Neuronal Network Disturbance After Focal Ischemia in Rats

Kazuo Kataoka, MD, Toru Hayakawa, MD, Kazuo Yamada, MD, Takeshi Mushiroi, Ryotaro Kuroda, MD, and Heitaro Mogami, MD

We studied functional disturbances following left middle cerebral artery occlusion in rats. Neuronal function was evaluated by [14C]2-deoxyglucose autoradiography 1 day after occlusion. We analyzed the mechanisms of change in glucose utilization outside the infarct using Fink-Heimer silver impregnation, axonal transport of wheat germ agglutinin-conjugated-horseradish peroxidase, and succinate dehydrogenase histochemistry. One day after occlusion, glucose utilization was remarkably reduced in the areas surrounding the infarct. There were many silver grains indicating degeneration of the synaptic terminals in the cortical areas surrounding the infarct and the ipsilateral cingulate cortex. Moreover, in the left thalamus where the left middle cerebral artery supplied no blood, glucose utilization significantly decreased compared with sham-operated rats. In the left thalamus, massive silver staining of degenerated synaptic terminals and decreases in succinate dehydrogenase activity were observed 4 and 5 days after occlusion. The absence of succinate dehydrogenase staining may reflect early changes in retrograde degeneration of thalamic neurons after ischemic injury of the thalamocortical pathway. Terminal degeneration even affected areas remote from the infarct: there were silver grains in the contralateral hemisphere transcallosally connected to the infarct and in the ipsilateral substantia nigra. Axonal transport study showed disruption of the corticospinal tract by subcortical ischemia; the transcallosal pathways in the cortex surrounding the infarct were preserved. The relation between neural function and the neuronal network in the area surrounding the focal cerebral infarct is discussed with regard to ischemic penumbra and diaschisis. (Stroke 1989;20:1226–1235)

The neuronal network, consisting of neurons and fibers, maintains the neuronal functions in the central nervous system. Therefore, even though an injury to the central nervous system is limited to a focal region, it may affect the blood flow, metabolism, and function of other brain regions. This pathophysiology has been named diaschisis.1 Recent advances in positron emission tomography (PET) may help to elucidate this phenomenon. In stroke patients, occlusion of a major intracranial artery results in ischemia of not only the cortical gray matter but also of subcortical structures. For example, PET clearly showed that a subcortical ischemic lesion and thalamic stroke affected the cerebral blood flow and glucose utilization of the overlying neocortex.2,3 A thorough understanding of ischemic injury to the neuronal pathway may lead to the development of therapeutic regimens to improve functional recovery after stroke.

In this study, we focused on neuronal network disturbances following focal ischemia induced by occlusion of the left middle cerebral artery (MCA) in rats. We evaluated neuronal activity by [14C]2-deoxyglucose autoradiography. To analyze the change in local glucose utilization, we used the Fink-Heimer silver impregnation method,4 which has been widely used to determine neuroanatomic pathways. This method facilitates inspection of the degenerated fibers and synaptic terminals following ischemic disruption of the neuronal network. We also examined the corticospinal and transcallosal tracts in the periphery of the focal ischemia by studying the retrograde axonal transport of wheat germ agglutinin-conjugated-horseradish peroxidase (WGA-HRP).5 Ischemic injury of axons may produce retrograde degenerative changes in the neuronal soma that may be revealed by the application of succinate dehydrogenase (SDH) histochemical methods. Histochemistry may provide macroscopic

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Supported in part by a Japan Heart Foundation research grant for 1987.

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information concerning mitochondrial integrity because SDH is one of the enzymes of the tricarboxylic acid cycle and is found in the inner membrane of mitochondria.\textsuperscript{6}

Materials and Methods

Twenty-seven mature female Wistar rats, each weighing approximately 300 g, were anesthetized with 120–150 mg/kg i.p. ketamine hydrochloride. A 0.5% lidocaine solution was topically applied to the surgical wounds. After enucleation of the left eye, the MCA was exposed via the transorbital approach\textsuperscript{7,8} and coagulated from its origin to approximately 3 mm from the olfactory tract. In sham-operated rats, the arachnoid surrounding the MCA was divided, but the artery was not coagulated. We always cooled the surgical wounds to avoid thermal cortical injury as a result of bipolar cautery. Throughout this procedure, special care was taken to protect the vibrissae and the trigeminal nerve. The entire procedure took approximately 30 minutes.

For \textsuperscript{[\textit{14}C]}2-deoxyglucose autoradiography, 11 rats (seven with MCA occlusion and four with sham operation) were anesthetized with 100 mg/kg i.p. ketamine hydrochloride 1 day after the operation. A 1% lidocaine solution was topically applied to the surgical wound for additional anesthesia. Left femoral artery and vein catheters were placed, and the rats were restrained with a plaster cast on a board. Heparin (0.5 unit/g) was used to avoid coagulation of the catheters. When the rats became fully alert (approximately 6–8 hours after anesthesia), they received a bolus injection (100 µCi/kg) of \textsuperscript{[\textit{14}C]}2-deoxyglucose in the left femoral vein. Arterial blood pressure and arterial blood gases were measured just before the isotope injection. Timed arterial blood samples were taken to determine plasma glucose and carbon-14 concentrations. The rats were killed 45 minutes later with an intravenous overdose injection of sodium pentobarbital, and their brains were immediately removed and frozen. A cryostat was used to obtain 20-µm sections of the brain. These were placed on glass coverslips, dried on a hot plate at 60° C, and exposed for 5–7 days to x-ray film (Kodak SB-5, Rochester, New York) together with a calibrated carbon-14–embedded methylmethacrylate standard. Local cerebral glucose utilization of the noninfarced area was calculated using the equation of Sokoloff et al.\textsuperscript{9} No attempt was made to calculate glucose utilization of the infarced area. In this deoxyglucose study, we used seven rats for MCA occlusion and four rats as sham-operated controls. The distribution of the data was tested by analysis of variance. The difference between MCA occlusion and sham-operated control was evaluated using the nonparametric Mann-Whitney \textit{U} test. Interhemispheric differences were investigated by Student’s paired \textit{t} test. Values are given as mean±SD.

For SDH histochemistry, the rats were placed under deep pentobarbital anesthesia (50 mg/kg i.p.) 1 or 5 days after the operation, transcardiac perfusion with 500 ml cooled 10% glycerin solution was performed, and the brains were immediately removed and frozen. Frozen sections (40 µm) were placed on chrome-alum subbed glass slides and stored overnight in a cryostat at −20° C. The sections were then dried at 37° C and incubated for 40–50 minutes to demonstrate SDH in a solution consisting of 0.55 mM nitro blue tetrazolium and 0.05 M sodium succinate in 0.05 M phosphate buffer (pH 7.4) at 37° C. The reaction was stopped by immersing the slides in 10% buffered formalin for at least 1 hour. The slides were then mounted with glycerin.\textsuperscript{6,10} We used seven rats for this histochemical study. Two were sham-operated controls; one was examined 1 day and the other 5 days after the operation. Three rats were used 1 day after occlusion and two rats were used 5 days after occlusion.

For the Fink-Heimer method for silver impregnation, two rats with MCA occlusion and two with sham operation were deeply anesthetized with sodium pentobarbital 4 days after the operation. Transcardiac perfusion was performed with 100 ml of saline followed by 500 ml of 10% formalin. The brains were then taken out and after storage for 2 weeks in 10% formalin, they were immersed overnight in a 30% sucrose solution for cryoprotection, and 30-µm brain slices were cut and processed according to the second method of Fink-Heimer.\textsuperscript{4}

For the axonal transport study, rats were anesthetized with 120 mg/kg i.p. ketamine the day after the operation and the skull or thoracic vertebrae were fixed to a stereotactic apparatus. A tracer of 5% WGA-HRP (0.5 µl) in saline was injected with a microsyringe into both sides of the corticospinal tracts at the level of the T10 thoracic vertebra. Two rats with MCA occlusion and one sham-operated rat were used. In two other rats with MCA occlusion, 0.1 µl of 5% WGA-HRP in saline was injected into the contralateral cerebral cortex; 48 hours later, these rats were deeply anesthetized with sodium pentobarbital and transcardiac perfusion was performed with 50 ml of saline followed by 500 ml of 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and 500 ml of 10% sucrose in phosphate buffer. The brains were immersed overnight in 30% sucrose in phosphate buffer, and serial

<table>
<thead>
<tr>
<th>Variable</th>
<th>MCA occlusion (\textit{n}=7)</th>
<th>Sham operation (\textit{n}=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td>118±13</td>
<td>123±3</td>
</tr>
<tr>
<td>Plasma glucose concentration (mg/dl)</td>
<td>192±66</td>
<td>122±16</td>
</tr>
<tr>
<td>\textit{Paco} \textsubscript{2} (mm Hg)</td>
<td>95±6</td>
<td>94±3</td>
</tr>
<tr>
<td>\textit{Paco} \textsubscript{2} (mm Hg)</td>
<td>37±3</td>
<td>36±2</td>
</tr>
<tr>
<td>pH</td>
<td>7.34±0.06</td>
<td>7.36±0.04</td>
</tr>
</tbody>
</table>

\textsuperscript{MCA, middle cerebral artery. Values are mean±SD.}
Rat MCA Occlusion (1 Day)

(14C) 2-Deoxyglucose Autoradiography

40-μm brain sections were cut. These sections were processed with tetramethylbenzidine by the method of Mesulam.

**Results**

In the autoradiography study, there were no significant differences in physiological variables between the MCA occlusion and the sham-operated groups (Table 1). In the four sham-operated controls (in which the left eyes were removed without MCA occlusion), structures on the right side related to the visual system showed reduction of glucose utilization compared with structures on the left side. Occlusion of the left MCA resulted in a well circumscribed infarct of the striatum and/or the development of infarction in the parietal cortex (Figure 1). The ipsilateral ventral thalamus of these seven rats showed a significant decrease in glucose utilization compared with the controls and compared with the contralateral hemisphere, and the cortical areas close to the infarct exhibited a significant reduction of glucose utilization compared with the contralateral hemisphere (Table 2). In the cortical area overlying the infarct, the band of high [14C]2-deoxyglucose accumulation in layer IV that was present in the contralateral hemisphere and in the sham-operated rats was absent (Figure 2).

SDH activity in the infarcted area had decreased on the day after MCA occlusion; both thalami stained symmetrically. Five days after MCA occlusion, the ipsilateral thalamus showed reduced SDH activity compared with the contralateral side (Figure 3).

**TABLE 2. Local Cerebral Glucose Utilization in 11 Rats Studied With [14C]2-Deoxyglucose Autoradiography**

<table>
<thead>
<tr>
<th>Structure</th>
<th>MCA occlusion (n=7)</th>
<th>Sham operation (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>Frontal cortex (motor area of face)</td>
<td>37±16*</td>
<td>62±9</td>
</tr>
<tr>
<td>Parietal cortex (somatosensory area of face)</td>
<td>62±12†</td>
<td>71±8</td>
</tr>
<tr>
<td>Auditory cortex</td>
<td>67±12‡</td>
<td>84±15</td>
</tr>
<tr>
<td>Visual cortex</td>
<td>58±15</td>
<td>57±13</td>
</tr>
<tr>
<td>Anterior cingulate cortex</td>
<td>44±15*</td>
<td>64±9</td>
</tr>
<tr>
<td>Posterior cingulate cortex</td>
<td>55±10*</td>
<td>63±8</td>
</tr>
<tr>
<td>Medial caudate-putamen</td>
<td>57±6†</td>
<td>62±12</td>
</tr>
<tr>
<td>Lateral caudate-putamen</td>
<td>65§</td>
<td>62±11</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>26±10‡</td>
<td>30±9</td>
</tr>
<tr>
<td>Ventral thalamus</td>
<td>34±11*</td>
<td>55±11</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>33±10</td>
<td>35±9</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>50±15*</td>
<td>36±9</td>
</tr>
</tbody>
</table>

SDH, succinate dehydrogenase; MCA, middle cerebral artery. Values are mean±SD μmol/100 g/min.

*Significantly different from contralateral side (p<0.01, paired t test).
†Mean of 3 rats; the other 4 developed infarction.
‡Mean of 6 rats; the 7th rat developed infarction.
§Value for 1 rat; the other 6 developed infarction.
||Significantly different from sham-operated control (p<0.05, Mann-Whitney U test).
FIGURE 2. $[^{14}C]2$-Deoxyglucose autoradiogram obtained on day after left middle cerebral artery occlusion in rat shows decrease in glucose utilization in cortical area overlying infarct. Note disappearance of high activity in layer IV in ischemic hemisphere. In contrast, this band of high activity was clearly present in contralateral hemisphere (arrows). Left side of brain is on right side of figure.

FIGURE 3. Photographs of succinate dehydrogenase (SDH) histochemistry 5 days after left middle cerebral artery occlusion in rat. Note reduced SDH activity in left thalamus (arrow) compared with contralateral side. On the other hand, in cortical areas close to infarct, changes in SDH activity were not remarkable. Arrowheads indicate margin of infarct. Left side of brain is on right side of figure.

FIGURE 4. Top: $[^{14}C]2$-Deoxyglucose autoradiogram obtained 1 day after left middle cerebral artery occlusion in rat shows remarkable reduction of glucose utilization in ipsilateral thalamus. Bottom: Fink-Heimer silver impregnation 4 days after occlusion. Note dense staining of thalamus on side of occlusion. Ipsilateral internal capsule and cortical areas are also impregnated by silver grains. Impregnation of right optic tract is due to removal of left eye.
lateral cortical areas transcallosally connected to the ischemic lesion were also affected by terminal degeneration (Figure 6).

Injection of WGA-HRP into both corticospinal tracts at T10 retrogradely labeled the symmetrical corticospinal neurons in both the hind leg and the trunk areas within the motor cortex in a sham-operated rat. Although these cortical areas did not develop infarction, WGA-HRP failed to stain the cortical neurons on the side of the occlusion in two rats because the subcortical infarction intersected the corticospinal tract (Figure 7).

Injection of WGA-HRP into the contralateral cortex clearly labeled the neurons in layer V of the cortical tissue close to the infarct through the transcallosal pathway (Figure 8). In this area close to the infarct, Fink-Heimer staining demonstrated extensive synaptic terminal degeneration and degenerated fibers (Figure 9).

Discussion
MCA occlusion did not critically reduce blood flow in the ipsilateral thalamus and cingulate cortex because in rats the MCA does not supply blood to those areas. However, we noted decreased glucose utilization and massive degeneration of synaptic terminals in those areas. Reduction of glucose utilization in the noninfarcted area may reflect the absence of synaptic inputs from the ischemic area. Schwartz et al reported that autoradiographic values for glucose utilization in rats mainly reflect synaptic activities in the normal conscious state. The evaluation of glucose utilization under pathologic conditions is problematic. Nedergaard et al observed a rim of high glucose accumulation in the peri-infarct area until 6 hours after MCA occlusion in rats; this may have resulted in lactic acidosis, leading to sporadic neuronal loss. We did not observe
high \[^{14}C\]2-deoxyglucose accumulation 1 day after ischemia. Decreased glucose utilization in the peri-infarct area mainly reflects or coincides with functional disturbances. Our study suggested the coexistence of a decrease in glucose utilization and degenerated synaptic terminals in similar brain structures. We did not quantify the silver grains and could not study glucose utilization and Fink-Heimer histochemistry in the same rats. We suppose that terminal degeneration explains only a part of the mechanisms of functional disturbances. We measured glucose utilization 1 day after occlusion, while rats used for Fink-Heimer histochemistry were killed 4 days after occlusion. Degenerated synaptic terminals became well impregnated by silver grains between 1 and 3 days after induction of the lesion; however, complete determination of degenerating fibers requires a survival time of 3–5 days in rats.\(^{16}\) Because vascular occlusion takes time to develop into complete cerebral infarction, we chose 4 days for postoperative survival to inspect both terminal degeneration and degenerating axoplasm. The neurofunctional disturbances following brain damage sometimes resolve with time. Therefore, because the presence of degenerated synaptic terminals and axoplasm may reflect initial neurofunctional disturbances before their resolution, we compared glucose utilization 1 day after occlusion and terminal degeneration 4 days after occlusion.

The Fink-Heimer silver impregnation method mainly provides information about anterograde changes.\(^{16}\) We used the axonal transport of WGA-HRP to evaluate disruption of the efferent pathway. Subcortical ischemia was found to intersect the ipsilateral corticospinal tract. On the other hand, the transcallosal neurons in the cortical area close to the infarct maintained their function of retrograde axonal transport and their connections to the contralateral hemisphere. This means that both intact neuronal networks (i.e., the transcallosal...
pathway) and disrupted neuronal networks (i.e., the corticospinal pathway) exist in the peri-infarct area. The callosal and corticospinal neurons are very close to each other in layer V although there is no evidence of collaterals between the two types of neurons in normal rats.\textsuperscript{17} Considering enhanced neurologic recovery after cerebral infarction, the role of the surviving intact neuronal network in the peri-infarct area needs to be investigated further.

After axonal injury, the cell body of some neurons becomes retrogradely damaged; this is known as retrograde degeneration. The degeneration of specific nuclei in the thalamus after cortical ablation has been well documented.\textsuperscript{18–22} In focal ischemia such a degenerative process may also occur. Although Cooper et al\textsuperscript{22,23} reported that cortical ablation in rats resulted in neuronal shrinkage and decreased glucose utilization in the ipsilateral thalamus 3–4 days after surgery, there have been few investigations concerning experimental cortical ischemia and thalamic degenerative changes. In our study, SDH histochemistry (possibly reflecting mitochondrial integrity) revealed reduced staining in the ipsilateral thalamus 5 days after occlusion, although symmetrical staining of both thalami was observed 1 day after the insult. Electron microscopy has revealed degenerative changes 3–5 days after cortical ablation,\textsuperscript{20} and in view of these findings, disturbances in the SDH histochemistry of the thalamus reflect degenerative changes following focal ischemia. The SDH activity in the cortical area surrounding the infarct was not markedly changed. This method does not permit the precise quantification of mitochondrial activity. Glial cells, macrophages, and other inflammatory cells in the cortical area surrounding the infarct may affect SDH activity.

Occlusion of a single MCA produces cerebral infarction in rats, but the extent of infarction may vary from only striatal infarction to massive corticostriatal infarction.\textsuperscript{24} We coagulated the MCA for

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8}
\caption{Top: Coronal section at level of globus pallidus 48 hours after injection of wheat germ agglutinin-conjugated-horseradish peroxidase into contralateral cortex of rat after left middle cerebral artery occlusion. Note retrogradely labeled transcallosal neurons in cortical area near infarct (arrow). Bottom: Microscopic enlargement of area indicated by arrow. ×100.}
\end{figure}
the focus of many studies concerning cerebral ischemia. Since the introduction of the concept of the ischemic penumbra, which is based on the local blood flow--functional relation, there have been efforts to explain functional disturbances in the tissue surrounding the cerebral infarct by this concept. However, there is some controversy about how to understand subtle neural cell loss in the area surrounding the infarct. Strong et al suggested that the patchy ischemic cell change on the marginal gyrus acutely following MCA occlusion in cats made it unnecessary to postulate retrograde degeneration due to white matter infarction, the mechanism proposed by Mies et al. We previously showed that the residual local blood flow did not correlate with neuronal function evaluated by sensory evoked potentials. This is especially evident in the periphery of focal ischemia induced by MCA occlusion in cats. In our present axonal transport study subcortical ischemia clearly intersected the ipsilateral corticospinal tract, and we propose that the ipsilateral thalamocortical pathway was also affected. The thalamus provides a large output to cortical layer IV, in which a prominent band of metabolic activity is observed on [14C]2-deoxyglucose autoradiograms. The cortical area overlying the infarct lost its high-uptake band in the present [14C]2-deoxyglucose study because MCA occlusion affected the ipsilateral thalamocortical pathway. The pathophysiology of the ischemic penumbra, based on the local blood flow--functional relation, may exist in the subcortical white matter as well as in the cortical center of noncritical focal ischemia.

The concept of ischemic diaschisis should be considered in neurofunctional disturbances produced by ischemic lesions. Ischemic deafferentation and/or de-efferentation may be included in the concept of diaschisis. This concept has been used in connection with changes in regional cerebral blood flow, glucose utilization, and neuronal function throughout the neuronal pathways. For example, Ginsberg et al suggested that mild suppression of glucose utilization in the contralateral hemisphere might be mediated by transhemispheric projections after MCA occlusion in cats. Results of our study, which revealed degenerated synaptic terminals in the contralateral hemisphere, support the suggestion of Ginsberg et al. These degenerated synaptic terminals are derived from degenerated transcallosal fibers that originate from the ischemic neurons; however, we did not find decreased glucose utilization in the contralateral hemisphere compared with sham-operated rats. In the rat MCA occlusion model, the neostriatum developed ischemia. Tamura et al reported that in halothane-anesthetized rats, blood flow in the ipsilateral globus pallidus and substantia nigra increased significantly 30 minutes after MCA occlusion. In our study, glucose utilization in the ipsilateral substantia nigra decreased slightly compared with the
contralateral side 1 day after occlusion in conscious rats. The production of unilateral striatal electrolytic lesions in rats does not result in apparent changes in glucose utilization of the ipsilateral substantia nigra 1 day after the operation. It does, however, result in increased glucose utilization in the ipsilateral substantia nigra 7 days after the operation in conscious rats. These results indicate that the observed influences exerted through the neuronal pathways probably change depending on the time since experimental occlusion and on experimental conditions. We noted decreases in glucose utilization in the frontal cortex on the side of the occlusion, probably due to ischemic deafferentation and/or de-efferentation. Similarly, unilateral cholinergic deafferentation in the frontal cortex caused by an ibotenate or electrocoagulated lesion of the nucleus basalis of Meynert results in a temporary increase in glucose utilization in rats and baboons. 44

Our study clearly shows that disruption of the neuronal network plays an important role in the neurofunctional disturbances in the area surrounding ischemia. We suggest that ischemic diaschisis (i.e., areas of deafferentation and/or de-efferentation as a result of focal ischemia) widely surround areas that are considered the cortical ischemic penumbra.

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**KEY WORDS** cerebral ischemia • diaschisis • metabolism • rats
Neuronal network disturbance after focal ischemia in rats.
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Stroke. 1989;20:1226-1235
doi: 10.1161/01.STR.20.9.1226

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