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INFLUENCE OF SHORT–TERM ISCHEMIA ON THE ULTRASTRUCTURE OF HIPPOCAMPAL GYRUS IN MONGOLIAN GERBIL. III. SYNAPSES IN LATE STAGE OF THE PATHOLOGICAL PROCESS.

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Electron microscope analysis of the CA_1 Ammon's horn sector was performed in Mongolian gerbils three days after an incident of short-term ischemia of the forebrain. CA_1 pyramidal neurons showed advanced disintegration. Some GABA-ergic interneurons revealed ultrastructural alteration of variable degree. The latter finding contradicts the generally helt view on the relative resistance of CA_1 sector interneurons to the ischemic injury. Synapses localized in all cortical layers of the CA_1 sector exhibited ultrastructural abnormalities involving both pre-and postsynaptic parts. They consisted in marked swelling and accumulation of unbound electron dense material, considered as calcium deposits. Presynaptic parts revealed additionally reduced number of synaptic vesicles and their abnormal distribution. Contrary to the early postischemic period, the most severe synaptic alterations appeared in *stratum pyramidale*, *radiatum* and *oriens*, involving both small dendritic branchings and their spines as well as large shafts of both basal and apical pyramidal dendrites.

Synaptic alterations especially features of the postsynaptic damage correspond to those indicating excitotoxic neuronal lesions. Presynaptic alterations may indicate both cessation of neurotransmission function as well as direct ischemic damage. The presence of calcium deposition seems to favour the former possibility.

Key words: *Brain ischemia, hippocampus, CA_1 sector of Ammon's horn, synapses.*

Delayed death of the pyramidal neurons in the CA_1 hippocampal sector after short-term cerebral ischemia is preceded by their hyperactivity (Suzuki et al. 1983; Mossakowski et al. 1989), and accumulation of Ca^{2+} ions in their cytoplasm (Van Reempts et al. 1986; Deshpande et al. 1987). Accumulation of calcium ions leads to severe neuronal damage (Łazarewicz et al. 1975; Siesjö 1981) through stimulation of lipolysis (Strosznajder 1980), proteolysis (Baudry et al. 1981), phosphorylation of proteins (Baudry, Lynch 1980) and to dispersion of cytoskeletal elements (Siesjö, Wieloch 1985).

Numerous experimental data suggest that the delayed death of the pyramidal neurons in the CA_1 sector of Ammon's horn results from the excitotoxic action of amino acid neurotransmitters, mostly glutamate (Kirino

1982; Jörgensen, Diemer 1982; Pulsinelli 1985a). Selective vulnerability of this particular hippocampal area is connected with its specific synaptic organization, namely, with rich innervation of CA_1 pyramidal neurons by glutaminergic Schaffer's collaterals (Collingridge et al. 1983). This hypothesis was convincingly proven in experiments revealing the cytoprotective effect on the pyramidal neurons of both glutaminergic deafferentation of the area (Pulsinelli 1985b) and application of specific blockers of NMDA receptors (Simon et al. 1984).

In the light of this concept an analysis of the synaptic changes in the CA_1 sector resulting from brain ischemia seems to be fully justified.

This study is a continuation of the previously carried out experiments analysing ultrastructural changes in the CA_1 sector of the hippocampus in Mongolian gerbils after an ischemic incident (Gajkowska et al. 1988, 1989), in which detailed morphological evaluation of synapses at an early postischemic period (12 and 24 h) was done. The studies disclosed in all layers of the CA_1 hippocampal sector ultrastructural features of increased synaptic activity of excitatory character with reduced synaptic activity of inhibitory type. These observations inclined us to perform a detailed ultrastructural analysis of synapses of the hippocampal CA_1 sector at a later period after the ischemic insult. The third postischemic day, when most of the pyramidal cells exhibit severe ultrastructural changes, was chosen. All layers of the CA_1 sector of Ammon's horn were analysed.

MATERIAL AND METHOD

Experiments were performed on 10 male, 3-month-old Mongolian gerbils, which were subjected to 5-min forebrain ischemia, produced by bilateral common carotid artery ligation according to the method described previously (Gajkowska et al. 1988). Following the experimental procedure the animals remained in normal laboratory conditions for three days and then were sacrificed under ether anesthesia by transcardiac perfusion with 2% glutaraldehyde. The tissue blocks, containing all cortical layers of Ammon's horn CA_1 sector were taken and processed in a routine way for electron microscope examination. Brains of 5 other animals not subjected to any experimental procedure served as control material.

RESULTS

The ultrastructure of subsequent layers of CA_1 sector of Ammon's horn is described separately (Gajkowska et al. 1988).

Stratum pyramidale. Pyramidal neurons occupying this layer display alterations of various intensity. In most of them there is disintegration of cytoplasm and organelles. Many cellular organelles are greatly swollen. This concerns mostly the Golgi apparatus and mitochondria. The cytoplasm contains large electron-lucent vacuoles, numerous polymorphic lysosomes and a variable number of ribosomes and polyribosomes. Elements of the cytoskeleton are fragmented and haphazardly distributed (Fig. 1). In both neuronal cytoplasm and dendritic processes there are small unbound aggregates of

Fig. 1. Stratum pyramidale. Fragment of cytoplasm of pyramidal neuron containing swollen Golgi complex (AG), mitochondria (M), electron-lucent vacuoles (V) and aggregations of electron-dense material (arrows). The majority of synapses on the neuron and in the neuropil are normal. Occasional astrocytic processes (A) are swollen. × 30 000

electron dense material, identified as calcium deposits. Intercellular spaces are sometimes dilated with loss of synaptic contacts. The remaining neurons of this layer, corresponding to interneurons, generally retain their normal ultrastruc-

ture. Only in few interneurons, swelling of some mitochondria and peripheral portion of the cytoplasm are observed; the number of lysosomes is also increased (Fig. 2). On the surface of pyramidal neurons and interneurons there is a great number of symmetric type synapses with normal ultrastructural

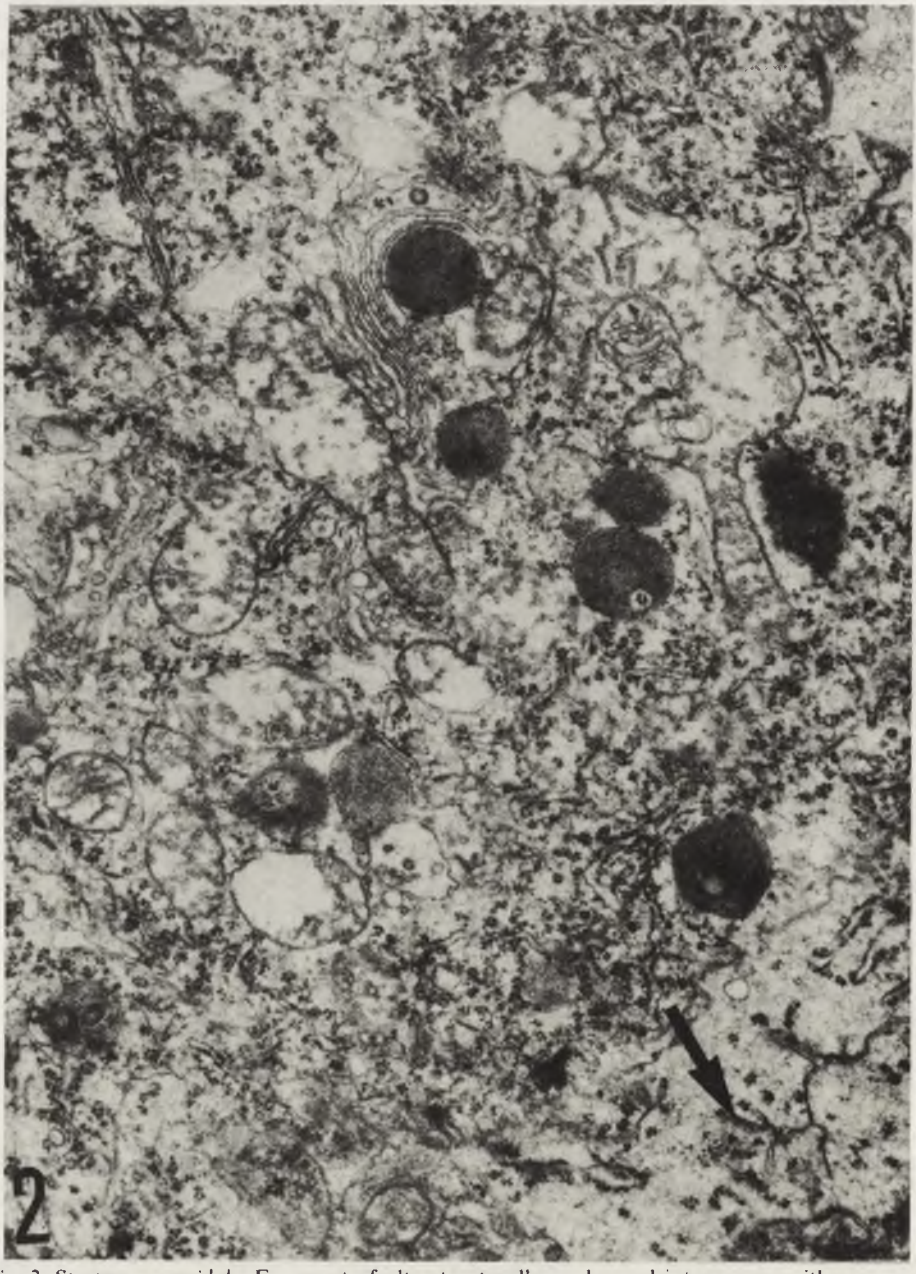

Fig. 2. Stratum pyramidale. Fragment of ultrastructurally unchanged interneuron with numerous lysosomes. Peripheral part of the cytoplasm swollen with reduced endoplasmic reticulum (arrow). × 22 500

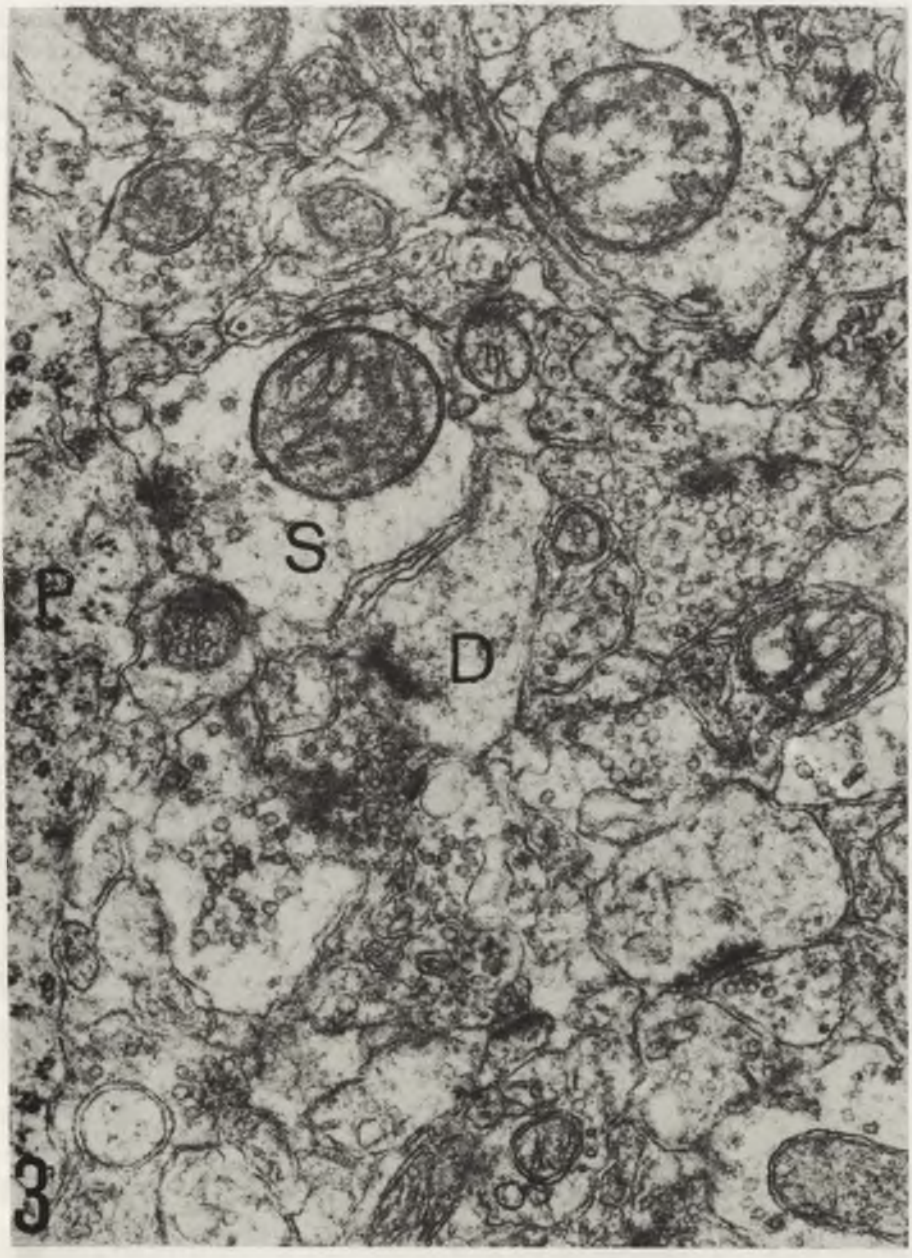

Fig. 3. Stratum pyramidale. Swollen axonal ending (S) contacting surface of the pyramidal neuron (P). In the neuropil some swollen dendritic spines (D) with normal presynaptic parts are visible. × 45 000

appearance (Fig. 1 and 2). A great part of asymmetric synapses, present in the neuropil surrounding pyramidal neurons, reveal severe ultrastructural abnormality, mostly in the form of swelling of the dendritic spines. Synapses are also encountered, although less frequently, which are swollen in the presynaptic

part, with only few synaptic vesicles usually aggregated near the synaptic density (Fig. 3). In the neuropil of this layer there are many greatly swollen dendrites with electron lucent vacuoles, few short, haphazardly dispersed neurotubules and swollen mitochondria (Fig. 4). The synaptic endings on their surface do not display any ultrastructural changes. Swollen astrocytes are also present in the neuropil of this layer (Figs. 1 and 4).

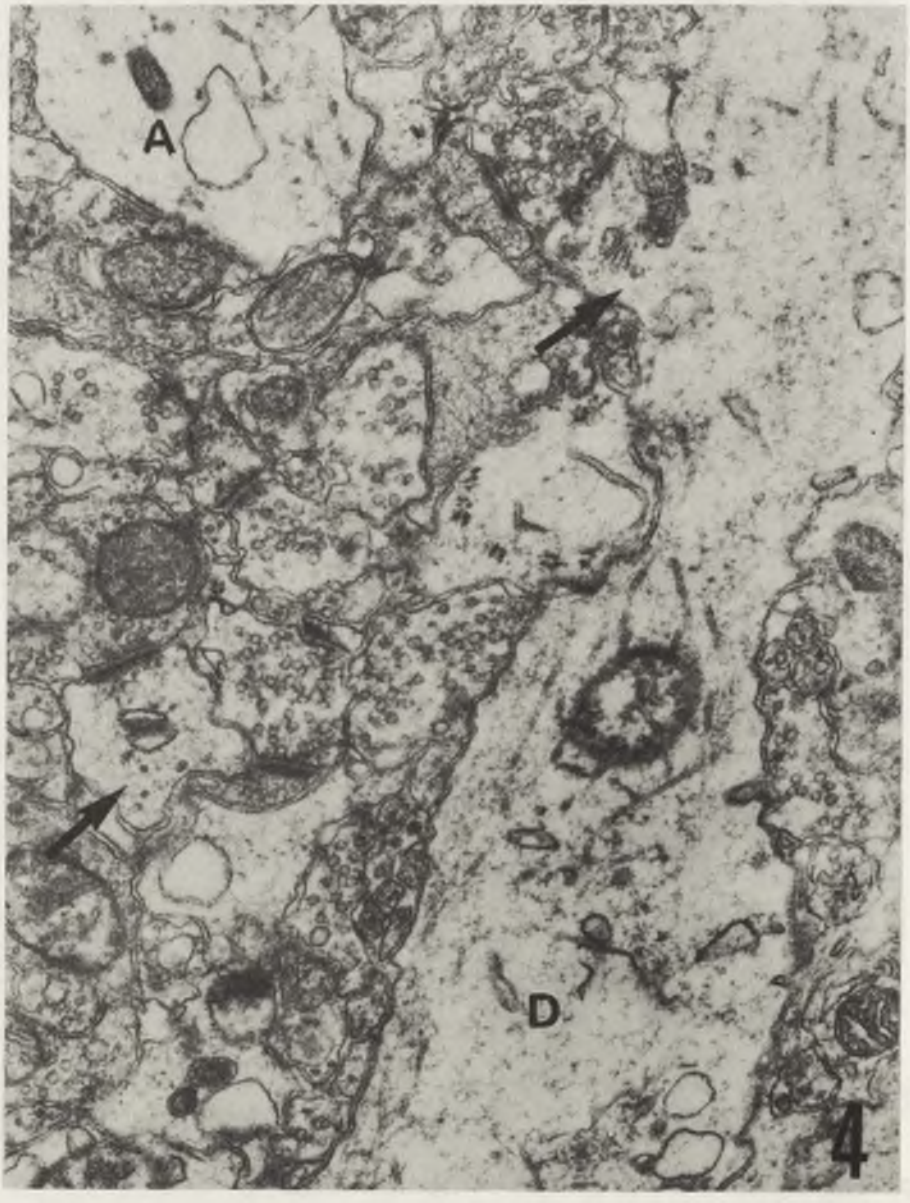

Fig. 4. Stratum pyramidale. Swollen dendrite (D) with fragments of cytoskeletal elements, swollen mitochondria and electron-lucent vacuoles. Note swollen dendritic spine (arrow). Most of the axonal endings are unchanged. Some astrocytic processes (A) are swollen. × 27 000

Stratum oriens. In this layer the great majority of synapses contacting basal dendrites of the pyramidal cell represent axonal endings of interneurons and granular cells. Most of symmetrical and asymmetrical synapses preserve normal structure in both pre- and postsynaptic parts (Fig. 6). However, there

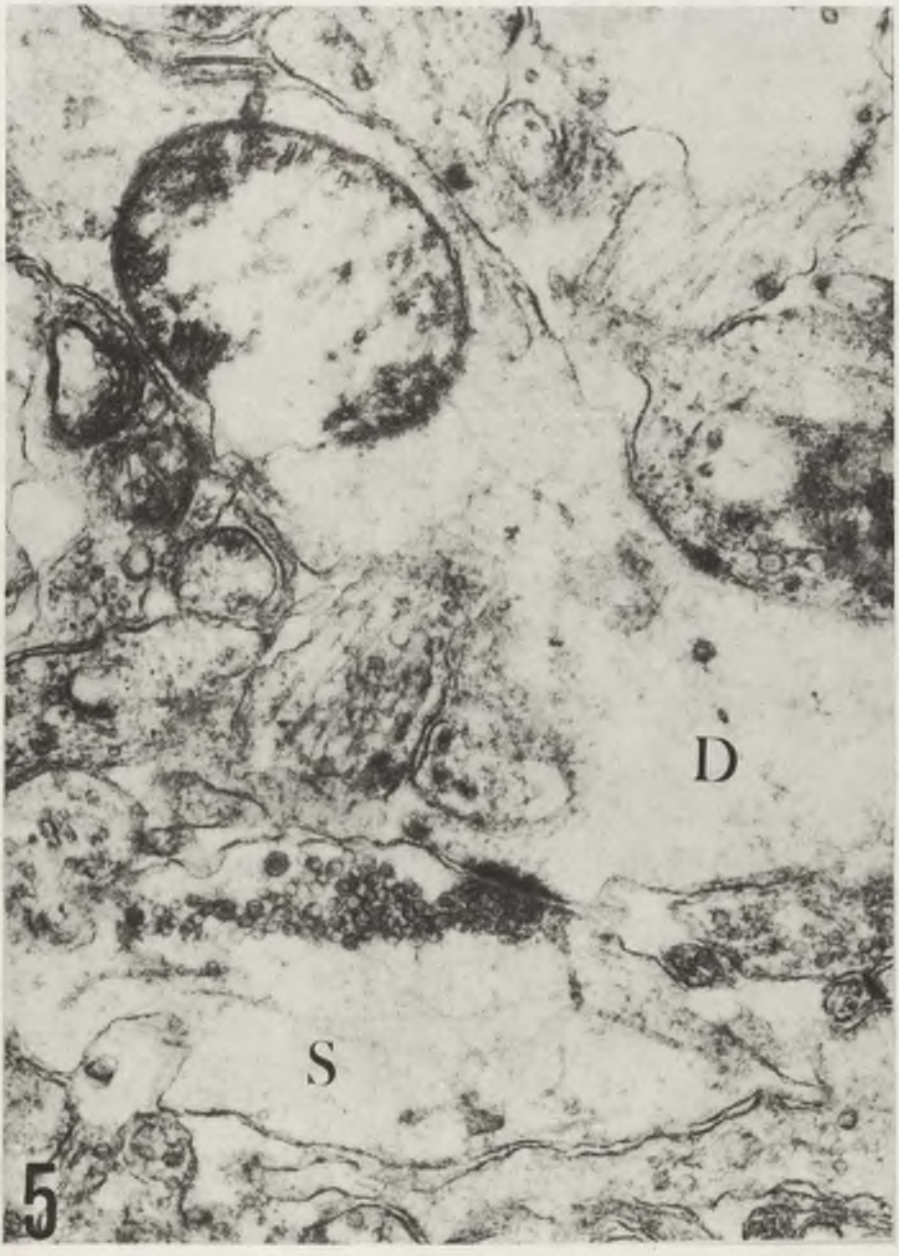

Fig. 5. Stratum oriens. Remarkable swelling of presynaptic (S) and postsynaptic parts (D) of a synapse. Note abnormal clumping of synaptic vesicles in the axonal ending and mitochondrial swelling in the dendrite. × 45 000

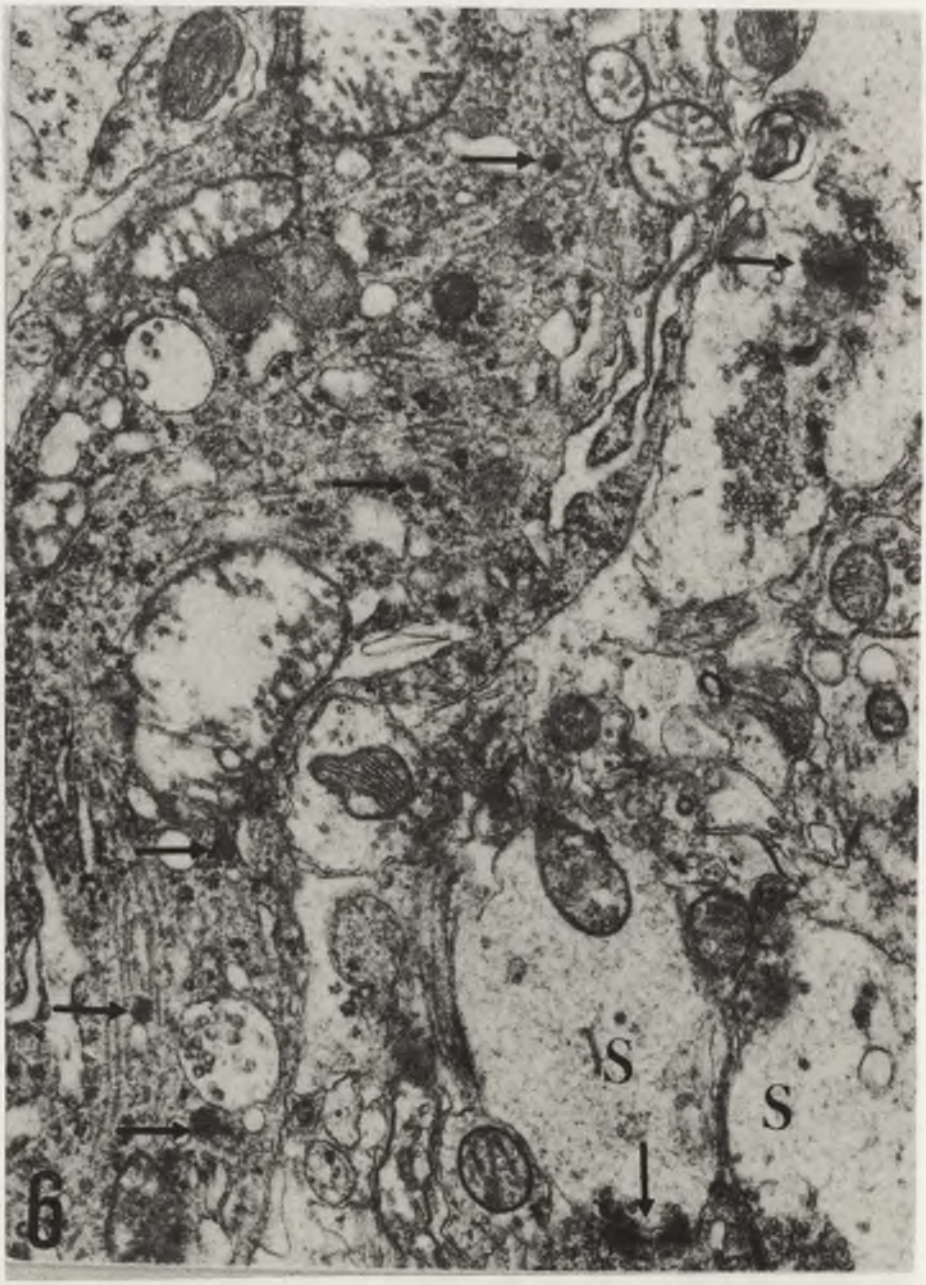

Fig. 6. Stratum radiatum. Fragment of apical dendrite with condensed cytoplasm. Dendritic cytoplasm contains abundant organelles, swollen mitochondria and unbound clusters of electron-dense material (arrows). In swollen presynaptic parts (S) abnormal aggregations of synaptic vesicles and small clusters of dense material (arrows) are present. × 30 000

also are some swollen synapses. Swelling involves both basal pyramidal dendrites and their branchings as well as presynaptic endings in which most of the vesicles accumulate in the vicinity of the active region of the synaptic cleft.

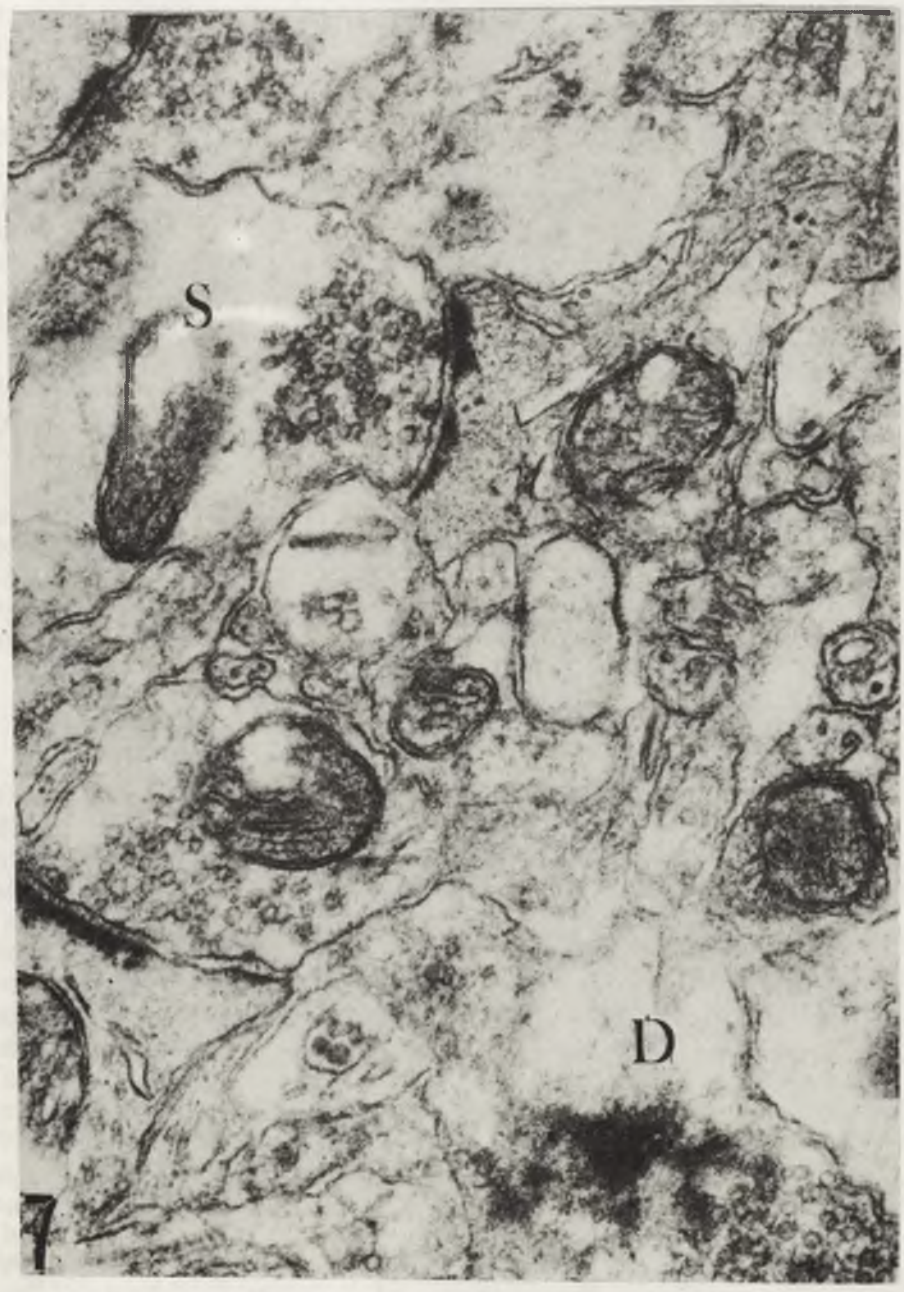

Fig. 7. Stratum lacunosum-moleculare. In the neuropil numerous synapses with swollen both pre- (S) and post-synaptic (D) parts are visible. × 60 000

This concerned the symmetrical as well asymmetrical synapses (Fig. 5). Many astrocytes in this layer display remarkable swelling.

Stratum radiatum. Synaptic contacts present in this layer are mostly formed by nerve endings of Schaffer's collaterals and interneurons as well as nerve

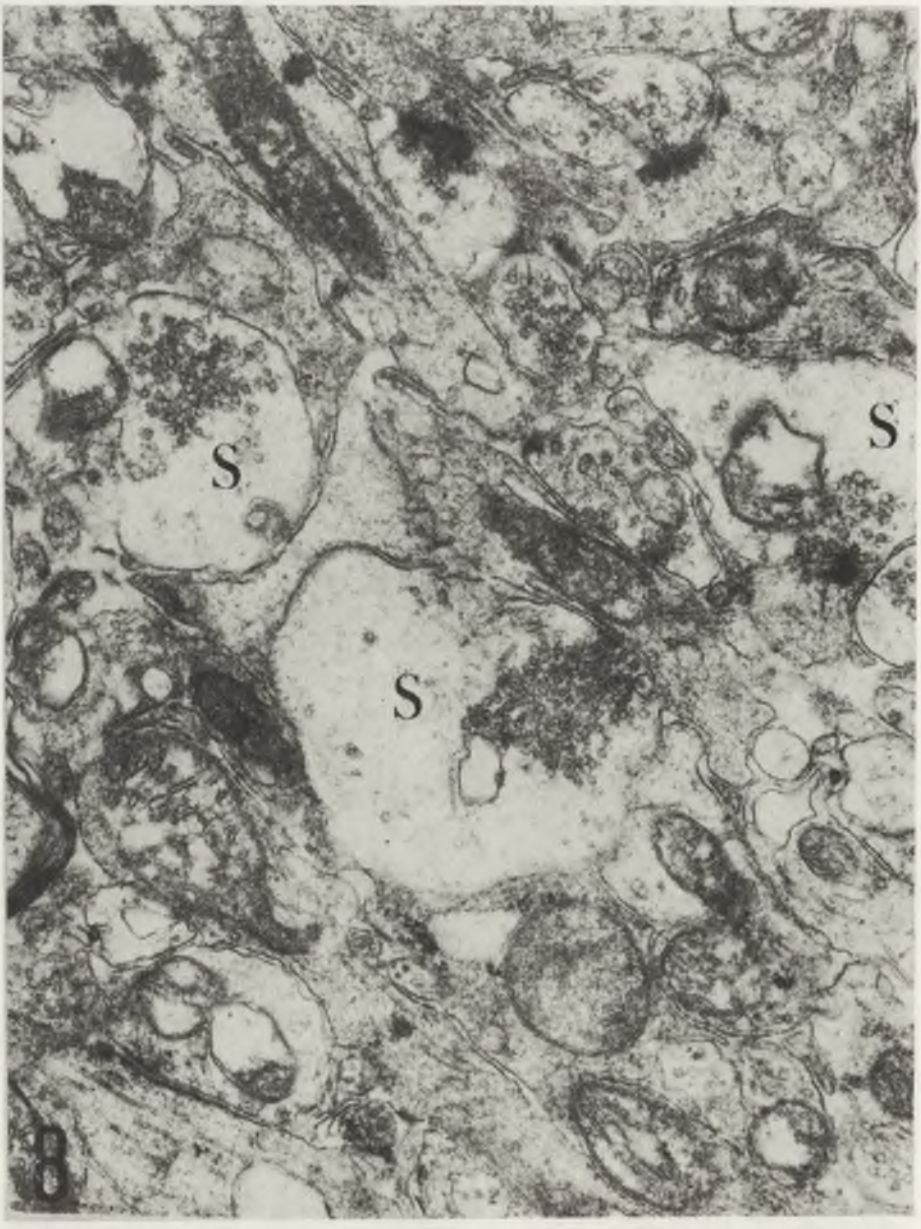

Fig. 8. Stratum moleculare. In the neuropil numerous synapses with swollen presynaptic (S) and unchanged postsynaptic parts are visible. Note clumping of synaptic vesicles. × 36 000

fibers deriving from the dentate gyrus. Apical dendrites of pyramidal neurons, accumulated here usually display some ultrastructural changes. Their condensed cytoplasm contains many organelles and elements of cytoskeleton. Small, unbound aggregates of high electron density are present (Fig. 6). Almost all mitochondria are swollen. Synaptic bulbs contacting such dendrites are also swollen. They contain few synaptic vesicles which accumulate near the active

synaptic region and deposits of electron-dense material (Fig. 6). In the same layer there are also normal synapses filled with a great abundance of synaptic vesicles. Some glial cells also show swelling.

Stratum lacunosum-moleculare. Synapses in this layer are formed by nerve endings of commissural fibers, Schaffer's collaterals and interneurons. They contact here branches of apical dendrites of pyramidal cells. A characteristic feature of the layer are "en passant" synapses formed by fibers deriving from interneurons. Most of the synapses present here, representing both symmetrical and asymmetrical types, usually preserve normal ultrastructure. However, some synapses with remarkably swollen both pre- and postsynaptic part are visible (Fig. 7).

Stratum moleculare. The anatomical composition of this layer is almost identical with *stratum lacunosum-moleculare.* The postsynaptic pool is formed by distal branchings of apical dendrites of the pyramidal cells. The nerve endings contacting them derive from interneurons and Schaffer's collaterals. The additional source of nerve endings consists of fibers originating from the entorhinal cortex. The ultrastructure of this layer is similar to that in *stratum lacunosum-moleculare.* In numerous presynaptic bags swelling of synaptoplasm with abnormal accumulation of synaptic vesicles is observed. (Fig. 8). Changes in postsynaptic parts are less common. In quite a number of otherwise normally looking dentrites clusters of electron-dense material are present.

DISCUSSION

Electron microscopic analysis of the CA_1 sector of Ammon's horn 3 days after the short-term ischemic incident revealed ultrastructural abnormalities in all cortical layers, involving both neuronal perikarya and synapses. Changes of pyramidal neurons were identical with those described in our previous studies (Mossakowski et al. 1989). Comparing with earlier postischemic stages (Gajkowska et al. 1989) they were more advanced and consisted mostly in disintegration of cellular cytoplasm, accompanied by accumulation of electron-dense, unbound aggregates, considered as calcium deposits. Great variance in the intensity of neuronal damage was noted. The ultrastructural picture of small interneurons located in the pyramidal layer deserves a short comment. These GABA-ergic neurons are usually considered as relatively resistant to the ischemic incident (Johansen et al. 1983; Mossakowski et al. 1989; Nitsch et al. 1989). Our previous study, concerning early postischemic changes in the CA_1 sector of Ammon's horn (Gajkowska et al. 1989) showed the appearance of marked, although reversible, alterations of the pyramidal layer interneurons. They consisted in severe swelling of cytoplasm and processes 12 h after the ischemic incident. Their ultrastructural picture returned to normal 24 h after ischemia. Our present study showed that 3 days after short-term ischemia some of the interneurons showed pronounced abnormalities in the form of mitochondrial swelling, an increased number of lysosomes and rarefaction of the peripheral part of their cytoplasm with reduction of endoplasmic reticulum channels, their denudation and decreased number of mono- and polyribosomes. This indicates that, contrary to previous opinions (Francis, Pulsinelli, 1982; Johansen et al. 1983, Mossakowski et al. 1989), interneurons of the CA_1

sector are also sensitive to the ischemic incident. Comparison of their early and late postischemic changes indicate that ultrastructural features of their alteration appear in the early recirculation stage and are less intensive as compared with lesions of pyramidal neurons.

Ultrastructural abnormalities of synapses concern both those of symmetrical type, located mostly on neuronal perikarya and dendritic shafts and asymmetrical type connected first of all with dendritic spines. Pre- and postsynaptic parts are involved, although their alterations vary in intensity, ultrastructural expression and localization. Two ultrastructural features were common for both pre- and postsynaptic parts. These were swelling and appearance of electron-dense unbound material, identified as calcium aggregates. Swollen axonal endings revealed additionally reduced contents of synaptic vesicles, being usually clumped in the vicinity of active zones. Postsynaptic lesions took the form of cytoplasmic swelling of dendrites and/or their spines, with accompanying damage of cytoskeletal structures, mitochondrial swelling and dilatation and fragmentation of endoplasmic reticulum channels. The great variability of the above described synaptic changes is to be stressed. Alongside with normal synapses there were synapses with remarkably swollen both pre- and postsynaptic parts or synapses in which ultrastructural abnormalities appeared only in the presynaptic or postsynaptic parts. In some cases calcium deposits were present in otherwise normally looking synapses.

Abnormalities observed 3 days after the ischemic incident differed from those occurring in the early postischemic period (Gajkowska et al. 1989). At that time swelling of postsynaptic parts with mostly intact axonal endings dominated. This corresponded to axon-sparing synaptic damage, typical for abnormalities resulting from the excitotoxic action of neurotransmitters (Schwarcz et al. 1983). Distribution of the ultrastructural abnormalities observed was also different. In an early postischemic period pathological changes prevailed *in stratum lacunosum-moleculare* and *stratum moleculare*, in which most of the axonal endings of Schaffer's collaterals are located. In the presently examined material abnormalities in these layers, although present were less conspicuous than in the *stratum pyramidale*, *oriens* and *radiatum*. Here, in addition to alterations involving small dendritic branchings and spines, lesions of large shafts of basal and apical dendrites were observed. The nature of the latter differed in both layers. In *stratum oriens* swollen basal dendrites of the pyramidal neurons dominated, while in *stratum radiatum* apical dendrites with condensed cytoplasm prevailed.

In the light of advanced damage of pyramidal neurons in the examined postischemic period, the question arises to what extend dendritic abnormalities, especially those concerning large apical and basal shafts can be considered just as an element of progressing neuronal injury involving both the perikarya and processes and leading to cellular death or as an exponent of the specific mechanism of this damage connected with neurotransmitter toxicity. Delimitation of these two components seems impossible, due to the complexity of structural changes and mechanisms involved. Involvement of both synaptic parts indicates that we are dealing with a process more complicated than pure axon-sparing synaptic damage, related with the excitotoxic action of an amino acid neurotransmitter.

Swelling of nerve endings, changes in the content and distribution of synaptic vesicles may reflect both disturbances in neurotransmission in the sense of exhaustion due to oversecretion or disturbances in membrane permeability resulting from ischemia. The same may concern postsynaptic changes. Mitochondrial abnormality may support the latter mechanism. Horseradish peroxidase studies performed by Diemer and Ekström von Lubitz (1983) revealed abnormalities in synaptic membrane permeability in the CA_1 hippocampal sector in the case of cerebral ischemia. However, the permeability changes described by them concerned mostly postsynaptic membranes with very little involvement of axonal endings and they appeared in much earlier postischemic period.

It seems that the synaptic abnormalities observed in our material correlate well with the general concept of the excitotoxic mechanism of delayed neuronal death. This concerns both elements of synaptic pathology – calcium deposition and swelling of the pre- and postsynaptic parts.

The metabolic cascade initiating delayed death of hippocampal pyramidal neurons begins by a decrease of adenosine triphosphate reserves (Norberg, Siesjö 1975; Onodera et at. 1986), accompanied by accumulation of calcium ions in presynaptic endings and increased production of glutamate (Benveniste et al. 1984). Influx of Ca^{2+} at the nerve terminals triggers synaptic release of neurotransmitter (Rubin 1970). Increased release of glutamate and its reduced re-uptake due to lack of energy resources (Choi 1987) result in an enhanced neurotransmitter content in the extracellular spaces. This phenomenon was observed during ischemia in selectively vulnerable areas of the hippocampus by Benveniste et al. (1984) and Hagberg et al. (1985). Moreover, there seem to exist a close correlation between disorders in calcium homeostasis and the level of excitotoxic amino acids preceding delayed neuronal death in the CA_1 hippocampal sector following cerebral ischemia (Sakamoto et al. 1986; Ogura et al. 1988).

Glutamate accumulated in the synaptic clefts induces activation of glutaminergic receptors, this being accompanied by an increased influx of calcium ions to the postsynaptic part (Sakamoto et al. 1984). Initiated in that way intracellular calcium accumulation activates Ca-dependent enzymes, among others proteases and phospholipase A_1 leading in turn to disintegration of cellular membranes (Abe et al. 1987) and cytoskeletal elements (Yanagihara et al. 1985). Progressing cellular disintegration, connected with calcium accumulation leads finally to neuronal death (Siesjö 1981; Van Reempts et al. 1986; Deshpande et al. 1987). All the above presented data clearly indicate that calcium entry into the cell is a major mediator of the glutamate destructive effect.

Intracellular calcium entry is accompanied by influx of other ions from the extracellular space, mostly sodium and chlorides. This is followed by water redistribution between extra- and intracellular compartments resulting in cellular swelling.

Most probably the process concerns synapses of excitatory character, although it is difficult to exclude synapses of inhibitory nature. Some immunocytochemical studies indicate the coexistence of neurotransmitters of both excitatory and inhibitory character in synapses, deriving from interneurons and contacting the perikarya and dendritic shafts of pyramidal neurons (Somogyi et al. 1984; Kosaka et al. 1985). These observations suggest the need of modification of the traditional morphological classification of hippocampal synapses, even more so, since there are many controversial morphological descriptions of them (Chang, Greenaught 1984). These controversies concern mostly differences in distribution and density of synaptic vesicles considered as morphological exponents of neurotransmission processes (Applegate et al. 1987).

WPŁYW KRÓTKOTRWAŁEGO NIEDOKRWIENIA NA ULTRASTRUKTURĘ ZAKRĘTU HIPOCAMPA U CHOMIKÓW MONGOLSKICH III SYNAPSY W PÓŹNEJ FAZIE PROCESU PATOLOGICZNEGO

Streszczenie

Przeprowadzono elektronowo-mikroskopową analizę sektora CA_1 rogu Ammona u chomików mongolskich po upływie 3 dni od krótkotrwałego niedokrwienia kresomózgowia. Wykazano cechy rozpadu neuronów piramidowych oraz zróżnicowanego nasilenia uszkodzenia interneuronów. Spostrzeżenie to podważa pogląd o oporności na niedokrwienie GABA-ergicznych interneuronów sektora CA_1 hipokampa. Synapsy położone we wszystkich warstwach sektora Ca_1 wykazywały ultrastrukturalne cechy uszkodzenia, obejmujące zarówno ich części przed- jak i posynaptyczne. Wyrażały się one obrzmieniem oraz nagromadzeniem nieobłonionego, elektronowo gęstego materiału, ocenianego jako złogi wapnia. Części presynaptyczne wykazywały ponadto zmniejszenie liczby pęcherzyków synaptycznych i ich nieprawidłowe rozmieszczenie. W odróżnieniu od wczesnego okresu poniedokrwiennego nasilone zmiany synaps występowały przede wszystkim w *stratum pyramidale*, *radiatum* i *oriens* i dotyczyły obok drobnych rozgałęzień dendrytycznych ich kolców, również dużych pni dendrytów podstawowych i szczytowych. Nieprawidłowości obrazu synaps, zwłaszcza przyjmujące postać uszkodzeń postsynaptycznych, mogą stanowić wykładnik ekscytotoksycznego mechanizmu zmian neuronalnych. Zmiany części presynaptycznych mogą zarówno wyrażać zaburzenia procesów neurotransmisji w sensie ich wyczerpania, jak i uszkodzenia związane bezpośrednio z niedokrwieniem. Nagromadzenie w nich złogów wapnia wydaje się przemawiać na korzyść pierwszego mechanizmu.

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