Exo-Focal Postischemic Neuronal Death in the Rat Brain

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Introduction

It is well known that the extent of brain damage caused by focal ischemia depends on the degree of the ischemic insult and its duration[1,2]. In the case of a high degree of ischemia, even if recirculation is achieved, the disorder of energy metabolism in the ischemic tissue cannot be remedied, and the tissue undergoes irreversible changes as a result of energy failure. On the other hand, in the case of a low degree of ischemia, it has been considered that postischemic neuronal damage occurs selectively in the ischemic areas, depending on the degree of ischemia, and only in the more vulnerable cells, as a result of the injury to the cell membrane which contains the signal transducing systems such as, glutamate-phosphatidylinositol (PI) cycle-Ca^{2+} system [3,4,5].

In recent years, we found that delayed neuronal damage occured in the ipsilateral remote areas outside the ischemic areas of rat brain after transient focal ischemia. In the present study, we investigated the distribution and the chronological changes of the postischemic neuronal damage outside the ischemic areas, and investigated the mechanism involved by measuring local cerebral glucose metabolism.

Materials and Methods

Ischemia Model

Adult male Wistar rats of the SPF strain weighing 280 - 300 g were allowed free access to food and water before and after all procedures. Five rats were used in each experiment. A detailed description of the surgical procedure has been reported[6]. In brief, after induction of anesthesia with a gas mixture of 70% N_2O and 2% halothane (the balance O_2), the right middle cerebral artery (MCA) was occluded with a silicone rubber cylinder attached to nylon surgical thread introduced from the bifurcation of the internal carotid artery immediately after ligation of the ipsilateral common and external carotid arteries. The cylinder was made of 4 - 0 nylon surgical thread (Nitcho Kogyo Co., Ltd) 16 mm long coated with silicone (Xantopren, Bayer Dental) mixed with a hardener (Elastomer Activator) to thicken

the distal 5 mm to 0.25 - 0.30 mm. After introducing the embolus, the internal carotid artery was ligated just distal to the point of insertion. The embolus extended from the bifurcation of the internal carotid artery to the proximal portion of the anterior cerebral artery (ACA). The origin of the right MCA and posterior communicating artery was occluded by the silicone rubber cylinder. In five sham-operated control rats, the right internal and external carotid arteries were ligated. The surgery was performed within 15 minutes with no bleeding. Body temperature was kept at normal limits with a heating pad. Following surgery, anesthesia was discontinued and all rats exhibited neurologic deficits characterized by left hemiparesis with upper extremity dominant and right Horner's syndrome. After 15, 30, 60, and 90 minutes of MCA occlusion, recirculation was achieved by pulling the thread out of the internal carotid artery under the same anesthetic conditions as during surgery. Once again, the rats were allowed free access to food and water until the next procedure. Although the ipsilateral common and external carotid arteries had been ligated, the ischemic area could be reperfused via the cerebral arterial circle (circle of Willis) through the contralateral carotid and basilar arteries, and by collateral circulation of the cortical branches of the cerebral arteries.

^{45}Ca Autoradiography

Immediately after recirculation and then after 1 day, 3 days, 1 week, and 2 weeks of recirculation following different periods of MCA occlusion, 300μCi $^{45}CaCl_2$ (Amersham) in aqueous solution (0.3 ml of 0.9% NaCl) was administered intravenously followed immediately by a 0.2-ml saline flush by the method of Dienel[7]. Six hours after ^{45}Ca injection, the rats were decapitated. The brains were quickly removed and frozen in powdered dry ice. Serial coronal sections 20 μm thick were cut from the frozen brain in a -20°C cryostat and dried at 60°C on glass coverslips. Autoradiograms were prepared from these sections by exposing them to X-ray film (Kodak NMC-1) for 4 weeks in standard X-ray cassettes.

Neuropathological Study

After 6 hours, 1 day, 3 days, 1 week, and 2 weeks of recirculation following different periods of MCA occlusion, the rats were anesthetized and their brains were perfusion-fixed with 40% formaldehyde: glacial acetic acid: methanol (1:1:8, FAM) via the ascending aorta after briefly (30 seconds) washing out the cephalic circulation with heparinized physiological saline[8]. The brains were removed from the skulls and stored in FAM until they were embedded in paraffin. Brain sections (5 μm) were stained with cresyl violet and Luxol fast blue and with hematoxylin and eosin. The sections were examined under a light microscope, and regional neuronal damage was graded according to the number of cells with morphological changes and by the nature of the staining[8].

Regional Cerebral Blood Flow

In order to verify the degree of ischemia induced in the ischemic model, regional cerebral blood flow (rCBF) was measured in the rats after 60 minutes of MCA occlusion. Under the same anesthetic conditions as during surgery, a tracheotomy was performed and the rats were ventilated. Pancronium bromide (0.6 mg/kg i. p.) was administered, and both femoral arteries and vein were cannulated to allow the continuous monitoring of mean arterial blood pressure, the repeated sampling of arterial blood and the administration of fluids. After the surgical preparation of the rats, 2% halothane administration was discontinued and the rats were ventilated with 70% N_2O and 30% O_2 allowing normoxia and normocapnia. CBF was measured by the [^{14}C] iodoantipyrine (Amersham) quantitative autoradiographic technique according to Sakurada et al.[9].

Regional Cerebral Glucose Metabolism

Regional cerebral glucose utilization (rCGU) was measured in the rats after 90 minutes of MCA occlusion followed by 1 week of recirculation. After the same surgical preparation as for the measurement of CBF, samples of arterial blood were taken immediately prior to measurement of rCGU for determination of blood glucose level, PaO_2, $PaCO_2$, and pH. CGU was measured by the 2-[^{14}C]deoxyglucose (Amersham) quantitative autoradiographic technique according to Sokoloff et al.[10].

Statistical Analysis

Cerebral [^{14}C] tissue concentrations of the autoradiograms were determined by means of computer-based microdensitometer system (Chromoscan). Data regarding rCBF and rCGU were analyzed using a t-test with $p<0.05$ and $p<0.01$ considered statistically significant.

Results

The value of rCBF in the rats after 60 minutes of MCA occlusion is shown in Table 1. In this model, the anterior neocortex (FrPaSS) and lateral part of the caudate putamen (CPu-L), which were supplied by the occluded MCA, were the regions most frequently damaged. Sixty minutes of MCA occlusion resulted in a significant reduction in CBF of 0.04 ml/g/min ($p<0.01$) in the lateral segment of the caudate putamen and 0.19 ml/g/min ($p<0.01$) in the cortex of the occluded MCA area compared with the corresponding regions in the contralateral hemisphere. No significant reduction in CBF was observed in the ipsilateral hippocampus, thalamus, or substantia nigra compared with the corresponding regions in the

contralateral hemisphere.

Chronological Changes of ^{45}Ca Accumulation

In the 15-minute MCA occlusion group, no ^{45}Ca accumulation was detected until 2 weeks after ischemia (Fig. 2). In the 30-minute-and-more MCA occlusion groups, ^{45}Ca accumulation became more prominent in proportion to the duration after reperfusion, and abnormal accumulation was also detected in remote areas which were not initially affected by the ischemic insult. The chronological changes of ^{45}Ca accumulation after 30 minutes of MCA occlusion are shown in Fig.1. In this group, after 6 hours of recirculation, only a slight degree of ^{45}Ca accumulation was detected in the lateral margin of the caudate putamen; after 1 day and 3 days, it was extended to the lateral segment of the caudate putamen, and the accumulation images became more prominent as well. Moreover, after 3 days of recirculation, ^{45}Ca accumulation was first detected in the ipsilateral substantia nigra as well. After 2 weeks, ^{45}Ca accumulation was detected in the cortex supplied by the occluded MCA, and also in the ipsilateral ventral posterior nucleus of the thalamus. In the 60 and 90 minutes MCA occlusion groups, after 6 hours of recirculation, remarkable ^{45}Ca accumulation was observed in the cortex and lateral segment of the caudate putamen in the occluded MCA areas. In both groups, no abnormalities were detected in the ipsilateral thalamus or the substantia nigra even after 1 day of recirculation, and ^{45}Ca accumulation was first detected in both remote areas after 3 days. A remarkable accumulation of ^{45}Ca was detected after 2 weeks in the thalamus and the substantia nigra, only in the ischemic side (Fig. 2).

Neuropathology

Histologic examination of the brains from the five sham-operated rats showed no pathological alterations. In the 15-minute MCA occlusion group, no abnormal findings were detected until 2 weeks of recirculation. In the 30-minute MCA occlusion group, no ischemic changes were detected until 1 week of recirculation, and after 2 weeks, a mild degree of ischemic change as described by Brown and Brierley[11] in other ischemic models was observed in the cortex and the lateral segment of the caudate putamen in the occluded MCA areas. Moreover, after 2 weeks, a mild degree of selective neuronal cell damage and mild gliosis were observed in the ventral posterior nucleus of the thalamus and the substantia nigra (pars reticularis) in accordance with the distribution of ^{45}Ca accumulation. These histologic findings in the thalamus and the substantia nigra were characteristic: neuronal cells were selectively reduced in number and became atrophic with gliosis to a varying extent and no ischemic changes were detectable in the gliacytes and blood vessels. These histologic effects became severer in proportion to the duration of both the ischemia and the time interval after recirculation. The effect of the different periods of ischemia on the distribution and grade of

neuronal cell damage is shown in Table 2. The histologic appearance of the substantia nigra (pars reticularis) after 90 minutes of MCA occlusion followed by 2 weeks recirculation is shown in Fig. 3. No histological abnormalities were seen in the contralateral hemisphere.

Glucose Metabolism

After 90 minutes of MCA occlusion followed by 1 week of recirculation, representative 2-[^{14}C] deoxyglucose autoradiograms of the thalamus and the substantia nigra are presented in Fig. 4. Regional CGU in the ventral posterior nucleus of the thalamus of the ischemic side decreased inhomogeneously, to a variable extent, but there was no significant reduction compared in the corresponding region of the contralateral hemisphere. On the other hand, rCGU in the substantia nigra of the ischemic side increased significantly ($p<0.01$) in comparison with the corresponding region of the contralateral hemisphere (Table 3).

Discussion

We studied the distribution of tissue damage after transient focal ischemia by using the ^{45}Ca autoradiographic technique, and found that ^{45}Ca accumulated not only in the lateral segment of the caudate putamen and the cerebral cortex, which were directly affected by the ischemic insult, but also in the ipsilateral ventral posterior nucleus of the thalamus and the pars reticularis of the substantia nigra, which lay outside the ischemic areas. In the cerebral cortex (FrPaSS) and the lateral segment of the caudate putamen (CPu-L) which were supplied by the occluded MCA, after 60 minutes of MCA occlusion, the level of rCBF was reduced by approximately 12% and 3%, respectively, compared with that in the corresponding regions of the control rats. It is believed that the acute necrosis of the neurons and neuroglia cells observed in these ischemic foci is a result of the disorder of energy metabolism which followed the ischemia. Histologic examination revealed very little ischemic brain edema in this model and the durations of the ischemia were not long enough to promote such edema[6]. In the thalamus and the substantia nigra, in which ^{45}Ca accumulation was detected after 3 days of recirculation following the transient MCA occlusion, no significant reduction of CBF was found even after 60 minutes of MCA occlusion in comparison with the corresponding regions of the sham-operated rats. The mechanism of injury in both remote areas is not explained by the disorder of energy metabolism which followed the ischemia. Johansson reported that histologic changes were observed in the regions outside the ischemic foci up to 3 weeks after the ischemia, using a perpetuated MCA occlusion model with no recirculation, and that the damages in the remote areas (ipsilateral cerebral cortex, internal capsule, corpus callosum, etc) could be caused by vasogenic edema originating from the ischemic foci[12]. The areas observed in her study, however, were adjacent to the ischemic areas, and as the duration of ischemia was also long, it seems that the effect of the brain edema induced by the

ischemia should not be neglected. In our study, on the other hand, the durations of ischemia were quite transient, and there was no evidence of brain edema even after 60 minutes of MCA occlusion followed by recirculation[6]. So brain edema is probably not responsible for delayed neuronal death in remote areas.

It is known that both remote areas that were affected, the thalamus and the substantia nigra, have transsynaptic connections with the cerebral cortex and the caudate putamen, respectively, via the cortico-thalamic pathway and the striato-nigral pathway [13,14]. In the thalamus, the damage was limited to the ventral posterior nucleus (VPM, VPL), and the selective neuronal death in this area may be explained by a process of retrograde degeneration as a result of neuronal cell damage caused by the precedent ischemic insult in the postcentral gyrus of the cerebral cortex, which forms anatomically close fiber connections with this area[15]. It is known that the ablation of the cerebral cortical fibers reduces glutamate uptake in the synaptic terminals of the cortico-striatal fibers and cortico-thalamic fibers, and it is thus believed that these fibers can act as a glutaminergic [16,17]. Therefore, it seems that the non-physiological release of a large quantity of neurotransmitters could occur at the axon terminals of the thalamus, when neuronal cells in the cortex are damaged by an ischemic insult. As a result, it could induce a collapse of homeostasis, which is related to the postsynaptic membrane ion permeability, and this may then lead to cell death in the thalamus [18,19,20]. Moreover, from the 2-[^{14}C]deoxyglucose autoradiogram obtained after 90 minutes of MCA occlusion followed by 1 week of recirculation, rCGU in the thalamus showed a tendency to decrease, to a variable extent, in comparison with the corresponding region in the contralateral hemisphere. This fact suggests the mechanism of the selective neuronal death observed in the thalamus is quite different from that observed in the substantia nigra via the striato-nigral pathway, which is mentioned below. Based on our study, we speculate that the selective neuronal death in the thalamus is caused by retrograde degeneration originating from the precedent ischemic lesion of the cortical neurons. In our experiment, however, this mechanism was still unclear, and a further detailed study over time is required in order to clarify this point.

As for the pars reticularis of the substantia nigra, it consists of fibrous connections originating in the caudateputamen and projecting over the globus pallidus, forming the striato-nigral pathway[21]. The caudate nucleus of the rat contains high concentrations of several neurotransmitters, such as acetylcholine, dopamine, glutamate, substance-P and gamma-aminobutyric acid (GABA). The neuroinhibitory transmitter GABA plays an important functional role in the striato-nigral pathway[22,23,24,25]. Moreover, glucose metabolism was increased approximately 30% in the substantia nigra of the ischemic side, compared with the corresponding region in the contralateral hemisphere, after 90 minutes of MCA occlusion followed by 1 week of recirculation. The increase of the rCGU in this area may thus be explained as a result of altered neuronal function and hypermetabolism, and is caused by

diminished inhibitory output from the caudate putamen, which was affected by the precedent ischemia. Onodera, using a receptor autoradiographic method, reported that the benzodiazepine receptor density in the pars reticularis of the substantia nigra increased approximately twice as much as that of a control group after the ischemic neuronal death of the lateral part of the caudate putamen[26]. He deduced that this phenomenon is reflected denervation hypersensitivity in the substantia nigra resulting from suppression of the GABAergic output from the ischemic lesion of the caudate putamen[26]. Moreover, the disinhibition can induce a postsynaptic longterm potentiation or continuous excitatory state in the substantia nigra. As a result, it could lead to a collapse of the homeostasis of the cell membrane and to neuronal cell death in this area[27]. Tamura, using a MCA occlusion model with no recirculation, reported that a postischemic hyperemia was observed in the ipsilateral substantia nigra of the rat brain and that this hyperemia was related to the hyperactivity of the substantia nigra, which resulted from the disinhibition via the striato-nigral pathway[28]. But hyperemia was observed in the hippocampus, in connection with delayed neuronal death after a transient global ischemia in gerbils[29]. The hyperemia may be caused not by disinhibition, but by changes in the tissue environment of the dying cells, such as lactic acidosis[30] etc. On the other hand, another possibility is that the selective neuronal death in the substantia nigra is caused by retrograde axonal degeneration from the ischemic lesion of the caudate putamen via the nigro-striatal pathway; such a mechanism is not ruled out by the present study. Further detailed investigation is required to clarify the mechanism. In any case, the postischemic selective neuronal death was observed in remote areas which have close transsynaptic connections with the ischemic foci.

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Table 1. Carebral blood flow measured in various regions of the rat brain following 60 min of MCA occlusion

Structure	ischemic side	non-ischemic side
FrPaM	0.94 ± 0.10*	1.49 ± 0.15
FrPaSS	0.19 ± 0.08*	1.69 ± 0.16
CPu(L)	0.04 ± 0.02*	1.63 ± 0.12
CPu(M)	0.62 ± 0.15*	1.73 ± 0.13
Hippocampus	1.20 ± 0.25	1.35 ± 0.14
Thalamus	1.12 ± 0.13	1.28 ± 0.19
Substantia nigra	1.42 ± 0.19	1.30 ± 0.16
Pons	1.20 ± 0.12	1.23 ± 0.15

Values are given in mean ± SD ml/g/min. Five animals.
FrPaM: frontoparietal cortex, motor area, supplied by ACA;
FrPaSS: frontoparietal cortex, somatosensory area, supplied by MCA;
Cpu(L): lateral segment of caudate putamen;
Cpu(M): medial segment of caudate putamen.
*: $p<0.01$, compared with value in the same row using a t-test.

Table 2. Distribution and grade of neuronal damage in rats after two weeks following different periods of middle cerebral artery occlusion

Region	Control	Occlusion			
		15min	30min	60min	90min
Ipsilateral					
FrPaM	0	0	0	0	0
FrPaSS	0	0	1	2	3
CPu(L)	0	0	2	2	3
CPu(M)	0	0	0	0	0
Hippocapmus	0	0	0	0	0
Thalamus (VPN)	0	0	1	1	2
SN	0	0	1	2	2
Contralateral					
FrPaM	0	0	0	0	0
FrPaSS	0	0	0	0	0
CPu(L)	0	0	0	0	0
CPu(M)	0	0	0	0	0
Hippocampus	0	0	0	0	0
Thalamus (VPN)	0	0	0	0	0
SN	0	0	0	0	0

Data are mean grade for five rats compared with corresponding regions of sham-operated control rats. O, normal brain; 1, few neurons damaged; 2, many neurons damaged; 3, majority of neurons damaged. FrPaM, frontoparietal cortex, motor area, supplied by anterior cerebral artery; FrPaSS, frontoparietal cortex, somatosensory area, supplied by middle cerebral artery; CPu(L), lateral segment of caudate putamen; CPu(M), medial segment of caudate putamen; Thalamus (VPN), ventral posterior nucleus of thalamus; SN, pars reticularis of substantia nigra.

Table 3. Local cerebral glucose utilization measured after 90 min of MCA occlusion followed by 1 week recirculation

Structure	ischemic side	non-ischemic side
Thalamus (VP nucleus)	7.20 ± 0.29	8.20 ± 0.88
Substantia nigra	10.12 ± 0.76*	7.68 ± 0.64

Values are given in mean ± SD mg/100g/min. Five animals.
VP nucleus: Ventral posterior nucleus
*: $p<0.01$, compared with value in the same row using a t-test.

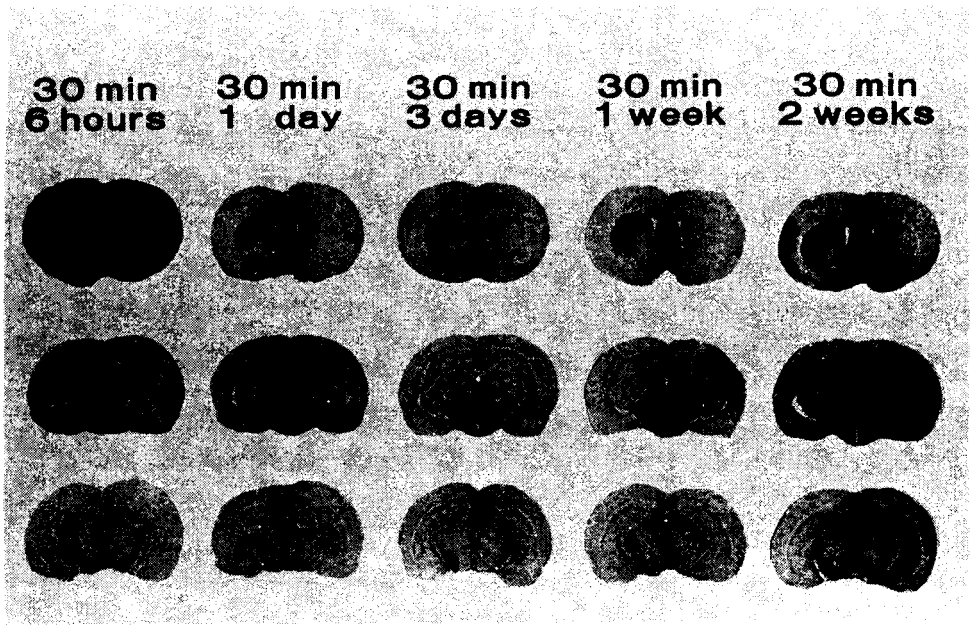

Fig. 1 ^{45}Ca autoradiograms of rat brain obtained after 30 minutes of MCA occlusion followed by 6-hr, 1-day, 3-day, 1-week, and 2- week recirculation. Representative autoradiograms show coronal sections at the level of caudate putamen (top), Thalamus (middle)., and substantia nigra (bottom).

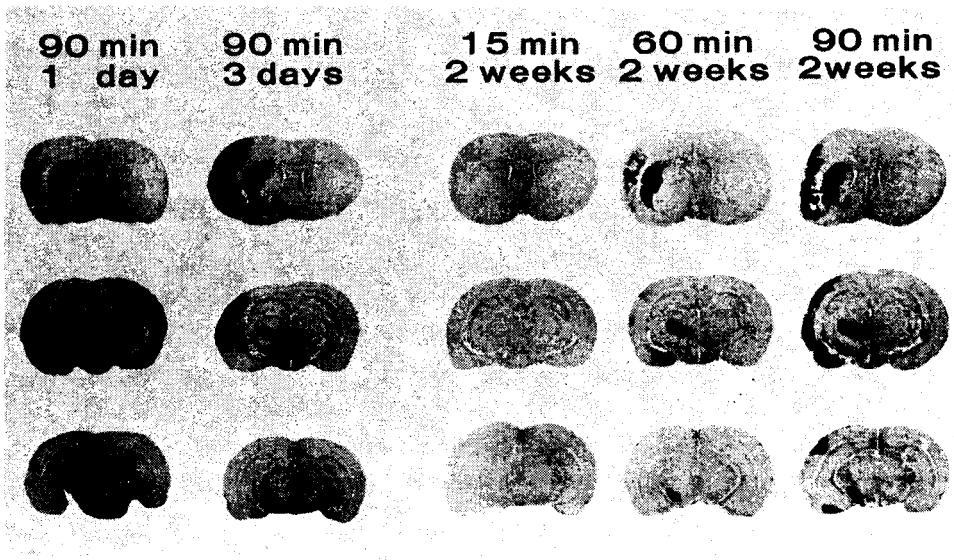

Fig. 2 ^{45}Ca autoradiograms of rat brain obtained after 90 minutes of MCA occlusion followed by 1-day, 3-day, and 2-week recirculation, and after 15-min and 60-min of MCA occlusion followed by 2-week recirculation . The levels of coronal sections are the same as described in Fig. 1.

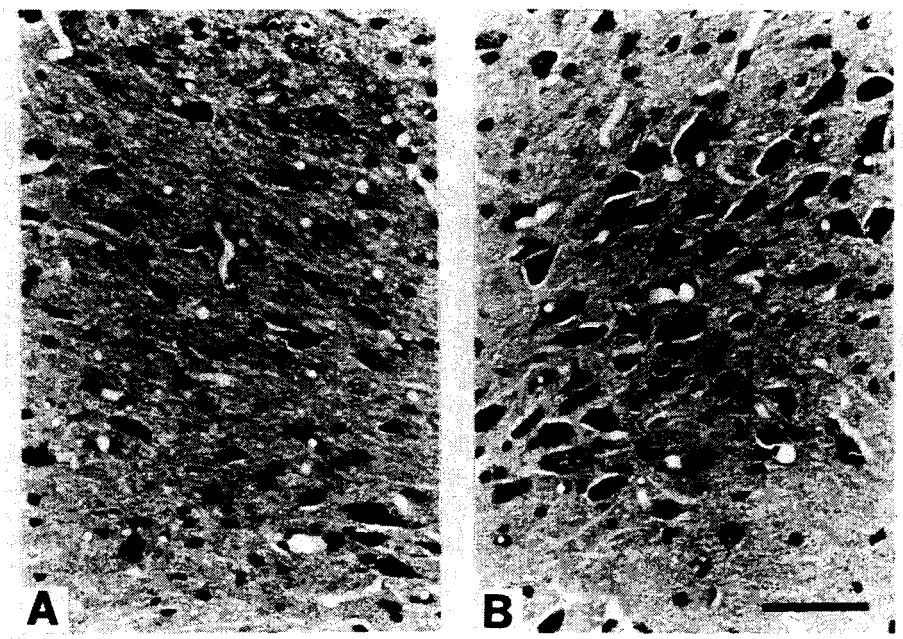

Fig. 3 Representative histological appearances of the pars reticularis of the substantia nigra after 90 minutes of MCA occlusion followed by 2 weeks of recirculation. (A) ischemic side, (B) non-ischemic side. The neurons in (A) are reduced in number and shrunken, whereas the neurons in (B) are normal. Hematoxylin and eosin. Scale bar represents 50 μm.

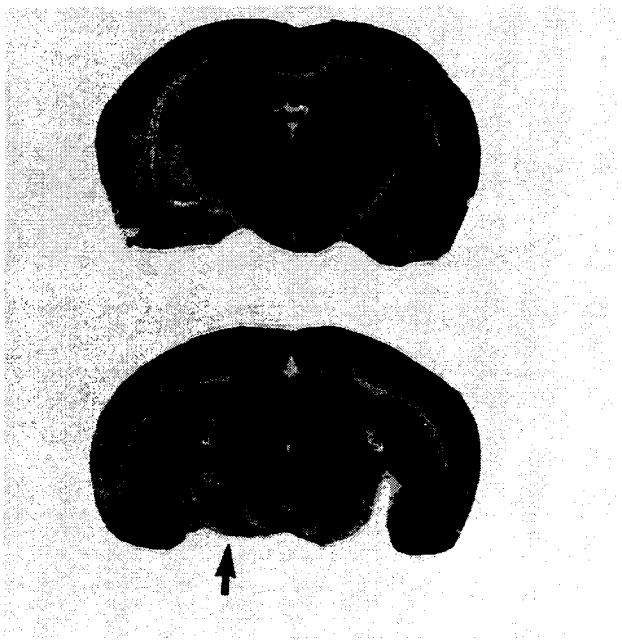

Fig. 4 2-[^{14}C]deoxyglucose autoradiographic images obtained after 90 minutes of MCA occlusion followed by 1 week of recirculation. Regional cerebral glucose utilization (rCGU) decreases slightly and inhomogeneously in the ventral posterior nucleus of the thalamus of the ischemic side (arrowhead), whereas an increase in rCGU is seen in the ipsilateral substantia nigra (arrow).