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;33:3006-3011.)

Key Words: brain ischemia ■ neuronal damage ■ thalamus ■ rats

Focal cerebral ischemia may cause delayed and selective neuronal cell death in nonischemic, remote brain areas that have synaptic contacts to the primary lesion site. Such secondary lesions can be observed in the substantia nigra pars reticulata and in specific nuclei of the thalamus dorsalis.1,2 In most studies of secondary thalamic damage, neuronal cell loss was localized within the ventroposterior nucleus (VPN).3–5 This nucleus is connected to the ischemic cortex via thalamocortical and corticothalamic projections. The accepted hypothesis is that this secondary damage is due to retrograde degeneration of thalamocortical projections.6 In addition, neuronal damage of the reticular thalamic nucleus (RTN) has been described in some studies but was not investigated systematically.5,7 This thalamic nucleus receives collateral afferents from corticothalamic and thalamocortical fibers, eg, of the connection between the VPN and cerebral cortex. Anatomic studies found efferent projections from the RTN into nearly all other thalamic nuclei via inhibitory GABAergic terminals.8 In contrast to the VPN, efferent projections from the RTN to the cerebral cortex do not exist. Those efferent projections from the RTN to other thalamic nuclei modulate transmission of neuronal information between the thalamus and the cortex.8,9 Thus, focal lesions in the extremely vulnerable RTN in humans, which could be detected after short durations of cardiac arrest, were suspected to be responsible for attentional and cognitive deficits occurring after that event.10–12 An extreme sensitivity to global ischemia is assumed to play a role in the selective loss of RTN neurons after cardiac arrest in humans or global ischemia in animals. However, mechanisms of possible secondary RTN damage after focal cerebral ischemia are unclear. Retrograde degeneration of RTN neurons after focal cerebral ischemia cannot be considered because efferent projections from the RTN to the cortex do not exist.13 In the present study RTN and VPN damage after focal cerebral ischemia was investigated under 2 different conditions. Focal cerebral ischemia was induced by transient middle cerebral artery occlusion (MCAO). In addition, focal cerebral ische-
mia was induced by photothermolysis of the temporoparietal cortex, which is known to produce a pure cortical lesion. 14 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 photothermolysis 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 O 2 /N 2 (1:2) and placed in a stereotaxic frame. 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.

Photothermotropic Ischemia

Photothermotropic cerebral infarction was induced in the rat parietal cortex according to the method of Watson et al., as described in detail elsewhere. 15 For this purpose rats were anesthetized with 1.3% halothane in O 2 /N 2 (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 with a mouse monoclonal antibody to the astrocyte antigen 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% H 2 O 2 to quench endogenous peroxidase activity for 10 minutes. After they were washed, the sections were blocked with 10% normal horse serum. The sections were washed 3 times and incubated with secondary antibodies (biotinylated horse anti-mouse, 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% H 2 O 2 . 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 μm 2 . Quantification of neuronal cell density within the ipsilateral and contralateral globus pallidus was done 1 day (MCAO) or 14 days (photothermolysis ischemia) after ischemia at −1.4 mm from bregma. Only vital neurons with intact membranes and nuclei were counted. Five MCAO- and photothermolysis-treated rats plus 1 sham-operated control animal were analyzed. To obtain representative results, 5 sections per animal (separated by a distance of 20 μm) were analyzed. Mean and SEM values per 200 μm 2 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 term 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 and RTN 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.

**Photothrombosis**

**Primary Lesion Site**

Sham-operated rats did not exhibit neuronal damage in the cerebral cortex, basal ganglia, or thalamus. Photothrombosis resulted in infarction of the parietal cortex, including all cortical layers down to the white matter. Neither the striatum nor the globus pallidus was affected by this kind of ischemia (Figure 1B).

**Thalamus**

Fourteen days after photothrombosis, on Nissl-stained slices, the ipsilateral VPN displayed a reduction of neurons of 31.85% (Figures 3A, 5A, and 5B). The ipsilateral RTN displayed no signs of neuronal damage or neuronal cell loss compared with the contralateral side (Figures 3B, 5C, and 5D). Only the VPN revealed an increased number of glial cells. The number of glial cells within the RTN remained constant.

While the enhancement of GFAP and OX-42 immunoreactivity, beginning 1 day after photothrombosis, within the ipsilateral VPN revealed a time course similar to that after MCAO, no microglial or astrocytic activation could be seen within the ipsilateral RTN 14 days after photothrombosis (Figure 6E and 6F).

**Globus Pallidus**

The globus pallidus of animals that received photothrombotic ischemia did not show any changes after focal ischemia (Figure 7B). Fourteen days after ischemia, on Nissl-stained slices, no neuronal cell damage could be detected. Quantification of neuronal cell density 14 days after ischemia revealed no statistical difference between the ipsilateral and contralateral globus pallidus ($P=0.87$). GFAP and OX-42 immunoreactivity revealed no astrocytic or microglial activation.

**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. Occlusion of the MCA alone does not induce a significant reduction in regional cerebral blood flow within the thalamus. 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 vasoergic 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 vasoergic 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 vasoergic 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. This 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 photothermalbosis, 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 input. Aachen. This work was supported by the START program of the medical lateral striatum.28,29 In this case, the loss of GABAergic reticulata after infarction or excitotoxic damage of the ipsilateral striatum.28,29 In this case, the loss of GABAergic projection from the globus pallidus into substantia nigra pars reticulata. 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 input.

**Figure 8.** Connections between the cortex, basal ganglia, and thalamus. Glu indicates glutamatergic; GABA, GABAergic.

**Acknowledgment**

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

**References**


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Stroke. 2002;33:3006-3011; originally published online October 17, 2002;
doi: 10.1161/01.STR.000039406.64644.CB

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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