Histochemical Changes of Brain Dopamine
In an Acute Stage of Cerebral Ischemia in Gerbils

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Summary
The fluorescence histochemical method of Falck et al. was applied to 40 gerbil brains after ligation of a unilateral common carotid artery to investigate alterations of brain dopamine in the acute stage of cerebral ischemia. The distribution of dopaminergic terminals and cell bodies in gerbils is the same as in other mammals. On the ligated side after one hour of ischemia, diffuse green fluorescence of dopaminergic terminals showed only a slight decrease in intensity when compared to the nonligated side. But white matter and bundles of myelinated fibers adjacent to and in the dopamine-rich regions had an intense green fluorescence in contrast to the non-ligated side where they are normally non-fluorescent. This is considered to indicate the extraneuronal leakage and diffusion of dopamine. The intensity of extraneuronal green fluorescence was especially high in glial cells. Occasionally, there was also an unusual green fluorescence in the lumen of small vessels in dopamine-rich regions on the ligated side. Dopaminergic cell bodies in the substantia nigra on the ligated side revealed a conspicuous reduction in the fluorescence intensity in severely affected cases. After 2 or 3 hours of ischemia, there was a marked reduction or disappearance of the diffuse green fluorescence on the ligated side. This may be attributed in part to further diffusion of leaked dopamine.

Materials and Methods
Forty mature gerbils, each weighing between 60 and 80 gm, were anesthetized with ethylether. Using a stereoscopic microscope, we doubly ligated and transected the left common carotid artery. All animals were divided into a symptomatic and an asymptomatic group according to the neurological signs of cerebral ischemia described by Kahn. Three gerbils were sham-operated. Each animal was decapitated either 1, 2 or 3 hours after the ligation, and the brain was rapidly removed and processed by the monoamine fluorescence histochemical method of Falck et al. Sections of 10 µm thickness were made and observed with a fluorescence microscope, a Schott BG12 filter as an excitation filter and a Zeiss 50 filter as a barrier. Fluorescence differences were determined by comparing the cerebral hemispheres of gerbils with ligated carotid arteries and non-operated controls. One observer (M.I.) determined the change in fluorescence intensity without knowing which sections were taken from the brains of gerbils with or without neurologic abnormalities. Light microscopic observation was performed in some sections after Klüver-Barrera staining in order to identify anatomical structures and ischemic changes. The normal distribution of

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Dopaminergic neuron systems was investigated in 2 non-operated gerbils and in another 2 gerbils after the administration of Nialamide and l-dopa.

Results

Following the left common carotid artery ligation, 13 gerbils (33%) showed neurological signs such as lethargy, hemisensory inattention, hemiparesis, ipsilateral circling behavior and convulsive seizures. In these animals light microscopic observation after Klüver-Barrera staining revealed ischemic tissue changes such as neuronal shrinkage, pyknosis and perivascular edema in the ipsilateral hemisphere 2 or 3 hours after ligation. Unilateral cerebral hemispheric ischemia involved most areas which have been reported as dopamine-rich in other mammals except for the median eminence and, occasionally, the medial part of the nucleus accumbens. Ischemic areas were clearly demarcated at the medial border by a narrow band of neuropil vacuolations (fig. 1). After one hour of ischemia there were slight ischemic changes only in severely affected cases. No apparent histological alteration was observed in the contralateral hemisphere of a symptomatic animal, nor in either hemisphere of an asymptomatic nor a sham-operated animal.

Fluorescence Histochemical Study

The control study with non-operated gerbils showed intense diffuse green fluorescence in the nucleus caudoputamen, the nucleus accumbens and the tuberculum olfactorium, and many green fluorescent cell bodies in the substantia nigra. Because of the same appearance and distribution as in other mammals and the marked increase in intensity after administration of Nialamide and l-dopa such diffuse green fluorescence and cell bodies with green fluorescence were considered to be dopaminergic terminals and cell bodies. Alterations of green fluorescence of dopamine in cerebral ischemia are described.

Symptomatic Group

After One Hour of Ischemia

In moderately affected animals which showed no significant histological changes, there was no apparent reduction in the intensity of diffuse green fluorescence.

FIGURE 1. Schematic representation of the typical extension of ischemic area. Frontal sections are depicted in illustrations A-D sequenced in rostro-caudal order. The narrow band of neuropil vacuolations is represented as shaded area. Lateral to this band there were many ischemic tissue changes which were conspicuous in 2 or 3 hours of ischemia but hardly visible in 1 hour of ischemia. Dots and black spots respectively indicate dopaminergic terminals and cell bodies. Squares 2–8 indicate the positions of photographs in the following figures 2–8. Drawings are modified after Loskota et al. a: nucleus accumbens ac: nucleus amygdaoides centralis c: nucleus caudoputamen ca: commissura anterior cc: crura cerebri ci: capsula interna co: chiasmaticus opticus cp: commissura posterior f: fornix lm: lemniscus medialis m: fasciculus mamillothalamicus pm: fasciculus prosencephali medialis r: fasciculus retroflex snc: substantia nigra, zona compacta snr: substantia nigra, zona reticulata s: stria terminalis st: nucleus interstitialis striae terminalis to: tuberculum olfactorium.
Figure 2. Nucleus caudoputamen after 1 hour of ischemia. (×80) (A) Non-ligated side. Note the non-fluorescent areas of various sizes in the diffuse green fluorescence of dopaminergic terminals. Of the non-fluorescent areas, relatively large areas, short curved lines and a part of the small spots indicate the bundles of myelinated fibers, capillaries in the longitudinal plane and capillaries in the transverse plane. (B) Ligated side of the same animal. Diffuse green fluorescence covers all areas of the nucleus caudoputamen and so the bundles of myelinated fibers and capillaries are hardly identified. They become visible again as non-fluorescent areas after application of ultraviolet rays (C).

Figure 3. Pars anterior of commissura anterior in the nucleus accumbens after 1 hour of ischemia. (×80) (A) Non-ligated side. Myelinated fibers are non-fluorescent. Darker spots indicate glial cells intervening between them. (B) Ligated side of the same animal showing intense green fluorescence. It is rapidly reduced by irradiation of ultraviolet rays but the glial cells still have significant fluorescence (C).
In the nucleus caudoputamen, however, bundles of myelinated fibers, which were normally non-fluorescent on the non-ligated side (fig. 2-A), had an intense green fluorescence to such a degree that they could hardly be distinguished from the surrounding fluorescent tissue of the nucleus caudoputamen (fig. 2-B). This fluorescence was rapidly faded by an application of sodium borohydride or ultraviolet rays (fig. 2-C). The glial cells intervening between the myelinated fibers remained significantly fluorescent against the markedly decreased fluorescence in the surroundings after the irradiation of ultraviolet rays. This was also observed more conspicuously in the anterior commissure (fig. 3). The margin of the nucleus caudoputamen became obscure due to the fluorescence diffusion into the deep subcortical white matter (fig. 4). Capillaries, which were normally seen as dark spots or short curved lines scattered in diffuse green fluorescence on the non-ligated side, also became indistinct on the ligated side. There was occasionally green fluorescence in arterioles and venules (fig. 5). In some severely affected areas, slight reduction in intensity of diffuse green fluorescence of dopaminergic terminals was noted, especially in the marginal zone of the ischemic area where more remarkable zonal reduction of the fluorescence intensity was observed (fig. 6). In the contralateral hemisphere and the medial region of the ipsilateral hemisphere, which were spared from ischemia, no apparent changes in diffuse green fluorescence were found. In the substantia nigra, although many intensely fluorescent cell bodies were seen on the non-ligated side, there was sometimes marked reduction of the fluorescence in cell bodies with slightly increased background fluorescence in the lateral part on the ligated side (fig. 7).

After 2 or 3 Hours of Ischemia

Diffuse green fluorescence in ischemic areas was markedly reduced or almost lost, especially near and in the marginal band of neuropil vacuolations at the border of the ischemic area (fig. 8). Equivocal de-
A zonal reduction of the fluorescence at the medial border of ischemic area (three arrows) after 1 hour of ischemia. (X 40)

A band of neuropil vacuolations has already appeared but it is still mild (two arrows). M: medial side L: lateral side D: dorsal aspect V: ventral aspect.

creases in fluorescence intensity were seen in the non-ischemic contralateral hemisphere.

Asymptomatic Group and Sham-Operated Animals

These animals had no fluorescence histochemical abnormalities.

Discussion

Recently, studying Mongolian gerbils after unilateral cerebral hemispheric ischemia, many workers have reported changes in brain monoamines and marked depiction of brain dopamine has been demonstrated after 24 hours of ischemia. During the few hours of cerebral ischemia, although some investigators found a marked reduction of cortical dopamine content, hemispheric dopamine content has shown only minor, if any, alteration and ranged from normal to 70% of normal value.

After one hour of cerebral ischemia, the present histochemical study revealed an intense green fluorescence in bundles of myelinated fibers in the dopamine-rich areas on the ligated side. This fluorescence was believed to be dopamine because of the rapid reduction in its intensity after the application of sodium borohydride or ultraviolet rays and its topography. Such findings indicate that brain dopamine may leak from nerve terminals and diffuse into the bundles of myelinated fibers in an ischemic area. Since energy substances such as ATP, ADP and P-creatine are more than half depleted in the ischemic cortex of gerbils, the storage function of monoamine neurotransmitters in ischemic neurons appears to be disturbed, making extraneuronal
leakage of stored dopamine conceivable. Reports have also attributed the reduction of monoamine content in ischemic brain to a leakage or an increased release, but there has been no histochemical demonstration of the extraneuronal leaked dopamine in situ until the present study. Inhibition of degradation and removal of monoamines in ischemic brain also appears to play a role in the appearance of fluorescence visualized. Fluorescence in the deep subcortical white matter around the nucleus caudoputamen is believed to be dopamine for the same reasons and, chemically, an increased content of dopamine in ischemic cortex has been reported at an earlier stage. The present study has shown leaked dopamine in small vessels in dopamine-rich regions. Release of serotonin into cerebral veins has been demonstrated in the baboon after cerebral ischemia and such a phenomenon is also likely for dopamine. An increased level of serotonin and catecholamines in the cerebrospinal fluid (CSF) of patients with cerebral infarction has also been reported.

Although it is sufficiently probable that the leaked dopamine diffuses into CSF spaces, no positive support for this was obtained in the present study. Remaining fluorescence in glial cells intervening between the myelinated fibers after the application of ultraviolet rays suggests that the leaked dopamine may be concentrated to some degree in glial cells. It has been demonstrated that normal rabbit brain fractions rich in glial cells accumulate neurotransmitter amines and that exogenous norepinephrine injected into the corpus callosum of a normal rat is taken up by the interfascicular glial cells. A zonal reduction in the fluorescence intensity in the periphery of an ischemic area precedes the eventual boundary zone necrosis.

After 2 or 3 hours of cerebral ischemia, there was a marked decrease in the intensity or a disappearance of diffuse green fluorescence in ischemic areas. The fluorescence histochemical method is much less sensitive in detecting a change of monoamine content than chemical assays. According to Kopin et al., in the nucleus caudoputamen, diffuse green fluorescence of dopamine terminals shows no visible reduction in intensity until the dopamine content is reduced to 70% of control value and it disappears when dopamine content is reduced to 20% of control. The results of many studies imply that at least 70% of normal dopamine content remains in an ischemic hemisphere after a few hours of ischemia. Thus, if dopamine in this amount is concentrated around or in nerve terminals, the intensity of the fluorescence would not be decreased. The marked reduction of fluorescence as shown in the present study may be at least in part attributed to further diffusion of the extraneuronally leaked dopamine.

Chemical assays measure the extraneuronal as well as the intraneuronal dopamine. There is a report which has shown only slight additional reduction of hemispheric dopamine content from 1 to 3 hours after ischemia, while the present study has demonstrated a marked additional decrease during this interval. Loss of fluorescent cell bodies in the substantia nigra involved by ischemia may be in part responsible for the reduction of hemispheric dopamine content. An equivocal reduction in fluorescence intensity in non-ischemic areas suggests that an alteration of
dopaminergic neuron systems may not be confined to the ischemic area. Some authors have also reported a change of monoamine content in the contralateral cerebral hemisphere\(^6,14,20\) but neither electron\(^a\) nor light\(^b\) microscopic study has revealed ischemic changes on the non-ligated side within 3 hours of ischemia.

The circling behavior of the animals following ligation has been attributed to asymmetry of dopaminergic neuron systems as shown from studies in the rat after destruction of the unilateral nigrostriatal pathway\(^7\), \(^8\) and in gerbils with unilateral cerebral ischemia.\(^9\) Harisson et al.\(^10\) concluded that dopamine changes did not directly contribute to circling behavior because there were no significant changes in dopamine content after 3.5 hours of ischemia. The present study, in which all symptomatic gerbils had an alteration of dopamine fluorescence, also supports the significant participation of dopaminergic neuron systems in this behavior. It should be emphasized that a normal content of dopamine is not necessarily associated with normal distribution in ischemic brain, as shown by the present histochemical study.

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