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CALCIUM ACCUMULATION IN SYNAPSES OF THE RAT HIPPOCAMPUS AFTER CEREBRAL ISCHEMIA

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The ultrastructural localization of calcium deposits in the synapses of rat hippocampus after 10 min global cerebral ischemia was evaluated. Oxalate-pyroantimonate technique was applied. After 24 hours of postischemic recirculation enhancement of intracellular (pre- and postsynaptic parts) and extracellular (synaptic clefts) calcium deposits was found in great proportion of synapses in CA_1 sector. Abundant Ca-precipitates appeared specially in synaptic clefts and in the postsynaptic parts near synaptic densities. Increased calcium deposits in some changed mitochondria were also observed. The results presented in this paper suggest synaptic modulation of Ca^{2+} homeostasis, disturbed after ischemic incident. Presence of Ca-precipitates in synaptic clefts and postsynaptic parts seems to be a sensitive indicator of increased calcium influx from the extracellular to the intracellular compartments.

Key words: *hippocampus, synapses, Ca-pyroantimonate technique, ischemia.*

Delayed neuronal death is a characteristic type of irreversible nerve cell injury resulting from cerebral ischemia. It appears in some selectively vulnerable neuronal groups of the brain, among which pyramidal cells of the CA_1 sector of Ammon's horn have been most extensively studied. Delayed neuronal death, first described by Ito et al. (1975) in Mongolian gerbils as an exponent of the maturation phenomenon, was later shown to occur both in gerbils and rats in various experimental models of cerebral ischemia (Kirino 1982; Kirino et al. 1984; Kirino, Sano 1984; Yamaguchi, Klatzo 1984; Suzuki et al. 1985; Mossakowski et al. 1989). It is generally accepted that delayed neuronal death results from the excitotoxic action of aminoacid neurotransmitters, mostly glutamate and aspartate (Olney et al. 1983; Griffith et al. 1984).

Two main mechanisms of neuronal changes due to the action of excitatory aminoacid neurotransmitters (EAAs) are postulated (Rothman, Olney 1986; Siesjö, Bengtsson 1989). The first is represented by acute cellular swelling, resulting from abnormal ions and water influx into neurons, following opening of ion channels by EAAs (Choi 1985, 1987; Rothman 1985). The second type is represented by a delayed mechanism mediated by the increased intracellular level of free calcium, which activates a number of metabolic processes leading

to cell disintegration and death (Choi 1987, 1988; Siesjö, Bengtsson 1989). Two major features distinguish both cellular responses to the action of EAAs. These are selectivity and reversibility. Delayed neuronal death is known to be selective and irreversible. Acute cellular swelling accompanied by a transient increase of cytosolic Ca^{++} concentration is totally reversible and involves all types of neurons and dendritic fields, which receive excessive EAAs stimulation (Olney et al. 1983; Griffith et al. 1984). The synaptic mechanism operated by EAAs in the hippocampus interacts with other neurotransmitter systems, in which GABA, acetylocholine, adenosine, serotonin, noradrenaline, histamine and/or neuropeptides are involved. Functional balance between inhibitory and excitatory stimulation plays an important role in preventing cellular damage due to ischemia. It seems, therefore, possible that the pathological process ending as delayed neuronal death of CA_1 hippocampal pyramidal neurons may at least be initiated by disturbances of this balance caused by the decreased inhibition appearing against the background of normal or enhanced excitation by EAAs during the postischemic period. Our previous electron-microscope studies revealed features of an early and reversible alteration of CA_1 sector interneurons, which may result in insuffiency of GABA-ergic inhibitory processes (Gajkowska et al. 1989). Yasumoto et al. (1988) described a decrease of GABA content in the CA_1 sector after cerebral ischemia and postulated that reduced inhibitory processes may be one of the factors responsible for neuronal damage. The delayed neuronal death of pyramidal neurons in the CA_1 hippocampal sector is preceded by accumulation of calcium in the tissue (van Reempts et al. 1986; Deshpande et al. 1987). Its cellular compartmentation has so far not been clarified. These alterations of calcium homeostasis evoked by excitatory synaptic stimulation in the selectively vulnerable area of Ammon's horn in cerebral ischemia inclined us to perform ultrastructural and cytochemical studies of synaptic contacts in the CA_1 hippocampal sector in case of global cerebral ischemia resulting from temporary cardiac arrest in rats. Application of the highly sensitive oxalate-pyroantimonate histochemical technique of Borges et al. (1977) as modified by Mata et al. (1987) seemed to be most appropriate for specific visualization of calcium distribution of various compartments of the synaptic system.

MATERIAL AND METHODS

Studies were carried out on 10 male Wistar rats, weighing 160–180 g. In the experimental animals cardiac arrest was achieved for 10 min according to Korpachev et al. (1982). A detailed description of experimental procedure and pathophysiological data were presented previously (Mossakowski et al. 1986).

24 h after resuscitation 5 experimental animals were sacrificed by transcardiac perfusion with a 2.5% solution of glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Blocks of brain tissue containing the CA_1 sector of dorsal hippocampus were taken and additionally fixed for 1 h in 2.5% of glutaraldehyde solution, washed in 0.1 M cacodylate buffer and postfixed in 2.0% osmium tetroxide in cacodylate buffer. They were dehydrated routinely in alcohol solutions and propylene oxide and embedded in Epon 812. Ultrathin

sections were counterstained with uranyl acetate and lead citrate, and examined in a JEOL 1200E electron microscope.

The remaining 5 experimental animals were 24 h after resuscitation perfused transcardially for 2 min with 90 mM potasium oxalate in 1.9% sucrose, adjusted to pH 7.4 with KOH at 37°C. This was followed by perfusion with a solution composed of 3% glutaraldehyde 0.5% paraformaldehyde, 90 mM potasium oxalate and 1.9% sucrose adjusted to pH 7.4 with KOH. Perfusion lasting approximately 1 h was performed at a constant rate. At the end of perfusion, the tissue samples containing the CA_1 sector of dorsal hippocampus were taken and kept in the fixative solution for 2 h at 4°C. Then they were rinsed in 90 mM potasium oxalate in 1.9% sucrose, pH 7.4, postfixed in 1% osmium tetroxide and 2% potasium pyroantimonate for 2 h at room temperature. Unbound potasium pyroantimonate was washed out by rinsing the tissue samples for 15 min in water adjusted with KOH to pH 10. Subsequently, samples were dehydrated in graded ethanol solutions and embedded in Epon 812. Ultrathin sections, counterstained with uranyl acetate and lead citrate were examined in a JEM 1200E electron microscope.

In order to check the spacificity of the reaction, ultrathin sections mounted on grids were washed in 10 mM EGTA (ethylene glycol-bis-β-aminoethyl-ether) – N,N,N′,N′-tetraacetic acid), the calcium chelating agent, for 1 h at 60°C. EGTA-treatment in turn was controlled by incubation of the sections in distilled water for 1 h at 60°C.

Two animals not subjected to any experimental procedures served as control material. Tissue blocks containg the CA_1 sector of dorsal hippocampi were processed indentically as those from the experimental animals.

RESULTS

Ultrastructural observations.

The great majority of the synaptic contacts in the CA_1 sector examined 24 h after the ischemic incident were ultrastructurally unchanged. This concerned symmetric and asymmetric synapses of axo-somatic and axo-dendritic types. No abnormalities were observed either in the axonal endings, synaptic clefts or postsynaptic areas (Figs 1, 2). However, among the dominating ultrastructurally unchanged synaptic population, some synapses revealed obvious abnormalities, concerning both the pre- and postsynaptic parts.

Some presynaptic bags were swollen, they were devoid of synaptic vesicles or contained a remarkably reduced number of them (Fig. 3). Abnormal arrangement of synaptic vesicles in the direct vicinity of the synaptic cleft was a common feature. In some cases swollen mitochondria represented the only organelles of swollen axonal endings. So changed synaptic contacts were located mostly on ultrastructurally altered perikarya of pyramidal cells and their dendritic shafts or spines (Figs 3, 4). Some of the pyramidal cells revealed marked dilatation of channels of the rough endoplasmic reticulum and disaggregation of polyribosomes and cytoskeleton elements. However, most of

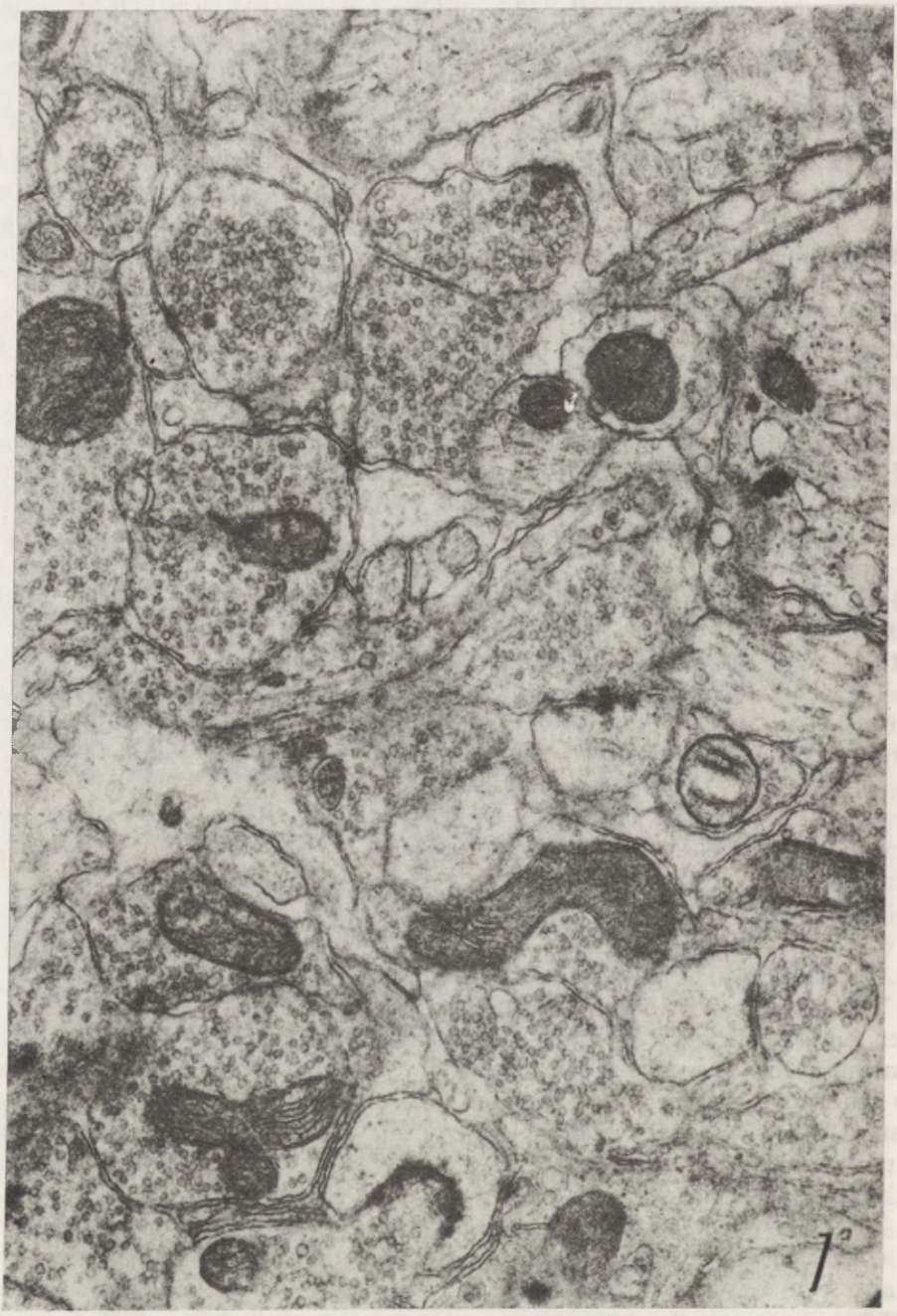

Fig. 1. CA_1 sector, 24 h after cerebral ischemia. Fragment of neuropil with ultrastructurally unchannged synapses both in pre- and postsynaptic parts. × 30 000

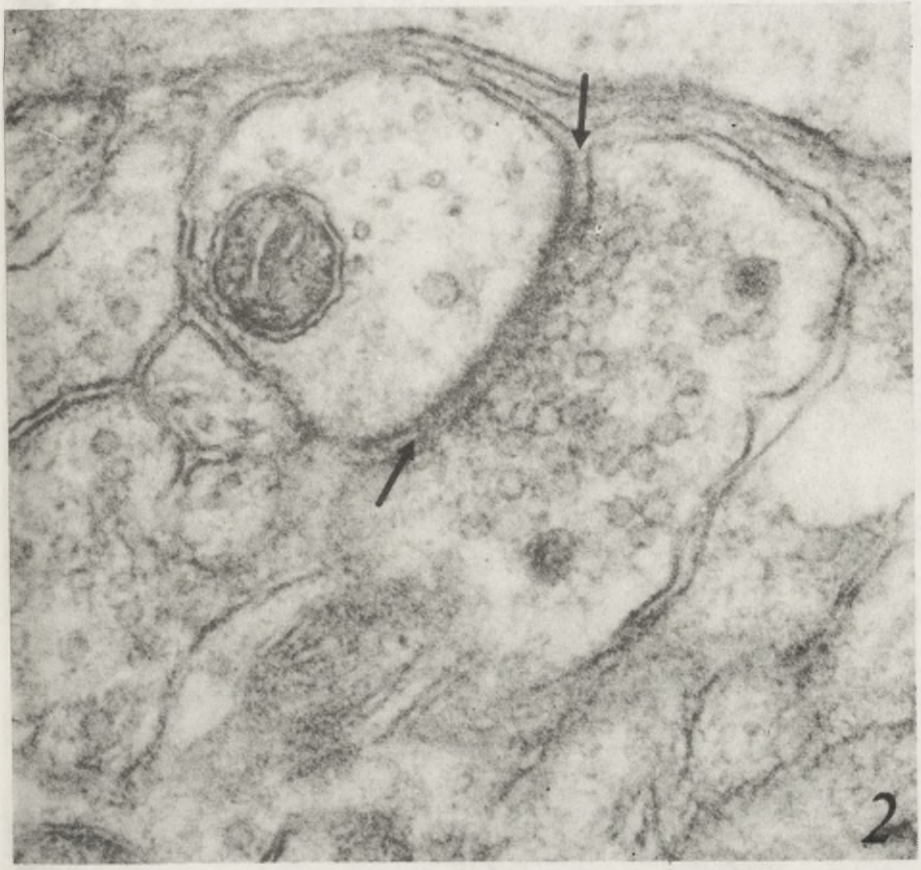

Fig. 2. CA_1 sector, 24 h after ischemia. Unchanged axo-dendritic asymmetric synapse. Note clearly visible synaptic cleft, filled with delicate floccular filaments (arrows). ×90 000

the mitochondria and other cytoplasmic organelles seemed to be relatively well preserved. Some of the dendritic profiles in the neuropile of the CA_1 sector were greatly swollen, containing distended vacuolar structures (Fig. 3).

Cytochemical observations.

In control animals, not subjected to any experimental procedure, cytochemical pictures resembled that described previously by Probst in 1986. Delicate, fine calcium deposits were present mostly in the extracellular compartment first of all within the synaptic clefts. Some fine granular material was also visible in the cytoplasm of both pre- and postsynaptic parts (Fig. 5). Intramitochondrial calcium deposition was a rather unfrequent feature.

The only difference between control and experimental animals as far as cytochemical picture is concerned consisted in significant increase of calcium deposition within the synaptic compartments in animals which survived the ischemic incident. In quite a large proportion of the synapses abundant calcium aggregates were present. Most of them were localized within synaptic clefts and in the postsynaptic parts (Figs 6, 7, 8, 9). In the latter location they were either

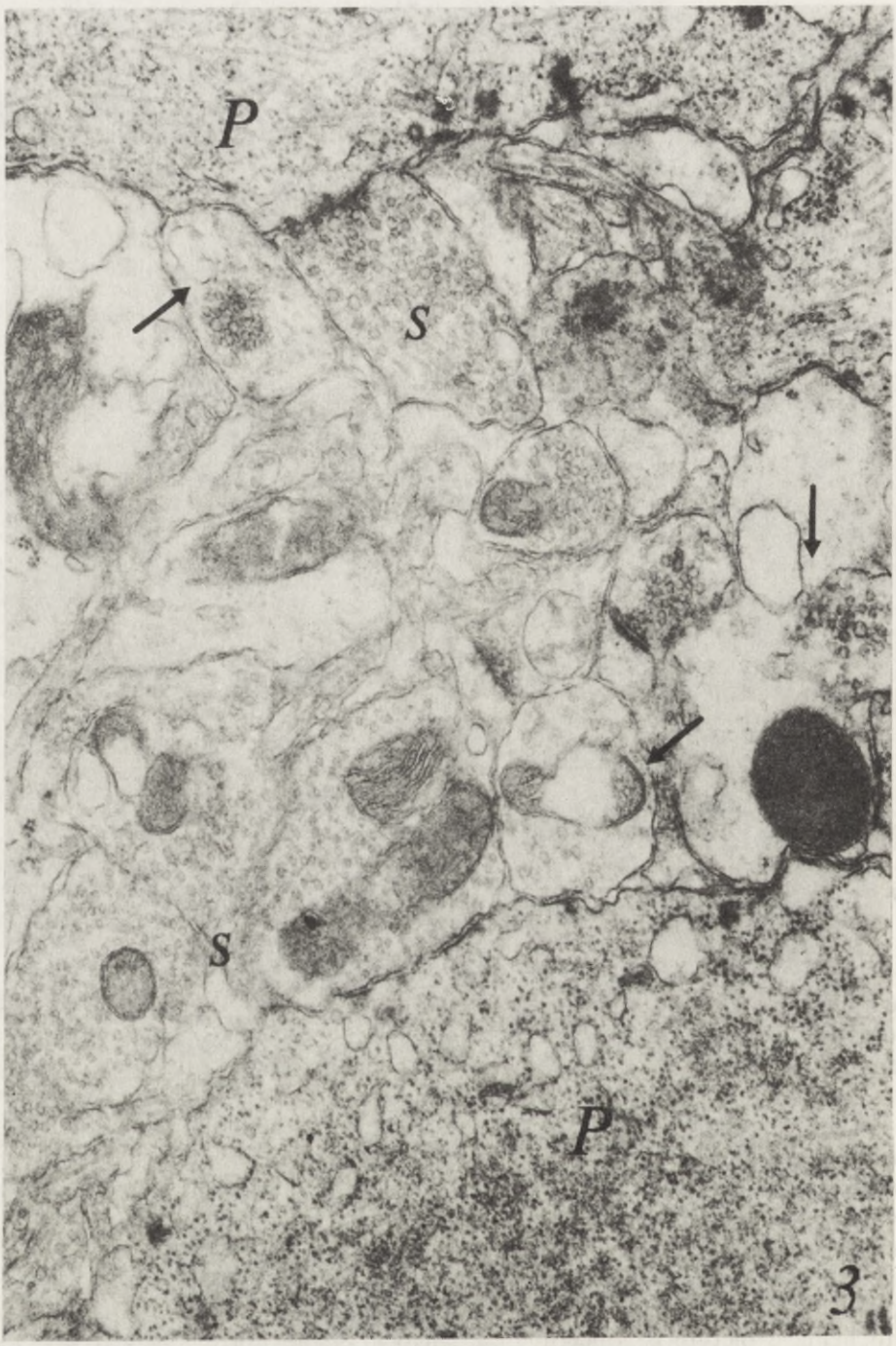

Fig. 3. CA_1 sector, 24 h after ischemia. Numerous synaptic contacts are present on the perikarya of two ultrastructurally altered pyramidal cells (P). Among apparently normal synapses (S), some display considerable swelling, reduction in number of synaptic vesicles and abnormal arrangement as well as appearance of large vesicular structures (arrows). × 30 000

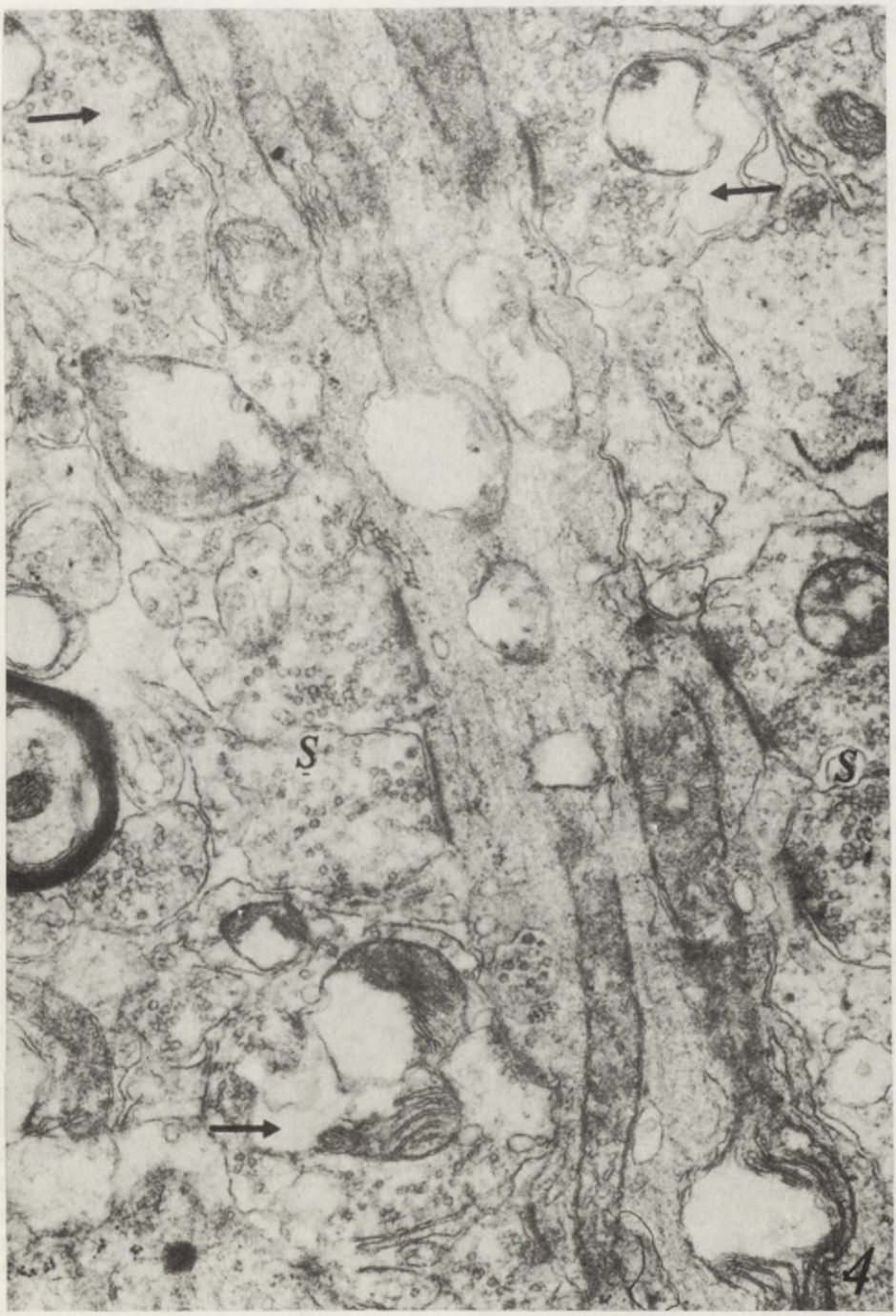

Fig. 4. CA_1 sector, 24 h after ischemia. Slightly abnormal dendritic shaft surrounded by mostly unchanged synaptic bags (S). Some of them are swollen and display abnormalities concerning number and arrangement of synaptic vesicles (arrows). × 30 000

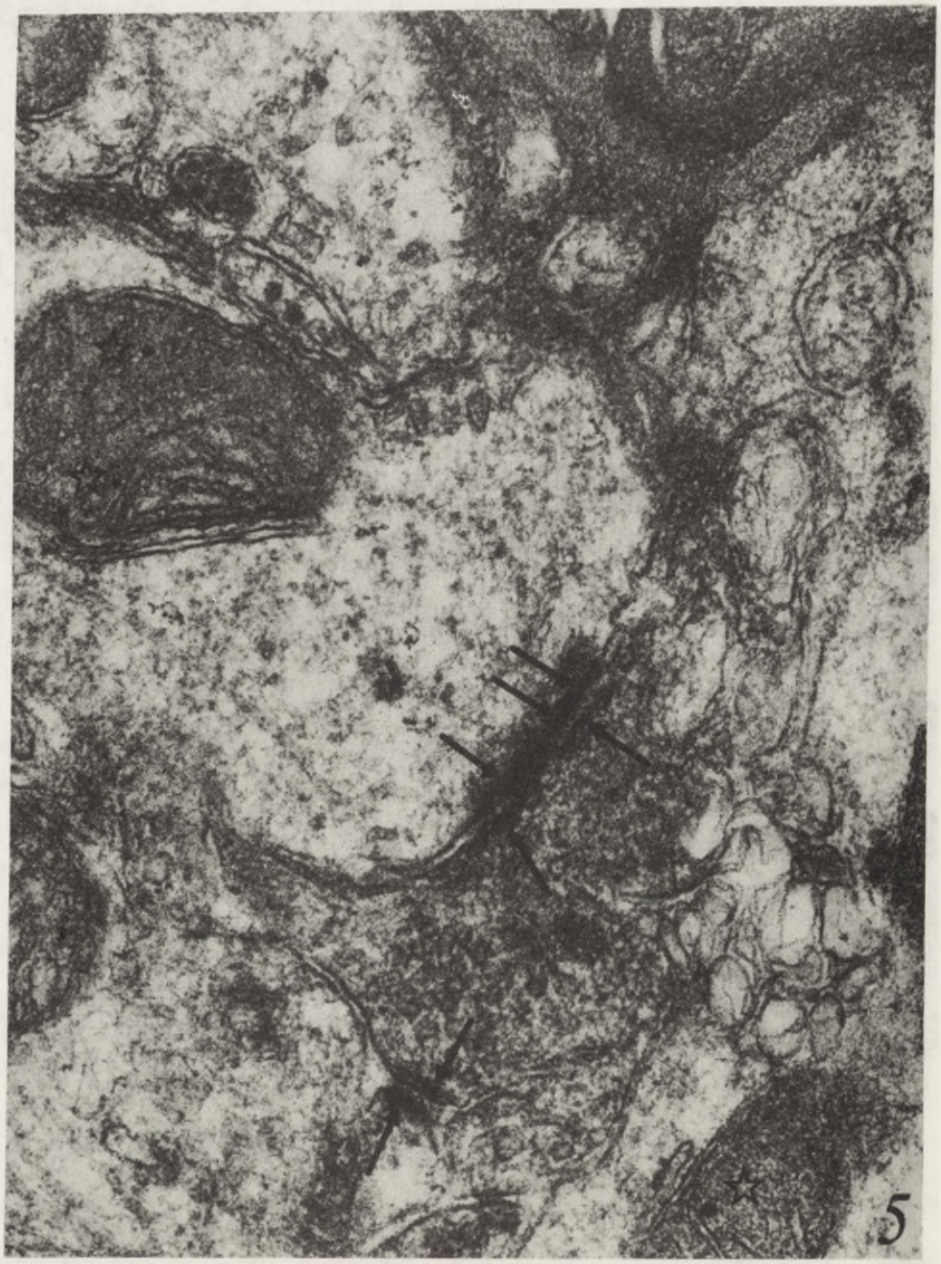

Fig. 5. CA_1 sector, control animal. Oxalate-pyroantimonate reaction. Fine-granular calcium deposits are spread in both pre- and postsynaptic parts in the vicinity of the synaptic densities (arrows). Calcium deposits are also visible in some mitochondria (asterisk). × 60 000

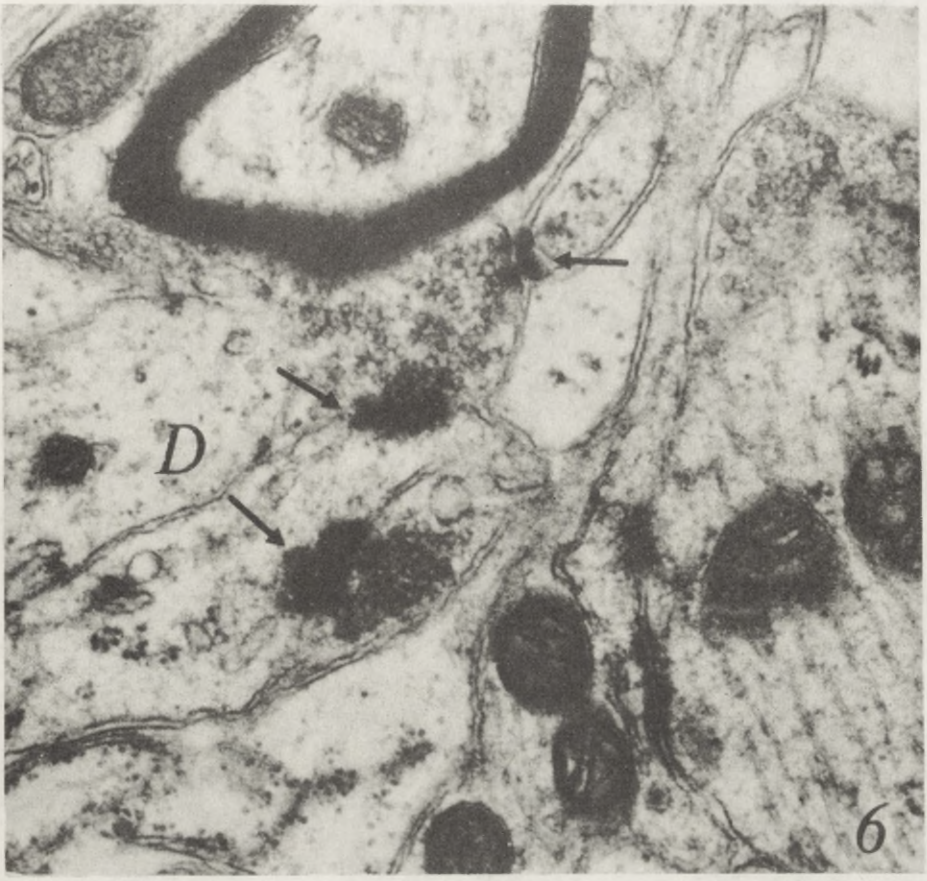

Fig. 6. CA_1 sector, 24 h after ischemia. Oxalate-pyroantimonate reaction. Dense calcium deposits (arrows) are present within the synaptic clefts and in the postsynaptic parts of swollen dendritic shafts (D). × 50 000

diffusely spread or clumped in larger accumulations (Fig. 6). It is noteworthy that the direct vicinity of postsynaptic density was the most common site of calcium aggregation in intracellular synaptic compartments. Occasionally mitochondria displaying ultrastructural alterations contained either fine granular or dense calcium deposits (Figs 7, 9). Presynaptic bags very seldom displayed calcium aggregation. Synaptic structures containing calcium deposits either in synaptic clefts or postsynaptic parts were usually contacting ultrastructurally changed perikarya of the pyramidal neurons or their abnormal dendritic shafts and spines.

DISCUSSION

Our cytochemical observations showed that application of the oxalate-pyroatimonate technique of Borges et al. (1977) permits reproducible demonstration of tissue calcium deposits in the central nervous system in both

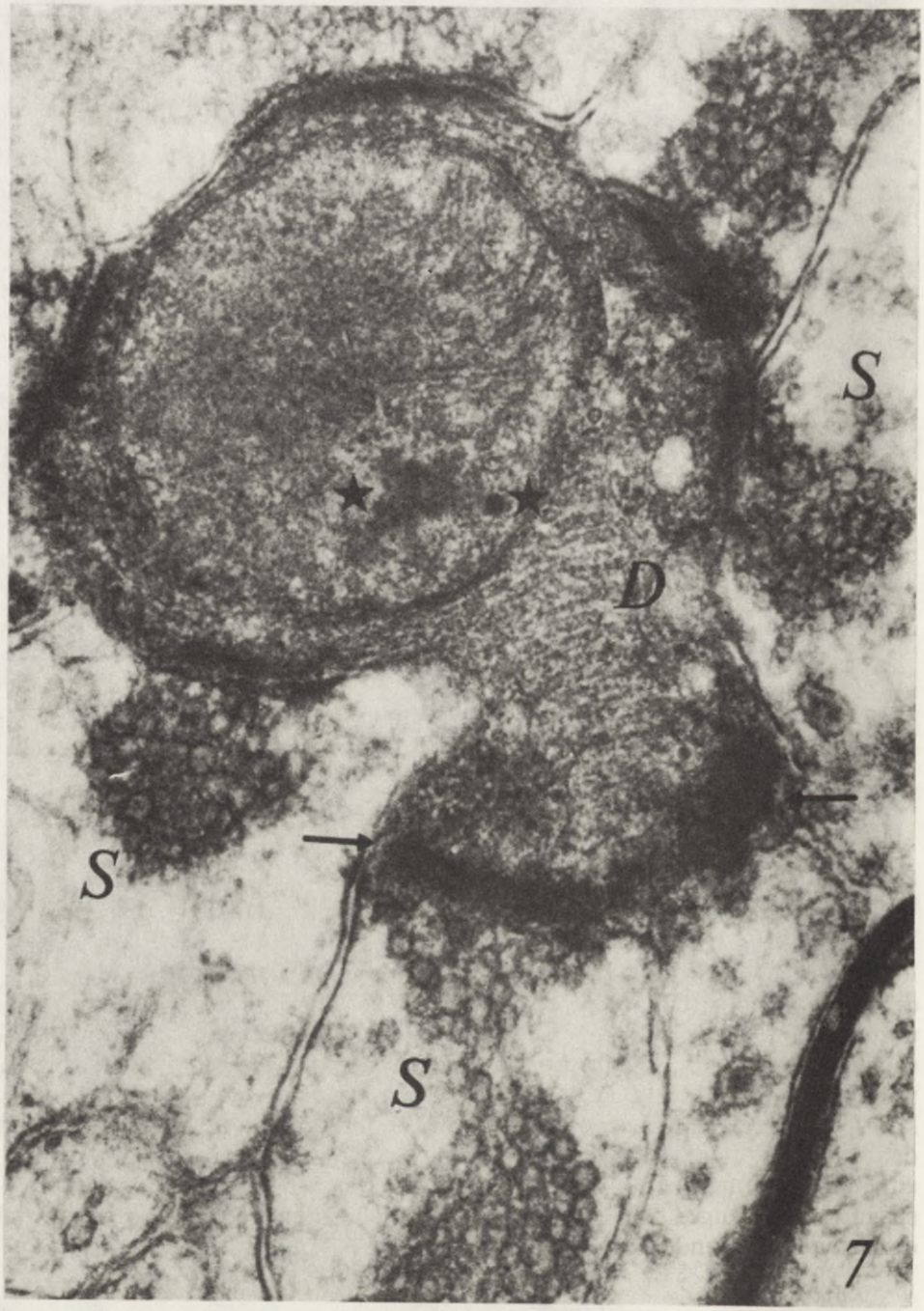

Fig. 7. CA_1 sector, 24 h after ischemia, oxalate-pyroantimonate reaction. High magnification of synaptic contacts. Accumulation of calcium deposits within synaptic clefts (arrows) and in postsynaptic part in the vicinity of synaptic density. Note swollen nerve endings with aggregations of synaptic vesicles. Granular and diffuse calcium precipitates are present within mitochondrium (asterisk) of postsynaptic part (D). × 75 000

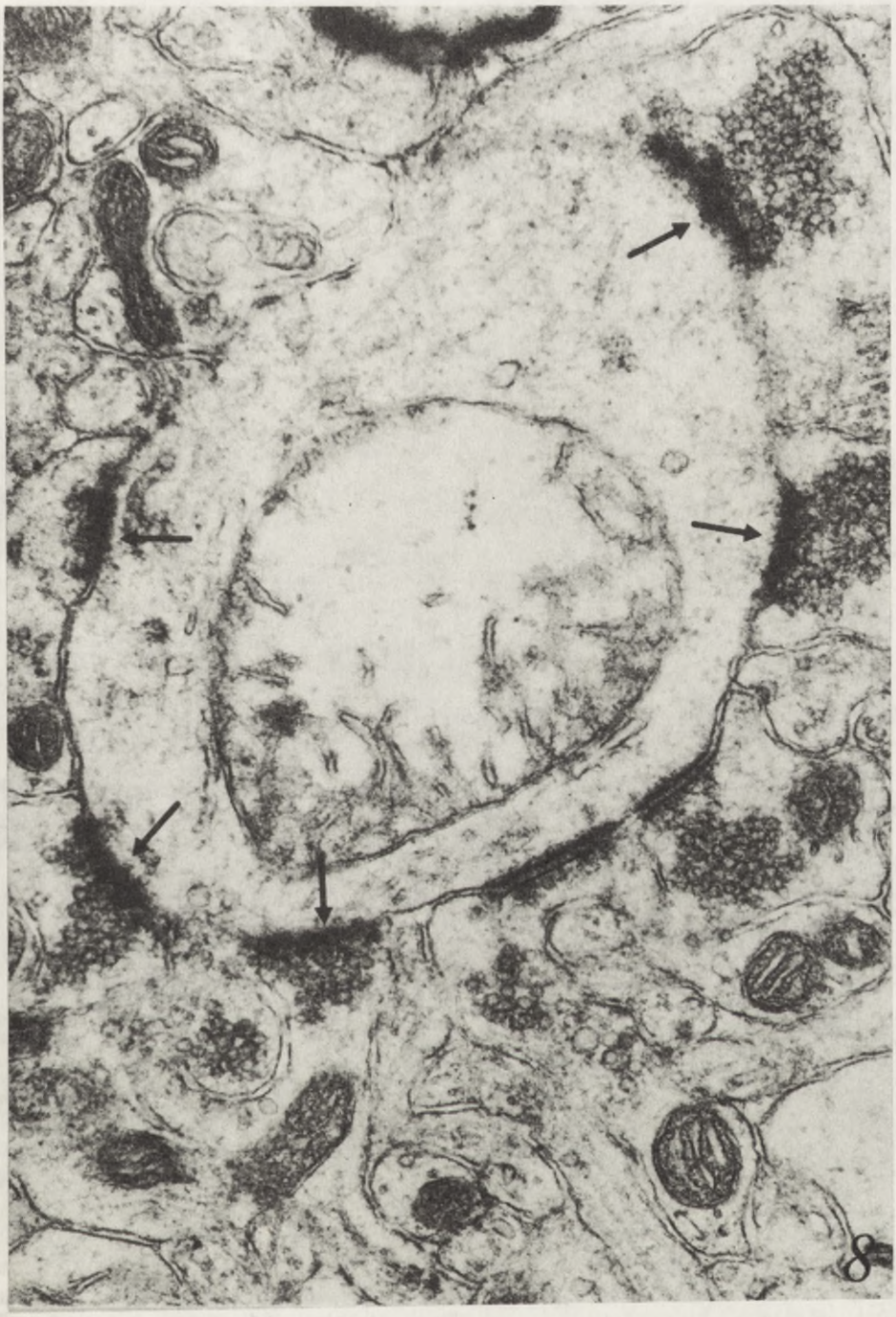

Fig. 8. CA_1 sector, 24 h after ischemia, oxalate-pyroantimonate reaction. Large swollen dendrite surrounded by slightly swollen synaptic bags with irregular aggregations of synaptic vesicles. Some synaptic contacts show dense calcium deposits within synaptic clefts (arrows). ×45 000

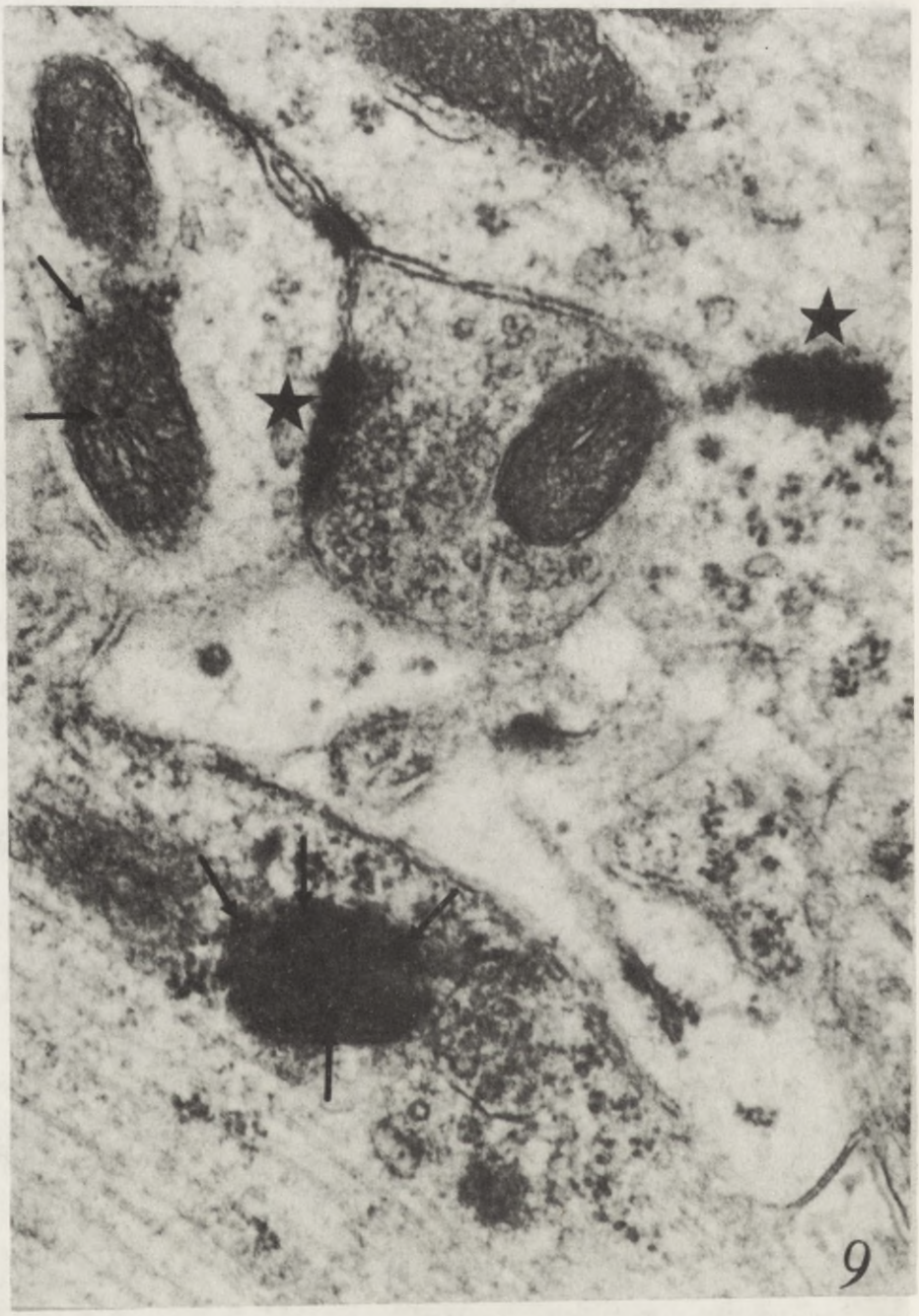

Fig. 9. CA_1 sector, 24 h after ischemia, oxalate-pyroantimonate reaction. Dendritic profiles containg mitochondria with large (ultrastructurally changed) and granular (ultrastructurally normal) calcium deposits (arrows). Note calcium precipitates within synaptic clefts (asterisk). × 60 000

normal and pathological conditions. In the presented series of studies we concentrated our attention on the CA_1 sector of Ammon's horn under conditions of global cerebral ischemia resulting from experimentally induced cardiac arrest in rats, on the basis of the generally accepted hypothesis concerning the damaging effect of intracellular influx of calcium following the excitatory action of amino acid neurotransmitters operating in this particular brain area (Choi 1988; Siesjö, Bengtsson 1989).

The specific target of our observations were synaptic contacts in this region 24 h after restoration of the cerebral blood flow. Selection of this particular postischemic period was connected with earlier literature data indicating the beginning of increased tissue calcium content within 24–48 h following temporary brain ischemia (Deshpande et al. 1987). According to their observations maximmal calcium concentration was concomitant with progression or irreversible neuronal changes. The other reason of this choice were our electron microscopic observations indicative that ultrastructural exponents of calcium deposition in the cytoplasm of sector CA_1 neurons after short lasting forebrain ischemia in Mongolian gerbils started to appear 24 h after restoration of normal cerebral blood flow (Mossakowski et al. 1989). In earlier postischemic stages ultrastructural abnormalities of CA_1 interneurons suggested insufficiency of GABA-ergic inhibition. This inclined us to postulate that the process leading finally to delayed neuronal death may result from imbalance between inhibitory and excitatory stimulation of the CA_1 pyramidal neurons, the former due to temporary insufficiency of GABA-ergic interneurons, the latter – following excessive glutaminergic stimulation *via* Schaffer's collaterals (Gajkowska et al. 1989).

Our cytochemical studies showed that the essential difference between control and experimental studies was basically quantitative and consisted in more abundant calcium precipitation in animals which survived the ischemic insult. The second feature distinguishing both animal groups was distribution of calcium deposits. In the control animals calcium aggregates were more or less equally distributed in the pre- and postsynaptic parts of synapses with marked accentuation in the synaptic clefts. This calcium distribution was previously described by Ohara et al. (1979) and more recently by Probst (1986). In our experimental animals most of the calcium deposits were localized either in the synaptic clefts or in postsynaptic parts, with relatively slight accumulation in presynaptic bags. The increased postsynaptic calcium content indicates its massive influx from the extracellular space to intracellular compartment, related with reduced energy metabolism and damage of transport mechanisms (Siesjö, Wieloch 1985; Wieloch, Westerberg1989). Postsynaptic calcium deposition confirmed the previous observation of van Reempts et al. (1986), who described this phenomenon in another model of cerebral brain ischemia. Very slight calcium accumulation in presynaptic bags in the ischemic animals, lower than in the control ones seems to be related with the examined stage of postischemic events. It has been established by Blaustein et al. (1978) that in the presynaptic terminal calcium influx leads to the fusion of synaptic vesicles with the membranes and to release of neurotransmitters to the synaptic clefts. Vesicles clumping in the direct vicinity of presynaptic densities and significant reduction of the amount of the synaptic vesicles in the axonal endings

may suggest that we were observing final stages of the neurotransmitter secretion. It seems, however, impossible to exclude that swollen and empty synaptic bags belong to damaged inhibitory interneurons, as described in our previous studies (Gajkowska et al. 1989). This hypothesis finds support in their location on the cell bodies of the pyramidal neurons, which is the typical site of inhibitory nerve endings in this part of the hippocampus.

LOKALIZACJA JONÓW WAPNIA W SYNAPSACH HIPOKAMPA SZCZURA PO ISCHEMII

Streszczenie

Oceniono ultrastrukturalną lokalizację złogów wapnia w synapsach hipokampa szczura po 10 min całkowitym niedokrwieniu, stosując cytochemiczną metodę pyroantymonową. Stwierdzono wzrost wewnątrzkomórkowych (w odcinkach pre- i postsynaptycznych) oraz zewnątrzkomórkowych (w szczelinach synaptycznych) złogów wapnia w większości badanych synaps odcinka CA_1 hipokampa. Obfitość złogów wapnia była szczególnie zaznaczona w szczelinach synaptycznych oraz w odcinkach postsynaptycznych. Mitochondria wykazywały również nagromadzenie w nadmiarze złogów Ca. Przedstawione wyniki sugerują zaburzenie synaptycznej homeostazy wapnia po przebytym incydencie niedokrwiennym i wzmożony napływ jonów Ca z przestrzeni zewnątrzkomórkowych do wewnątrzkomórkowych.

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