'REACTIVE CHANGE' OF THE SUBSTANTIA NIGRA NEURONS SUBSEQUENT TO STRIATAL INFARCTION IN RATS

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ABSTRACT

The substantia nigra pars reticulata (SNr) receives the inhibitory γ -aminobutyric acid (GABA)ergic input from the striatum and globus pallidus, deafferentation of which is supposed to lead to anterograde transneuronal degeneration of the neurons in the nucleus. In the present study, we studied the pathological changes in the deafferented neurons in the rat SNr following massive striatopallidal ischemic lesion produced by transient middle cerebral artery (MCA) occlusion. At 3 days post-ischemia, in association with a decrease of GABAergic afferent fibers, over one-fourth of the neuronal population in the ipsilateral SNr consisted of swollen chromatolytic cells, while no apparent changes were found at day 2. Electron microscopy revealed characteristic ultrastructural findings, including the breakdown of the Golgi apparatus, an accumulation of swollen mitochondria, and disruption of the polysomal clusters of ribosomes. The pathology of these chromatolytic neurons clearly coincides with that of so-called 'reactive change (RC)'. Shrunken dark neurons were widely distributed at 7 days after surgery, and a significant reduction in the number of neurons was found in the SNr at 15 days ($P < 0.01$). The present study suggested that chromatolytic feature of the SNr neurons may be a consequence of cellular hyper-excitation after the removal of GABAergic inputs, which may lead these neurons finally to cell death.

Injuries to certain parts of the brain may induce neuronal cell death in distant areas innervated by the sites of primary lesion. This form of cell death characteristic to the nervous system is termed 'anterograde transneuronal degeneration' (5). Such pathological changes may occur at the next and more distant synaptic levels (4), which probably play a part in the slow progression of some 'system degenerations' (5). It is also plausible, however, that transneuronal cell death could act as a natural process of control over the maladaptive activity of deafferented neurons (10, 21, 22).

Loss of striatonigral or pallidonigral afferents also causes transneuronal degeneration of neurons of the substantia nigra pars reticulata (SNr) both in humans (7, 18) and in experimental animals (11, 15, 20, 24). Saji and Reis (20) suggested that the delayed transneuronal degeneration of the deaffer-

ented SNr neurons may be produced by neuronal disinhibition as a consequence of loss of inhibitory γ -aminobutyric acid (GABA)ergic input. Indeed, the long-term replacement of the GABA receptor agonist muscimol completely prevented neuronal cell loss in the SNr after striatal lesion (20).

In contrast to the rapid degeneration such as 'ischemic cell change (ICC)' (1, 12, 17), pathological alteration of deafferented SNr neurons is very slow and was thought to first appear 7 days after deafferentation, showing the shrunken dark neurons in the ischemia model in rats (24). However, our recent studies showed that, corresponding to the marked decrease in GABAergic inputs, a certain metabolic change should occur in the deafferented SNr neurons, inducing synthesis of heat shock protein (26) or growth-associated protein (9) at 3 days after cerebral hemitransection or transient

middle cerebral artery occlusion. Thus, the degenerating process of the deafferented SNr neurons is considered to start earlier than expected.

The aim of the present study was to reappraise the pathological alterations of delayed trans-synaptic degeneration of the deafferented SNr neurons, especially at the early stage prior to the shrinkage process. For this purpose, light and electron microscopic studies were carried out on the substantia nigra of rats which had undergone unilateral transient middle cerebral artery occlusion to produce massive striatopallidal ischemic injury. Characteristic pathology of the deafferented SNr neurons provided here should contribute to our further understanding of the mechanism(s) underlying the delayed transneuronal degeneration.

MATERIALS AND METHODS

Transient Middle Cerebral Artery (MCA) Occlusion

We employed the reversible rat ischemia model, as described previously (9, 26). Briefly, male Wistar/albino rats, weighing 230–270 g, were anesthetized with 2.5% halothane and oxygen. After the right common carotid artery (CCA) was surgically exposed, the external carotid artery (ECA) was ligated, cut off 2 to 3 mm distal to the CCA bifurcation and inverted. An 18.5 mm-long silicon-coated 4–0 nylon thread was introduced into the ECA lumen through a small incision 1 mm distal to the bifurcation and advanced from the ECA to the internal carotid artery for the entire length just short of the proximal tip so that the advanced tip of the thread reached the proximal segment of the anterior cerebral artery and obstructed the origin of the MCA. The ECA with the thread embolus was ligated to prevent bleeding. Rectal temperature was continuously monitored with a thermometer and maintained between 37.5 and 38.5°C, with circling movement to the left confirmed after discontinuation of the anesthesia. Restoration of the MCA blood flow was performed by removal of the thread embolus under brief halothane anesthesia following 2-h MCA occlusion.

Tissue Preparation for Histological Study

The animals were allowed to survive 1 ($n = 6$), 2 ($n = 6$), 3 ($n = 6$), 7 ($n = 6$) or 15 ($n = 6$) days at which time they were deeply anesthetized by intraperitoneal administration of pentobarbital and perfused transcardially with 200 ml of phosphate-

buffered saline (PBS) followed by 300 ml of 4% of paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains were removed and kept in the same fixative solution for at least 12 h, followed by embedding in paraffin and vibratome-sectioning. The prepared sections were subjected to hematoxylin-eosin, Nissl's or Klüver-Barrera's staining, and to immunohistochemical examination.

Tissue Preparation for Ultrastructural Study

Specimens obtained from the substantia nigra of 3 animals sacrificed at day 3 after the operation were processed for electron microscopic study. The rats were deeply anesthetized and perfused transcardially with 200 ml of PBS followed by 300 ml of 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH; 7.4). The brains were removed, cut into small blocks and postfixed in the same fixative overnight at 4°C. Specimens were postfixed with 1% OsO₄ for 2 h. After dehydration through a graded alcohol series, blocks were transferred to propylene oxide and embedded in Epon. Ultra-thin sections, stained with uranyl acetate and lead citrate, were examined with a HU12A electron microscope (Hitachi, Japan).

Immunohistochemistry

A specific cDNA-generated polyclonal antibody against 67 kDa glutamic acid decarboxylase (GAD67, Chemicon; working dilution 1:3,000), the limiting enzyme of GABA synthesis characterizing the GABAergic neurons (8, 13), were used. As described in our previous paper (26), vibratome sections with 40 μ m thickness were incubated in the serial media with a free-floating method. Bound primary antibodies were visualized by with an avidin biotin complex (ABC) kit (Vector). 3,3'-Diaminobenzidine tetrahydrochloride (0.5 mg in 1 ml of PBS) was used as the chromogen.

Cell Counting Method

Six- μ m-thick transverse microtome sections were obtained within the ipsilateral SNr from rats which were sacrificed at 1 ($n = 3$), 3 ($n = 3$), 7 ($n = 3$) and 15 ($n = 3$) days, and the numbers of neuronal cells stained for Nissl's substances were examined. The total number of neurons was counted in the entire area of the SNr sectioned at the level of the oculomotor nerve. The neurons were classified into the 4 morphological categories of those with normal-

Fig. 1 Schematic representation of the extents of ischemic lesions (black-shaded areas), which were determined by means of Nissl staining, beginning from 3 days after MCA occlusion of rats. The ipsilateral side is shown on the right. Massive ischemic lesion is observed in the ipsilateral striatopallidal regions (a) as well as in the cerebral cortex (a, b), but not in the mesencephalon (b). Abbreviations: AC, anterior commissure; CC, corpus callosum; CPu, caudate-putamen; CTX, cortex; GP, globus pallidus; HPC, hippocampus; SC, superior colliculus; SN, substantia nigra

appearance, dark neurons, reactive change (RC), and burst up-like neurons, as defined in Results. Data obtained from 3 sections in each rat are expressed as the means $\pm$ SD. Two-way analysis of variance followed by unpaired *t*-test was used to assess the statistical significance of differences in cell numbers in the SNr at each time during the course.

RESULTS

Time Sequence of Histological Changes of the Ipsilateral SNr Neurons

The ischemic lesions were widely distributed in the striatopallidal region and in the cerebral cortex from 1 to 15 days after MCA occlusion (Fig. 1). While the substantia nigra of the contralateral side showed strong immunoreactivity (IR) for GAD67 throughout the time period examined, marked depletion in GAD67-IR was observed ipsilaterally

at 3, 7 and 15 days (Fig. 2, a, c, d and e). By 2 days postoperatively, a number of neurons with normal-appearance, e.g., round nuclei, prominent nucleoli and Nissl bodies in the perikarya, were seen in the ipsilateral substantia nigra (Fig. 2b). At 3 days post-MCA occlusion, in association with a significant reduction in afferent fibers immunostained for GAD67 (Fig. 2c, arrow), many neurons showing swollen chromatolytic features and vacuoles were noted in the ipsilateral SNr (reactive change, RC; Fig. 2d, arrows). Their nuclei were eccentric and swollen, showing a decrease in the staining intensity. Some SNr neurons had burst and presented disruption of the cell membrane and spreading of the cytoplasmic materials (burst up-like neurons, Fig. 2d, arrowheads). However, the nuclear membranes in these cells were barely preserved, with the exception of a neuronal subpopulation showing complete destruction. Scattered shrunken dark neurons, were also seen at this stage. These changes were more prominent in the lateral portion of the SNr compared to the medial portion.

At 7 days after ischemic insult, the number of swollen chromatolytic neurons was markedly decreased, and shrunken dark neurons with triangular nuclei were widely distributed in the ipsilateral SNr (Fig. 2f). After 15 days, atrophy of the deafferented substantia nigra was noted (Fig. 2g, arrow), in which a marked loss of neurons and a marked decrease in the number of myelinated fibers were seen, together with proliferation of reactive glial cells (Fig. 2h).

Ultrastructural Findings

The ultrastructural characteristics of the SNr neuron with chromatolytic features are shown in Fig. 3. The nucleus is eccentric, but it has a clearly visible nucleolus and well-preserved nuclear membrane. The cell membrane has disintegrated in places. The most conspicuous findings in these cells were the almost total absence of Golgi apparatus and either partial or complete disintegration of the cisternae of the rough endoplasmic reticulum (RER). A prominent redistribution of mitochondria and other organelles led to their accumulation in the central cytoplasm formerly occupied by the nucleus. There were also many stacks of 10-nm neurofilaments (arrowheads) in the cytoplasm. Mitochondria were swollen and frequently formed vacuoles (Fig. 3, arrows), and vacuoles derived from swollen components of the RER were also

Fig. 2

seen (Fig. 4). The polysomal clusters of ribosomes attached to the RER tended to disaggregate and form free single ribosomes (Fig. 4, arrowheads).

Cell Density Analysis

At 1 day post-ischemia, the number of neurons with normal appearance and with dark discoloration was 148 ± 14 and 6 ± 2 , respectively. At 3 days after MCA occlusion, over one-fourth of the SNr neurons presented RC; at this time the number of neurons with normal, dark, RC and burst appearance was 105 ± 8 , 11 ± 4 , 43 ± 8 and 5 ± 4 , respectively. Dark neurons also constituted 30% (43 ± 16) of neuronal population at 7 days later, while the number of neurons with RC and burst appearance was markedly decreased (7 ± 8 and 0, respectively), at this time, normal neurons numbered 96 ± 20 . At 15 days postoperatively, dark neurons and neurons with RC had almost disappeared (5 ± 2 and 3 ± 3 , respectively) and those with normal appearance constituted 91% (89 ± 6) of the total number of SNr neurons (98 ± 9). The total number of SNr neurons at 15 days was significantly decreased compared to that at every other observation time ($P < 0.01$, unpaired *t*-test).

DISCUSSION

Although the swollen chromatolytic feature of the

nigral neurons seemingly resembles 'central chromatolysis', which is a typical feature of retrograde reaction as a consequence of axonal injury, the present ultrastructural observations strongly suggest that the pathology of these neurons closely coincides with RC (3, 12, 14), also known as 'selective chromatolysis (SC)' (2). RC/SC has been found as a reversible nature, appearing in the neurons in CA2 or CA3 subfields of the hippocampus as well as in the deeper layers of the cerebral cortex 20 h after brief (15 to 60 min) cerebral ischemia in gerbils (3, 12, 14). The most conspicuous difference between RC/SC and central chromatolysis appears to be related to the Golgi apparatus (3). Retispersion and retisolution as variations of the morphology of the Golgi apparatus are characteristic of central chromatolysis (16, 19), whereas its breakdown, as observed in this study, is observed in RC/SC (3). In addition, disruption of the polysomal clusters of ribosomes tends to form free single ribosomes predominantly in RC/SC, a finding generally considered to indicate cessation of RNA and protein synthesis.

Saji and Reis (20) suggested that removal of the GABAergic input originating from the striatum (and/or from the globus pallidus) (6, 23) induces lethal excitation of the SNr cells, leading to transneuronal death of those neurons. This hypothetical pathomechanism may also underlie RC/SC of the deafferented SNr neurons. It is consistently found that a decrease in heavy polyribosomes and an increase in single ribosomes of the damaged neurons are also seen in the hyper-excitation conditions such as status epilepticus in rats (25). Indeed, Brown *et al.* (2) have suggested that RC/SC is related to epileptic activity rather than ischemic insult *per se* in the gerbil ischemia model.

Time sequence of change of neuronal cell numbers based on the morphological classification (Fig. 5) strongly suggested that neuronal population exhibiting RC at day 3 would lead to dark neurons at day 7, which may consequently die up to 15 days after MCA occlusion. Although RC/SC has been suggested to be reversible (3, 12, 14), persisting insults may lead these cells to irreversible degeneration as shown in this study. Various morphological features including the mild chromatolytic change, burst up-like feature or shrunken dark neurons may correspond to the various degrees of severity or stage of the neuronal damage in the deafferented SNr.

In conclusion, following striatal infarction, SNr neurons exhibit the characteristic features of

Fig. 2 Time sequence of changes in GAD67 immunoreactivity (IR) and the histology in the ipsilateral substantia nigra pars reticulata (SNr) at 2 (a, b), 3 (c, d), 7 (e, f) and 15 days (g, h) after striatopallidal ischemia. At 2 days, only a mild decrease in GAD67-IR is observed in the ipsilateral SNr (a, arrow), and the SNr neurons shows normal features (b). In association with a marked reduction in GAD67-IR (c, arrow) at 3 days, chromatolytic swollen neurons with vacuolation are noted in the ipsilateral SNr. Their nuclei are also swollen and show decreased staining. The number of Nissl bodies is decreased at this stage (d, arrows). The perikaryal borders of the swollen neurons are frequently indistinct, occasionally showing disruption of the cell membrane and spreading of the cytoplasmic materials (burst up-like neurons, d, arrowheads). Seven days after ischemia, as GAD67-IR shows further reduction (e), shrunken dark neurons with triangular nuclei are observed throughout the ipsilateral SNr (f). At 15 days, many neurons are destroyed, and reactive gliosis is also seen (h). Marked atrophic change is observed in the ipsilateral SNr with decreased labelling for GAD67 (g). Klüber-Barrera. a, c, e and g, $\times 8$; b, d, e and f, $\times 670$

Fig. 3 Electron micrograph of the ipsilateral substantia nigra pars reticulata 3 days after striatopallidal ischemia. The nucleus (N) is eccentric, but the nuclear membrane is well-preserved. The Golgi apparatus have disappeared. A striking redistribution of mitochondria and other organelles leads to their accumulation in the central cytoplasm formerly occupied by the nucleus. Mitochondria are swollen and occasionally form vacuoles (arrows). The cisternae of the rough endoplasmic reticulum (RER) have disintegrated. The larger vacuoles (V) are derived from swollen components of the RER. There are also many stacks of 10-nm neurofilaments (arrowheads) in the cytoplasm. Scale bar, 1.0 μ m

Fig. 4 Electron micrograph of the region corresponding to the area marked with a square in Fig. 3. The vacuole is revealed to be a swollen component of the broken rough endoplasmic reticulum (RER). The polysomal clusters of ribosomes attached to RER have disaggregated and tend to form free single ribosomes (arrowheads). Scale bar, 1.0 μ m

RC/SC, which presumably leads to shrinking and discoloration of the neurons and, finally, to cell death. Chromatolytic feature may result from cellular hyper-excitation in consequence of loss of the inhibitory input mediated by GABA (20). The present results may provide a histological basis for pathomechanisms underlying the exo-focal postischemic death of SNr neurons.

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Fig. 5 Time sequence of change of neuronal cell numbers in the ipsilateral substantia nigra pars reticulata (SNr). Three days after MCA occlusion, over one-fourth of the total number of the SNr neurons show RC. Dark neurons were predominant at 7 days post-MCA occlusion, and marked cell loss was seen at day 15. In contrast, the proportion of neurons with normal appearance increased from 3 to 15 days. The total number of SNr neurons at day 15 showed significant decrease compared to that at every other observation time (*, $P < 0.01$; two-way analysis of variance followed by unpaired t -test).

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