymerase chain reaction, used to amplify DNA, was performed using the ABI Prism 7000 (Applied Biosystems). Target gene expression was normalized to endogenous gluteraldehyde-3-phosphate dehydrogenase (GAPDH). Primer/probe sets were purchased as premade TaqMan Assays on Demand: gluteraldehyde-3-phosphate dehydrogenase (Rn99999916_s1); BDNF (Rn00560868_m1) and thermal cycling conditions were as described by the manufacturer in a total volume of 25 µL. All samples were assayed in triplicate.

Relative quantification of target mRNA, normalized to gluteraldehyde-3-phosphate dehydrogenase expression, was performed using the comparative cycle threshold (CT) method. The relative target gene expression was expressed as 2^{-ΔΔCT}, where ΔΔCT is ΔCT test animal – ΔCT calibrator animal. Two animals in the vehicle-no rehab condition were randomly chosen as the calibrator sample (ie, target gene expression averaged and arbitrarily given the value of 1). The ΔCT was defined as C_{target} – C_{GAPDH} and the target gene was BDNF. To determine the amount of template required for each target gene, a validation curve was constructed to verify that ΔC_{target} was proportional to the template amount. The slope for BDNF was 0.09, which was within the acceptable limit of <0.10.

Statistical Analyses
Behavioral analyses were performed by experimenters blind to group identity. Statistical analyses were conducted using SPSS software (version 14). Repeated-measures ANOVAs were used to analyze behavioral data. A 2-factor ANOVA (main effects of blocking BDNF and rehab) was used to analyze infarct volume and BDNF mRNA. Least significant difference posthoc comparisons were used when appropriate. Significance was set at P<0.05 for all analyses and values are expressed as mean±SEM.

Results
BDNF mRNA Levels
Four rats were excluded from the study because of damaged catheter tubing or pump failure, leaving 28 animals included in the analysis (block–no rehab n=6; block–rehab n=8; vehicle–no rehab n=6; vehicle–rehab n=8).

Animals that received the BDNF antisense oligonucleotide (intracerebroventricular infusion) had significantly lower brain
levels of BDNF mRNA (2.55 arbitrary units) than those with vehicle infusion (1.15; P<0.05).

**Effects of BDNF Antisense Oligonucleotide on Tissue Loss, Body Weight, and Foraging Ability**

The volume of tissue lost did not differ significantly among groups (Table; Figure 3). Body weight and the time to obtain all food pellets in the foraging test were also similar among groups (Table).

**Skilled Reaching Performance**

All groups exhibited substantial reaching impairments in the staircase task 4 days after ischemia (Figure 4). A 3-way ANOVA (within-subjects factor: time; between-subjects factors: blocking BDNF, rehab) revealed a significant time×block×rehab interaction (P<0.05), and a significant time×rehab interaction (P<0.05), indicating that performance in the rehab condition improved over time, whereas performance in the block condition did not (Figure 4). Further analyses (2-factor between-subjects analysis at each time point, followed by least significant difference posthoc comparisons when appropriate) revealed a significant block×rehab interaction at week 4 (P<0.05), which indicated that rehabilitation significantly improved reaching success, but that blocking BDNF attenuated this improvement. Animals in all groups maintained or improved skilled reaching ability with the ipsilateral (less affected) forelimb over the course of the experiment (data not shown).

**Forelimb Use for Postural Support**

Analysis of contralateral forelimb use in the forelimb-use asymmetry (cylinder) task revealed that all groups were significantly impaired after ischemia (P<0.05, day 4 vs baseline; data not shown) and that the level of impairment was similar among groups (P>0.05). A repeated-measures ANOVA on limb use from 4 days to 4 weeks after ischemia revealed that there were no significant main effects of block or rehab and no interactions (P>0.05), suggesting that the frequency with which rats used their contralateral forelimb was similar regardless of treatment condition (Figure 5). Furthermore, the effect of time was not significant, indicating that there was no significant recovery in limb use over time (P>0.05).

**Discussion**

Several therapeutic interventions such as exercise, exposure to an enriched environment, and rehabilitation enhance functional recovery after stroke. Beneficial effects of these therapies include improved learning and memory,27,28 improved motor function,13,16 and enhancement of proteins involved in brain plasticity such as BDNF, cAMP response-element-binding protein, and synapsin-1.11,27,29–31 Consistent with these findings, we found that a challenging and graduated rehabilitation therapy consisting of running exercise and reach training significantly improved skilled reaching in the staircase task after focal ischemia in rats. Importantly, attenuating BDNF mRNA via intracerebroventricular infusion of antisense oligonucleotide prevented behavioral recovery. Pre-
vious investigations have found that increasing BDNF levels or activating BDNF associated signaling pathways leads to better recovery after stroke; however, the present results provide the first direct demonstration that BDNF is crucial for mediating the motor relearning that takes place as a result of poststroke rehabilitation.

The lack of recovery of skilled reaching in the block-rehab group was not attributable to an exacerbation of injury because there was no difference in infarct volumes between groups. Furthermore, the BDNF oligonucleotide did not induce nonspecific deficits in the animals (eg, general malaise, anhedonia) because body weight was comparable among groups as were the latencies to consume food rewards in the foraging test. Thus, there appears to be no motivational impairment in the rats with decreased expression of BDNF. This is an important consideration because increased levels of BDNF have been linked to the mood elevating actions of antidepressant drugs as well as exercise.

The recovery profile observed in the cylinder forelimb use asymmetry test differed from that in the staircase test. Because very little recovery occurred over time, and all groups were similarly impaired, we did not detect an effect of reducing BDNF mRNA levels. It is somewhat surprising that the vehicle-rehab group recovered nearly to prestroke levels in the staircase test because typically reaching impairments in both humans and rodents are very resistant to any therapeutic intervention. However, in the present study the cortical damage, although extensive, typically spared most of the forelimb motor cortex, thereby allowing reorganization processes (eg, increased dendritic branching, reconfiguration of motor maps, etc) to take place. Also, it must be remembered that the rats are highly motivated to use the impaired limb in the staircase test to obtain highly palatable food rewards, unlike the cylinder task that is a test of spontaneous, non-skilled limb use.

Although BDNF appears to underlie many forms of memory, it is not involved in all types of learning and memory.

For example, animals with BDNF deletions learn fear extinction within sessions but are unable to consolidate new learning between sessions. In addition, inhibition of hippocampal BDNF associated CAMKII signaling impaired memory retention but did not affect learning in the Morris water maze. Recently Gomez-Pinilla et al identified a critical role for BDNF in a model of simple spinally-mediated motor learning. In that study, blocking BDNF levels attenuated a learned flexion response and decreased expression of signaling molecules CaMKII, cAMP response-element-binding protein, and synapsin mRNA. BDNF infusion into the subarachnoid space restored learning, which was significantly correlated with BDNF mRNA levels. These data and our own findings suggest that BDNF also plays a crucial role in motor learning orchestrated at both cortical and spinal cord levels. Indeed, relearning motor skills after stroke is multifaceted incorporating or relying on spatial context, retrieval of previously learning movement strategies, motivation, working memory, and internal and external sensory feedback. Therefore, strategies that increase BDNF broadly within the nervous system, such as exercise or BDNF infusion, may enhance neuroplasticity processes in multiple neuronal systems involved in motor relearning during stroke rehabilitation.

Our data clearly show that rehabilitation in combination with endogenous growth factor release is essential for post-stroke recovery. Many complex processes contribute to both injury and recovery after stroke. Thus, combination therapies may provide the greatest benefit for stroke patients. The optimal rehabilitation paradigm, which will critically depend on timing, intensity, and duration of treatment, as well as stroke severity and subtype, has yet to be identified. Furthermore, although we have determined a critical role for BDNF in this study, the restorative potential of other growth factors, or combinations of growth factors, must also be assessed. It is essential that preclinical studies evaluate how such therapies interact, and how they can be further optimized for stroke patients.

In summary, attenuating BDNF levels in the brain after middle cerebral artery occlusion in rats completely negated recovery of skilled motor movements. These data illustrate the critical role of BDNF in recovery of motor function in response to rehabilitation. The challenge is to develop new methods for harnessing the therapeutic potential of BDNF in ways that can optimize the “neuroplastic milieu” of the ischemic brain to achieve more complete functional recovery.

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Disclosures
None.

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