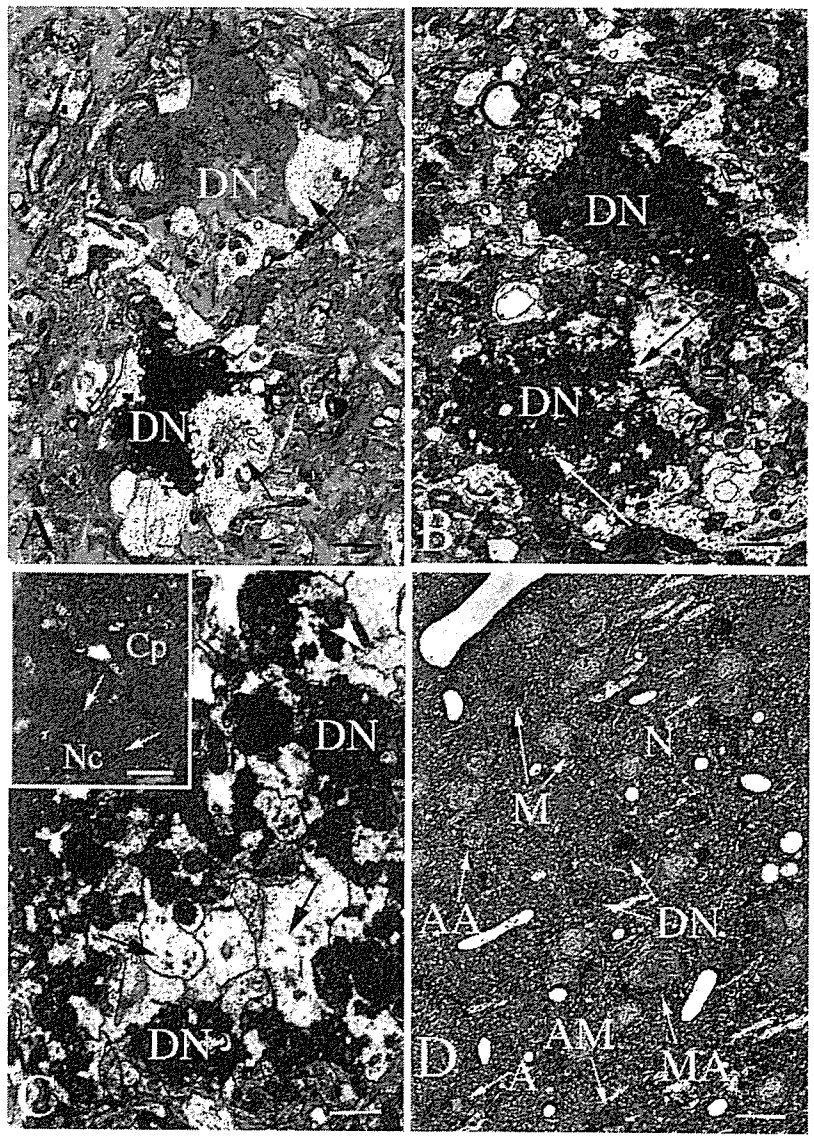


Fig. 2

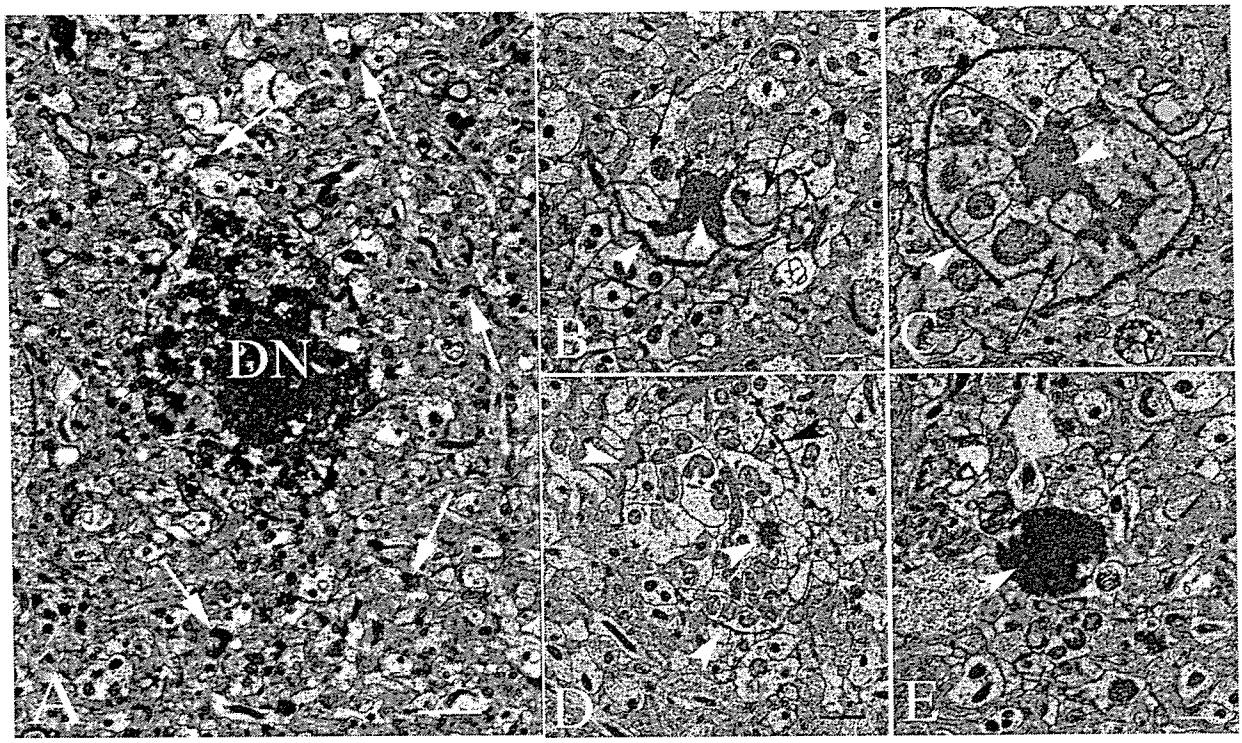


Fig. 3

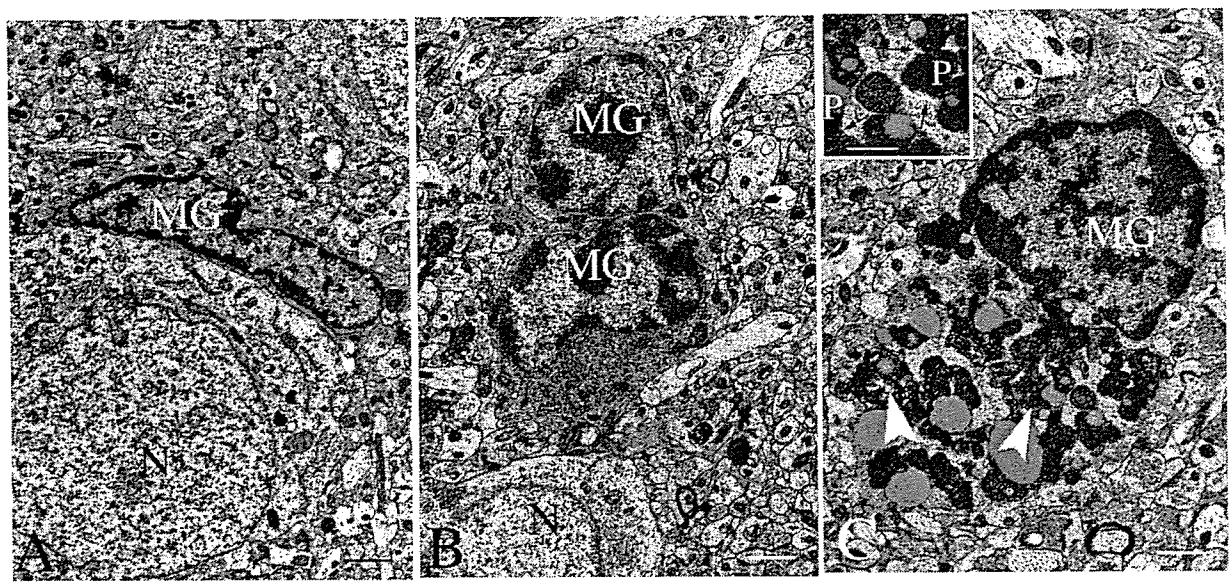


Fig. 4

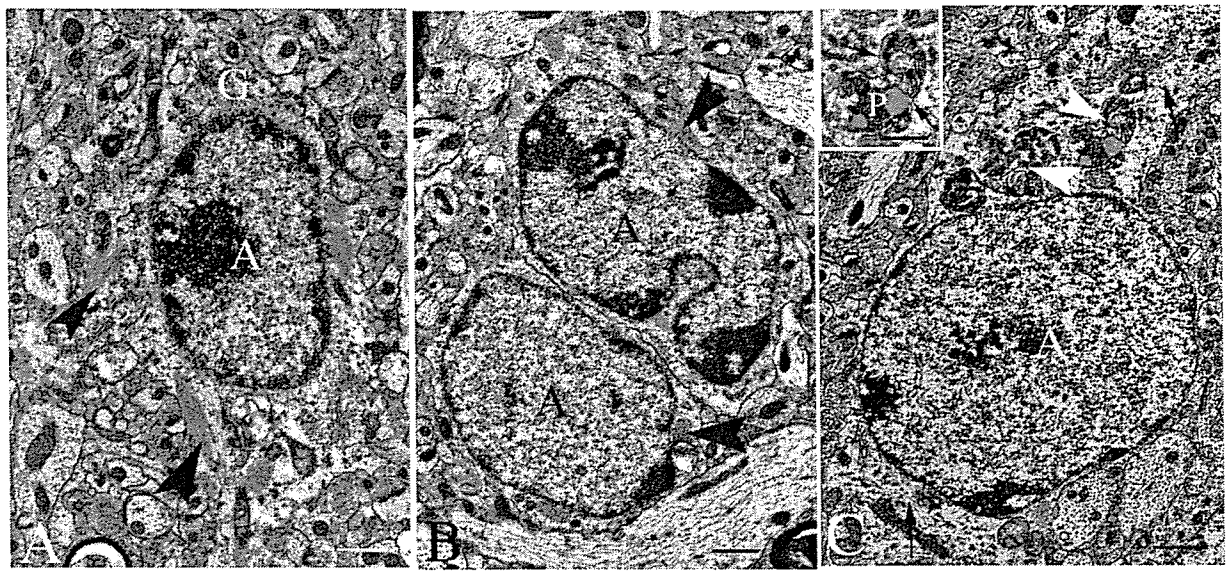


Fig. 5

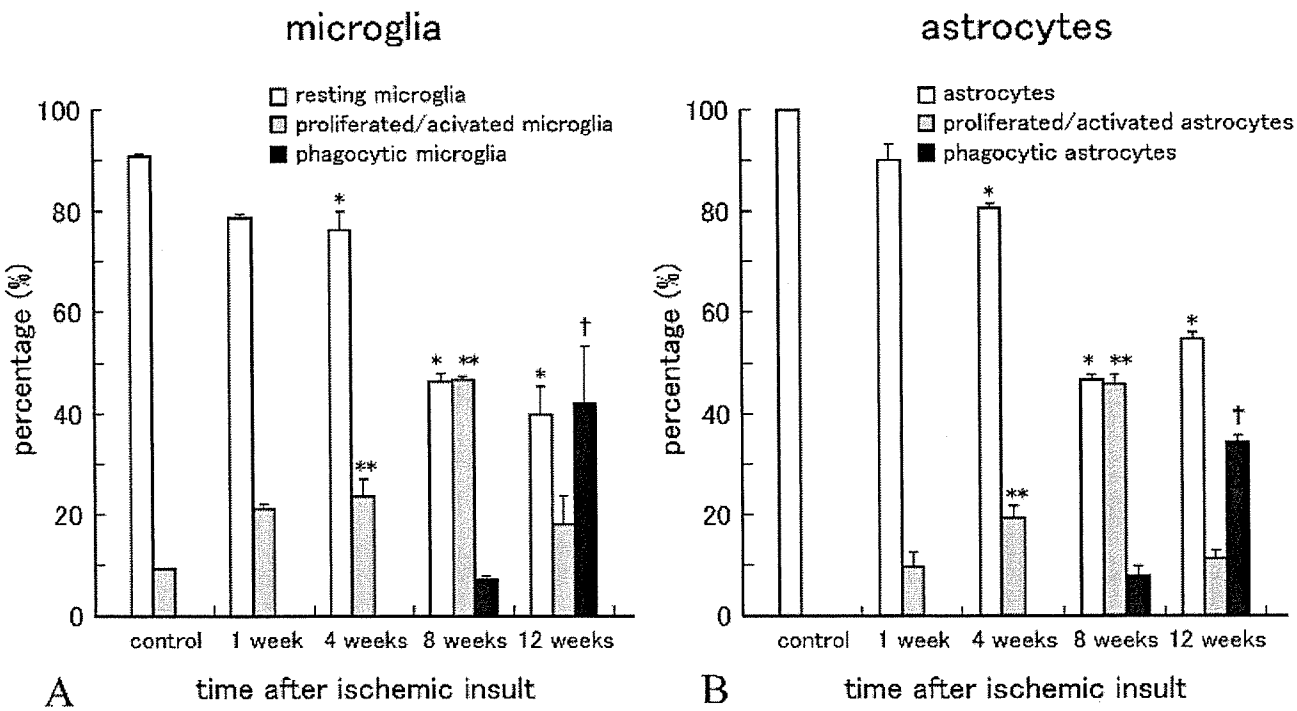




Fig.6

# Temporal Profiles of Axon Terminals, Synapses and Spines in the Ischemic Penumbra of the Cerebral Cortex

## Ultrastructure of Neuronal Remodeling

Umeo Ito, MD, PhD, FAHA; Toshihiko Kuroiwa, MD, PhD; Jun Nagasao, DVM; Emiko Kawakami, BS; Kiyomitsu Oyanagi, MD, PhD

***Background and Purpose***—Because the recovery process of axon terminals, synapses, and spine-dendrites in the ischemic penumbra of the cerebral cortex is obscure, we studied the temporal profile of these structures up to 12 weeks after the ischemic insult, using a gerbil model.

***Methods***—Stroke-positive animals were selected according to their stroke index score during the first 10-minute left carotid occlusion done twice with 5-hour interval. The animals were euthanized at various times after the second ischemic insult. Ultra-thin sections including the 2nd to 4th cortical layers were obtained from the neocortex coronally sectioned at the infundibular level, in which the penumbra appeared. We counted the number of synapses, spines and multiple synapse boutons, measured neurite thickness, and determined the percent volume of the axon terminals and spines by Weibel point counting method.

***Results***—The number of synapses, synaptic vesicles and spines and the total percent volume of the axon terminals and spines decreased until the 4th day. From 1 to 12 weeks after the ischemic insult, these values increased to or exceeded the control ones, and neuritic thickening and increase in number of multiple synapse boutons occurred.

***Conclusions***—In the ischemic penumbra, the above structures degenerated, with a reduction in their number and size, until 4 days and then recovered from 1 to 12 weeks after the ischemic insult. (***Stroke*. 2006;37:2134-2139.)**

**Key Words:** axon terminal ■ dendrites ■ spine ■ synapses ■ transient cerebral ischemia

Cerebral infarction develops rapidly after a large ischemic insult. Earlier we developed a model of temporary ischemia in which a focal infarction surrounded by a large penumbra was produced in the cerebral cortex of Mongolian gerbils by giving a threshold amount of ischemic insult to induce focal infarction.[1,2]

Concerning the neuronal recovery, recent findings have revealed that synapses and their networks express a high degree of functional and structural plasticity.[3] Ultrastructural changes in the postsynaptic density in hippocampal CA-1 were investigated after temporary ischemia.[4–6] Degenerated boutons and multiple synapse boutons (MSBs) in this region were investigated by electron microscopy (EM) after temporary ischemia,[7–9] as well as after temporary hypoxia/hypoglycemia in hippocampal slices.[10] Changes in spines and dendrites were studied by time-lapse microscopy after temporary anoxia/hypoglycemia in cell culture,[11,12] by light microscopy (LMS) of Golgi stain-impregnated sections of the 3rd to 5th cortical layers of the cerebral cortex after temporary ischemia,[13] and by EM after temporary hypoxia/glycemia in hippocampal slice.[10] Changes in CA-1 dendrites after temporary ischemia were investigated by light microscopy of horseradish peroxidase-injected specimens,[14] by EM after temporary ischemia in CA-1,[15,16] and by EM of Golgi stain-impregnated cerebral cortex after temporary ischemia for 20 minutes.[17]

Almost all of the above studies were performed in connection with delayed ischemia-induced injury to CA-1 neurons, and observations were made during a short period after ischemia. However, clinically, most patients show gradual recovery from behavioral dysfunctions after a stroke. The long-term integrated profile of axon terminals, synapses, spines, and dendrites during the recovery stage after an ischemic insult has remained obscure, especially in the ischemic penumbra of the cerebral cortex.

Because no functional recovery is anticipated in the infarction itself,[18–20] we aimed to elucidate neuronal remodeling process in the ischemic penumbra in which neuronal death progresses in a disseminated fashion,[2,18,20] by focusing on the temporal profiles of axon terminals, synapses, spines, and neurites.

## Materials and Methods

Under anesthesia with 2% halothane, 70% nitrous oxide, and 30% oxygen, the left carotid artery of adult male Mongolian gerbils (60 to

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80 g) was twice occluded with a Heifetz aneurismal clip for 10 minutes each time, with a 5-hour interval between the 2 occlusions, anesthesia was discontinued immediately after each cervical surgery, the animals soon became awake and moved spontaneously.

Ischemia-positive animals registering >13 points were selected based on the stroke index score determined during the first occlusion.[21] The gerbils were euthanized at various times, ie, at 5, 12, 24, 48-hour, 4 days, and 1, 5, 8, and 12 weeks after the ischemic insult. Anesthetization was followed by intracardiac perfusion with diluted fixative (1% paraformaldehyde, 1.25% glutaraldehyde in 0.1 mol/L cacodylate buffer) for 5 minutes, followed by perfusion with concentrated fixative (4% paraformaldehyde, 5% glutaraldehyde in 0.1 mol/L cacodylate buffer) for 20 minutes for EM (3 animals in each time group), or with 10% phosphate-buffered formaldehyde fixative for 30 minutes for LMS (5 animals in each time group).

In this model, after the restoration of blood flow, only ischemic penumbra with progressing disseminated selective neuronal necrosis (DSNN) appeared in the coronal face sectioned at the infundibular level (Face B) and focal infarction evolved among the DSNN in the coronal face sectioned at the chiasm (Face A). Ultra-thin sections including the 2nd to 4th cortical layers were prepared from the left cerebral cortex at the mid-point between the interhemispheric and rhinal fissures on Face B, penumbra >1 mm caudal to infarction edge. The sections were double stained with uranyl acetate and lead solution, and observed with an electron microscope (H9000, Hitachi). Paraffin sections of both faces were separately stained with hematoxylin-eosin (HE) or periodic acid fuchsin Schiff (PAS) or by Bodian silver impregnation or used for immunohistochemical detection of glial fibrillary acidic protein.

Placing 1.0 cm×1.0 cm quadratic lattices of points on 5000×2.67 times enlarged EM photographs, we measured the number of synapses (synapses: consist of the pre- and postsynaptic densities associated with their cytoplasmic faces, and the synaptic cleft between them) and spines (spines: an ovoid bulb that is filled with a fluffy material and connected to dendrite directly or by a stalk) in the neuropil in a 100-$cm^2$ area (56 $\mu m^2$, by real size) by examining 1800±364 $cm^2$ in the neuropil of 3 animals in each time group. We determined the percent volume of the axon terminals (axon terminals: presynaptic expansion of the axon that contains synaptic vesicles and mitochondria) and spines by using the point counting method[22] in which the number of intersecting points touched by the axon terminals and/or spines were counted among 1000 to 15 000 points (counting number varied according to the equation of the relative error for different volumetric proportions) of the quadratic lattice in each time group. We measured the thickness of 194±38 neurites (neurites: axons and dendrites those contain microtubules, neurofilaments and mitochondria; differentiation between them in transverse section is often difficult, especially in small ones) in each time group, as the maximal diameter perpendicular to their neurofilaments and/or microtubules. We also measured the percentage of MSBs by counting 327±38 synapses in each time group, all on the same EM pictures. The statistical differences between each of the time groups were analyzed by ANOVA, followed by Bonferroni-Dunn test. All data in the Table and Figure 6 were presented as average±SEM and a statistical difference was accepted at $P<0.05$ level.

## Results

In the ischemic penumbra of the cerebral cortex in Face B, eosinophilic ischemic neurons (HE staining) appeared in disseminated fashion among the normal-looking neurons in the 2nd to 6th cortical layers by LMS, around 5 hours after the ischemic insult. Some of these eosinophilic cell bodies became remarkably shrunken and died during the period of 12 to 48 hours, which indicates DSNN (Figure 1A). The eosinophilic ischemic neurons were found by EM to be disseminated electron-dense dark neurons that increased in number during the period of 12 to 48 hours after the ischemic insult.

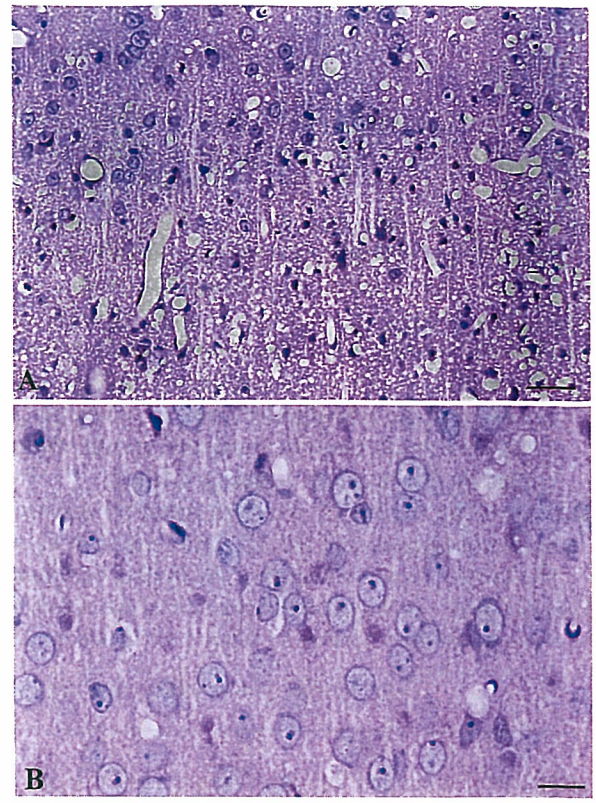


**Figure 1.** Light-microscopy of 2nd to 4th cortical layers of the cerebral cortex in Face B. A, Twenty-four hours after the ischemic insult, some of the eosinophilic ischemic neurons show marked shrinkage compared with the more normal-looking neurons, indicating disseminated selective neuronal necrosis. These abnormal neurons increased in number until day 2 to 3 postischemia (HE, Bar 31.3 $\mu m$). B, Eight weeks after the ischemic insult. The eosinophilic ghost cells of faintly formed cell bodies are reduced in size and are found in the 3rd cortical layer (PAS, Bar 12.5 $\mu m$).

From 4 days until 8 weeks, these condensed electron-dense dark neurons became fragmented into an accumulation of electron-dense granular fragments, which were observed by LMS as eosinophilic ghost cells of faintly formed cell bodies by HE and PAS staining (Figure 1B). During 2 to 12 weeks, these eosinophilic ghost cells accumulated in the 3rd and occasionally in the 5th cortical layer decreasing in size because of loss of their periphery (Figure 1B). In Face A, focal infarction evolved and developed among the DSNN from 12 hours to 4 days.

From 4 days to 12 weeks after the ischemic insult, the axon terminals of the surviving neurons were found being attached

**Percent of MSBs Among Synapses**

| | Control | 5 Hours | 4 Days | 1 Week | 5 Weeks | 8 Weeks | 12 Weeks |
|---|---|---|---|---|---|---|---|
| % MSBs | 1.76±0.82 | 0.85±0.84 | 0.86±0.67 | 1.18±0.75 | 0.45±0.45 | 3.14±0.85 | 6.93±2.24* |

*Compared with control, 4 days, and 8 weeks; $P<0.05$.

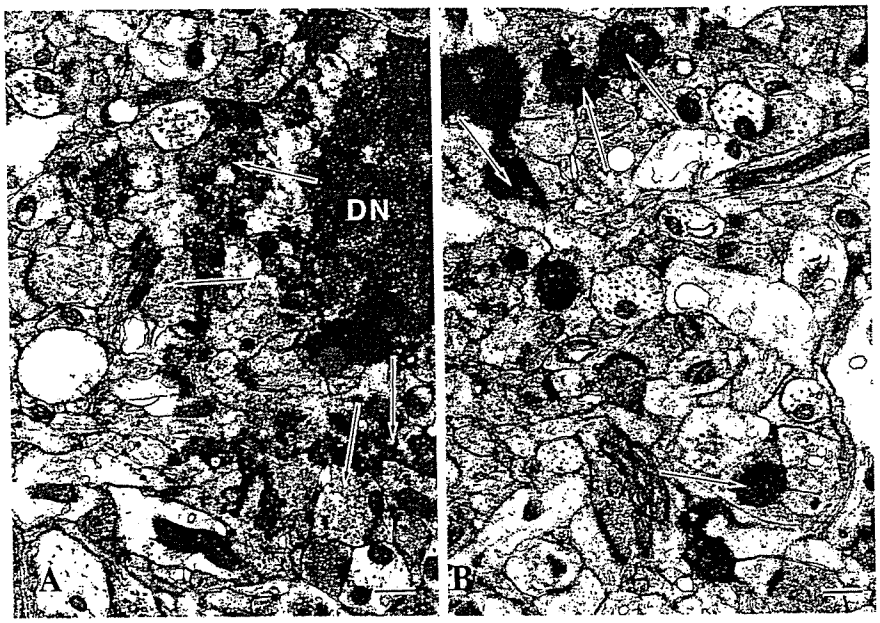


**Figure 2.** Electron microscopy of 3rd layer of the cerebral cortex in Face B, 1 week after the ischemic insult. A, The axon terminals of the surviving neurons make contact with the periphery of the accumulated fragments of electron-dense granules of dead neurons (DN). Some of them appeared to have pinched off pieces of the dead neurons, and are encrusted by the electron-dense granular fragments of the dead neurons (arrows). Bar 2.3 μm. B, Some axon terminals are found attached to the electron dense thick neurites of dead neurons (arrows). Bar 2.6 μm.

to the peripherally located electron-dense granular fragments and dendritic portions of the shrunken dark neurons. Some of these terminals appeared to have pinched off pieces of the dead neurons, and they bore a crust of the electron-dense granular fragments of the dead neurons (Figure 2A). Other axon terminals were found attached to the electron-dense thick neurites of dead neurons (Figure 2B). Some of the fragment-encrusted axon terminals were occasionally observed to have synapsed with the spines and neurites of the surviving neurons (Figure 3A). Some axons connected to the dying neurons showed globular and abnormal distensions of their terminals as seen by silver impregnation (Figure 4A). These structures appeared as degenerated axon by EM. The amplified degenerated axon contained degenerated mitochondria, laminated dense bodies, and irregularly located neurofilaments and microtubules; the degenerated axon occasionally made synapses onto adjacent structures (inset of Figure 4A).

Such axons were often observed around the accumulations of the fragmented electron-dense granular pieces of the dead neurons (Figure 4B).

By 12 weeks after the ischemic insult, neuritic shafts and their branches were remarkably thickened (Figure 5B) compared with those at day 4 (Figure 5A), and they made synapses with voluminously enlarged and occasionally sprouting polygonal axons terminals filled with synaptic vesicles (Figure 5B). MSBs[23] of the axons terminals (Figure 3B) increased in frequency compared with those of the control animals (Table).

The percent volume of the total axon terminals (Figure 6A) and spines (Figure 6B) in the neuropil decreased drastically to 30.9% and 24.8%, respectively, of the control value at that

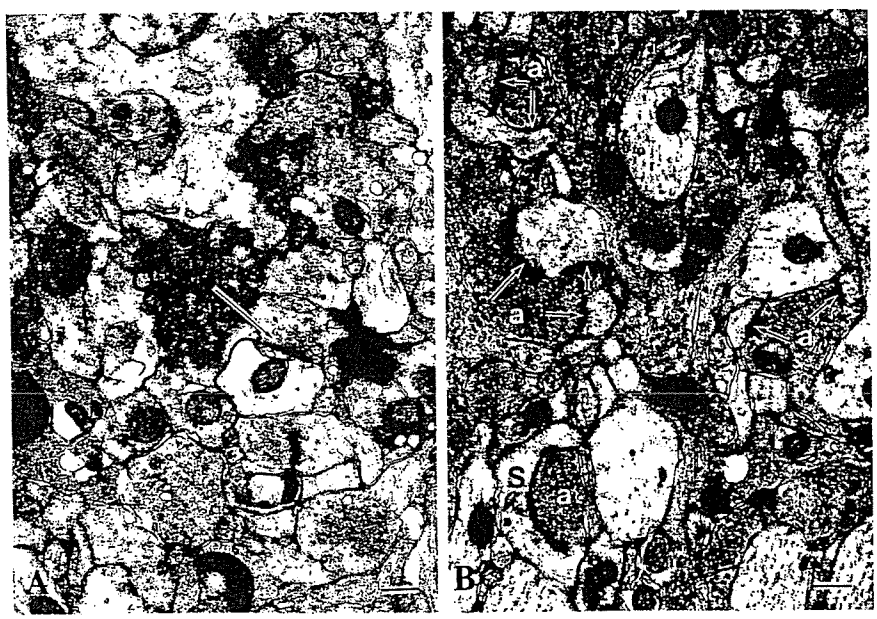


**Figure 3.** A, Electron microscopy of the 3rd cortical layer in Face B, 1 week after the ischemic insult. Some axon terminals encrusted by the electron-dense granular fragments of the dead neurons are occasionally observed to have made synapses with the spines and neurites of the surviving neurons (arrow). Bar 3.1 μm. B, Electron microscopy of 3rd cortical layer of the cerebral cortex in Face B, 12 weeks after the ischemic insult. MSBs with >2 spines synapsed (arrows) to 1 axon terminal (a). A widened spine(s) with multiple synapses on axon terminal (a) is seen. Bar 3.2 μm.

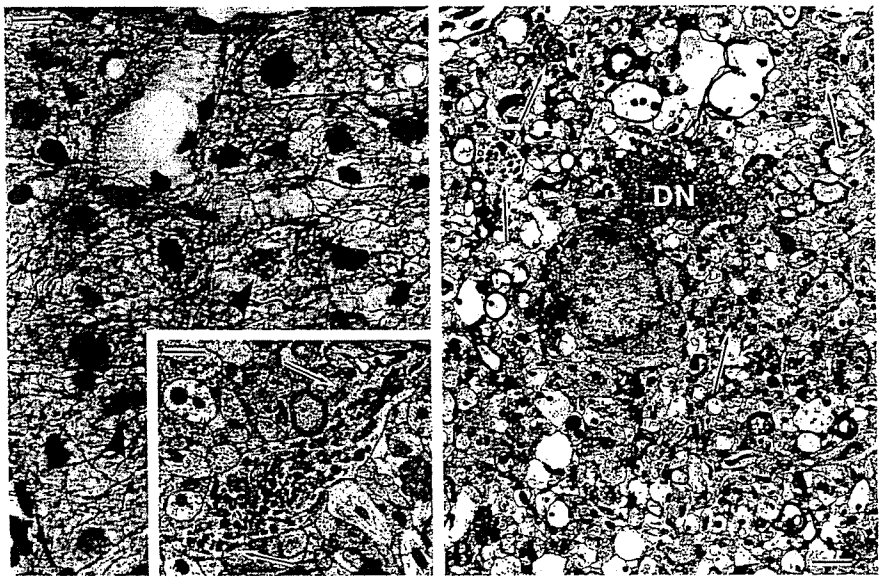


**Figure 4.** A, Light-microscopy. Four days after the ischemic insult, some axons attached to dying neurons show globular and abnormal distension of their terminals (arrow-heads). Bar 8.9 μm. Bodian's silver impregnation. Inset: EM observation of the distended degenerated axon 3 weeks after the ischemic insult. It contains degenerated mitochondria, laminated dense bodies, and irregularly located neurofilaments and microtubules and has synapses along its wall (arrows). Bar 1.3 μm. B, Electron microscopy of the 3rd layer of the cerebral cortex in Face B, 2 weeks after the ischemic insult. These amplified degenerated axons (arrows) are observed around accumulations of the fragmented electron-dense granular pieces of the dead neurons (DN). Bar 0.6 μm.

time after the ischemic insult, with a decrease in frequency of synaptic vesicles especially those close to the synapses (Figure 5A). The number of synapses also decreased to 73.5% of the control value at 4 days, after a temporary increase up to 135% at 5 hours after the ischemic insult (Figure 6A). The number of spines also decreased to 35.8% of the control value (Figure 6B), by 4 days. From 1 to 12 weeks after the ischemic insult, the percent volume of the total axon terminals (Figure 6A) and the percent volume of the total spines (Figure 6B) increased, being 162.8% and 86.7%, respectively, of the control value at 12 weeks. The number of synapses (Figure 6A) and spines (Figure 6B) also rose, becoming 113.2% and 91.9%, respectively, of it at that time.

The average thickness of neurites in the neuropil of the control animals were 0.607 μm. This value was unchanged at 0.587 μm at day 4, 0.604 μm at 1 week, and 0.665 μm at 8 weeks, and increased to 0.934 μm at 12 weeks after the ischemic insult (Figure 6C).

## Discussion

In the neuropils of the ischemic penumbra in the cerebral cortex, we found a marked decrease in the number of the synapses and volume of the axon terminals from 5 hours to 4 days after the ischemic insult, along with a decrease in the number of synaptic vesicles. These changes may be attributed to a demolished synaptic neurotransmission attributable to calcium-dependent neuronal hyperexcitation[4-6] and could be reduced by NMDA (*N*-methyl-D-asparate) receptor antagonists as was reported in a morphological study recording excitatory postsynaptic potential from hippocampal slice cultures subjected to brief anoxia-hypoglycemia.[10]

Almost in accordance with our present study, the LMS study of Golgi silver impregnated spine and dendrites showed that the number of spines and thickness of dendrites decreased maximally in 4 to 7 days after the temporary ischemia and recovered around 5 weeks in the 2nd to 3rd cortical layers of the rat cerebral cortex.[13] Earlier studies on cultured

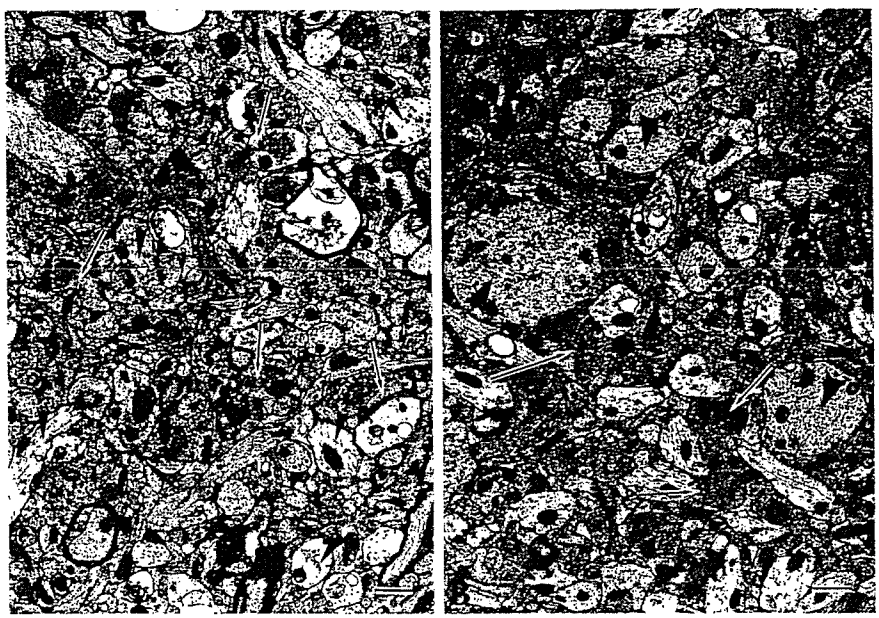

**Figure 5.** Electron microscopy of the 3rd layer of the cerebral cortex in Face B. A, Four days after the ischemic insult. The volume of axon-terminals and spines has decreased with a decrease in frequency of synaptic vesicles, especially those close to the synapse (arrows). The thickness of degenerated neurites has decreased slightly compared with that of the control (arrowheads). Bar 1.5 μm. B, Twelve weeks after the ischemic insult. The neuritic shafts and their branches are remarkably thickened (arrowheads) and make synapses with voluminously enlarged and occasionally sprouting polygonal axon terminals filled with synaptic vesicles (arrows). Bar 1.5 μm.

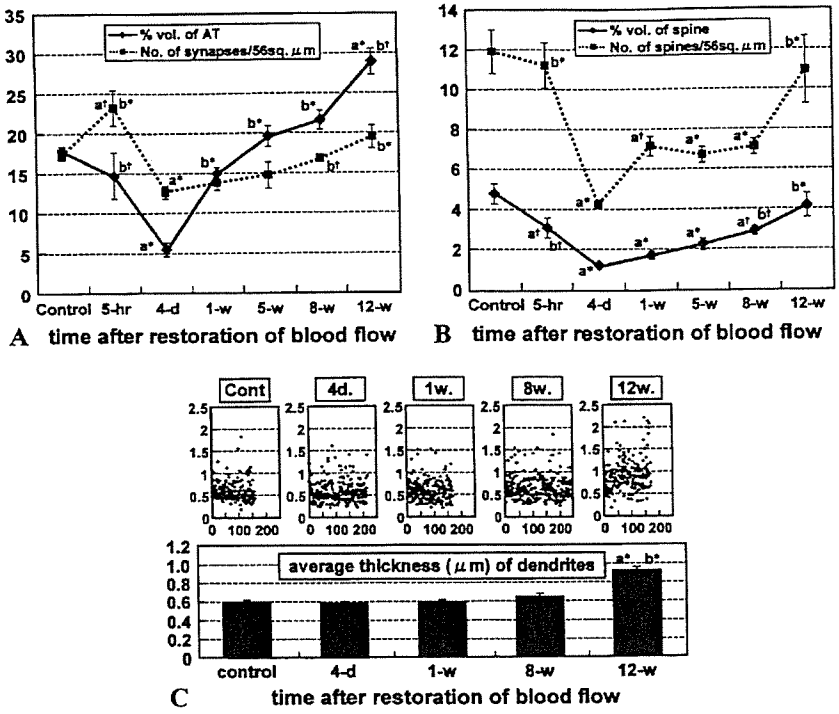


**Figure 6.** A, Time course of percent volume of axon terminals and number of synapses in the neuropil at various time after ischemic insult. B, Time course of percent volume and number of spines in the neuropil at various time post ischemia. C, Time course by scatter graph (upper) and average thickness (lower) of neurites in the neuropil after the ischemic insult. [a]Compared with control; [b]compared with 4 days; $^{*}P<0.001$, $\dagger P<0.05$.

neurons showed a decreased spine number and segmental dendritic beading after temporary hypoxia/hypoglycemia followed by recovery,[11,12] and a LMS study using horseradish protein injection showed beading of dendrites in the CA-1 of the hippocampus after temporary ischemia.[14] Also an EM study on the CA-1 showed degeneration and shrinkage of dendrite around 3 to 4 days after temporary ischemia.[15–17] In the present study, we found that the neurites degenerated around 4 days and that their thickness increased, in association with the recovery to normal of the number and percent volume of spines, at 12 weeks after the insult.

From 1 to 12 weeks after the ischemic insult, we found that the synaptic number increased gradually in association with an increase in the volume of axon terminals showing sprouting. The present study also showed an increase in the number of the MSBs from 8 to 12 weeks after the ischemic insult, which increase was associated with one in the number and volume of axon terminals and spines. The MSBs represent 2 independent dendritic spines contacting the same axon terminal.[23] One spine branched to make synapses at >2 portions of 1 axon terminal is considered to facilitate neurotransmission.[10] In another study, there was an increase in the number of MSBs in CA-1, paralleling the marked increase in the number of synaptic vesicles after temporary ischemia[7] and after temporary hypoxia/hypoglycemia in hippocampal slices.[10]

From 4 days to 12 weeks after the ischemic insult, some axons attached to the dying neurons showed an abnormal distension of their terminals, which contained degenerated mitochondria, laminated dense bodies, and irregularly located neurofilaments and microtubules (degenerated axon).[8,9] They were frequently observed around accumulations of the electron-dense granular fragments of the dead neurons.

Some axon terminals encrusted with the electron-dense granular fragments of the dead neurons became connected to the spines and neurites of the surviving neurons. These axon terminals, previously attached to the dying and/or dead neurons, seemed to become newly connected to the spines and to the thickened dendrites of the surviving neurons associated with synaptogenesis in the neuropil.[24] However, it could also be that some of these axon terminals were originally contacting more than one dendrites or spines.

Clinically, most stroke survivors show recovery from behavioral dysfunctions. The short-term recovery may be attributable to the resolution of brain edema. A more gradual recovery, promoted by exercise for rehabilitation, may be attributed to the anatomical and functional recovery of the penumbra. Stroemer[24] reported behavioral recovery after neocortical infarction in rats, which recovery was associated with neuronal sprouting followed by synapto-genesis, as demonstrated by immunohistochemical staining for GAP-43, a growth-associated protein expressed on axonal growth cones, and for synaptophysin. Functional remodeling of the cerebral cortex remote from the infarction was detected by intracortical microstimulation mapping of the hand of the squirrel monkey.[25,26]

Activation of the complement system was shown to promote neuronal survival and tissue remodeling.[27] Postischemic treatment with brain-derived neurotrophic factor and physically exercised animals had better functional motor recovery, attributable to induction of widespread neuronal remodeling, as demonstrated by MAP1B and synaptophysin expression.[19] Clinical introduction of novel agents and functional methods to promote synaptogenesis and neuronal networks in the iscehmic penumbra, is highly anticipated.

## Summary

In the penumbra around a focal infarction of the cerebral cortex, synapses, synaptic vesicles, axon terminals, spines

degenerated, with a reduction in their number and size, until 4 days and then recovered from 1 to 12 weeks after the ischemic insult.

## Source of Funding

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# Restitution of ischemic injuries in penumbra of cerebral cortex after temporary ischemia

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## Summary

We investigated, at both light and ultrastructural levels, the fate of swollen astrocytes and remodeling of neurites connected to disseminated, dying neurons in the ischemic neocortical penumbra. Specimens from left cerebral cortex were cut coronally at the infundibulum and observed by light and electron microscopy. We measured synapses and spines, and the thickness of neuritic trunks in the neuropil on electron microscopy photos. We also determined percent volume of axon terminals and spines by Weibel's point-counting method. Astrocytic swelling gradually subsided from day 4 after the ischemic insult, with increases in cytoplasmic glial fibrils and GFAP-positive astrocytes. Disseminated dying electron-dense neurons were fragmented by invading astrocytic cell processes and accumulated as granular pieces. The number of synapses and spines and total percent volume of axon terminals and spines decreased with an increasing sparsity of synaptic vesicles until day 4. One to 12 weeks after the ischemic insult, these values increased to or exceeded control values, and sprouting and increased synaptic vesicles were seen. Axons that had been attached to the dying neurons appeared to have shifted their connections to the spines and the neurites of the surviving neurons, increasing their thickness. Astrocytic restitution and neuronal remodeling processes started at 4 days continuing until 12 weeks after ischemic insult.

*Keywords:* Maturation phenomenon; cerebral ischemia; neuronal remodeling.

## Introduction

Cerebral infarction develops rapidly after a major ischemic insult. Earlier, we developed a model to induce an ischemic penumbra around a small focal infarction in the cerebral cortex of Mongolian gerbils [5, 8] by giving a threshold amount of ischemic insult to induce cerebral infarction. The histopathology of this model revealed disseminated eosinophilic ischemic neurons by light microscopic observation, and disseminated electron-dense neurons seen ultrastructurally (disseminated selective neuronal necrosis) increased in number in the penumbra of the cerebral cortex after restoration of blood flow. A focal infarction developed later in a part of this area of disseminated selective neuronal necrosis within 12 to 24 hours after ischemic insult, due to massive astrocytic death. This area expanded gradually, involving dead and still-living eosinophilic neurons, and normal-looking neurons progressing to death 4 days after the ischemic insult [8, 9, 11]. No additional new infarction (pan necrosis) was found later than 4 days after the ischemic insult in our coronal as well as para-sagittal sections of the forebrain [3, 19].

In previous studies of the cortical penumbra [7, 9, 11], we found that the cytoplasm and cell processes of living astrocytes in the penumbra were actively swollen and that brain edema, determined by tissue gravimetry, was maximum around 3 days after ischemic insult, subsiding gradually by 7 days [4, 11]. Isolated dark neurons with different grades of high-electron density increased in number among the normal-looking neurons from 5 to 24 hours. These dark neurons were surrounded by severely swollen astrocytic cell processes. As a general pathological sign of irreversible cellular damage, granular chromatin condensation was apparent in the nuclear matrix and along the nuclear membrane of some of these dark neurons [12]. The dark neurons increased in number rapidly until day 4, and new ones continued to appear 12 weeks after the ischemic insult. These observations correspond to the maturation phenomenon of ischemic injuries [3, 6, 19], which is the same as the delayed neuronal death described for CA1 neurons [3, 15, 19].

In the present study, we investigated the fate of swollen edematous astrocytes and dead neurons at the ultrastructural level, as well as remodeling of axons connected to the dead neurons in the ischemic penumbra.

## Materials and methods

Stroke-positive Mongolian gerbils were selected according to their stroke index score [18] during left carotid clipping for 10 minutes, followed by another 10 minutes of clipping with a 5-hour interval between the 2 occlusions. The gerbils were sacrificed at 5, 12, and 24 hours, at 4 days, and at 3, 5, 8, 12, and 24 weeks following the last ischemic insult by intracardiac perfusion with cacodylate-buffered glutaraldehyde fixative (3 animals in each group) for electron microscopy and with 10% phosphate-buffered formaldehyde fixative for light microscopy (5 animals in each group).

Ultrathin sections including the second through fifth cortical layers were obtained from the neocortex at the mid-point between the interhemispheric and rhinal fissures on the left coronal face sectioned at the infundibular level, in which only the penumbra appeared. The sections were double-stained with uranyl acetate and lead solution, and observed with a Hitachi electron microscope (H9000). Separate paraffin sections were stained with hematoxylin-eosin, periodic acid fuchsin Schiff, or by Bodian silver impregnation or immuno-histochemistry for glial fibrillary acidic protein (GFAP).

Placing 1 cm × 1 cm lattices on the 5,000 × 2.67 enlarged EM photographs, we measured the number of synapses and spines in the neuropil in a 100-square cm (56 sq.μ, by real size), and determined the percent volume of the axon terminals and spines using the point-counting method [22] by counting intersections of the lattice dropped on the axon terminals and/or spines. We also measured neuritic thickness as the maximal diameter perpendicular to their neurofilaments and/or microtubules on the same EM pictures.

## Results

Astrocytic swelling gradually subsided starting on day 4 after ischemic insult, then an increase was observed in the number of cytoplasmic glial fibrils in astrocytes seen ultrastructurally and in GFAP-positive cells seen by light microscopy. Astrocytes in mitosis or with 2 nuclei were occasionally seen.

The disseminated dying electron-dense neurons had been fragmented into granular pieces by invading astrocytic cell processes (Fig. 1A). These accumulations of fragmented dark neurons were observed as eosinophilic ghost cells by light microscopy. The electron-dense granular pieces were dispersed around the extracellular spaces and phagocytized by microglia, astrocytes, and neurons. There was no evidence of macrophages in the penumbra.

The number of synapses and spines, and the percent volume of the axon terminals and spines (Table 1) decreased with an increase in a sparsity of synaptic vesicles until day 4 (Fig. 2A). From 1 to 12 weeks after ischemic insult, however, they recovered to or exceeded the control values and were found surrounding the thickened neurites of the surviving neurons (Fig. 2B).

From 4 days to 8 weeks after the ischemic insult, most axon terminals that had been attached to dying neurons were found around the fragmented dead dark neurons. Some of them were separated from the dead neurons, being attached by a crust of granular electron-dense fragments (Fig. 1A). From 24 hours to 8 weeks after ischemic insult, some axons attached to dying neurons showed globular or spindle-shaped distension of their terminals, as seen by Bodian silver impregnation (Fig. 1B). Electron microscopic observation of these distensions showed amplified axon terminals containing degenerated mitochondria, lamellated dense bodies, and irregularly located neurofilaments and microtubules. They were frequently observed around accumulations of fragmented electron-dense granular pieces of dead neurons.

From 1 to 12 weeks, some axon terminals associated with crusts of electron-dense granular pieces became newly connected to the spines and neurites of the surviving neurons.

Neuronal death continued in the penumbra during these periods (maturation phenomenon). From 8 to 24 weeks after the ischemic insult, these structures and the accumulation of eosinophilic ghost cells remained confined to the third cortical layer, especially in some portions of the lateral part of the left coronal face sectioned at the infundibular level. Cortical thickness and cortical neuronal density were reduced evenly in the face during these periods.

## Discussion

Astrocytes swell in the acute phase after an ischemic insult, showing increases in the number of glycogen granules and mitochondrial size and number, indicating an active reaction of astrocytes to prevent ischemic neuronal injury [7, 11]. Four days after ischemic insult, astrocytic swelling subsides and glial fibrils, stained by GFAP antibodies, increase in number. These GFAP-positive reactive astrocytes are increased in number by mitotic division, especially those surrounding the focal infarction (pan necrosis), which evolves and develops from 12 hours to 4 days after the insult. Necrotic tissue is then scavenged by macrophages and

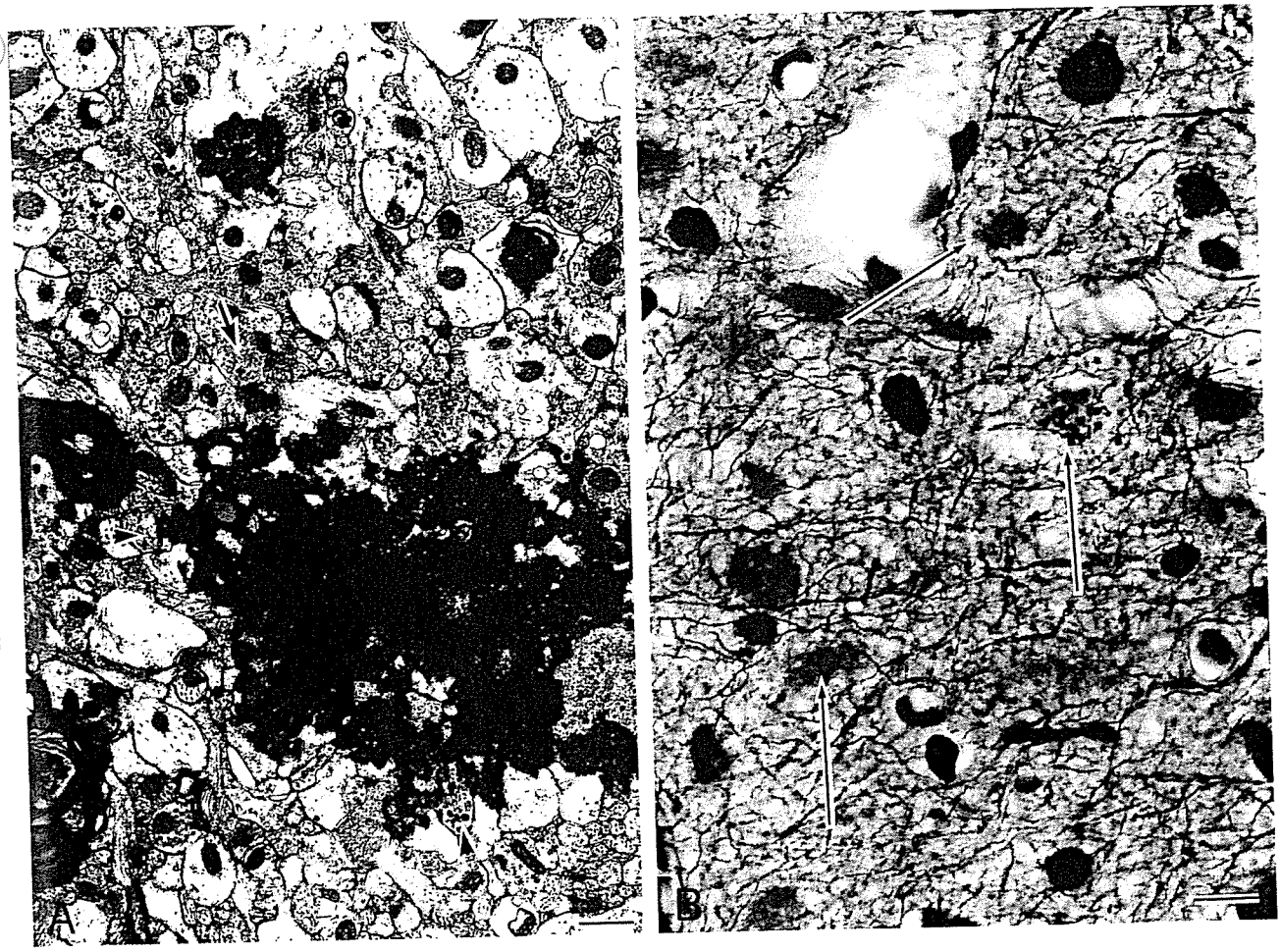

Fig. 1. (A) Electron microscopy of cerebral cortex 4-days after restoration of blood flow. The disseminated dying electron-dense neuron has been fragmented into granular pieces by invading astrocytic cell processes. Some axon terminals that were attached to the dying neurons are found around the fragmented dead dark neurons (arrowheads). Bottom bar 0.1 micron. (B) Light microscopy of cerebral cortex 24 hours after restoration of blood flow. Some thick axons attached to dying neurons showed globular or spindle-shaped distension of their terminals surrounding the dead neurons (arrows). Bodian silver impregnation. Bottom bar 2 micron

Table 1. *Data showing astrocytic restitution and neuronal remodeling processes up to 12 weeks after ischemic injury.*

| Average value | Time after last ischemic insult | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | 5 hours | 4 days | 1 week | 5 weeks | 8 weeks | 12 weeks |
| % vol. of axon terminal | 17.75 ± 1.32 | 14.69 ± 5.79† | 5.48 ± 1.71* | 14.89 ± 1.69† | 19.55 ± 2.62† | 21.62 ± 2.24† | 28.90 ± 3.55*† |
| % vol. of spine | 4.80 ± 1.12 | 3.09 ± 0.89*† | 1.19 ± 0.20* | 1.70 ± 0.25* | 2.25 ± 0.45* | 2.91 ± 0.42*† | 4.16 ± 1.04† |
| No. of synapses/56 sq μ | 17.21 ± 1.09 | 23.24 ± 4.52*† | 12.65 ± 1.67* | 13.80 ± 1.92† | 14.71 ± 3.26 | 16.68 ± 1.55† | 19.48 ± 3.10† |
| No. of spines/56 sq μ | 11.89 ± 2.50 | 11.20 ± 2.33† | 4.26 ± 0.40* | 7.14 ± 0.78* | 6.69 ± 0.63* | 7.10 ± 0.68* | 10.93 ± 3.04† |
| Thickness of neuritis (μ) | 0.61 ± 0.02 | – | 0.59 ± 0.02 | 0.60 ± 0.02 | – | 0.67 ± 0.02*† | 0.93 ± 0.03*† |

Average ± standard error, $p < 0.05$: * compared with control; † compared with 4 days.

becomes liquefied [3, 8, 19]. The infarcted focus is surrounded by gliosis induced by reactive astrocytes. GFAP-positive reactive astrocytes increase moderately in number, but do not induce gliosis in the penumbra. These are the restitutional processes of astrocytes in the ischemic tissue [8].

It has been thought that dead neurons and ischemically injured tissue are scavenged by macrophage inva-

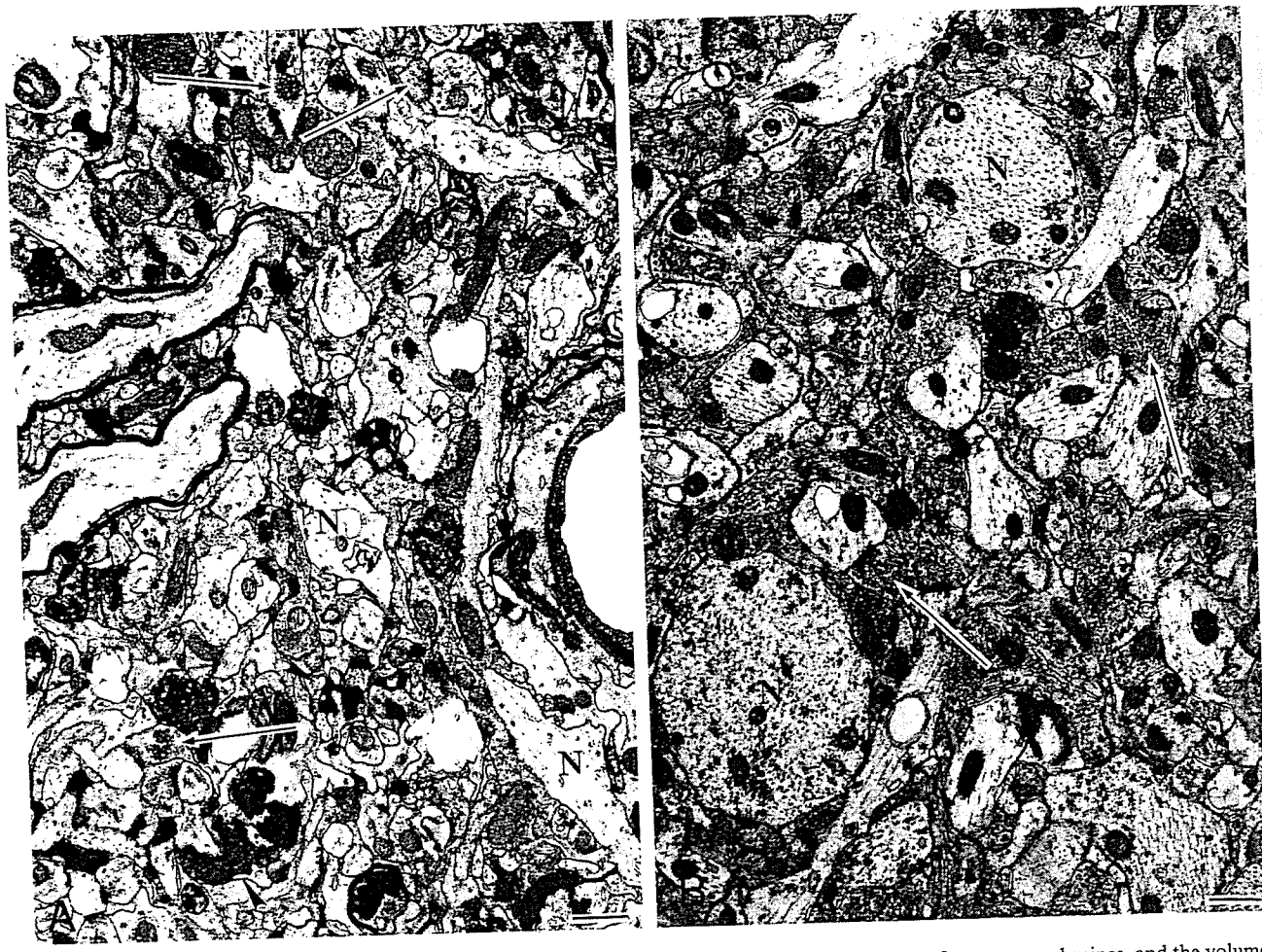


Fig. 2. (A) Electron microscopy of cerebral cortex 4 days after restoration of blood flow. The number of synapses and spines, and the volume of axon terminals and spines decreased with increase in sparsity of synaptic vesicles (arrows). Neurites (*N*) are degenerative. Electron-dense granular pieces are dispersed in the extracellular spaces of the neuropil (arrowheads). Bottom bar 0.1 micron. (B) Electron microscopy of cerebral cortex 12 weeks after restoration of blood flow. The number of synapses and spines, and volume of axon terminals and spines recovered, with increase in number of synaptic vesicles (arrows). Neurites (*N*) are thickened, surrounded and/or synapsed by axon terminals. Bottom bar 0.1 micron

sion into the injured tissue from the blood stream. However, in the present study, dead neurons were found disseminated among surviving neurons in the cortical penumbra. The axons and dendritic processes of the dying neurons were still connected to axon terminals and neurites of surviving neurons. Solitary dying neurons, which were connected by neuritic networks, were not phagocytized by a single macrophage. In contrast to infarction, i.e., massive necrosis, macrophages did not enter the neuropil of the penumbra where the network of the neuropil was still tight. In this situation, it is reasonable to assume that shrunken dead neurons become fragmented into granular debris (eosinophilic ghost cells seen by the light microscopy) and are removed by astrocytes, neurons, and perivascularly located microglia [10, 14, 16]. However, the tattered central cytosol of shrunken neurons remained for more than 5 weeks. No inflammatory cells or macrophages appeared in the ischemic penumbra wandering in the neuropils [10, 16].

We found a marked decrease in the number of synapses and volume of the axon terminals in the entire neuropil of the ischemic penumbra from 5 hours to 4 days after start of recirculation, along with marked shrinkage of axon terminals, which contained a decreased number of synaptic vesicles. These changes seemed to be due to calcium-dependent neuronal hyperexcitation [21] and were reduced by N-methyl-D-aspartate receptor antagonists in a morphological study recording excitatory postsynaptic potential

from hippocampal slice cultures subjected to brief anoxia-hypoglycemia [13].

The number of synapses increased gradually from 1 to 12 weeks after the ischemic insult, associated with an increase in the volume of axon terminals showing sprouting [20] and paralleling a marked increase in the number of synaptic vesicles. The number and volume of spines also increased in parallel. Axons that had been attached to the dying neurons were considered to have shifted their connections to the spines and the neurites of the surviving neurons, increasing their thickness associated with synaptogenesis in the neuropil [1, 2, 17]. The neuronal remodeling process progressed in the ischemic penumbra from its early stage to 12 weeks after the start of recirculation.

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# Transcriptional repression induces a slowly progressive atypical neuronal death associated with changes of YAP isoforms and p73

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Transcriptional disturbance is implicated in the pathology of polyglutamine diseases, including Huntington's disease (HD). However, it is unknown whether transcriptional repression leads to neuronal death or what forms that death might take. We found transcriptional repression-induced atypical death (TRIAD) of neurons to be distinct from apoptosis, necrosis, or autophagy. The progression of TRIAD was extremely slow in comparison with other types of cell death. Gene expression profiling revealed the reduction of full-length yes-associated protein (YAP), a p73 cofactor to promote apoptosis, as specific to TRIAD. Furthermore, novel neuron-specific YAP isoforms (YAPΔCs) were sustained during TRIAD to suppress neuronal death in a dominant-negative fashion. YAPΔCs and activated p73 were colocalized in the striatal neurons of HD patients and mutant huntingtin (*htt*) transgenic mice. YAPΔCs also markedly attenuated Htt-induced neuronal death in primary neuron and *Drosophila melanogaster* models. Collectively, transcriptional repression induces a novel prototype of neuronal death associated with the changes of YAP isoforms and p73, which might be relevant to the HD pathology.

## Introduction

Neurodegenerative disorders are characterized by the slow exacerbation of symptoms and by gradual progression of brain pathologies. Patients suffer for 5–20 yr from the onset of the disease to the bed-ridden state. Even fast-progressing amyotrophic lateral sclerosis takes 2–5 yr to render the patient bed ridden. Regarding the pathology, the total number of neurons and neural networks among them decrease. However, some of the neurons survive for an extensive period of time despite their expression of abnormal structures that are derived principally from the pathogenic disease-causing products. Typically, nigral neurons that express Lewy bodies in Parkinson's disease, hippocampal neurons that carry paired helical filaments in Alzheimer's disease, and motor neurons bearing Bunina bodies in amyotrophic lateral sclerosis can partially survive until the death of the patient. The mutant protein aggregates that characterize many of these diseases are known to trigger multiple cellular responses, including ER stress and mitochondrial abnormality. These stress responses are clearly sufficient to induce apoptosis in nonneuronal cell lines, whereas the brain pathology of patients indicates that neurons survive for a long period before their demise. A lengthy period of cell death is also observed in the polyglutamine (polyQ) diseases, a major group of neurodegeneration that includes nine disorders (for reviews see Gusella and MacDonald, 2000; Zoghbi and Orr, 2000; Ross, 2002; Taylor et al., 2002; Bates, 2003). Again, a fraction of the neurons that possess nuclear and/or cytoplasmic inclusions of mutant

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Abbreviations used in this paper: AMA, α-amanitin; CDDP, cisplatin; FL-YAP, full-length YAP; HD, Huntington's disease; Pol II, polymerase II; polyQ, polyglutamine; siRNA, short inhibitory RNA; TRIAD, transcriptional repression-induced atypical death; YAP, yes-associated protein.

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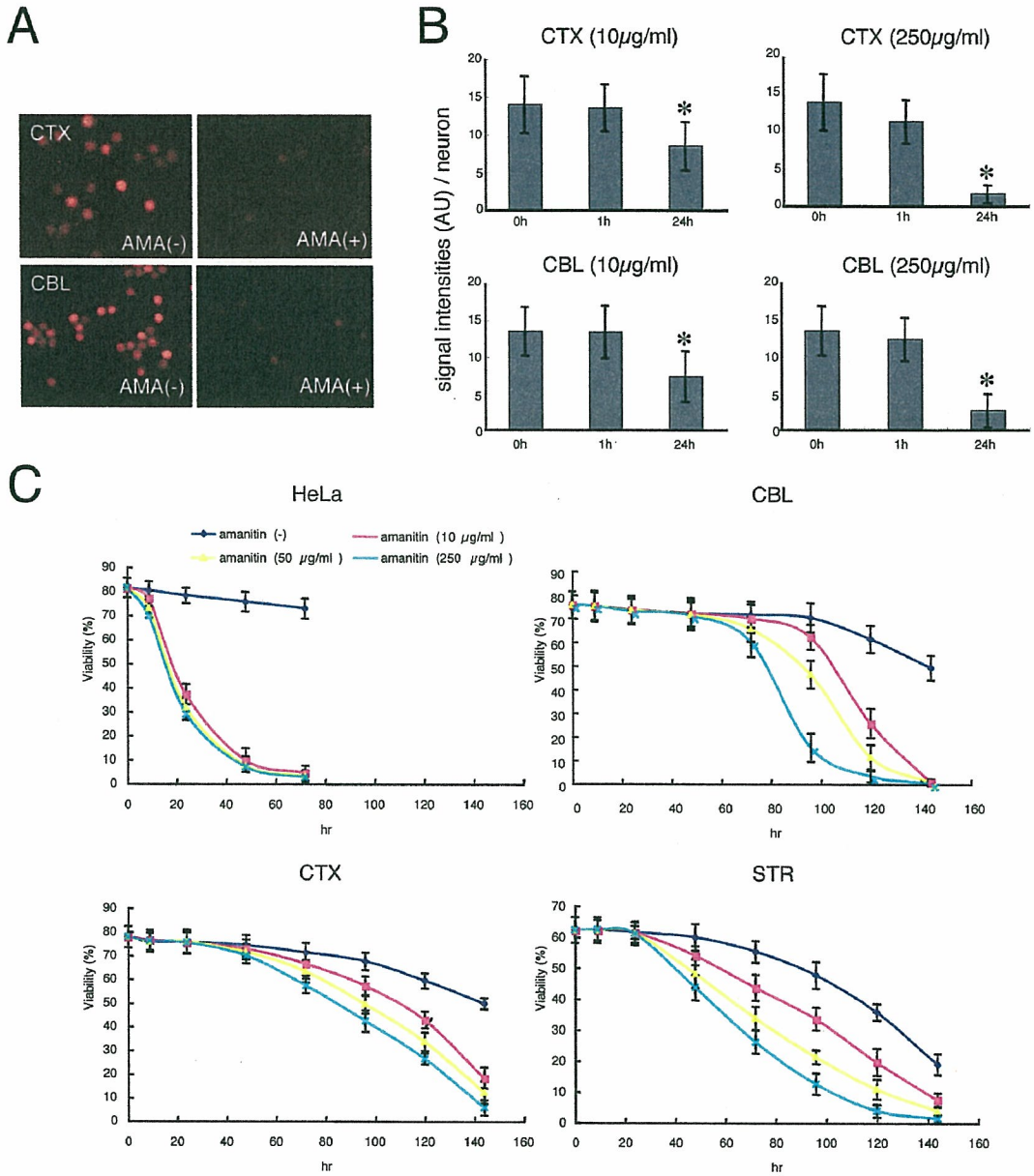


Figure 1. **Transcriptional repression by α-AMA induces a slowly progressive death of primary neurons.** (A) Uptake of BrdU in primary cortical (CTX) or cerebellar (CBL) neurons (Hoshino et al., 2004) was remarkably reduced at 6 h after the addition of 25 μg/ml AMA, indicating that AMA reduces transcription. (B) BrdU uptakes per neuronal nucleus at different concentrations of AMA (10 and 250 μg/ml) were analyzed chronologically. Signal intensities (AU, arbitrary unit) calculated with AQUACOSMOS (Hamamatsu) were obtained from >500 cells. Means ± SD are shown (error bars). Asterisks indicate significant reductions compared with 0 h ($P < 0.01$, *t* test). (C) Survival curves of HeLa cells and primary neurons (cortical, cerebellar, and striatal [STR] neurons) with AMA (10, 25, and 250 μg/ml). Viability (percent) indicates live cells/total cells by trypan blue assay (Tagawa et al., 2004) in six independent wells. Viability was shown as means ± SD of nonstained cells. Viability of HeLa cells declined at 24 h. In primary neurons, a significant reduction was first observed at 72 h at all concentrations of AMA. Of the three types of neuron tested, striatal neurons were the most vulnerable to AMA treatment for 48 h at 25 and 250 μg/ml AMA. $P < 0.05$ (*t* test).

polyQ peptides survives even in affected regions of the brain until the time of necropsy. So far, there is no model that fully explains the lengthy period of cell death in neurodegeneration.

In addition to ER and mitochondrial stresses, transcriptional dysfunction is suggested as a critical pathological component of polyQ diseases (for reviews see Gusella and MacDonald, 2000; Zoghbi and Orr, 2000; Ross, 2002; Taylor et al., 2002; Bates, 2003). Translocation of mutant proteins to the nucleus seems essential for neuronal dysfunction or cell death in polyQ diseases (Klement et al., 1998; Saudou et al., 1998; Katsuno et al., 2003). Numerous transcription-related factors, including LANP, PQBP-1, N-CoR, ARA24, p53, mSin3A, ETO/MTG8,

P160/GRIP1, A2BP1, $TAF_{II}130$, CA150, CRX, Sp1, CtBP, PML, $TAF_{II}30$, NF-κB, and SC35, are known to interact or colocalize with mutant polyQ disease proteins (for reviews see Okazawa, 2003; Sugars and Rubinsztein, 2003). Interaction with polyQ proteins may impair physiological functions of these transcription factors (Okazawa, 2003; Sugars and Rubinsztein, 2003), and, finally, even the general transcription level could be repressed (Hoshino et al., 2004). Some of these polyQ pathology-mediating factors bind directly to the core of transcription machinery, RNA polymerase II (Pol II; Okazawa et al. 2002). Therefore, one of the paramount issues in the field of polyQ diseases is the relationship between transcriptional dysfunction and neuronal death. However, as yet, the role of transcriptional disruption in neuronal death is unclear, as is the mode of neuronal death when transcription is severely impaired.

In this study, we found that inhibition of Pol II–dependent transcription leads neurons to undergo a slowly progressive atypical cell death (transcriptional repression-induced atypical death [TRIAD]) distinct from apoptosis, necrosis, or autophagy in morphological and biochemical analyses. Transcriptome analysis of TRIAD suggested that yes-associated protein (YAP), a known transcriptional cofactor, might be relevant to the death process. YAP, which was originally found as a binding protein to Src homology domain 3 of the yes proto-oncogene product (for review see Sudol et al., 1995), acts as a transcriptional cofactor of p73, mediates the expression of cell death–promoting genes, and induces apoptosis (Yagi et al., 1999; Basu et al., 2003; Melino et al., 2004). We found that in TRIAD, full-length YAP (FL-YAP) is down-regulated, and novel neuron-specific YAPΔC isoforms lacking the cell death–promoting activity sustain to protect neurons in a dominant-negative manner. The shift of balance in YAP isoforms seemed to slow down the cell death signaling pathway of p73 activated by α-amanitin (AMA), at least partially. We further questioned the relevance of YAP and p73 to Huntington's disease (HD) by using cellular, *Drosophila melanogaster*, and mouse models as well as human brain samples. Our data suggest that these molecules might be involved in neuronal death triggered by mutant Htt, the causative gene product of HD.

## Results

### Transcriptional repression induces an atypical slow neuronal death

To address the role of transcriptional disruption in neuronal death, we first made multiple short inhibitory RNAs (siRNAs) against RNA Pol II to suppress Pol II–dependent transcription. However, suppression of Pol II was inadequate and reminiscent of recent efforts to suppress basic transcription machinery by similar approaches (Ni et al., 2004). Therefore, we used a specific inhibitor of Pol II (AMA) whose three-dimensional molecular structure is exactly complementary to the groove of Pol II, through which mRNA is elongated (Cramer et al., 2001; Bushnell et al. 2002). Different concentrations of AMA were added to the culture medium of HeLa cells, primary rat embryonic (embryonic day [E] 15) cortical neurons, rat E15 striatal neurons, and rat pup cerebellar neurons (postnatal day [P] 7). BrdU up-take assay (Hoshino et al., 2004) showed significant repression of transcription at 6 h of AMA treatment in primary neurons (Fig. 1, A and B) and HeLa cells (not depicted). The survival of AMA-treated cells estimated by trypan blue assay (Fig. 1 C) revealed that AMA induces a slowly progressive cell death in a dose-dependent fashion. This was most pronounced in primary neurons, with half-lives of nearly 5 d. AMA-induced neuronal death was much slower than low potassium–induced apoptosis of cerebellar neurons, whose half-life was ~12 h (not depicted). The slow progression of AMA-induced neuronal death was confirmed independently by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200509132/DC1).

A population of HeLa cells (10–30%) began to show cytoplasmic vacuoles proximal to the nucleus (Fig. 2 A, HeLa-TRIAD) from 6–12 h after the addition of AMA. Similar vacuoles were also observed in cortical neurons treated with AMA for 2 d (Fig. 2 A, CTX neuron-TRIAD), although with a diminished frequency (1–5%). It is important to note that the vacuoles did not possess double-membrane structures reminiscent of autophagosomes. No classic apoptotic features such as chromatin condensation, nuclear fragmentation, or apoptotic bodies (Okazawa et al., 1996) were found in these neurons by electron microscopic analysis (Fig. 2 A). In addition, no necrotic features such as mitochondrial dilatation (Fig. 2 A, CTX neuron necrosis) or cytoplasmic ballooning and rupture (Fig. 2 A, CTX neuron necrosis) were observed in primary neurons under TRIAD.

Immunohistochemical analyses using organelle-specific antibodies excluded the idea that the cytoplasmic vacuole was derived from the Golgi apparatus, endosome, lysosome, and mitochondria (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200509132/DC1). Autophagosomes induced by rapamycin and labeled with EGFP-LC3, a marker protein of the autophagosome (Fig. 2 B, top and middle), failed to colocalize with AMA-induced vacuoles (Fig. 2 B, bottom). In addition, the size of AMA-induced vacuoles was larger than that of autophagosomes (Fig. 2 B, bottom). EGFP-LC3 actually expresses the LC3 peptide (Fig. 2 C, arrow), verifying the morphological result. Note that the immunoblot shows a nonspecific band that is consistently detected by this antibody (Fig. 2 C, asterisk; unpublished data). Furthermore, the addition of rapamycin to the medium increased LC3-positive vacuoles but did not affect the formation of LC3-negative vacuoles induced by AMA (Fig. S3 A). Collectively, these data suggested that AMA-induced cell death is distinct from autophagy. Finally, we found colocalization of the vacuoles with ECFP-ER fusion constructs (expressing calreticulin ER-targeting sequences and KDEL ER retrieval tags at the 5′ and 3′ ends, respectively, of ECFP; Fig. 2 D). It suggested that vacuoles might be derived from expanded ER.

In agreement with the absence of morphological features of apoptosis, genomic DNA analyses of cell lines and primary neurons did not show ladder formation after AMA treatment (Fig. 3 A). Caspase-3, -7, and -12 were not remarkably activated in primary neurons by AMA (Fig. 3 B). AMA induced neither the release of cytochrome c into the cytosol from these neurons (Fig. 3 C) nor the interaction of annexin-V with the membrane of these neurons at an early stage (Fig. S3 B). Caspase inhibitors

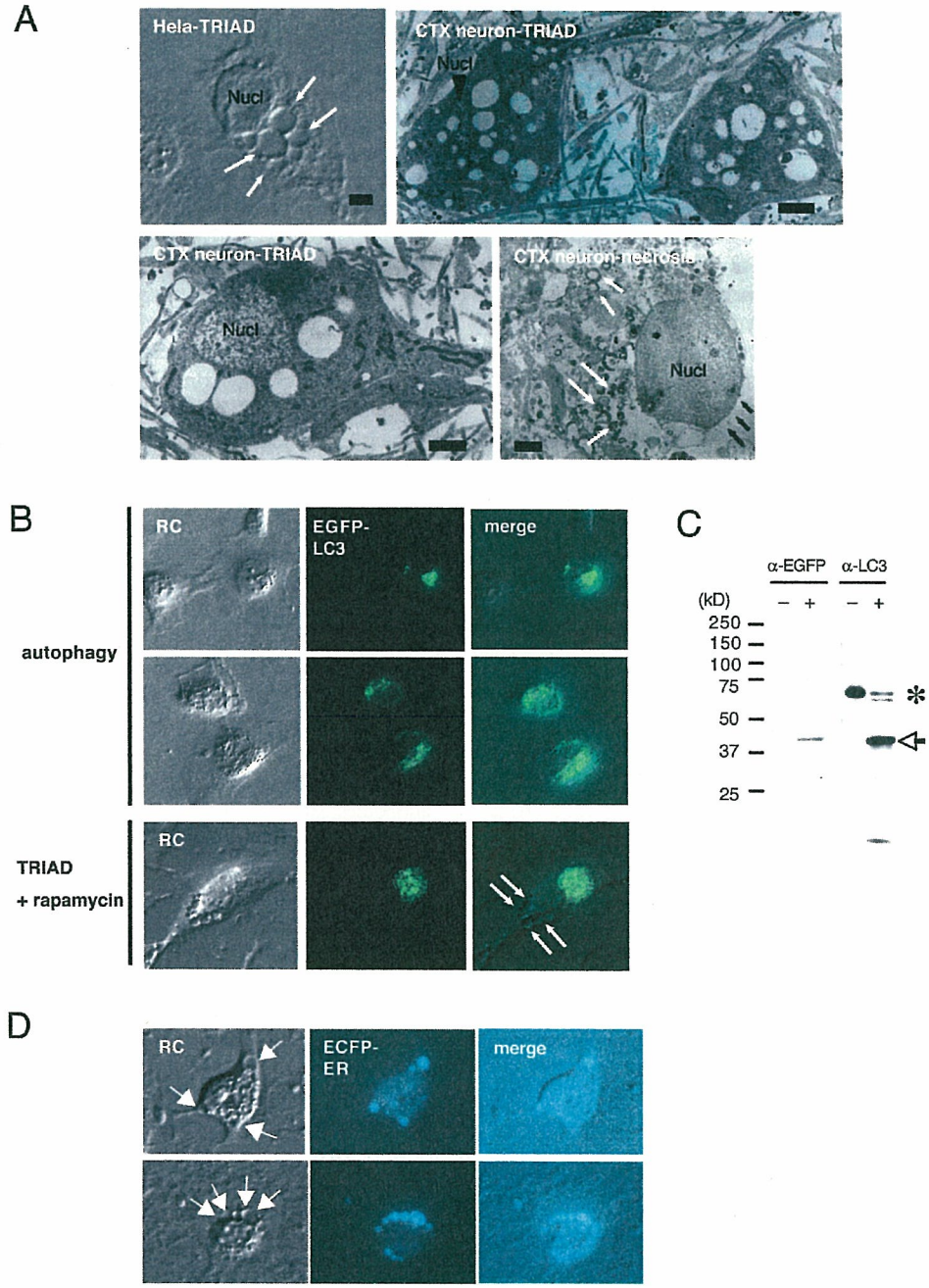


Figure 2. **Morphological features of TRIAD are distinct from those of apoptosis, necrosis, and autophagy.** (A) 10–30% of HeLa cells treated with 25 μg/ml AMA for 24 h (HeLa-TRIAD) showed cytoplasmic vacuoles (white arrows) proximal to the nucleus (Nucl). Electron microscopic analysis of cortical neurons treated with 25 μg/ml AMA for 48 h (CTX neuron TRIAD) revealed similar cytoplasmic vacuoles. Absence of chromatin condensation or nuclear fragmentation distinguishes TRIAD from classical apoptosis. The normal cytoplasm or mitochondria also excludes typical necrosis (bottom left). Electron microscopic analysis of primary cortical neurons in necrosis after freeze-thaw treatment (CTX neuron necrosis) showed the dilation of mitochondria (white arrows) and the rupture of cytoplasm (black arrows). Bars, 1 μm. (B) HeLa cells treated with 200 ng/μl rapamycin for 2 h showed autophagy (top and middle). AMA-induced vacuoles (arrows) did not merge with EGFP-LC3–labeled autophagosomes (bottom). RC, relief contrast. (C) Western blots to verify that pEGFP-LC3 expresses the LC3 peptide. Both anti-EGFP and anti-LC3 antibodies detect EGFP-LC3 (arrow), confirming that the EGFP-LC3 fusion protein is properly expressed. Asterisk indicates a nonspecific band. (D) A marker protein of ER, ECFP-ER (blue), was localized to AMA-induced vacuoles (arrows), suggesting that the vacuoles originate from the ER.