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Brain-Derived Neurotrophic Factor Improves Long-Term Potentiation and Cognitive Functions after Transient Forebrain Ischemia in the Rat

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We investigated the effect of brain-derived neurotrophic factor (BDNF) on hippocampal long-term potentiation (LTP) and cognitive functions after global cerebral ischemia in the rat. After four-vessel occlusion, BDNF was administered via an osmotic minipump continuously over 14 days intracerebroventricularly. Electrophysiological experiments were performed 14 days after cerebral ischemia. Test stimuli and tetanization were delivered to the Schaffer collaterals of the hippocampus and field excitatory postsynaptic potentials (fEPSP) were recorded in the CA1 region. Cognitive impairment was analyzed repeatedly with a passive avoidance test, a hole-board test, and with an activity center on the same animal. In sham-operated animals, LTP was consistantly induced after delivering a tetanus (increase of initial slope of fEPSP to 173 ± 12% of baseline; $n = 6$). After transient forebrain ischemia LTP could not be induced (117 ± 4% of baseline; $n = 7$). In ischemic animals treated with BDNF, LTP could be induced (168 ± 28% of baseline; $n = 8$). Transient forebrain ischemia resulted in a significant decrease in spatial discrimination performance but not of associative memory. The ratios for working memory (WM) and reference memory (RM) 15 days after ischemia were lower in the ischemic rats ($n = 10$) than in the sham-operated control animals ($n = 10$; WM: 22 ± 6 vs 72 ± 7; RM: 30 ± 7 vs 72 ± 5). Postischemic intracerebroventricular BDNF infusion increased both WM (63 ± 4; $n = 10$) and RM (58 ± 5; $n = 10$). The spontaneous locomotor activity did not differ significantly in the three groups. These data indicate a protective effect of BDNF for synaptic transmission and cognitive functions after transient forebrain ischemia. © 1999 Academic Press

Key Words: **long-term potentiation; global ischemia; neurotrophic factors; BDNF; working memory; reference memory.**

INTRODUCTION

Brain-derived neurotrophic factor (BDNF)[1] is a protein belonging to the nerve growth factor family of neurotrophic factors collectively referred to as neurotrophins (4). It is synthesized in the brain, particularly in the hippocampus, during development and in response to exogenous stimuli such as kindling or excitotoxic lesions. Addition of small amounts of BDNF increases the survival of a variety of cell types including primary sensory neurons, cholinergic neurons of the basal forebrain, dopaminergic neurons of the substantia nigra, and retinal ganglion cells *in vitro* (4). *In vivo,* BDNF promotes the survival of embryonic neurons and prevents atrophy and death of axotomized cholinergic neurons in the adult central nervous system (9). Therefore, there has been a great deal of interest in the use of neurotrophic factors as therapeutic agents for degenerative diseases of the nervous system (18). Substantial evidence indicates that neurotrophic factors can protect and even restore impaired functions resulting from trauma, aging, and ischemia (6, 20, 26, 30, 50). Recently, we observed a marked neuroprotective effect of BDNF administered intracerebroventricularly in focal ischemia. The size of infarction and neurological deficits were significantly reduced by BDNF (43).

In addition to its well-documented neuroprotective action, recent experimental data indicate a new role for BDNF in activity-dependent processes (21), such as synaptic development and plasticity (49). The expression of BDNF mRNA has been shown to be rapidly enhanced by neuronal activity in hippocampal neurons in culture (57) and in response to kindling, recurrent limbic seizures, and kainate-induced seizures *in vivo* (12, 13, 16). The hippocampal formation is centrally involved in the initial retention of information. The best-studied cellular model for hippocampal learning and memory is the long-term potentiation (LTP) of synaptic efficacy (8). Hippocampal LTP is induced by a brief tetanic stimulation, and the increase in synaptic efficacy can last for hours in anesthetized animals and

[1] Abbreviations used: BDNF, brain-derived neurotrophic factor; CSF, cerebrospinal fluid; EPSP, excitatory postsynaptic potentials; LTP, long-term potentiation; RM, reference memory; WM, working memory.

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for weeks in awake animals. It has been speculated that BDNF is involved in the maintenance of LTP (15, 25), which was further indicated by the impairment of hippocampal LTP in knockout mice deficient in BDNF (23). Reciprocally, reexpression of the BDNF gene in the CA1 region almost completely restores the severely impaired LTP in hippocampal slices of BDNF-deficient mice (24).

Pyramidal neurons in the CA1 region of the hippocampus degenerate selectively with a delay of a few days after transient carotid artery occlusion in the rodent (40). This "delayed neuronal death" has attracted interest since it may provide insight into the pathogenesis of ischemic brain damage and related disorders, such as memory deficit and dementia. Exogenous BDNF partially prevented neuronal death in the CA1 region of the hippocampus (6, 50). However, whether intracellular signal transduction remains intact in the rescued neurons resulting in unimpaired cognitive functions is not known. Therefore, we analyzed the effect of postischemic intracerebroventricular BDNF administration on cognitive functions and synaptic transmission in rats subjected to transient forebrain ischemia.

MATERIALS AND METHODS

Global cerebral ischemia was achieved in adult male Wistar rats (450–600 g) with four-vessel occlusion under halothane/nitrous oxide anesthesia as described by Pulsinelli and Brierley (39). Both vertebral arteries were coagulated, followed by the transient occlusion of both common carotid arteries for 10 min. In sham-operated control animals, both vertebral arteries were coagulated and the carotid arteries were exposed, but not occluded. 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. The temperature was recorded with a rectal thermistor and maintained between 37.0° and 37.5°C with a feedback-controlled heating lamp. Animals were returned to their cages after the surgical procedure with free access to water and standard rat chow.

BDNF was administered by an osmotic minipump (Alzet, 2002) which was implanted after the reopening of the carotid arteries. BDNF or artificial cerebrospinal fluid was delivered to the left ventricle via a catheter which was connected to a cannula fixed on the scull with bone cement. BDNF was administered continuously over 14 days at a constant rate of 0.06 μg/h. Ten sham-operated and 10 ischemic rats received artificial cerebrospinal fluid.

Electrophysiological Recording

Electrophysiological recordings were performed in three groups of animals: sham-operated controls receiving artificial cerebrospinal fluid ($n = 6$) and ischemic animals receiving cerebrospinal fluid ($n = 7$) or BDNF ($n = 8$). The animals were anaesthetized 14 days after ischemia with pentobarbitone, 40 mg/kg ip. Their head was fixed in a stereotaxic instrument with the incisor bar setting at 3.3 mm below the interauricular level. The rectal temperature was kept at 37–37.5°C by means of a feedback-controlled heating blanket. A tungsten recording microelectrode (A-M system, Everett, WA, U.S.A.) with an impedance between 3 and 5 MΩ was aimed at 5 mm posterior and 3.5 mm lateral to the bregma on the left side. The concentric bipolar stimulation electrode (tip separation of 0.5 mm) was lowered into the CA3 region of the hippocampus through small skull holes and dural openings and placed at 3 mm posterior to bregma and 1.5 mm lateral at a depth of 2.5 mm.

The recording electrode was initially placed at the depth where spontaneous firing of CA1 pyramidal cells was best recorded. The population spike responses to stimulation of Schaffer collaterals were then recorded. Test stimuli consisted of single cathodal square pulses of 0.1-ms duration and 2- to 5-mV amplitude given at 60-s intervals. The depth of the recording electrode was adjusted to obtain the largest amplitude of field excitatory postsynaptic potential (fEPSP). These were positive in stratum pyramidale (see Fig. 1) and nevative in stratum radiatum. LTP was induced by tetanic stimulation (four trains of tetani of 1-s duration, each consisting of 100 Hz of 2- to 5-mV amplitude, pulse width 0.1 ms, separated by 10 s) after at least 15 min of stable baseline recordings. Data were digitized, stored to disk, and analyzed off-line. Results are given as means ± SD of each time point before and after tetanus.

After the recordings, the animals were sacrificed by decapitation. The brains were rapidly removed and frozen in isopentane at −50°C. Frontal cryostat sections (16 μm) of the dorsal hippocampus were collected on gelatine-coated glass slides and the extent of neuronal damage was confirmed by cresyl violet staining (Nissl). Viable pyramidal cells in one square millimeter were counted in the CA1 subfield of the hippocampus in using a measuring grid. The mean of five sections per animal was averaged. The investigator performing the cell count was blinded to the treatment.

Behavioral Tests

Behavioral tests were performed in three groups of animals: sham-operated controls receiving artificial cerebrospinal fluid ($n = 10$) and ischemic animals receiving cerebrospinal fluid ($n = 10$) or BDNF ($n = 10$). Three tests were used: a hole-board test and a single-trial step-through passive avoidance test to investigate learning and memory, in addition to a closed-field activity center to study the locomotor activity.

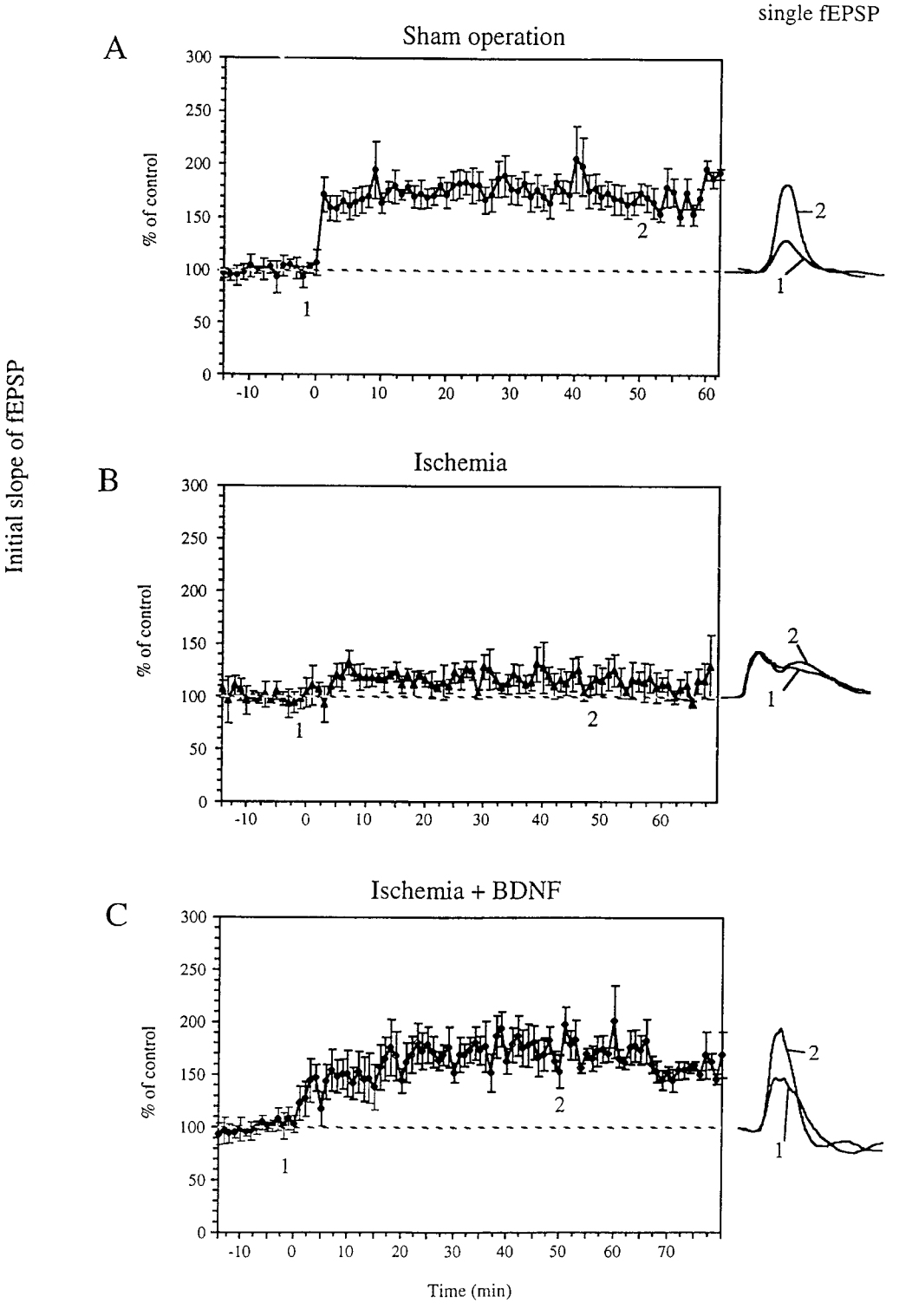

FIG. 1. BDNF attenuation of ischemia-induced impairment of LTP at CA1 synapses. Field EPSP were recorded in the CA1 region of the hippocampus 14 days after 10-min four-vessel occlusion or sham operation. Graphs plot the mean fEPSP slopes ± SD and single fEPSP recorded 1 min before (1) and 50 min after tetanus (2) in sham-operated rats (A; $n = 6$), ischemic rats (B; $n = 7$), and BDNF-treated ischemic rats (C; $n = 8$). After 15 min of baseline recording, a tetanus was applied at time point zero and data were normalized to baseline level. The stippled line indicates 100%. LTP was observed in sham-operated animals (A), but not in ischemic animals (B). BDNF, which was administered into the cerebral ventricle *after* reperfusion, recovered LTP after transient forebrain ischemia (C).

(1) The hole-board test. A hole-board test was adapted with minor modifications from Oades and Isaacson (36). An open field (70 × 70 cm) surrounded by plexiglass walls (40 cm high) contained 16 holes in a 4 × 4 array. In the middle of one wall, an attached startbox was separated from the testing area by a guillotine door, which could be operated from a distance. Each hole contained a metal cup (3 cm deep), which had a perforated bottom, under which food pellets of the type used for reinforcement (Altromin) were placed to minimize odoric cues. The test consisted of three phases: habituation, the test itself, and retest. A habituation phase (duration 4 days) was intended to get animals used to experimental conditions and for developing skills to find a bait in at least 15 cases of 16 trials. In the testing phase (7 days), animals were given a more complex level: the bait was put into only 4 holes in a certain combination. In the retest phase after four-vessel occlusion, the hole combination was reciprocal. The trial was terminated after 5 min or if the food pellets were consumed from all four cups.

Working memory (WM) and reference memory (RM) of reinforced visits were analyzed according to van der Staay *et al.* (52). Usually, WM and RM are operationally defined in terms of the number of specific errors (5). In addition to these error measures, WM and RM performance can also be expressed as ratios of different types of hole visits (11, 52). WM errors were defined as the number of revisits to the baited set of holes. WM ratio was defined as [number of food rewarded visits]/[number of visits and revisits of the baited set of holes]. Thus, WM represents the percentage of all visits to the baited set of holes that had been reinforced with food (51). Too frequent revisits in an initially filled but emptied hole during one trial worsens the results. RM errors were defined as the number of visits to the nonbaited set of holes. RM ratio was defined as [number of visits and revisits to the baited set of holes]/[number of visits and revisits to all holes]. This measure expresses the number of visits to the baited set of holes as a percentage of the total number of visits to all holes. WM is a measure for short-term memory, whereas RM represents long-term memory. The mean of four trials conducted on each test day was taken for statistical analysis.

(2) Single-trial passive avoidance learning. The step-through passive avoidance behavior was evaluated by using the light–dark box test. The inhibitory apparatus (70 × 45 × 40 cm wooden box) consisted of a light (30 × 45 × 40 cm) and a dark (40 × 45 × 40 cm) compartment. The light compartment was illuminated by a 60-W lamp fixed 40 cm above its floor. The floor of each compartment consisted of an individual stainless steel plate. Each plate had a separate connection to the transformer, which delivered footshocks (AC, 1 mA, 1 s) through the metal grid. In the beginning of each test, rats were placed in the light compartment. As soon as an animal had entered the dark part through an open guillotine door, a single electric shock was applied. The latent period was measured. At 24 h after the shock, the rat was placed in the illuminated starting box again and the step-through latency was determined. The test was concluded when the animal entered the dark compartment or after 5 min if it failed to enter.

(3) Locomotor activity. Spontaneous locomotor activity was assessed over a period of 300 s in a square closed-field arena equipped with a row of 12 infrared light-sensitive photocells. Interruptions of photocell beams were recorded by means of a microcomputer, allowing a record of all horizontal activity as measured by the total number of interruptions of the 12 photocell beams.

Single-trial passive avoidance learning and spontaneous locomotor activity were assessed 5, 10, 15, and 20 days after four-vessel occlusion.

Data Analysis

For estimation of LTP, the initial slopes of fEPSP after single pulses were averaged from every animal for 15 min before and for at least 70 min after tetanus. For comparison of the different groups of investigated animals, the slopes 1 min before and 50 min after tetanus were calculated. Statistical analysis was performed using the Wilcoxon rank test. The maximal amplitudes of all fEPSPs were also measured and revealed results similar to slope measurements. Psychometric data are displayed as mean values ± standard deviation (SD). The statistical comparisons of the working and reference memory ratios and of the locomotor activity were performed by means of the Mann–Whitney nonparametric *U* test. An alpha level of 0.05 was the criterion of statistical significance.

RESULTS

Histology

Global ischemia caused delayed neuronal death mainly in the CA1 area of the hippocampus. Histological evaluation 2 weeks after ischemia revealed that viable neurons in the CA1 region of the hippocampus of ischemic brains were reduced to 67.2% of control values. Postischemic, continuous intracerebroventricular administration of BDNF inhibited the neuronal degeneration (Table 1).

Electrophysiological Experiments

The recordings of the CA1 fEPSPs obtained from sham-operated, ischemic, and BDNF-treated ischemic animals are shown in Fig. 1. Brief repetitive stimuli of Schaffer collateral fibers induced a long-lasting synaptic potentiation of fEPSPs in sham-operated animals to

TABLE 1

Viable Pyramidal Cells in Postischemic Hippocampus after Chronic Infusion with BDNF

	Sham operation $n = 6$	Transient ischemia $n = 7$	Ischemia + BDNF $n = 8$
Viable neurons in CA1	434.9 ± 23	292.3 ± 10.67	439.6 ± 18.5

173 ± 12% 50 min after tetanus over control responses 1 min before tetanus. None of the seven ischemic animals expressed LTP after repetitive stimulation (117 ± 4% over baseline; Fig. 1). However, tetanic stimulation induced a stable increase of fEPSP slopes in all eight BDNF-treated ischemic animals to at least 120% of control (Fig. 1). Even though the mean slopes of fEPSPs recorded 50 min after tetanic stimulation did not reach the values of control animals, an increase to 153 ± 15% of control was observed in BDNF-treated ischemic animals, which was significantly higher than those of nontreated ischemic animals ($p = 0.03$).

Behavioral Tests

The ratios of RM and WM in the retest phase after ischemia are presented in Figs. 2A and 2B, respectively. Both WM and RM mean values increased during the retest phase in sham-operated control animals due to a training effect. This was not observed in rats after transient forebrain ischemia. The ratios for WM (22 ± 6) and RM (30 ± 7) 15 days after operation were significantly lower in the ischemic rats ($n = 10$) than in the sham-operated control animals (WM: 72 ± 7; RM: 72 ± 5; $n = 10$). WM was more severely impaired than RM. However, after continuous intracerebroventricular administration of BDNF ($n = 10$), WM (63 ± 4) and RM (58 ± 5) ratios were increased compared to those of ischemic rats ($p < 0.05$ for RM and $p < 0.001$ for WM) and did not differ significantly from those of sham-operated animals.

No differences were observed in the passive avoidance test among sham-operated, ischemic, and BDNF-treated ischemic rats. Also, the analyses of the spontaneous locomotor activity did not show any significant difference among the three examined groups (Fig. 3). This fact excludes the possibility that activity per se correlates with the changes in cognitive capacities.

DISCUSSION

The present data confirm that after brief transient global ischemia a partial degeneration of pyramidal neurons in the CA1 region of the hippocampus occurs (39). This is associated with a suppression of LTP and impaired spatial memory performance (35). For the first time we show that intracerebroventricular infusion of BDNF can restore almost completely both synaptic transmission and memory capacity. In keeping with previous results we observed a differential effect of transient forebrain ischemia on different forms of memory. The extent of impairment was somewhat higher for WM than for RM. This might be explained by the different mechanisms which those two parameters of spatial memory reflect. RM is thought to be based on processes involving protein synthesis and is less affected after ischemic lesions to the hippocampal CA1 region (55), whereas WM is impaired by strong irritants, neurotoxins, and hypoxia (44). In contrast, the nonspatial memory, investigated in our study by the single-trial passive avoidance learning, was not impaired after ischemia, which confirms similar results in other models of global ischemia (17, 56). The spontaneous locomotor activity in the closed field arena did not differ significantly between the two groups, which excludes the possibility that motor activity per se may have contributed to the changes in cognitive capacities.

LTP is considered a major synaptic mechanism underlying hippocampus-dependent learning and memory (8, 42, 47). This assumption is in part based on pharmacological investigations showing that both memory and LTP are blocked early on by antagonists of NMDA- or metabotropic glutamate receptors and by inhibitors of calcium/calmodulin-dependent protein kinase II and protein kinase C, whereas they are enhanced by glutamate and its receptor agonists (22). In agreement with previous investigations we demonstrate that synaptic transmission and the ability to induce LTP in the CA1 region of the hippocampus are impaired after transient forebrain ischemia (19). Taken together, our findings are in line with the view that the functional cognitive deficits after transient forebrain ischemia are associated with impairment of synaptic transmission.

Among the sequelae of ischemic hippocampal injury is the induction of BDNF mRNA expression (3, 27). Since also the receptor for BDNF, TrkB, is upregulated after ischemia in the same brain structures (6, 31), a neuroprotective role of endogenous BDNF has been suggested (30). Since LTP is lost in mutant mice lacking BDNF production and could be recovered after administration of exogenous BDNF (38), we investigated the therapeutic potential of continuous intracerebroventricular administration of BDNF on the recovery of cognitive functions and LTP induction after transient forebrain ischemia. In our experiments, postischemic BDNF treatment nearly completely restored LTP and spatial memory. Previously, several investigators have shown that BDNF modulates synaptic transmission in nonischemic cells. Thus, BDNF facilitates LTP induction in neonates by enhancement of synaptic response to high-frequency stimulation, not by direct effect on LTP triggering mechanisms (15), and in slice preparations of the visual cortex BDNF enhanced synaptic

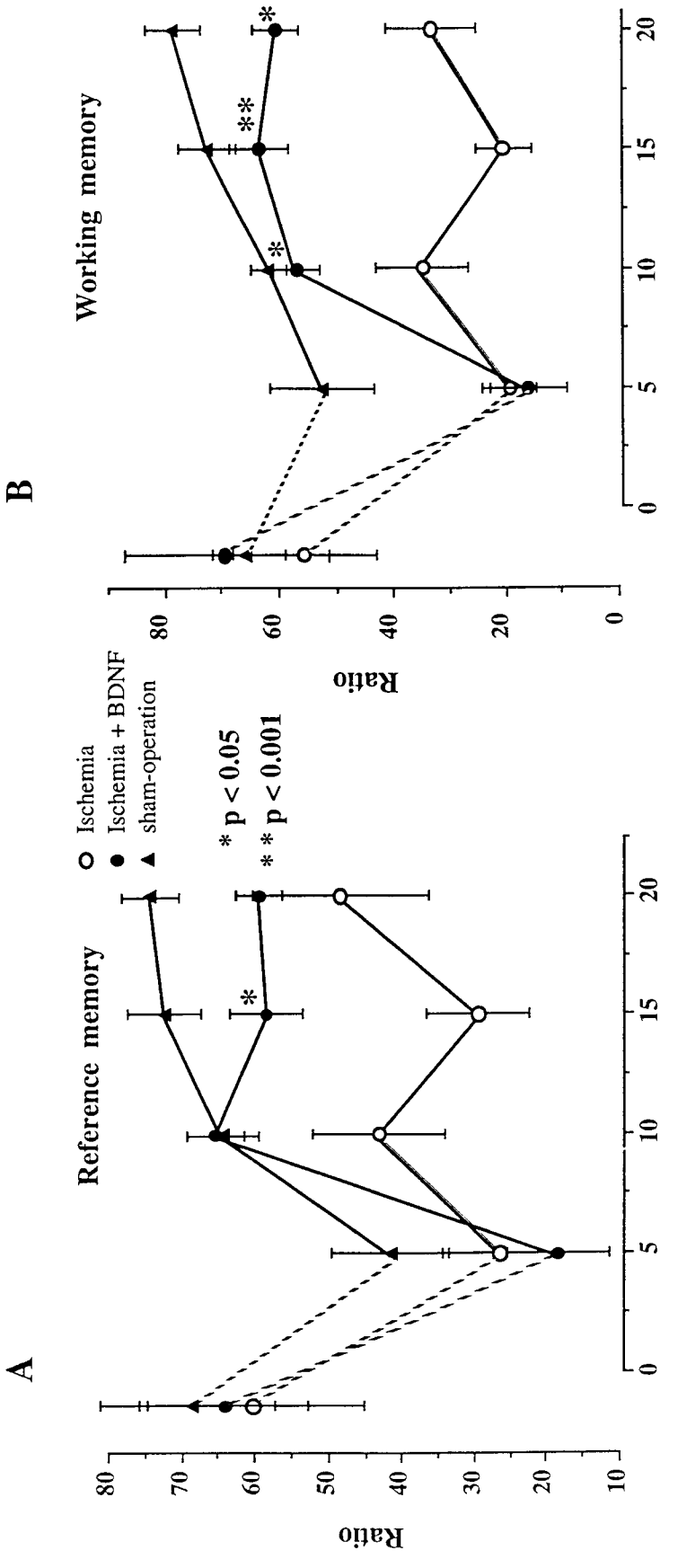

FIG. 2. The ratios of working memory and reference memory obtained for sham-operated (n = 10), ischemic (n = 10), and BDNF-treated ischemic (n = 10) rats. Rats were tested before, and 5, 10, 15, and 20 days after 10-min four-vessel occlusion. Ischemia resulted in an impaired learning capacity. The performance of BDNF-treated ischemic animals was significantly better than that of nontreated ischemic animals. The given values represent the mean ratios ± SD.

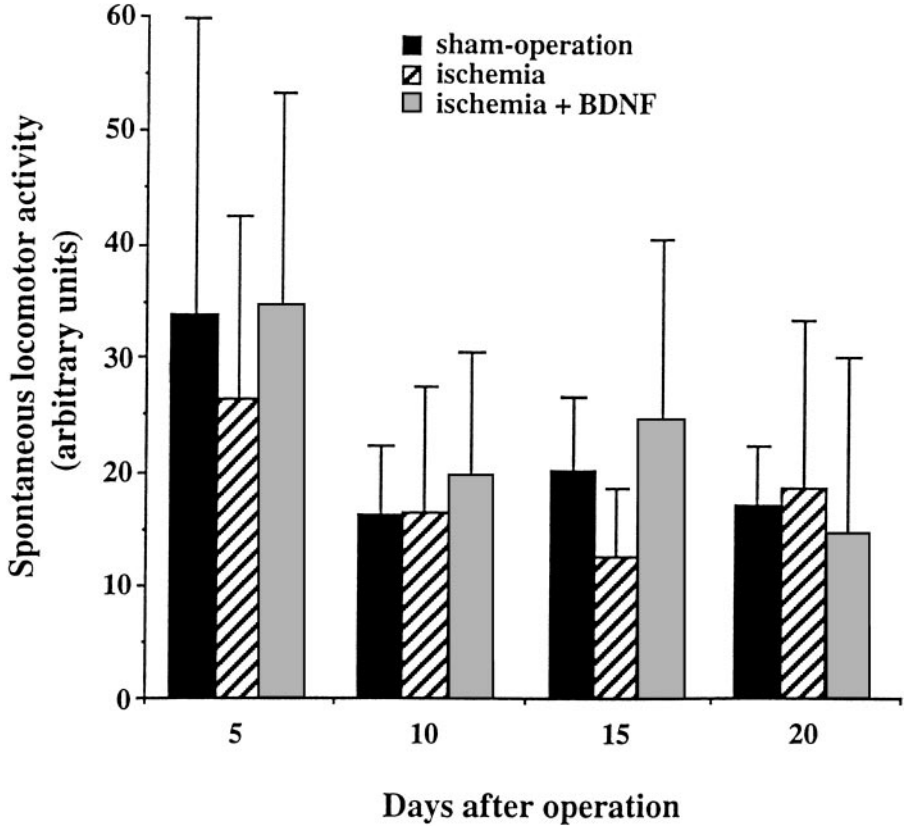

FIG. 3. Comparison of the locomotor activity for control, ischemic, and BDNF-treated ischemic rats. Rats were investigated 5, 10, 15, and 20 days after transient forebrain ischemia in an activity center. No significant differences between the two groups were observed. The given values represent the mean arbitrary units ± SD.

transmission and LTP (1). In our experiments we observed a clear neuroprotective effect of BDNF against the ischemic insult, which has been shown histologically previously (6, 50). However, we cannot exclude an enhancement of synaptic transmission in surviving neurons. The results of the behavioral tests suggest that reparative effects in addition to neuroprotective effects may also play a role, since memory performance 5 days after ischemia was equivalent in ischemic rats with or without BDNF treatment. Only at later time periods do BDNF-treated animals begin to show significant improvements in working and reference memory function.

The mechanisms underlying the protective activity of BDNF are still not well understood, but several functional pathways of its action can be suggested. Extracellularly, BDNF was shown to increase axonal branching *in vitro* and sprouting of cholinergic neurons in the hippocampus (9, 37). An important intracellular mechanism of BDNF action after ischemia may involve calcium homeostasis. BDNF elevates intracellular Ca^{2+} in hippocampal neurons (7), stimulates phospholipase C/ protein kinase C pathways (58), and increases the number of calbindin containing neurons in hippocampal slice cultures (29).

Several lines of evidence indicate that calcium ions play a central role in the delayed neuronal death observed in the CA1 region of the hippocampus after transient forebrain ischemia (10). The regulatory effects of calcium ions entering the cell are mediated by a number of calcium binding proteins and calcium-dependent enzyme systems (41), which may act as a buffering system against deleterious effects of excessive intracellular calcium. However, the activities of Ca^{2+}/calmodulin dependent and cyclic AMP-dependent protein kinases are significantly depressed after global ischemia in rat brains (2). Additionally, mutant mice deficient of the alpha subunit of Ca^{2+}/calmodulin dependent protein kinase II had twice as large infarct volumes than wild-type littermates in a focal model of cerebral ischemia. The reduced amounts of the alpha subunit of this kinase, which is a synaptic protein enriched in the hippocampus, predisposed neurons to increased damage following ischemia and any pertubation that decreases the amount or activity of this enzyme produced enhanced susceptibility to neuronal damage (53).

These calcium-dependent enzyme systems are also considered to be involved in learning and memory, and LTP (14, 46). The activity of hippocampal protein

kinase C was significantly reduced in poor spatial learners (34, 54) and pharmacological inhibition of calcium/calmodulin-dependent kinase II (33, 48) and protein kinase C (28) blocked LTP. Additional evidence derives from mutant mice deficient of the alpha subunit of the calcium/calmodulin-dependent kinase II or reduced calbindin D28K expression, which had no LTP (32, 45). These animals exhibited deficits of spatial learning, while other cognitive functions were unimpaired.

In conclusion, we described for the first time improved cognitive functions associated with a recovery of synaptic transmission after transient forebrain ischemia. These data provide further evidence for the neuroprotective effects of BDNF after lesions of the adult brain and indicate a therapeutic potential in acute neurovascular disease.

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