INTRODUCTION:

Previously, we investigated the neuronal remodeling process of the neurons that survived in the cortical ischemic penumbra after transient ischemia [1]. Presently, we investigated the scavenger mechanisms involved in removing the acidophilic dead neurons in the same penumbra. As regards the maturation of the lesions in ischemic stroke [2], numerous studies investigating the delayed neuronal death have been performed during the past 3 decades [3-6]. In the classical neuropathology seen after cerebral ischemia, the cytoplasm of the ischemic neuron becomes markedly eosinophilic; and the nucleus appears shrunken and darkly stained or develops clumped chromatin condensations. In the later stage, the cytoplasm is uniformly structure-less; and the nucleus shows advanced degeneration and appears homogeneous. Activated microglial cells accumulate around the dying neurons, and scavenge them by neuronophagia [3]. However, as Rosenblum stated in his review article [6], the histopathologic literature reveals uncertainty concerning the fate of all the acidophilic neurons after ischemia/hypoxia. The morphological description of the "eosinophilic ghost cell" is very limited in several papers [7-10], a vague term used for morphological description at light microscopic (LMS) level of necrotic neurons with a faintly stained nucleus and cytoplasm, in contrast to that of apoptotic neurons showing nuclear chromatin condensation.

In previous studies a clear separation of infarction and penumbra was not made. However, by giving a threshold amount of temporary ischemic insult to induce cerebral cortical infarction, we have developed a model in which a slowly developing large ischemic penumbra forms around a focal infarction in the cerebral cortex of Mongolian gerbils[11~12]. In this model, by dividing the ischemic insult into 2 parts, the mortality rate of the animals due

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to epileptic seizure is decreased drastically [11], the infarction size becomes uniform so that focal infarction appears in Face A and the penumbra in Face B [12], and the cellular response to ischemic injury is similar to the model of less than 20-min single ischemia, but progresses more uniformly [1, 2, 12~15].

In the present study, using this model and focusing on the scavenging mechanism, we investigated the fate of these disseminated dead neurons in the ischemic penumbra from 4 days to 24 weeks after the transient ischemic insult to answer the following questions: 1) Is such a neuron phagocytized by a single phagocytic cell or by many such cells? This question arises in light of the following facts: In the ischemic penumbra where neurons die compacted in a disseminated fashion, the neuropil is still tight with narrow and complicated extra-cellular spaces, and the blood-brain-barrier is not broken. Therefore, blood-born macrophages can not enter the neuropil to phagocytize them. These electron-dense dead neurons have long neuronal processes making a complicated network with the surviving neurons. 2) Are these structures phagocytized by the intrinsic resident microglia, which cells are reported to respond to injury gradually, first becoming activated and later being transformed into macrophages? [3, 16,17].

MATERIALS AND METHODS:

Following injection of atropine sulfate (10 microgram / kg), under anesthesia with 2% halothane, 70% nitrous oxide, and 30% oxygen, a midline cervical incision was made; and the left carotid artery of adult male Mongolian gerbils (60-80g) was twice occluded with a Heifetz aneurismal clip for 10 minutes each time, with a 5-hr interval between the 2 occlusions. Anesthesia was discontinued immediately after each cervical surgery, and

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the behavior of animals was carefully observed in the awake condition for 10 minutes after the first carotid occlusion.

Ischemia-positive animals were selected based on over 13 points of the stroke index score determined during the first occlusion [18]. These animals were sacrificed at 5, 12, 24 or 48 hours or 4 days or 1,4, 8,12 or 24 weeks following the last ischemic insult, by intra-cardiac perfusion with diluted fixative (1% paraformaldehyde, 1.25% glutaraldehyde in 0.1M cacodylate buffer) for 5 min, followed by perfusion with concentrated fixative (4% paraformaldehyde, 5% glutaraldehyde in 0.1M cacodylate buffer) for 20 min (3 animals in each time group for electron microscopy: EM) or with 10% phosphate-buffered formaldehyde fixative for 30 min (5 animals in each time group for LMS).

In this model, following restoration of blood flow, an ischemic penumbra with progressing disseminated selective neuronal necrosis (DSNN), appears in the coronal face sectioned at the infundibular level (face B); and focal infarction evolves among the DSNN in the coronal face sectioned at the optic chiasma (face A).

We used 3 animals for each time point in the EM study, and from each of them 2 adjacent ultrathin sections including the 2nd ~4th cortical layers were obtained from the left cerebral cortex at the mid-point between the inter-hemispheric and rhinal fissures on face B. The sections were double stained by uranyl acetate and lead solution, and observed with an electron microscope (H9000, Hitachi). Paraffin sections were separately stained with hematoxylin-eosin (HE) or periodic acid fuchsin Schiff (PAS).

Using an eye-piece micrometer(U-OCMSQ10/10), we calculated the percentage of the tiny PAS–positive vesicular particles among PAS–stained remnants of thinned-out dead neurons that appeared in all cortical layers on

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Face B, counting all of the dead neurons in one section from each of 5 animals at 8 (1132 ± 108) and at 24 weeks (618.3 ± 51.9) after ischemia.

Six ultrathin sections for each time group were observed by EM under 1,500 times magnification, and the number of microglia and astrocytes at various time points were counted by sliding the specimen cursor from one area to another, and increasing the magnification in the various areas for detailed observation. We determined the percentage of resting, proliferating/activated, and phagocytic microglia in the specimens obtained from the control and from the ischemic animals at 1, 4, 8, and 12 weeks after the ischemic insult (total number of all 3 kinds of microglia counted in each time group was 211.6 ± 26.2). Likewise, the percentage of astrocytes, proliferating/activated astrocytes, and phagocytic astrocytes in the same specimens was determined (total number of all 3 kinds of astrocytes counted in each time group was 104.6 ±14.6). The statistical difference between each time group were analyzed by ANOVA, followed by Bonferroni-Dunn test. All data in text and Fig.6 were presented as average ± SEM and a statistical difference was accepted at $p < 0.05$ level.

RESULTS:

A. Temporal profiles of fate of the dead neurons:

In the ischemic penumbra of the cerebral cortex in Face B viewed, by LMS, eosinophilic ischemic neurons appeared in disseminated fashion among the normal looking neurons in the 2nd to 6th cortical layers, around 5-hr after ischemic insult. These neurons gradually increased in
number and spread among the surviving neurons thereafter. Some of these eosinophilic cell bodies were remarkably shrunken with condensed and

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clumped nuclear chromatin at 12~48-hr (Fig. 1-A). These shrunken eosinophilic neurons were observed by EM as disseminated electron-dense dark neurons that were homogeneously condensed and surrounded by remarkably swollen astrocytic processes (Fig. 2-A).

By 4~7days after ischemic insult, the shrunken dark neurons had become fragmented into an accumulation of electron-dense granules by invading slender astrocytic cell processes containing glycogen granules and fibrils (Fig. 2-B, C). Small spots of condensed chromatin were scattered in the nucleus (Fig. 2-C-Inset). These fragmented dark neurons were scattered among the viable neurons, astrocytes and microglia (Fig. 2-D). By LMS, these fragmented neurons were observed to have a faint form without nuclear staining, and they looked like ghost cells when stained with PAS (Fig. 1-B). In Face A, focal infarction evolved and developed among the DSNN from 12-hr to 4 days, and foamy macrophages increased in number in it, that showed liquefaction necrosis, from 4 to 7 days.

By 2~8 weeks after ischemic insult, the fragmented electron-dense granular debris of the dead neuron had become dispersed throughout the narrow extra-cellular spaces in the neuropil and scattered eccentrically at a distance from the remainder of the dead neurons (Fig. 3-A). Thus, the peripheral cytoplasm was tattered and thinned-out, with only an electron-dense central portion remaining (Fig. 3-A). By LMS, the PAS-stained ghost cells were gradually reduced their size due to decay of their peripheral cytoplasm. At 8 weeks, tiny PAS-positive vesicular particles (3.5~5.5μm in diameter), each consisting of a PAS-positive thin circular frame and central dot, appeared occasionally among the thinned-out ghost cells (Fig. 1-C and -Inset).

During 12 to 24 weeks, remarkably slimmed tiny remnants of the

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eosinophilic ghost cells and the tiny PAS-positive vesicular particles (Fig. 1-D and -Inset) were observed by LMS to be grouped in the 3rd and occasionally in the 5th cortical layers (Fig.1-D). These vesicular particles were also observed disseminatedly independent of the above groups (Fig. 1-D- Inset). The average number of the dead neurons in the cerebral cortex of Face B decreased from 1132 ± 108 to 618.3 ± 51.9 ($p < 0.05$) at 8 and 24 weeks after ischemia, whereas the average percentage of the tiny PAS–positive vesicular particles among the dead neurons increased from 4.5 ± 0.11 to 57.1 ± 0.11 ($p < 0.05$).

EM observations of the tiny PAS–positive vesicular particles revealed particles composed of a central electron-dense core and a peripheral thin circular electron-dense frame, corresponding to the tiny PAS-positive vesicular particles with a central dot observed by LMS. Monolayers of clear cube-shaped structures had invaded the space between the 2 electron-dense structures and surrounded the frame. These cube-shaped structures consisted of axons with degenerated large mitochondria and electron-dense particles, astrocytic processes, and occasionally presynaptic axon terminals (Fig.3-B~D). Occasionally an electron-dense core was surrounded by multiple layers of processes with the same cube-shaped structures (Fig. 3-E).

The neuropil surrounding them was densely packed with voluminously enlarged polygonal axon terminals filled with synaptic vesicles, spines, and neurites of surviving neurons, and showed only a very few degenerated axons (Fig.3-B~E).

Small spots of condensed chromatin, like those seen in the nucleus of dead neurons (Fig. 2-C-Inset), were also observed in the above electron-dense cores (Fig.3-B~E).

B. Activation and phagocytic activities of resident microglia and

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astrocytes:

Rod-shaped resting microglia were observed along the capillaries, in the neuropil and capping the surviving neurons (Fig. 4-A). Their nucleus was oval or elongated with chromatin clumps beneath the nuclear envelope. The chromatin clumps were more prominent and occupied a larger proportion of the nuclear volume compared with those of the oligodendrocytes. The cytoplasm formed a thin rim around the nucleus and often extended out in processes, and displayed long narrow stringy granular endoplasmic reticulum and fewer microtubules than that of the oligodendrocytes.

Microglia showed activation as evidenced by a globular cytoplasm having a large round dark nucleus with a large amount of chromatin condensations along the nuclear membrane and a large nucleolus. This round nucleus was surrounded by a narrow cytoplasm rich in ribosomes and rough-surfaced endoplasmic reticulum. These cells often showed fusion of 2 cells (Fig.4-B). The percentage of these proliferating/activated microglia in the control was 9.1 ± 0.35%, and in the ischemic gerbils, 21.2 ± 0.8% at 1 week, 23.5 ± 3.5% at 4 weeks, 46.6 ± 0.9% at 8 weeks, and 18.0 ± 5.5% at 12 weeks after the ischemic insult (Fig. 6-A).

Astrocytes showed activation as evidenced by a large amount of chromatin condensation along the nuclear membrane and a large nucleolus (Fig. 5-A). Proliferating astrocytes were also observed along with fusion of 2 cells (Fig. 5-B). These proliferating/activated astrocytes were 0% in the control; and ischemic gerbils, 9.60 ± 3.1% at 1 week, 19.4 ± 2.4% at 4 weeks, 45.9 ± 2.1% at 8 weeks, and 11.1 ± 1.8% at 12 weeks after the ischemic insult (Fig. 6-B)

From 8 to 24-weeks, by LMS observation we frequently detected microglia filled with cytoplasmic PAS-positive materials (Fig. 1-D-Inset).

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EM observation of their cytoplasm revealed phagosomes containing laminated membrane structures, electron-dense debris and lysosomes (Fig.4-C). These heterolysosomes containing ingested materials and lysosomes were surrounded by single-membrane-bound structures (Fig. 4-C-Inset). These phagocytic microglia were first observed at 8 weeks (7.0 ± 0.8%) and increased in percentage at 12 weeks (42.1 ± 11.1%) after the ischemic insult (Fig. 6-A). Phagocytic astrocytes (Fig.5-C) showed the same type of heterolysosomes as the microglia during these periods (Fig. 5-C-Inset). They were first observed at 8 weeks (7.6 ± 2.3%) and increased in percentage at 12 weeks (34.2 ± 1.8%) after the ischemic insult (Fig. 6-B).

Throughout this study, no inflammatory cells or blood-borne macrophages appeared in the neuropils in the ischemic penumbra. Nor were apoptotic bodies observed in the extra-cellular spaces, or found in phagosomes of the microglia.

DISCUSSION:

A. Temporal profiles of the fate of dead neurons:

We found a novel scavenger mechanisms for the removal of disseminated dead neurons in the ischemic penumbra: The electron-dense dead neurons in the ischemic penumbra were fragmented to electron-dense granular debris by the invasion of multiple astrocytic cell processes. These fragmented and pulverized debris of dead neurons became dispersed throughout the narrow complicated extra-cellular space of the neuropils, with the thinned-out central core portions of the dark neurons remaining. A recent ultrastructural study showed that sporadically distributed compacted dead dark neurons in the non necrotic tissue, which death was induced by condenser-discharge electric shock, underwent cytoplasmic convolution and fragmentation [19].

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That report described that these fragments were engulfed by astrocytes and transported to the capillaries and small vessels and removed via blood vessels and that neither proliferation of microglial cells nor infiltration of hematogenous macrophages was observed. In the present study on the ischemic penumbra, where tissue necrosis did not occur, the fragmented debris were not removed immediately via blood vessels or by phagocytosis by microglia, which cells were in the process of becoming activated from 1 to 8 weeks after the ischemia, but these debris were later removed by microglia and astrocytes.

It has been thought that dead neurons and ischemically injured tissue are scavenged by activated resident microglia and/or macrophages that have invaded into the injured tissue from the blood stream [3, 5]. However, in the cortical ischemic penumbra, compacted dead neurons were found in a disseminated fashion (DSNN) among the surviving neurons. The dendrites and axons of the dying neurons were still connected to multiple axon terminals and dendrites of the surviving neurons. Thus, these dying neurons could not be phagocytized by a single microglia or macrophage. In this situation, it is not surprising that the shrunken dead neurons would become fragmented and pulverized into electron-dense granular debris.

B. Final remnants of the dead neurons

The average number of the dead neurons in the cerebral cortex of Face B decreased from 1132 ± 108 to 618.3 ± 51.9 at 8 and 24 weeks after ischemia, whereas the average percentage of the tiny PAS–positive vesicular particles among the dead neurons increased from 4.5 ± 0.11 to 57.1 ± 0.11. By 24 weeks, remarkably slimmed tiny eosinophilic dead neurons and the tiny PAS-positive vesicular particles remained in a group, and the latter were often scattered, in the 3rd and 5th cortical layers of the cerebral cortex. These

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vesicular particles seem to be the final remnants of dead neurons.

The neuropil surrounding these particles was densely packed with voluminously enlarged polygonal axon terminals filled with synaptic vesicles, spines, and neurites of surviving neurons, and showed only a very few degenerated axons (Fig.3-B~E)[1]. Therefore, we consider the cube-shaped structures with degenerated large mitochondria and electron-dense particles in and around the vesicular particles to be a degenerating part of axons of surviving neurons that had been in contact with the dead neurons prior to their death. Other synaptic connections present were those between axons and spines and dendrites of surviving neurons [1].

However, it is uncertain whether all slimmed ghost cells are destined to become these tiny vesicular particles. These particles have not been reported previously in the literature. Further longer term quantitative analysis of various ultra-structures showing the formation and fate of these tiny PAS-positive vesicular particles is necessary.

C. Activation and phagocytic activities of resident microglia and astrocytes:

Generally, the microglial response to various neuronal injuries occurs gradually. First, microglia proliferate and become activated; and secondly they are transformed into intrinsic brain phagocytes [3,16,17]. However, at the site of ischemic damage, microglial proliferation and activation has been reported to occur rapidly within the first 24 hours after an ischemic insult [20-22]. Within the infarct, phagocytes with a foamy cell appearance, those derived from local resting microglia and/or from the blood stream, are abundant within a few days [23]. All of these studies were done on ischemic lesions where the infarction and penumbra were not clearly separated. In the ischemic penumbra, in the present study, microglial proliferation and

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activation occurred gradually from 1 to 12 weeks after the transient ischemic insult, and phagocytic microglia were first prominent at 8~12 weeks [3,16,17].

Microglial and astroglial activation often occurs in concert induced by IL-1 during CNS injury [3]. Reactive astrocytes may contribute to neuronal remodeling of the surviving neurons by secreting neurotrophic factors [1, 3]. Phagocytic activity of adult astrocytes following various brain injuries is still controversial. Astrocytic phagocytosis of carbon particles inserted around the needle injury of the adult rat cerebral cortex [24] and uptake of Indian black particles by cultured human adult astrocytes [25] have been reported. In the present study, astrocytic proliferation and activation occurred from 1 to 12 weeks post ischemia; and heterolysosomes in which ingested materials and lysosomes were surrounded by a single-membrane-bound structure were observed at 8~12 weeks after the ischemic insult, almost in accordance with the microglial changes.

Conclusions: We found a novel scavenger mechanisms in the ischemic penumbra, one by which the dead shrunken neurons observed in disseminated fashion among the surviving neurons were fragmented to become an accumulation of granular debris, by the action of slender astrocytic cell processes that had invaded and separated the shrunken neurons into fragments. This debris was phagocytized by microglia and astrocytes late after the ischemic insult, i.e. at 8 ~ 24 weeks. The fragmented dead neurons became reduced in size due to decay of their peripheral cytoplasm. The tiny PAS-positive vesicular particles each with a central dot, appeared among the slimmed dead neuron at 8 weeks and later increased in percentage. By 24 weeks, remarkably slimmed tiny eosinophilic dead neurons and the tiny PAS-positive vesicular particles remained in groups,

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though the latter were often scattered, in the 3rd and 5th cortical layers of the cerebral cortex.

LEGENDS OF FIGURES:

Fig. 1 Light-microscopy of 3rd cortical layer of face B: A. Twenty-four hours after the ischemic insult: Some of the eosinophilic ischemic neurons are shrunken with condensed, aggregated nuclear chromatin (arrows). (HE, Bar =10.6 μm) B. Four days after the ischemic insult: Eosinophilic ghost cells with faint cell bodies without nuclear staining are seen (arrows). (PAS, Bar = 10.8 μm) C. Eight weeks after the ischemic insult, the ghost cells (arrows) are reduced in size and show decaying peripheral cytoplasm (PAS, Bar = 12.9 μm) Inset: Two tiny PAS-positive vesicular particles (arrows) consisting of a thin circular frame and central dot are seen among the dead neurons. (PAS, Bar = 20.5 μm). D. Twenty-four weeks after the ischemic insult, tiny remnants of the eosinophilic ghost cells and tiny PAS-positive vesicular particles are observed grouped regionally (arrowhead) in the 3rd cortical layer (PAS, Bar = 25.5 μm) Inset: vesicular particles (arrows) and phagocytic microglia (arrowhead) are found scattered in this layer. (PAS, Bar = 16.4 μm).

Fig. 2 EM of the 3rd cortical layer of face B: A. Twelve hours after the ishcmemic insult: The shrunken eosinophilic neurons (Fig. 1-A) are observed by EM as homogeneously condensed electron-dense dead neurons (DN) surrounded by remarkably swollen astrocytic processes (arrows). (Bar = 2.3 μm) B. Four days after the ischemic insult: The shrunken dead neurons have been fragmented by invading slender astrocytic processes (arrows). (Bar =1.6 μm) C. Dead neuron 4 days after ischemia. Glycogen

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granules (arrows) and glial fibrils (arrow heads) are noticed in these invading astrocytic cell processes. DN: dead neuron. (Bar = 0.71 μm) Inset: Small spots of condensed chromatin (arrows) are scattered in the nucleus (Nc). Cp: cytoplasm (Bar = 0.67 μm) D. One week after the ischemic insult: Fragmented shrunken electron-dense dead neurons (DN) are scattered among the viable neurons (N), astrocytes (A), activated astrocytes (AA), mitotic astrocytes (MA), microglia (M), and activated microglia (AM). (Bar = 19.3 μm)

Fig. 3 EM of a slimmed dead neuron and tiny PAS-positive vesicular particles consisting of a thin circular frame and central dot in the 3rd cortical layer. A. Four weeks after the ischemic insult. The peripheral cytoplasm of this dead neuron (DN) is tattered, and the central portion of the neuron has been thinned. The arrows point to disseminated granular debris. (Bar = 2.17 μm). B~E. Twelve weeks after the ischemic insult. EM observations of the variously shaped particles: B. The electron-dense central core and peripheral thin frame (arrowheads) are separated by the invading monolayers of clear cubic arrangement (arrows). The peripheral thin frame is also surrounded by the clear cubic arrangement. (Bar = 0.92 μm): C. A vesicular particle composed of a central electron-dense core and a peripheral thin circular electron-dense frame (arrowheads). A monolayer of clear cubic arrangement, consisting of axons with degenerated large mitochondria and electron-dense particles, astrocytic processes, and occasional presynaptic axon terminals, is seen around the frame and has invaded (arrow) between these structures, (Bar =0.70 μm): D. Disarranged vesicular particle. Between the small electron-dense central core and disrupted surrounding circular electron-dense frame, multi-layered clear cubic arrangements with large

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degenerated mitochondria have filled in the space. The circular electron-dense frame is also surrounded by a multi-layered cubic arrangement (Bar 1.25 μm): E. The central electron-dense core (arrowhead) is surrounded by multiple layers of cubic arrangement. (Bar = 0.82 μm).

Fig. 4 EM of the 3rd cortical layer. A. One week after the ischemic insult. A resting microglia (MG) has capped a neuron (N). (Bar = 1.5 μm). B. Four weeks after the ischemic insult. EM view of proliferating and activated microglia (MG). N: neuron. (Bar = 1.2 μm). C. Twelve weeks after the ischemic insult. EM shows evidence of phagocytosis by the microglia (MG). Arrowheads: heterolysosomes. (Bar = 1.0μm) Inset : Heterolysosomes (P) containing ingested materials and lysosomes are surrounded by single-membrane-bound structures (arrows) (Bar = 0.54 μm).

Fig. 5 EM of the 3rd cortical layer. A. Eight weeks after the ischemic insult. An activated astrocyte (A) is shown. G: Golgi complex. Arrowheads: Glial fibrils. (Bar = 0.81 μm). B. Eight weeks after the ischemic insult. Two proliferating astrocytes (A) that have fused are seen. Arrowheads: Glial fibrils. (Bar = 0.92μm). C. Twelve weeks after the ischemic insult. EM shows evidence of phagocytosis by an astrocyte. Arrowheads: heterolysosomes. Arrows: Glial fibrils. (Bar = 0.71μm) Inset: A heterolysosome (P) containing ingested materials and lysosomes are surrounded by a single-membrane-bound structures (arrows) (Bar = 0.71μm).

Fig. 6 Temporal profile of percentage of each of the 3 kinds of microglia and astrocytes. A. Percentages of resting, proliferating/activated, and

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phagocytic microglia. $^{*}p< 0.05$ compared with control, $^{**}p<0.05$ compared with control $^{\dagger}p<0.05$ compared with 8 weeks B. Percentages of activated, proliferating/activated, and phagocytic astrocytes. $^{*}p< 0.05$ compared with 1 week, $^{**}p<0.05$ compared with 1week $^{\dagger}p<0.05$ compared with 8 weeks

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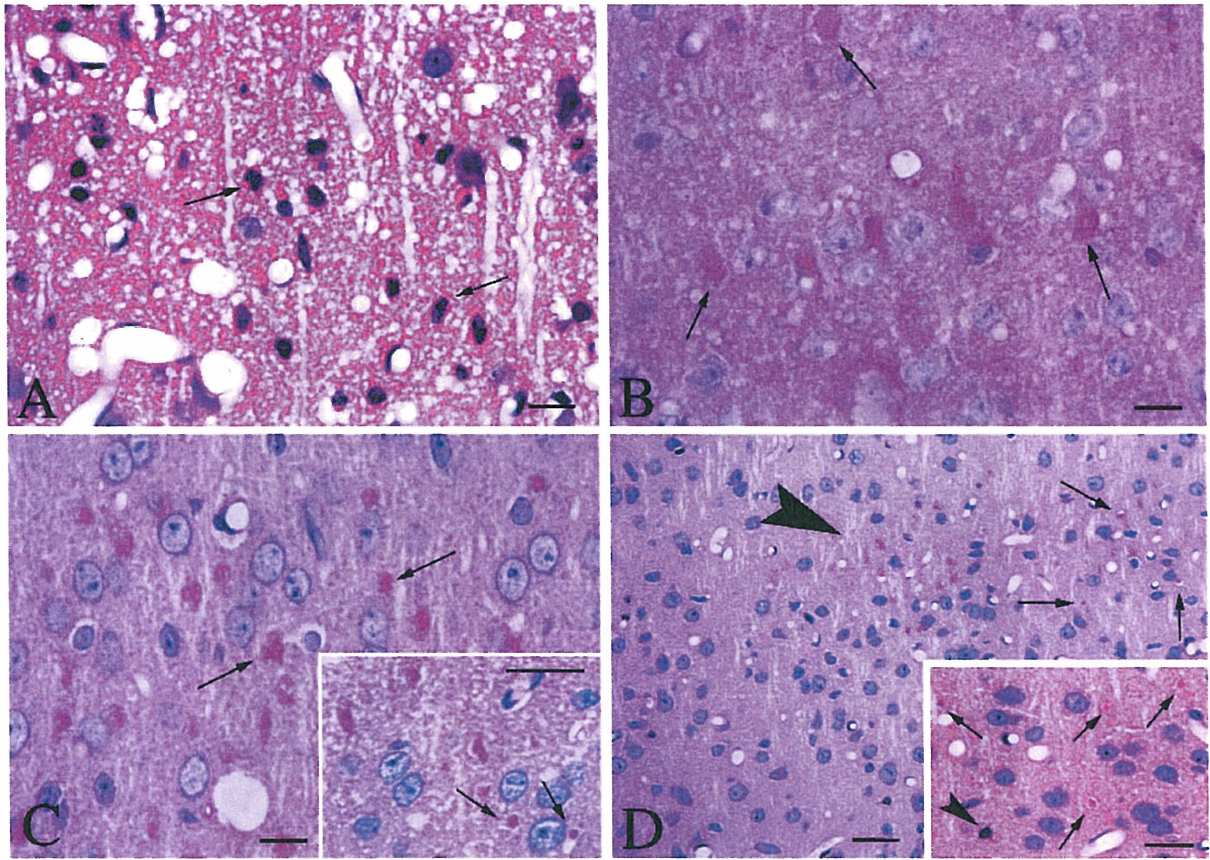

Fig. 1

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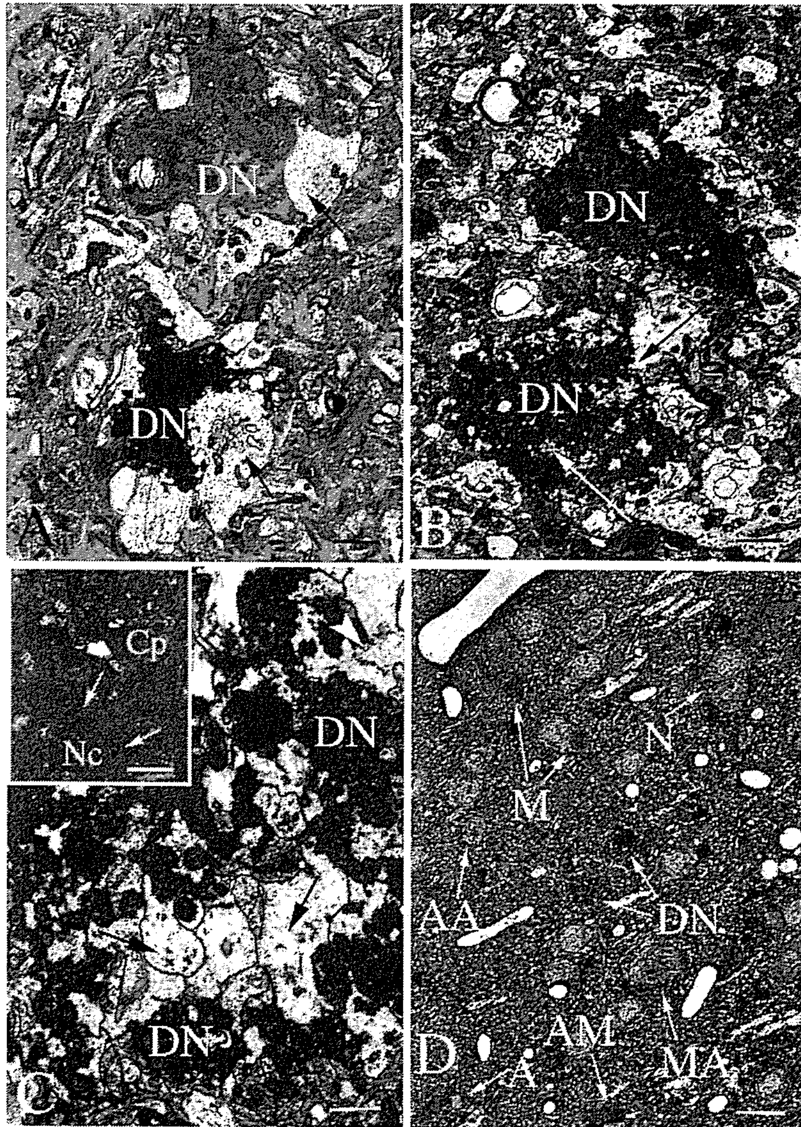

Fig. 2

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