Different Mechanisms of Secondary Neuronal Damage in Thalamic Nuclei After Focal Cerebral Ischemia in Rats

Marcel Dihné, MD; Christian Grommes, MD; Michael Lutzenburg, PhD; Otto W. Witte, MD; Frank Block, MD

Background and Purpose—After focal cerebral ischemia, depending on its localization and extent, secondary neuronal damage may occur that is remote from the initial lesion. In this study differences in secondary damage of the ventroposterior thalamic nucleus (VPN) and the reticular thalamic nucleus (RTN) were investigated with the use of different ischemia models.

Methods—Transient middle cerebral artery occlusion (MCAO) leads to cortical infarction, including parts of the basal ganglia such as the globus pallidus, and to widespread edema. Photothrombotic ischemia generates pure cortical infarcts sparing the basal ganglia and with only minor edema. Neuronal degeneration was quantified within the ipsilateral RTN and VPN 14 days after ischemia. Glial reactions were studied with the use of immunohistochemistry.

Results—MCAO resulted in delayed neuronal cell loss of the ipsilateral VPN and RTN. Glial activation occurred in both nuclei beginning after 24 hours. Photothrombotic ischemia resulted in delayed neuronal cell loss only within the VPN. Even 2 weeks after photothrombotic ischemia, glial activation could only be seen within the VPN.

Conclusions—Pure cortical infarcts after photothrombotic ischemia, without major edema and without effects on the globus pallidus of the basal ganglia, only lead to secondary VPN damage that is possibly due to retrograde degeneration. MCAO, which results in infarction of cortex and globus pallidus and which causes widespread edema, leads to secondary damage in the VPN and RTN. Thus, additional RTN damage may be due to loss of protective GABAergic input from the globus pallidus to the RTN or due to the extensive edema. Retrograde degeneration is not possible because the RTN, in contrast to the VPN, has no efferents to the cortex. (Stroke. 2002;33:lll–lll.)

Key Words: brain ischemia ■ neuronal damage ■ thalamus ■ rats
mia was induced by photothermalbosis of the temporoparietal cortex, which is known to produce a pure cortical lesion. Neuronal damage and glial reactions were studied in the VPN and RTN up to 14 days after induction of ischemia.

Materials and Methods
The experimental design (MCAO and photothermalbostic stroke) was in accordance with legal guidelines for animal care.

Middle Cerebral Artery Occlusion
In male Sprague-Dawley rats (weight, 220 to 250 g), focal cerebral ischemia was induced with the use of the intraluminal suture occlusion method. Under 2% halothane in 30% oxygen/70% nitrous oxide anesthesia, the right common carotid artery and the right external carotid artery were exposed through a midline neck incision. The distal common carotid artery, the external carotid artery, and the pterygopalatine artery were ligated. A 4-0 monofilament nylon suture (45 mm in length, its tip covered with poly-L-lysine) was placed into a guide sheath (30 mm in length) and then inserted through arteriotomy of the common carotid artery and gently advanced into the internal carotid artery to a point approximately 17 mm distal to the carotid bifurcation. In sham-operated animals the monofilament was only advanced 10 mm. Mild resistance to this advancement indicated that the suture had entered the anterior cerebral artery, thus occluding the origin of the middle cerebral artery (MCA). To establish reperfusion, after 3 hours the guide sheath together with 4-0 nylon suture was removed. The common carotid artery was then ligated distal to the arteriotomy, and the ligation of the pterygopalatine artery was removed. Finally, the wound at the neck was closed with sutures. During ischemia and the following 6 hours, rectal temperature was kept at 37±0.5°C with a heated operating table or with a heating lamp, respectively.

Photothermalbostic Ischemia
Photothermalbostic cerebral infarction was induced in the rat parietal cortex according to the method of Watson et al., as described in detail elsewhere. For this purpose rats were anesthetized with 1.3% halothane in O2/N2 (1:2) and placed in a stereotaxic frame. A fiberoptic bundle was positioned onto the skull 4.0 mm posterior to bregma and 4.0 mm lateral to the midline. The cortex was exposed to light from the fiberoptic bundle for 20 minutes, and during the first 2 minutes rose bengal (13 mg/kg, dissolved in saline) was injected into the femoral vein. Sham-operated animals were not exposed to the fiberoptic bundle light but received rose bengal.

Immunohistochemistry
After survival times of 1, 3, 7, and 14 days, rats (n=4±1 sham-operated animal per time point) were deeply anesthetized with pentobarbital and perfused transcardially with 50 mL saline followed by 400 mL 4% paraformaldehyde in PBS (0.1 mol/L, pH 7.4). The brains were then removed carefully and postfixed in the same fixative for 2 hours. After cryoprotection in 30% sucrose, the brains were rapidly frozen in isopentane and stored at −80°C. Free-floating 20-μm coronal cryostat sections at the level of the thalamus (anteroposterior −2.8 and −3.3 mm from bregma according to Paxinos and Watson17) were processed for immunohistochemistry with a mouse monoclonal antibody to the astrocyte antenene glial fibrillary acidic protein (GFAP) (Boehringer), diluted 1:250. To stain microglial cells/macrophages, a monoclonal mouse anti-rat CD11b (OX-42, Serotec), diluted 1:5000, was used. All washes between antibody incubations were made with PBS, which was also used for dilution of antibodies. First, the sections were reacted in 3% H2O2 to quench endogenous peroxidase activity for 10 minutes. After they were washed, the sections were blocked with 10% normal horse serum for 10 minutes. All normal serum solutions contained 0.3% Triton X-100. Serial adjacent sections from each animal and survival time were incubated overnight in the primary antibody solutions at 4°C with gentle agitation. After incubation with the primary antibody, the sections were washed 3 times and incubated with secondary antibodies (biotinylated horse anti-mouse, rat absorbed, Vector, diluted 1:500) for 1 hour at room temperature. After 3 rinses in PBS, the sections were incubated with the avidin-biotinylated horseradish peroxidase complex (ABC-Elite kit, Vector) for 30 minutes. The ABC kit was diluted 3 times more than recommended in the protocol. For visualization of peroxidase, all sections were routinely incubated with 0.05% diaminobenzidine (Sigma) and 0.02% H2O2. Finally, the sections were mounted on gelatin-coated slides, dehydrated in increasing ethyl alcohol concentrations, and cleared in xylene. Coverslips were applied with XEM mounting media (Vogel).

Sections incubated without the primary antibodies served as controls for the specificity of the immunostaining. In sections of both control rats and ischemic rats, no staining of cells could be observed under these conditions.

Quantification of Neuronal Cell Death and Statistical Analysis
Histological sections were examined by one of the authors (C.G.) without knowledge of the experimental protocol. Quantification of neuronal cell density ipsilateral and contralateral within the RTN and VPN 14 days after ischemia was done by counting intact neurons on cresyl violet (Nissl)-stained slices of the rostral part of the RTN and the VPN at −2.8 and −3.3 mm from bregma, respectively, within an area of 200 μm2. Quantification of neuronal cell density within the ipsilateral and contralateral globus pallidus was done 1 day (MCAO) or 14 days (photothermalbostic ischemia) after ischemia at −1.4 mm from bregma. Only vital neurons with intact membranes and nuclei were counted. Five MCAO- and photothermalbosis-treated rats plus 1 sham-operated control animal were analyzed. To obtain representa-tive results, 3 sections per animal (separated by a distance of 20 μm) were analyzed. Mean and SEM values per 200 μm2 were calculated. Group differences concerning the number of intact neurons were determined by the paired t test. Differences were regarded as statistically significant for P<0.01.

Evaluation of Glial Reactions
For the evaluation of reactive glial cells, the following generally accepted criteria were used. An enhancement of GFAP immunoreactivity within thickened astrocytic processes and cell bodies is denoted as astrocytic activation. The terminus microglial activation covers an increased number of microglial cells and a shift in microglial morphology toward a hypertrophic shape with stout processes.

Results

Middle Cerebral Artery Occlusion

Primary Lesion Site
Sham-operated rats did not exhibit neuronal damage in the cerebral cortex, basal ganglia, or thalamus. Three hours of MCAO resulted in reproducible infarction of the ipsilateral cerebral hemisphere including the striatum, globus pallidus, and parietal cortex. The area of infarction never included the thalamus or the internal capsule (Figure 1A and 1C).

Thalamus
On Nissl-stained slices, at days 1 and 3 after ischemia, no morphological changes could be observed within the ipsilateral VPN or RTN. At day 7 after ischemia, within the VPN and RTN, many neurons with shrunken cytoplasm and damaged nuclei could be seen. The number of glial cells increased. Fourteen days after ischemia, the number of intact neurons ipsilateral to the infarction was decreased within the VPN by 55.02% and within the RTN by 44.46% compared with the contralateral side (Figures 2A, 2B, and 4A through 4D). Furthermore, many damaged neurons could be seen, and a strong gliosis had developed. Pathological changes were
restricted strictly to thalamic structures. The internal capsule that surrounds the thalamus and that lies between the area of infarction and the RTN appeared to be normal.

From day 1 after ischemia, GFAP immunoreactivity was strongly enhanced in the ipsilateral VPN and RTN compared with the contralateral side (Figure 6A and 6B). Astrocytes displayed thickened perikarya and processes. On day 3, 7, and 14 after ischemia, the astrogliosis did not change substantially. While on day 1 after ischemia no clear changes of OX-42 immunoreactivity were seen in the ipsilateral VPN compared with the contralateral side, at 3 days after ischemia activated microglial cells with the typical hypertrophic shape and stout processes were seen (Figure 6C and 6D). Ameboid microglia was not seen. OX-42 immunoreactivity in the ipsilateral VPN and RTN increased to a slightly greater extent on days 7 and 14 after ischemia. Outside of the primary lesion site, all pathological changes in terms of immunohistochemical signs of glial activation were restricted to the thalamus and could not be seen within the internal capsule.

**Globus Pallidus**

The ipsilateral globus pallidus of MCAO-treated animals was included in the area of infarction in each animal (Figure 7A). Neuronal cell counts 1 day after ischemia revealed 93% cell loss within the ipsilateral globus pallidus ($P<0.01$). While GFAP and OX-42 immunostaining was decreased within the globus pallidus at 1 and 3 days after ischemia, at 7 days after ischemia a dense gliosis began to develop adjacent to the globus pallidus.

**Discussion**

This is the first study that demonstrates a consistent delayed neuronal cell death within the ipsilateral RTN after focal cerebral ischemia by transient MCAO. The number of intact neurons 14 days after ischemia was reduced by approximately 45% compared with the contralateral side. Reduction of neurons within the VPN was slightly higher (55%). The time course of microglial and astrocytic activation in the RTN and VPN revealed no major differences, as astrocytic activation in terms of enhanced GFAP immunolabeling in both nuclei started 1 day after ischemia and persisted up to 14 days. The microglial activation in both nuclei started 3 days after ischemia, as revealed by enhanced OX-42 immunolabeling. Because on Nissl-stained slices no changes in the ipsilateral VPN and RTN could be seen until day 7 after ischemia, this is a good example of the ability of cell-specific markers such as GFAP and OX-42 to detect pathological changes much earlier than simple Nissl staining.

The RTN, like other thalamic nuclei, is supplied by the posterior cerebral artery, which was not affected by the MCAO technique used in the present study. In the present study a 4-0 monofilament nylon suture was used to occlude the origin of the MCA. In contrast to 3-0 sutures, which were
often used in other studies on focal cerebral ischemia, the 4-0 sutures are not capable of unintentionally occluding the posterior communicans artery. Occlusion of this artery may lead to infarction within the territory of the posterior cerebral artery, which includes the thalamus.19 Occlusion of the MCA alone does not induce a significant reduction in regional cerebral blood flow within the thalamus.20,21 These anatomic conditions are in accordance with the fact that the area of infarction never extended into the RTN or other thalamic structures, suggesting that MCAO does not directly damage this thalamic nucleus. In addition, no secondary inflammatory reactions spread from the infarct to the thalamus because the internal capsule, which lies between those structures, showed no pathological changes in terms of glial activation. The delayed RTN damage, as well as VPN damage after MCAO, shows the typical features of secondary neuronal degeneration. Although Nissl staining, in principle, is not sufficient to distinguish between the primary lesion site, with neuronal necrosis, and secondary neuronal degeneration, with neuronal atrophy or neuronal cell loss, on Nissl-stained slices there are also clearly defined phenomena to distinguish between necrosis and secondary cell loss or atrophy. In pan necrosis, which can typically be seen within the primary damaged territory after cerebral infarction, neurons as well as glial cells...
are affected immediately after ischemia. Astrocytic and microglial cell loss can also be seen in GFAP and OX-42 immunostaining, which showed a rapid downregulation of those markers at the primary lesion site. In this study a glial scar developed only from the adjacent tissue at later time points. However, secondary neuronal degeneration in remote regions is characterized by delayed and selective neuronal cell death with sparing of glial cells. In addition, rapid glial activation can be seen. Thus, because those criteria can clearly be applied to the damage within the RTN as well as the VPN, it can be denoted as secondary neuronal degeneration after ischemia in the region of the MCA.

Retrograde degeneration, which has been suggested to be responsible for secondary neuronal cell death in the VPN, cannot account for the RTN damage because it is devoid of efferent projections to the cortex. A possible explanation of RTN damage could be the anterograde degeneration of axon terminals from the cortex, leading to a release of excessively high glutamate concentrations that affect RTN neurons. Ross and Ebner discussed this hypothesis concerning the VPN damage after cortical ablation in mice. Because RTN and VPN neurons receive the same cortical input, that hypothesis could account for RTN damage as well. However, the present study shows clearly that pure cortical infarcts induced by photothrombosis are not able to lead to secondary RTN damage. Thus, anterograde degeneration of corticothalamic fibers does not seem to be the pathological key factor in RTN degeneration after MCAO. In contrast, photothrombotic cortical ischemia is able to induce secondary neuronal degeneration within the VPN, which displayed a reduction of intact neurons of approximately 32%. In addition, in this model of ischemia early astrocytic and microglial changes were seen in the VPN, as revealed by cell-specific markers. This is in accordance with other reports that show astrocytic and microglial activation in the VPN after photothrombotic stroke. In regard to the situation after MCAO, retrograde degeneration, which likely accounts for secondary VPN degeneration or anterograde wallerian degeneration of glutamatergic corticothalamic axon terminals, seems insufficient to induce RTN damage. However, there is another conceivable mechanism. Nordborg and coworkers investigated secondary thalamic damage after MCAO. They found vasogenic edema fluid, spreading from the area of infarction, to be a cofactor for neuronal damage in the VPN. In that study rats treated with temporal MCAO also showed RTN damage. In contrast, animals with permanent MCAO did not display RTN damage. It is conceivable that the more profuse and widespread vasogenic edema after temporal MCAO compared with permanent MCAO is responsible for this difference. The absence of RTN damage in photothrombosis-treated animals could be in accordance with that hypothesis because, although a perifocal edema after photothrombotic ischemia is described, this edema is much smaller than that after temporal MCAO. Thus, the vasogenic edema after temporal MCAO could be a possible explanation or at least a cofactor for RTN damage.

The RTN is integrated into a network of afferents and efferents (Figure 8). RTN neurons receive glutamatergic input from the cerebral cortex and from other thalamic nuclei. In some studies, next to those afferents, GABAergic, inhibitory projections from the globus pallidus to the RTN were found. The finding of a direct connection between the basal ganglia and the RTN, which does not exist between the basal ganglia and the VPN, provides an additional route by which RTN neurons can be influenced. MCAO leads not only to cortical infarction but also to infarction of the globus pallidus, and thus the inhibitory GABAergic input to the RTN is reduced. An imbalance of excitatory (release of glutamate from degenerating cortical axon terminals) and inhibitory (reduced inhibitory basal ganglia input) RTN input could
therefore lead to neuronal cell death. This hypothesis is substantially supported by the fact that cortical infarction alone, which was induced in the present study by photothrombosis, does not lead to RTN damage, possibly because of the intact GABAergic projection from the globus pallidus into the RTN. A similar mechanism of secondary neuronal degeneration is known to occur in the substantia nigra pars reticulata after infarction or excitotoxic damage of the ipsilateral striatum.28-29 In this case, the loss of GABAergic striatal input is assumed to induce neuronal cell death in the substantia nigra pars reticulata. In regard to the RTN, degeneration of corticothalamic projections alone (photothrombosis) is not sufficient to induce degeneration of RTN neurons after 14 days. Because no signs of glial activation, which usually precede secondary neuronal degeneration, were seen within this nucleus after 14 days, it is highly unlikely that secondary neuronal degeneration could begin later than 14 days after pure cortical infarction. MCAO, however, which leads to infarction not only of the frontoparietal cortex but also of the globus pallidus (inhibitory GABAergic input), is sufficient to cause RTN degeneration. Thus, RTN degeneration after MCAO could be another example of neuronal cell death caused by an imbalance of neurotransmitters due to a loss of GABAergic input.

Acknowledgment

This work was supported by the START program of the medical faculty of Rheinisch-Westfälische Technische Hochschule—Aachen.

References

Different Mechanisms of Secondary Neuronal Damage in Thalamic Nuclei After Focal Cerebral Ischemia in Rats
Marcel Dihné, Christian Grommes, Michael Lutzenburg, Otto W. Witte and Frank Block

Stroke. published online October 17, 2002;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2002/10/17/01.STR.0000039406.64644.CB.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/