Levodopa Treatment Improves Functional Recovery After Experimental Stroke
Karsten Ruscher, MD; Enida Kuric, MSc; Tadeusz Wieloch, PhD

Background and Purpose—Delayed treatment of patients with stroke with levodopa/benserazide contributes to enhanced functional recovery, but the mechanisms involved are poorly understood. The present study was designed to investigate if levodopa/benserazide treatment improves recovery of lost neurological function and contributes to tissue reorganization in the rat brain after stroke.

Methods—Male Wistar rats were subjected to transient occlusion of the middle cerebral artery (120 minutes) and treated with levodopa (1, 5, and 20 mg/kg)/benserazide (15 mg/kg) or saline for 12 consecutive days starting on Day 2 after transient occlusion of the middle cerebral artery. Infarct volume was determined and sensorimotor function was assessed using the rotating pole test, a 28-point neuroscore, and a cylinder test on Days 2, 7, and 14 after transient occlusion of the middle cerebral artery. The spatiotemporal expression pattern of dopamine-1 and dopamine-2 receptors and the dopamine- and cAMP-regulated neuronal phosphoprotein in reactive astrocytes were analyzed in the ischemic hemisphere as well as in cultured astrocytes.

Results—Treatment with levodopa/benserazide significantly improved the recovery of sensorimotor function after transient occlusion of the middle cerebral artery without affecting the infarct volume. In addition, we found that different subpopulations of glial fibrillary acidic protein-positive astrocytes in the peri-infarct area express dopamine-1 receptors and dopamine-2 receptors as well as dopamine- and cAMP-regulated neuronal phosphoprotein.

Conclusions—Our results strongly corroborate the concept of recovery enhancing actions of levodopa treatment after stroke. Also, astrocytes in the peri-infarct area may contribute to the dopamine enhanced recovery mechanisms. (Stroke. 2012;43:507-513.)

Key Words: dopamine and cAMP-regulated neuronal phosphoprotein ■ dopamine receptor ■ levodopa ■ stroke recovery

Delayed treatment with levodopa contributes to recovery of sensorimotor function and to procedural motor learning in patients with stroke. It can therefore be anticipated that levodopa affects molecular and cellular mechanisms in the ischemic hemisphere that foster functional recovery after stroke. Such processes in the surviving brain tissue after a stroke are complex, slow, and incomplete but can readily be studied in experimental stroke models. They involve multiple cellular processes distinctly activated in time and space encompassing neuronal plasticity as well cell proliferation and modulation of inflammation. For example, we have shown that an enriched environment provides multimodal sensorimotor stimulation of the brain that markedly enhances recovery of lost neurological function.

Dopamine exerts a variety of physiological effects in the healthy or diseased brain. Hence, improvement of neurological function after stroke by levodopa treatment most certainly involves mechanisms of neuronal plasticity through actions on dopamine receptors (DR) in the primary motor cortex. However, DRs are also found on glial and immune cells putatively involved in a coordinated synthesis of neurotrophic factors, anti-inflammatory actions, and ion homeostasis.

We show that delayed treatment with levodopa significantly contributes to the recovery of neurological function after transient occlusion of the middle cerebral artery (tMCAO) in the rat beyond the therapeutic window for neuroprotection and we identified glial fibrillary acidic protein (GFAP)-positive reactive astrocytes in the peri-infarct area as possible targets for the action of levodopa.

Materials and Methods

Transient Rat Middle Cerebral Artery Occlusion
All animal experiments were approved by the Malmö-Lund ethical committee. Transient MCAO was induced as described. In brief, male Wistar rats (325–350 g, HsdBrHan, Harlan Scandinavia, Study...
Randomization and Treatment Protocols

Studies were carried out randomized and in a blinded fashion to the investigator who performed the surgeries and behavior assessments. For the first study, in total 29 animals were included; thereof 3 animals died within the first 48 hours before randomization and 4 animals were excluded from the behavioral analysis because they did not pass the selective sorting on Day 2 after tMCAO. On Day 2, all animals were randomized into treatment groups (uneven numbers: vehicle; even numbers: levodopa/benserazide) based on the performance in the rotating pole test. After tMCAO, animals showing a score >2 in the rotating pole test were excluded from the study. Sham-operated rats with a test score <3 in the rotating pole test were not included in the study. The same inclusion criteria were applied in the second study including 77 male Wistar rats; thereof 8 animals died before randomization and 18 animals did not pass selective sorting. Fifty-one rats were randomized into the indicated treatment groups. Every fourth animal was assigned to the same treatment after tMCAO or sham operation, respectively.

Subsequently, starting on Day 2 after tMCAO, rats were injected daily with levodopa/benserazide (1, 5, or 20 mg/kg/15 mg/kg intraperitoneally; Sigma-Aldrich, Stockholm, Sweden) or saline (referred to as vehicle; experimental design; Figures 1A and 2A).

Recovery of lost neurological function was assessed by the rotating pole test in both studies and in addition by a 28-point composite neuroscore and cylinder test in the second study.1,2,20

To study the spatiotemporal profile of dopamine-1 (D1R) and dopamine-2 receptors (D2R), and the dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) expression in the ischemic hemisphere rats were subjected to tMCAO (120 minutes) or sham operation and perfusion fixed at 2 days, 7 days, 14 days, or 30 days.

Infarct Size Measurement

Coronal brain 40-μm sections were immunostained for the neuronal-specific antigen neuronal nuclei (Millipore, Hampshire, UK; dilution 1:1000; Study 1) or stained for cresyl violet (Study 2). The nonlesioned area of the infarcted hemisphere and the nonlesioned contralateral hemisphere were outlined and the infarct volume was calculated as described previously.10

Astrocytic Cell Culture and Combined Hypoxia and Aglycemia

Astrogial cell cultures were prepared according to a modified method described previously.21 After 10 days in vitro, microglial cells were removed by shaking the culture and confluent subcul-

### Table. Physiological Parameters of Rats Subjected to tMCAO at the Time of Recirculation (Study 1)*

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<td>Temperature, °C</td>
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<td>Body weight, g‡</td>
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*Data are presented as means ± SD. No statistical differences were observed between the treatment groups.
†Before tMCAO.
‡Fourteen d after tMCAO.

1: 325–350 g, Taconic, Study 2) were housed under diurnal light conditions and were fasted for 12 hours before surgery. During surgery, physiological parameters were measured and controlled within physiological limits (Table; Supplemental Table I; http://stroke.ahajournals.org). Rats were anesthetized (initial 4% Fluothane in N₂O/O₂ [70:30], during surgery 2% Fluothane in N₂O/O₂; Astra Zeneca) and the right common carotid artery and external carotid artery were occluded permanently; the internal carotid artery was exposed and ligated. A nylon filament (top diameter 0.3–0.4 mm, Study 1; Doccol MCAOsuture monofilaments 4039RE, 4041RE, or 4043RE; Doccol Corporation, Study 2) was introduced into the internal carotid artery through a small incision into the distal end of the common carotid artery and pleaded up to occlude the origin of the middle cerebral artery. During occlusion, regional cerebral blood flow was monitored by an optical fiber probe (Probe 318-I; Perimed) connected to a laser Doppler flowmeter (Periflux System 5000; Perimed). Animals were included in the study if (1) regional cerebral blood flow decreased by at least 70% immediately after filament placement; (2) the regional cerebral blood flow reduction was sustained during the 120-minute occlusion time; and (3) if regional cerebral blood flow completely recovered within 5 minutes after filament removal. The same procedure was performed in sham-operated animals but no filament was introduced into the internal carotid artery.

Figure 1. A, Experimental design. B, Sensormotor function in levodopa/benserazide (L-dopa/bens, n=8) and vehicle-treated (vh, n=7) rats after tMCAO. Animals were tested on the rotating pole at 0, 3, and 10 rotations per minute to the left. Data are shown as differences between the scores obtained on Days 2 and 7 (d2 to d7) and between Day 2 and 14 (d2 to d14) and presented as medians with Q1, Q3, and the 95% CI. C, Infarct volume from L-dopa/benserazide (n=8) and vehicle-treated (n=7) rats presented as medians with Q1, Q3, and the 95% CI. tMCAO indicates transient middle cerebral artery occlusion; Q, quartile.
tures were exposed to combined hypoxia and aglycemia (HA). To induce HA, culture medium was washed out with phosphate-buffered saline and replaced by a deoxygenated aglycemic solution (HA solution: pH 7.4, Na\(^+\)/H\(^+\): 143.8 mmol/L, K\(^+\)/H\(^+\): 5.5 mmol/L, Ca\(^2+\)/H\(^+\): 1.8 mmol/L, Mg\(^2+\)/H\(^+\): 1.8 mmol/L, Cl\(^-\)/H\(^+\): 125.3 mmol/L, HCO\(_3\)/H\(^+\): 26.2 mmol/L, PO\(_4\)\(^3-\)/H\(^+\): 1.0 mmol/L, SO\(_4\)\(^2-\)/H\(^+\): 0.8 mmol/L) in a hypoxic atmosphere (1% oxygen). Hypoxia was generated in a humidified, gastight incubator (Electrotek, Shipley, UK) and flushed with gas of the following composition: 5% CO\(_2\), 85% N\(_2\), and 10% H\(_2\) as described previously.\(^{12}\)

**Western Blotting**

Samples were boiled for 5 minutes in a sodium dodecyl sulfate sample buffer and immediately after separated on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred onto polyvinyl difluoride membranes. Membranes were incubated overnight in a humidified, gas tight incubator (Electrotek, Shipley, UK) and flushed with gas of the following composition: 5% CO\(_2\), 85% N\(_2\), and 10% H\(_2\) as described previously.\(^{12}\)

**Immunofluorescence/Immunohistochemistry**

Brain sections (30 μm) from paraformaldehyde-perfused animals were incubated with rabbit polyclonal anti-D1R (1:400; Abcam), rabbit polyclonal D2R (1:400; Abcam), anti-DARPP-32 (1:1000; Invitrogen), and a monoclonal directly Cy3 conjugated anti-GFAP (1:5000; Sigma-Aldrich) and then with appropriate secondary antibodies. Fluorescent signals were visualized using a confocal microscopy system (LSM510; Zeiss). For bright-field immunohistochemistry, a standard peroxidase-based method using 3,3′-diaminobenzidine was applied,\(^{22}\) using a DARPP-32 antibody (1:20 000)\(^{23}\) and a biotinylated swine antirabbit secondary antibody (1:200; Dako Cytomation, Glostrup, Denmark).

**Statistics**

In cell culture experiments, unless otherwise stated, experiments were conducted in triplicate with 6 independent cultures each. Data are presented as means±SD. In the experimental stroke studies, behavioral data and infarct size measurements are displayed as differences between the scores obtained on Days 2 and 7 (Δd2 to d7) or 14 (Δd2 to d14; P<0.01, Kruskal-Wallis and Mann-Whitney U tests), respectively, and presented as medians with Q1, Q3, and the 95% CI. F, Cylinder test showing the percentage of impaired forelimb usage 14 days after tMCAO (1-way analysis of variance followed by the Scheffé correction). Q indicates quartile; tMCAO, transient middle cerebral artery occlusion.

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**Figure 2. A.** Experimental design. The number of animals per group included in infarct size measurements and behavioral analysis are indicated in parentheses. **B.** Infarct volumes from levodopa/benserazide and vehicle-treated rats presented as medians with Q1, Q3, and the 95% CI. **C.** Sensorimotor function in levodopa/benserazide and vehicle-treated rats after tMCAO. Animals were tested on the rotating pole at 0, 3, and 10 rotations per minute (rpm) to the left (P<0.05, Kruskal-Wallis and Mann-Whitney U tests). **D.** Improvement in the 28-point neuroscore at Day 7 after tMCAO. **E.** Improvement in the 28-point neuroscore at Day 14 after tMCAO. In C–E, data are shown as differences between the scores obtained on Days 2 and 7 (Δd2 to d7) or 14 (Δd2 to d14; P<0.01, Kruskal-Wallis and Mann-Whitney U tests), respectively, and presented as medians with Q1, Q3, and the 95% CI. F. Cylinder test showing the percentage of impaired forelimb usage 14 days after tMCAO (1-way analysis of variance followed by the Scheffé correction). Q indicates quartile; tMCAO, transient middle cerebral artery occlusion.

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Results

Levodopa/Benserazide Treatment Enhances Functional Recovery After tMCAO

Rats were subjected to tMCAO and randomized on Day 2 poststroke and thereafter treated with levodopa/benserazide (5 mg/kg/15 mg/kg) by daily intraperitoneal injection (Figure 1A). On Day 14 after tMCAO, sensorimotor function was assessed by the rotating pole test. As shown in Figure 1B, rats receiving vehicle injections (n=7) were not able to traverse the pole. In contrast, all levodopa/benserazide-treated rats (n=8) crossed the pole at 0 and 3 rotations per minute and 3 of 8 animals performed the test with a score 3 (traversing the pole without falling off) at 10 rotations per minute indicating a dramatic improvement of sensorimotor function after tMCAO. Importantly, the treatment had no effect on infarct volume (Figure 1C). In conclusion, the results clearly demonstrate the recovery promoting effect of delayed levodopa/benserazide treatment after tMCAO.

A second study was conducted to test if recovery enhancing effects observed by levodopa/benserazide treatment are dose-dependent. As shown in Figure 2A, animals were randomized on Day 2 after tMCAO (120 minutes) and thereafter injected daily with saline (n=8), 1 mg/kg/15 mg/kg levodopa/benserazide (n=7), 5 mg/kg/15 mg/kg levodopa/benserazide (n=8), or 20 mg/kg/15 mg/kg levodopa/benserazide (n=8) for 12 consecutive days. Physiological parameter did not differ between the study groups (Supplemental Table I). Moreover, measurement of infarct sizes showed no differences between the treatment groups (Figure 2B). Recovery of function assessed by the rotating pole test on Day 7 after tMCAO shows better recovery of the test scores with increased dosages of levodopa/benserazide (Figure 2C); however, no difference was observed between the treatment groups on Day 14 after tMCAO (Supplemental Figure I) due rapid spontaneous recovery and changes in the experimental protocol as stated in the “Methods” section. Hence, improvement of lost neurological function was observed in the 28-point neuroscore at 7 and 14 days after MCAO (Figure 2D–E). Levodopa/benserazide-treated animals also showed better performance in the cylinder test on Day 14 after tMCAO (Figure 4).

Figure 3. A, Coexpression of D1 receptors and GFAP in reactive astrocytes 7 days after tMCAO. Higher magnifications for D1 receptor and GFAP are presented to the right. Scale bars: low magnification, 200 μm; higher magnification, 20 μm; orthogonal projections, 5 μm. B, D2 receptor immunoreactivities in GFAP+ reactive astrocytes in the ischemic hemisphere. Almost all reactive astrocytes express D2 receptors. Scale bars: low magnification, 200 μm; higher magnification, 50 μm; orthogonal projections, 5 μm. Note, the D1R+ and D2R+ expression in GFAP− cells in the neocortex, presumable neurons (arrows). D1 indicates dopamine-1; GFAP, glial fibrillary acidic protein; tMCAO, transient ischemic middle cerebral artery occlusion; D2, dopamine-2; D1R, dopamine-1 receptor; D2R, dopamine-2 receptor.

Figure 4. D1 and D2 receptor levels in primary astrocytes after control stimulation and combined hypoxia/aglycemia. The molecular size of the protein standards (Lane M, kDa) is indicated (left). AU indicates arbitrary unit. *Significant difference compared with control stimulated astrocytes (n=6, P<0.05, Student t test). D1 indicates dopamine-1; D2, dopamine-2.
Day 14 (Figure 2F). No effects were observed in all treatment groups (saline, n=5; 1 mg/kg/15 mg/kg levodopa/benserazide, n=4; 5 mg/kg/15 mg/kg levodopa/benserazide, n=6; 20 mg/kg/15 mg/kg levodopa/benserazide, n=5) after sham surgery (data not shown).

Reactive Astrocytes Express Dopamine Receptors in the Peri-infarct Area After tMCAO and After HA

To identify target cells susceptible for the action levodopa in the ischemic hemisphere, we performed immunofluorescence analysis for D1R and D2R. As shown in Figure 3, an accumulation of D1R+/GFAP+ and D2R+/GFAP+ astrocytes was observed in the peri-infarct area on Day 7 after tMCAO. Interestingly, D1R and D2R immunoreactivity appeared not to be homogeneously distributed among reactive astrocytes. Although D1R+ astrocytes were mainly found in the very proximal and cortical peri-infarct area, D2R+ astrocytes were spread more homogeneously throughout the peri-infarct area. High immunoreactivity for both receptors also was found in the subventricular zone (Figure 3A–B). Expression of D1R and D2R in GFAP+ astrocytes in the ischemic hemisphere increased during the first 2 weeks poststroke (Supplemental Figure II). Up to 48 hours after tMCAO, D1R and D2R were mainly expressed in neurons. Receptor expression in GFAP+ astrocytes increased with the number of cells in the peri-infarct region from Day 7 onward. In contrast, no expression of D3R receptors was found in GFAP+ astrocytes (data not shown). De novo D1 and D2 receptor expression was confirmed by Western blot analyses from control and HA-stimulated astrocytes. As shown in Figure 4, a slight expression of D1 receptors was detected in control stimulated astrocytes but was markedly increased after HA. Similar to D1 receptors, astrocytes also express D2 receptors after HA. Together, we conclude that subpopulations of reactive GFAP+ astrocytes in the ischemic hemisphere express D1R and D2R and might be modulated by levodopa treatment.

The Expression of DRs Is Accompanied With an Increase of DARPP-32 in Reactive Astrocytes After tMCAO

One of the regulated proteins involved in DR signaling cascades is the DARPP-32. As previously shown,24 we found DARPP-32+ cells in neurons in all layers of the neocortex.
with cells exhibiting a more pronounced immunoreactivity in Layer VI (Figure 5A). Those cells also were detected in the remote peri-infarct area in rats subjected to tMCAO. In addition, strong immunoreactivity was observed in stellate astroglia like cells around the infarct core on Day 7 after tMCAO (Figure 5A). Those cells were identified as GFAP+ reactive astrocytes (Figure 5B). Importantly, the spatial expression pattern of DARPP-32 coincided with the expression of D1R and D2R (Figure 5C) with a maximum expression on Day 7 after tMCAO. In conclusion, delayed upregulation of DRs and DARPP-32 suggests a functional dopaminergic signaling cascade in GFAP+ -reactive astrocytes in the peri-infarct area after tMCAO.

Discussion

Based on previous studies showing that levodopa treatment improves sensorimotor function and procedural motor learning after stroke,1,2 we investigated the effect of combined levodopa/benserazide treatment on recovery of neurological function after experimental stroke. We show recovery-promoting effects of combined levodopa/benserazide treatment in 2 independent studies after tMCAO. Importantly, studies were performed randomized and in a blinded fashion. Our data also demonstrate that levodopa-induced effects can be achieved in rats with different infarct volumes. The major findings we discuss are (1) the recovery-promoting effects of levodopa treatment; (2) mechanisms affected by levodopa/benserazide treatment in the peri-infarct area of rats subjected to tMCAO; and (3) the role of dopamine signaling in GFAP+ astrocytes.

The neocortex, and in particular the motor cortex,25,26 is an area with dense dopaminergic nerve terminals. Here, the D1R is the most abundant DR.27 The D2R is found in Layers V and VI of the rat neocortex on pyramidal neurons.28 However, dopamine also activates other types of neurons through various DRs with variable receptor affinity, regulating the excitability and function of local neuronal networks.25 On the cellular level, the activation of D1R and D2R may modulate the activity of N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in the peri-infarct area through the activation of protein kinase A.29 Furthermore, D1R activation increases the expression of surface N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors30 and D1R and D2R have been shown to regulate intracellular calcium levels.31 Overall, the concerted action of DR signaling and activation of ionotropic glutamate receptors have been implicated in long-term plasticity processes after brain injury.32 Hence, it is reasonable to assume that the recovery-promoting effects of levodopa treatment is achieved by an enhanced activation of the DA system in the motor cortex to promote motor skill learning.33

We propose that the glia cells may play an equally important role in the action of levodopa during recovery after stroke. Astrocytic DA receptor activity may regulate interstitial glucose and lactate in postischemic tissue.34 Furthermore, DR activation may induce the expression of different growth factors in the recovery phase after ischemia. Hence, DR agonists regulate expression of fibroblast growth factor-2,35 brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor.36 factors also involved in mechanisms of functional recovery after brain injury. The finding that DARPP-32 is expressed in reactive astrocytes in the peri-infarct area further suggests functional DR signaling cascades.36 Thus, the phosphorylation status of DARPP-32 may control the activity of protein phosphatase 1 and protein kinase A involved in the regulation of proliferation, differentiation, and morphogenesis of reactive astrocytes37 in the peri-infarct area.

After stroke, brain resident and peripheral immune cells infiltrate in the ischemic hemisphere, possibly modulated by dopamine.38 DRs are expressed in leukocyte subpopulations and the maturation and function of those cells can be regulated by dopamine or DR agonists,39 strongly suggesting levodopa treatment affects the number and function of immune cells in the ischemic hemisphere. Moreover, D1R and D2R are found on microglia in the ischemic territory, but the functional relevance of the receptors remains to be determined. Hence, dopamine may also regulate the migration of microglia.39

In conclusion, our experimental data provided in this study strongly support previous clinical trials using levodopa treatment to enhance sensorimotor function in patients with stroke. In addition, we found that GFAP+ -reactive astrocytes in the peri-infarct area express D1R, D2R, and DARPP-32. Targeting dopamine signaling in reactive astrocytes during the first few weeks after stroke may affect processes of reorganization in the ischemic hemisphere important to enhance functional recovery after stroke.

Acknowledgments

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Disclosures

None.

References


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Supplements
Table S1.

Physiological parameters of rats subjected to tMCAO at the time of recirculation (study 2). Data are presented as means ± standard deviation. No statistical differences were observed between the treatment groups.

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\(^1\)before tMCAO, \(^2\)14 days after tMCAO.
Figure S1.

Sensorimotor function in L-Dopa/benserazide and vehicle treated rats following tMCAO. Animals were tested on the rotating pole at 0, 3 and 10 rotations per minute (rpm) to the left on day 14 after tMCAO (120 min). Data are shown as differences between the scores obtained on day 2 and 14 (Δd2 to d14) and presented as medians with Q1, Q3 and the 95% CI.
Figure S2.

Temporal expression profile of D1 and D2 receptors in GFAP\(^+\) astrocytes after MCAo. Coronal section from a sham operated rat and from rats on day 1, on day 2, day 7, and day 14 after MCAo, respectively. Scale bar 50 µm. Abbreviations: IC – Infarct core, PI – peri-infarct area.