Brain-Derived Neurotrophic Factor But Not Forced Arm Use Improves Long-Term Outcome After Photothrombotic Stroke and Transiently Upregulates Binding Densities of Excitatory Glutamate Receptors in the Rat Brain

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Background and Purpose—Both application of neurotrophic factors like brain-derived neurotrophic factor (BDNF) and constraint-induced movement therapy like forced arm use have been shown to potentially improve outcome after stroke. The aim of the present study was to check whether postischemic long-term outcome correlates to specific modifications in the abundance of various neurotransmitter receptors.

Methods—Adult male Wistar rats were subjected to photothrombotic ischemia and assigned to various treatment groups (n=5 each) with end points at 3 and 6 weeks: (1) ischemic control (saline); (2) BDNF (ischemia, 20 μg BDNF); (3) forced arm use (ischemia, saline, and ipsilateral plaster cast for 5 or 14 days for the 3- and 6-week groups, respectively); and (4) combined treatment (combi; ischemia, 20 μg BDNF, forced arm use). Animals received intravenous bolus infusions of saline or BDNF 1 hour 3 and 5 days after ischemia, respectively. A group of sham rats (n=2) served as a control. A battery of behavioral tests was performed before and up to 6 weeks after ischemia. Quantitative in vitro receptor autoradiography was performed on 12-μm-thick cryostat sections using [3H]MK-801, [3H]AMPA, and [3H]muscimol for labeling of NMDA, AMPA, and GABA_A receptors, respectively.

Results—Best functional outcome was seen after BDNF treatment, whereas vice versa rats with forced arm use did worse in behavioral performance. Improved behavioral outcome was associated with increased perilesional binding densities of NMDA and AMPA receptors 3 weeks after stroke.

Conclusions—Our findings suggest that transient enhanced neurotransmission as reflected by increased ligand binding of NMDA and AMPA receptors may participate in successful postlesional reorganization processes. (Stroke. 2008;39: 1012-1021.)

Key Words: forced arm use ■ receptors ■ stroke ■ trophic factors

For a long time, clinicians have recognized that after cerebral injury, over time there is, at least to some degree, spontaneous recovery referred to as plasticity or reorganization (for review, see Chen et al^1). Based on this phenomenon, basic research focuses on manipulating these processes to accelerate and enhance recovery after stroke. We could recently demonstrate that intravenously administered brain-derived neurotrophic factor (BDNF) significantly improves reorganization processes and neurological long-term outcome in a phototothrombotic stroke model in the rat. A completely different approach to enhance postischemic reorganization is subserved under the term “constraint-induced movement therapy” or “forced arm use” (FAU; for review, see Taub et al^3). The molecular basis and the long-term effects of these different therapeutic strategies are largely unknown. Because one mechanism possibly contributing to deficits and recovery is an altered balance between excitatory and inhibitory neurotransmitter receptors,^4 our study was designed to analyze potential correlations between functional outcome in BDNF- versus FAU-treated rats and receptor densities of excitatory glutamate receptors and the inhibitory GABA_A receptor by quantitative receptor autoradiography. A third group with combined treatment of BDNF and FAU was analyzed to check for potential additional or synergistic effects.

Materials and Methods

Experimental Groups

All animal procedures were carried out according to the guidelines of the German animal protection law and were approved of by the local ethics committee. Experiments were performed on adult male Wistar rats (Charles River, Sulzfeld, Germany; 280 to 320 g body weight),
which had free access to food and water before experiments. Animals were randomly assigned to groups (n=5) with end points at 3 and 6 weeks: (1) control group (ischemia, treatment with 0.5 mL saline 0.9% as intravenous bolus infusion starting 1 hour after ischemia); (2) BDNF group (ischemia, treatment with 20 μg BDNF [Amgen] dissolved in 0.5 mL saline 0.9% as intravenous bolus infusion starting 1 hour after ischemia followed by a plaster cast of the ipsilateral side); and (4) combi group (ischemia, treatment with 0.5 mL saline 0.9% as intravenous bolus infusion starting 1 hour after ischemia followed by a plaster cast of the ipsilateral side). All animals received subsequent repetitive intravenous bolus infusions through the tail vein at days 3 and 5 (groups 1 and 3: saline; groups 2 and 4: BDNF). A sham group (n=2) was subjected to all experimental procedures except for induction of photothrombotic ischemia.

**Photothrombotic Ischemia**

All animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg body weight; WDT, Garbsen, Germany) and rompun (8 mg/kg/body weight; Bayer, Leverkusen, Germany), and anesthesia was maintained if necessary. The left femoral artery was cannulated with PE-50 polyethylene tubing for continuous monitoring of arterial blood pressure and blood sampling for analysis of arterial blood gases. During the experiment, rectal temperature was monitored and maintained at 37°C by a thermostat-controlled heating pad (Föhr Medica Instruments). Photothrombotic ischemia was induced in the frontal cortex. For illumination, a fiberoptic bundle with a 1.5-mm aperture was placed stereotactically onto the skull 4 mm posterior to the bregma and 4 mm lateral from the midline (white light beam, 150 W, 20 minutes). During the first 2 minutes of illumination, the dye, Rose bengal (0.5 mL/kg body weight, 10 mg/mL saline), was injected intravenously. After surgery, catheters were removed and the animals were allowed to recover from anesthesia.

**Forced Arm Use**

Before recovery from anesthesia, FAU-treated animals were fitted with one-sleeve plaster casts. The upper torso was wrapped in soft felt, and the ipsilateral forelimb was wrapped in felt and positioned in a naturally retracted position against the animal’s sternum. Plaster of Paris strips were wrapped around the immobilized limb and upper torso. FAU treatment with a one-sleeve plaster cast of the ipsilateral side lasted 5 days for the 3-week groups and 14 days for the 6-week groups.

**Behavioral Testing**

In all animals of the 6-week groups, a battery of behavioral tests was performed before ischemia (baseline) as well as 3, 4, 5, and 6 weeks after ischemia by an investigator (K.H.) who was blinded to the experimental groups. Beam balance performance of animals was measured. The speed was slowly increased from 4 to 40 rpm within 5 minutes. The trial ended whenever the animal fell off the rungs or gripped the device and spun around for 2 consecutive revolutions without attempting to walk on the rungs. An arbitrary limit of time was set for the rats at 500 seconds on the Rotorod cylinder during training as well as during testing procedures. The animals were trained 3 days before ischemia, and the mean duration (seconds) on the device was recorded with three measurements.

For the adhesive-removal test, somatosensory deficit was measured both before and after ischemia. All rats were familiarized with the testing environment. In the initial test, 2 small pieces of adhesive-backed paper dots of equal size (113.1 mm²) were used as bilateral tactile stimuli occupying the distal–radial region at the wrist of each forelimb. The rat was then returned to its cage. The time to remove each stimulus from forelimbs was documented by 5 trials per day for each forepaw. Individual trials were separated by a time shift of at least 5 minutes. Before surgery, the animals were trained for 3 days. Once the rats were able to remove the dots within 10 seconds, they were subjected to ischemia.

**Neurological function** was graded according to a Neurological Severity Score on a scale of 0 to 10 (normal score=0; maximal deficit score=10). This score is a composite of motor, sensory, and reflex tests. In the severity scores of injury, one score point is awarded for the inability to perform the test or for the lack of a tested reflex. Hence, the higher the score, the more severe the injury.

**Brain Tissue Calculations**

After 3 or 6 weeks, respectively, rats were neurologically assessed, reanesthetized with ketamine (150 mg/kg intraperitoneally), and decapitated. Brains were rapidly removed, frozen in isopentane at −30°C for 10 minutes, and stored at −80°C until analysis. For receptor autoradiography and assessment of infarct volume, coronal cryostat sections of 12 μm thickness were serially cut at −20°C at the level of the striatum and mounted on TESPA-coated slides. Additional sections were taken at the level of the dorsal hippocampus. Because photothrombosis only produces well-defined cortical infarcts, infarct volumes after 3 and 6 weeks were estimated by measurement of the maximum diameter and measurement of the maximum infarct area on the slides, as previously described. Because infarct size and tissue loss do not always match, an additional analysis of the remaining cortical tissue was performed at the level with the largest infarct extension.

**Receptor Autoradiography**

Quantitative in vitro receptor autoradiography studies were performed using [3H]MK-801, [3H]AMP, and [3H]muscimol as ligands for NMDA, AMPA, and GABA_A receptors, respectively. Ligands were purchased from PerkinElmer, Inc. Labeling of in incubation procedures for the different binding sites were performed according to protocols of Zilles et al previously described. In brief, incubation with [3H]MK-801, [3H]AMP, and [3H]muscimol was always preceded by a preincubation period with the respective buffer to remove endogenous ligands. To demonstrate the maximal binding of [3H]MK-801 to NMDA receptors, the binding assay was performed in a magnesium- and zinc-free solution (50 mmol/L Tris-HCl buffer, pH 7.2) and in the presence of 30 μmol/L glycine and 50 μmol/L spermidine with 5 nM [3H]MK-801 (specific activity 28.1 Ci/mmol) at 22°C for 60 minutes. Incubation was terminated by washing in cold buffer (2×5 minutes) and in H2O (2 sec). AMPA receptors were labeled with 10 nM [3H]AMP (specific activity 55.5 Ci/mmol) in 50 mmol/L Tris-acetate buffer (pH 7.2, containing 100 mmol/L KSCN) for 45 minutes at 4°C. Incubation was terminated by rinsing (3×4 seconds) in cold buffer and by postfixation with rinses (2×2 seconds) in acetone/glutaraldehyde (100:2.5) solution. GABA_A receptors were incubated with 3 nM [3H]muscimol (50 mmol/L Tris-citrate buffer, pH 7.0) for 40 minutes at 4°C. Incubation was terminated by rinsing (3×4 seconds) in cold buffer. Unspecific binding was determined by coinubation of alternating sections with labeled ligands and an excess of appropriate unlabeled competitor. After the final rinsing procedure, slides were carefully dried in either a stream of cool air ([3H]MK-801 and [3H]muscimol) or hot air ([3H]AMP). Air-dried, tritium-labeled sections were coexposed with [3H]plastic standards (Autoradiographic [3H] Microscale; Amersham Biosciences, Freiburg, Germany) and brain-paste standards to a [3H]-sensitive film (Hyperfilm-H; Amersham Biosciences) for 5 weeks. Autoradiographies were scanned in equal light conditions with a digital camera (Roper Scientific, Olympus, Munich, Germany) and digitized with the MCID image analysis system (Imaging Research Inc, St. Catharines, Ontario, Canada). Gray value images of the coexposed plastic standards were used to compute a nonlinear calibration curve, which defined the relation-
ship between gray values in the autoradiographs and concentrations of radioactivity. Plastic standards were calibrated to tissue standards with known concentrations of radioactivity. Final values were normalized to sham control levels (mean±SEM). Quantitative analysis of ligand binding was performed in cortical areas Fr1, Par1, and Par2 both ipsilateral and contralateral to the ischemic lesion (Figure 1). The respective brain areas have been identified according to the atlas by Paxinos. Regions of interest were marked on the monitor and the gray values automatically assessed by the imaging software. In all cases, nonspecific binding was just above background labeling or not visible at all. Therefore, background density could be used as an estimate of unspecific binding and subtracted from total binding.

**Statistical Analysis**

One-way analysis of variance (ANOVA) and post hoc least significant difference was used for comparison of postmortem infarct volumes and analysis of the remaining cortical tissue. Ligand binding was analyzed by calculating mean concentration values for each ligand and region. Significant group effects between different ischemic groups were confirmed by one-way ANOVA and Bonferroni error protection. Values of behavioral testing are presented as means±SEM. A 2-way repeated measure ANOVA and subsequent post hoc least significant difference tests were used for statistical analyses. Analysis was performed using the General statistics module of Analyze-it for Microsoft Excel (Leeds, UK). For all tests, P<0.05 was considered statistically significant.

**Results**

**Behavioral Testing**

BDNF-treated animals had a more favorable motor recovery (Beam-Balance, Rotorod, Neurological Severity Score) over time compared with controls, FAU-treated animals, and rats of the combined treatment group (see Figure 2). This effect was significant (P<0.05) for all tests after 6 weeks with the exception of the Rotorod test. FAU-treated rats performed worst in all tests over time. Because combined sensorimotor deficits develop in the photothrombotic lesioning model used, it is important to test for both qualities. Sensorimotor function, as established by the adhesive removal test, again, achieved the best results in BDNF-treated rats compared with the lowest results in FAU-treated rats (Figure 2).

**Brain Tissue Calculations**

Infarct volumes were 11.3±4.4 mm³ (control), 14.7±3.4 mm³ (BDNF), 12.9±2.7 mm³ (FAU), and 18.4±8.5 mm³ (combi) after 3 weeks and 8.9±4.8 mm³ (control), 13.6±4.5 mm³ (BDNF), 13.3±4.9 mm³ (FAU), and 6.1±2.2 mm³ (combi) after 6 weeks, respectively. Differences were not significant among
groups (means ± SEM; *P < 0.05, ANOVA). Analysis of the remaining cortical tissue in the various experimental groups showed no significant differences compared with NaCl-treated rats at both time points. The total cortical area of the hemisphere containing the infarct was significantly reduced in all treatment groups compared with sham rats, both at 3 and 6 weeks (Figure 3).

Receptor Autoradiography

[3H]MK-801 Ligand Binding
Within the ischemic core, [3H]MK-801 binding was widely abolished 3 and 6 weeks after photothrombotic stroke in all experimental groups (see Figure 4). The corresponding cortex of the contralateral hemisphere (Fr1) showed largely the same
binding densities within the various experimental groups both after 3 and 6 weeks. In layers I and II of the cortex adjacent to the ischemic lesion (Par1), labeling of NMDA receptors by tritium-labeled MK-801 was significantly increased in BDNF-treated rats compared with the other treatment groups 3 weeks after ischemia, whereas, after 6 weeks, labeling significantly decreased in layers I to III in the BDNF group compared with ischemic controls. In layer I, a significant reduction of [3H]MK-801 ligand binding was also seen in the FAU and combi groups compared with ischemic controls. In the corresponding contralateral cortex, [3H]MK-801 ligand binding was increased in layer V after 3 weeks. No other significant changes were observed both after 3 and 6 weeks. After 3 weeks, binding densities in remote cortical areas

Figure 3. Remaining cortical tissue (mean ± SEM) (A, C) and alterations of the total cortical hemispheric area (B, D) after 3 (A, B) and 6 weeks (C, D), respectively. The hemisphere with the infarct is indicated in gray; the contralateral unlesioned hemisphere is hatched. Absolute values are presented at the top and differences in percent at the bottom of the respective diagram. At 3 (A) and 6 (C) weeks, no significant differences in the remaining cortical tissue of the various treatment groups are present compared with the NaCl control group. Similarly, both after 3 (B) and 6 weeks (D), the total cortical hemispheric area is reduced in all experimental groups to a similar extent (*significant; P < 0.05, ANOVA and least significant difference).
(Par2) differed only slightly among experimental groups both ipsi- and contralateral to the lesion. After 6 weeks, no significant changes were detectable in the ipsilateral remote cortex. Contralateral to the ischemic lesion, however, [3H]MK-801 ligand binding was significantly upregulated at this time point in layers II to VI in the BDNF group compared with the combi group, and the same applied to layer VI compared with ischemic controls (Figure 4).

Figure 4. Quantitative analysis of [3H]MK-801 ligand binding 3 and 6 weeks in the ipsi- and contralateral regions Core/Fr 1, Par 1, and Par 2, respectively. Values are means ± SEM presented as percent of sham-operated controls (N, B, F, C: significant difference compared with NaCl, BDNF, FAU, or combi group, respectively; P<0.05, ANOVA all pairwise, Bonferroni).

[3H]AMPA Ligand Binding
[3H]AMPA binding density in the ischemic lesion was massively reduced after 3 and 6 weeks in all experimental groups.
[3H]AMPA ligand binding

(see Figure 5). Within the corresponding cortex of the contralateral hemisphere (Fr1), no significant changes among the various treatment groups became evident both after 3 and 6 weeks. Adjacent to the lesion (Par1), [3H]AMPA ligand binding increased after 3 weeks in all experimental groups. At the same time point, ligand binding to AMPA receptors was significantly higher in the contralateral cortex of FAU-treated rats in layers II and V compared with ischemic controls and in layers I, II, III, and V compared with the BDNF group. This pattern changed after 6 weeks. Now, highest binding densities were present both ipsi- and contralaterally in the deeper layers of the BDNF and combi groups. In the remote

Figure 5. Quantitative analysis of [3H]AMPA ligand binding 3 and 6 weeks in the ipsi- and contralateral regions Core/ Fr 1, Par 1, and Par 2, respectively. Values are means±SEM presented as percent of sham-operated controls (N, B, F, C: significant difference compared with NaCl, BDNF, FAU, or combi group, respectively; \( P < 0.05 \), ANOVA all pairwise, Bonferroni).
cortex (Par2), ligand binding ipsilateral to the lesion was reduced in all layers of all experimental groups after 3 weeks. This reduction was most pronounced in rats with combined treatment but reached significance only in layer IV. Contralaterally, no considerable differences in binding densities were present among the various experimental groups. [3H]AMPA binding densities were also quite similar in the various treatment groups after 6 weeks both ipsi- and contralaterally (Figure 5).

**[3H]Muscimol Ligand Binding**

Although 3 weeks after photothrombotic lesion, [3H]muscimol binding in the ischemic core was partially present in all experimental groups, this residual binding to GABAA receptors was widely abolished after 6 weeks (see Figure 6). Contralateral to the lesion, no considerable differences in cortical binding densities among experimental groups could be observed after 3 and 6 weeks. Also, the perilesional cortex (Par1), ipsi- and contralateral to the damaged side, did not reveal any differences in [3H]muscimol binding among the various treatment groups at both time points investigated. Similarly, no substantial differences in binding densities among the treatment patterns were detectable in remote cortical areas (Par2) both ipsi- and contralaterally (Figure 6).

### Discussion

In our present study, we analyzed the long-term effect of BDNF treatment versus FAU training after photothrombotic stroke on functional recovery using a battery of behavioral tests. The potential contribution of glutamatergic and GABAergic neurotransmitter receptors was analyzed by receptor autoradiography. In all functional tests carried out, BDNF-treated rats showed better performance 6 weeks after photothrombotic stroke than ischemic control rats, FAU-treated rats, and rats with combined therapy consisting of BDNF application and FAU. Conversely, FAU-treated rats performed worst in all tests, which reached significance even compared with ischemic rats in the Rotorod test. FAU-treated rats with additional application of BDNF showed a tendency to perform better than rats with FAU only but worse than untreated ischemic controls. These results corroborate our previous findings that postischemic BDNF treatment is superior to that of FAU. The positive effect of BDNF is also indicated by a trend to improve the outcome of rats with constraint-induced movement. Importantly, the effects observed are not attributable to significant differences in infarct size or reductions of the remaining or global cortical tissue between the various treatment groups (Figure 3).

Results from functional MRI and positron emission tomographic studies demonstrating plasticity, both on the damaged and the undamaged hemisphere after physical therapy paradigms, would suggest specific changes in cortical receptor-binding densities in FAU-treated rats. In fact, the importance of the contralateral hemisphere for improvement of functional outcome is corroborated by numerous experimental studies. Damaging the forelimb representation area of the sensorimotor cortex in the rat induces increased dendritic arborization of pyramidal neurons in the contralateral hemisphere. Furthermore, the recovery-enhancing effect of growth factors may, at least in part, be mediated by reorganization in the intact contralateral cortex. Using quantitative autoradiography, a selective increase in [3H]AMPA binding densities in the contralateral cortex could recently be shown in a model of transient focal cerebral ischemia in rats housed in an enriched environment. In addition, a strong correlation between [3H]AMPA binding densities and the rate of recovery could be observed. Similarly, in our study, compared with ischemic controls and BDNF-treated rats, there was significant upregulation of [3H]AMPA binding densities only in the FAU group 3 weeks after ischemic injury, although only on the contralateral undamaged hemisphere. This, however, was not accompanied by functional improvement. Possibly, this up-regulation might be a compensatory one related to the decrease of glutamate in the contralateral hemisphere due to the long immobilization period of the respective limb. An alternative explanation for this unexpected finding is that upregulated [3H]AMPA densities in this situation are merely a marker for the rat’s continuing attempt to move its casted forelimb. This pattern changed after 6 weeks. Then BDNF-treated rats showed significantly increased binding densities compared with the FAU group both ipsi- and contralateral to the lesion. This coincides exactly with a bilateral upregulation of synaptophysin in BNDF-treated animals, possibly reflecting AMPA receptor-mediated neurite growth and synaptogenesis.

The constellation of receptor binding densities, which was associated with the best functional outcome, ie, BDNF treatment, was a very specific one. Although 3 weeks after the photothrombotic insult, AMPA receptor binding densities were increased in all treatment groups in the ipsilateral cortex, an additional, significantly higher binding density for [3H]MK-801 in the ipsilateral perilesional cortex was detectable in BDNF-treated rats only. Inhibitory GABAA receptors detected by [3H]muscimol were largely unchanged and did not differ among the various treatment groups. Thus, a relative shift to excitatory neurotransmission seems to be a prerequisite for functional improvement. These findings further corroborate the hypothesis that postischemic hyperexcitability may enhance functional outcome in the long run. Although it is well established that excitotoxic overactivation of glutamate receptors is a key factor for consecutive neuronal death in the hyperacute postischemic phase, although preventable by NMDA receptor antagonists (for review, see Lee et al), there is increasing evidence that this time period of excitotoxicity is very short. In 1990, Barth et al hypothesized that MK-801 can have either beneficial or detrimental effects after brain damage, depending on the time point of application. Biegon et al could recently convincingly demonstrate in a mouse model of head injury that hyperactivation of NMDA receptors occurs only during the first hour after injury and is followed by a profound and longlasting functional loss. Consequently, in their model, stimulation of NMDA receptors 24 and 48 hours after injury significantly increased functional outcome. In our present study, however, upregulation of [3H]MK-801 ligand binding in the perilesional cortex in BDNF-treated rats was not static but decreased over time, resulting in significantly lowered levels compared with FAU and combi groups after 6 weeks and, thus, suggested the beneficial effect of a postlesional increase of excitatory neurotransmission being
transient and limited to a specific timeframe. Although the most favorable neurological outcome excellently correlates with this specific binding pattern of excitatory glutamate receptors, one has to concede that there is some overlap in binding patterns of FAU- and combi-treated rats, which, however, do also not exhibit any significant differences in any of the behavioral tests (Figure 2).

Summary
Our present study corroborates our recent data that BDNF treatment convincingly enhances functional recovery after photothrombotic stroke in the long run, whereas FAU treatment has no beneficial effect at all. The slightly more favorable neurological outcome in the combined therapy group further indicates the potential of BDNF for regenera-
tive processes. As demonstrated by receptor autoradiography, the simultaneous perilesional upregulation of NMDA and AMPA receptors may reflect the structural basis of an improved functional outcome, suggesting a positive effect of increased excitation for a limited time period.

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Disclosures
None.

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