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IMMUNO-ELECTRON MICROSCOPIC DEMONSTRATION OF GABA AND GLUTAMATE SYNAPSES IN MONGOLIAN GERBILS HIPPOCAMPUS AFTER ISCHEMIA

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The subcellular distribution of glutamate and GABA in synapses of Mongolian gerbils hippocampus was examined using post-embedding immunogold staining method for electron microscopy. Immunolabelling was performed with 10 nm gold-antibody complexes for glutamate and GABA. The gold particle densities gave reliable information about the relative concentrations of these amino acid neurotransmitters. Our results indicate that ischemia leads to the temporal decrease of GABA-like immunoreactivity in symmetric synapses and a slight enhancement of the level of glutamate-like reactivity in asymmetric ones. The striking finding was a redistribution of glutamate-like immunoreactivity from neurons to glia. This suggests the capacity of glia to metabolize the excess of glutamate after ischemia. The disturbances at the level of neurotransmitters and their possible role in hippocampal neuronal injury were stressed.

Key words: *forebrain ischemia, GABA, glutamate, immunogold immunocytochemistry*

The amino acids: glutamate (Glu) and γ-aminobutyric acid (GABA) play an important function in vertebrate brain. It is generally accepted that GABA is the major inhibitory transmitter in the central nervous system and Glu and aspartate (Asp) are the most prevalent excitatory transmitters.

The hippocampus has drawn much attention as a typical place where a delayed neuronal death develops after brief transient ischemia. In the recent papers the major role for synapses as regulators of pathological postischemic processes is suggested. The most deleterious effect can be attributed to the excitotoxic amino acid neurotransmitters (Jorgensen, Diemer 1982). Excitatory synapses on hippocampal pyramidal cells are mostly located on dendritic spines. It is estimated that there are as many as 12,000 excitatory boutons per one CA1 pyramidal cell in rats (Andersen et al. 1990). Glutamate has been shown to fulfill several of the criteria for a neurotransmitter in the synapses between the Schaffer collateral/commissural fibers and the CA1 pyramidal cells (Nadler et al. 1976; Storm-Mathisen, Ottersen 1984). Ten percent of the hippocampal neuronal population are interneurons (Olbrich, Braak 1985). The majority of them (80-95%) are believed to utilize GABA as neurotransmitter (Babb et al. 1988; Woodson et al. 1989). These intrinsic GABAergic interneurons play an important role in the hippocampus by offsetting excitatory inputs to the pyramidal cells.

Our previous morphological investigations revealed early postischemic changes of the CA1 GABAergic interneurons reversible in nature (Gajkowska et al. 1989). These findings inclined us to perform the immunocytochemical studies on the distribution of the two classical neurotransmitters: glutamate and GABA in this hippocampal area. The aim of the study was:

1) the analysis of the distribution of Glu and GABA in synapses of the CA1 sector of hippocampus,
2) the investigation of the profile of presynaptic terminal damage in the gerbil model of bilateral cerebral ischemia in which the characteristic selective neuronal damage (i.e. delayed neuronal death) can be seen in the CA1 region of hippocampus following a brief forebrain ischemia,
3) the confirmation with an application of immunocytochemical studies of the transient insufficiency of interneuron function, postulated in previous studies.

Material and methods

Adult Mongolian gerbils of both sexes weighing 50-80 g were used in the present study. Forebrain ischemia was induced as described previously by bilateral ligation of carotid arteries (Gajkowska et al. 1989). After 7.5 min lasting ischemia the ligatures

were removed and 6, 12, 24 hours after recovery of blood circulation animals were sacrificed. Brains of the animals were fixed by a transcardiac 15 min perfusion with a solution of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) and subsequently rinsed (1 min) with 0.9% NaCl. At the end of the postfixation period blocks of tissue were taken from the *stratum radiatum* and *stratum pyramidale* of CA1 sector of hippocampus, rinsed overnight in buffer, treated with 1% osmium tetroxide for 1 hour, dehydrated in a graded series of ethanol and embedded in Epon. Ultrathin sections were prepared according to the postembedding immunogold technique. The primary antisera to GABA (Biogenesis cat. No. 4610-0004) and Glu (Biogenesis cat. No. 4670-5021) were diluted in TRIS phosphate buffered saline to 1:400 and 1:5 respectively. The antisera were applied to the sections mounted on the formavar-coated golden grids for 130 min. The sections were pretreated with 10% H_2O_2 to alleviate the masking effect of OsO_4 and Epon. To reveal the primary antibodies the sections were exposed for 30 min to colloidal gold particles (diameter 10 nm) coated with goat anti-rabbit IgG diluted to 1:20 (Janssen, Belgium). Sections were stained with 1% uranyl acetate for 15 min and lead citrate for 2 min. Electron micrographs from *stratum radiatum* and *stratum pyramidale* of CA1 sector of hippocampus of control and experimental animals were taken by JEOL 1200Ex electron microscope.

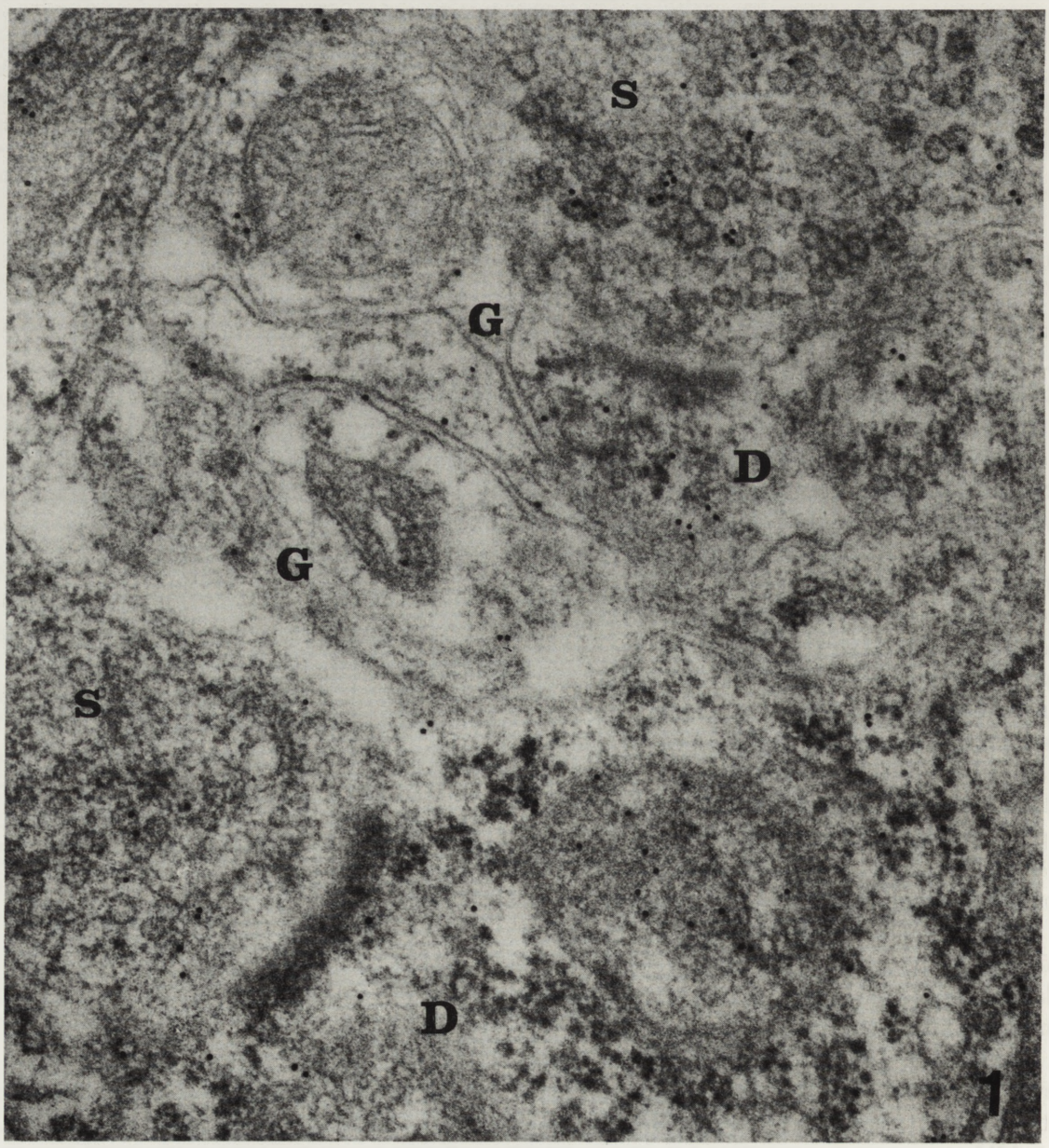

Fig. 1. Control animal. Glutamate-like immunoreactivity (black gold particles) located on synaptic vesicles appears mainly in asymmetrical synapses (S). Very low immunoreactivity in glial process (G) and postsynaptic parts (D). × 30 000

Results

I. Glutamate

Control animals. The gold particle densities were found to be abundant in nerve terminals forming asymmetric contacts with dendritic spines in *stratum radiatum* of CA1 (i.e. terminals of Schaffer's collaterals and commissural fibers. The gold particle densities were of moderate intensity in dendrites, dendritic spines and cell bodies of CA1 pyramidal cells and very slight in glial processes. The densities were more intensive in glutamatergic nerve terminals than in any other tissue elements (Fig. 1). In here the gold particles appeared to be closely related to synaptic vesicles (often seen over vesicle profiles) indicating that glutamate immunoreactivity was located in the synaptic vesicles. In neuronal and glial cell bodies the gold particles demonstrating glutamate-like immunoreactivity, were found over all cellular components, except some cytoplasmic inclusion bodies. The mitochondrial profiles were slightly richer in gold particles as compared with the surrounding cytoplasm (Fig. 1).

Putative GABAergic terminals forming symmetric contacts with CA1 pyramidal cell bodies or stem dendrites showed no immunolabelling or very low particle density, comparable to that over glial processes.

Experimental animals. Six hours after ischemia the distribution of glutamate-like immunoreactivity was

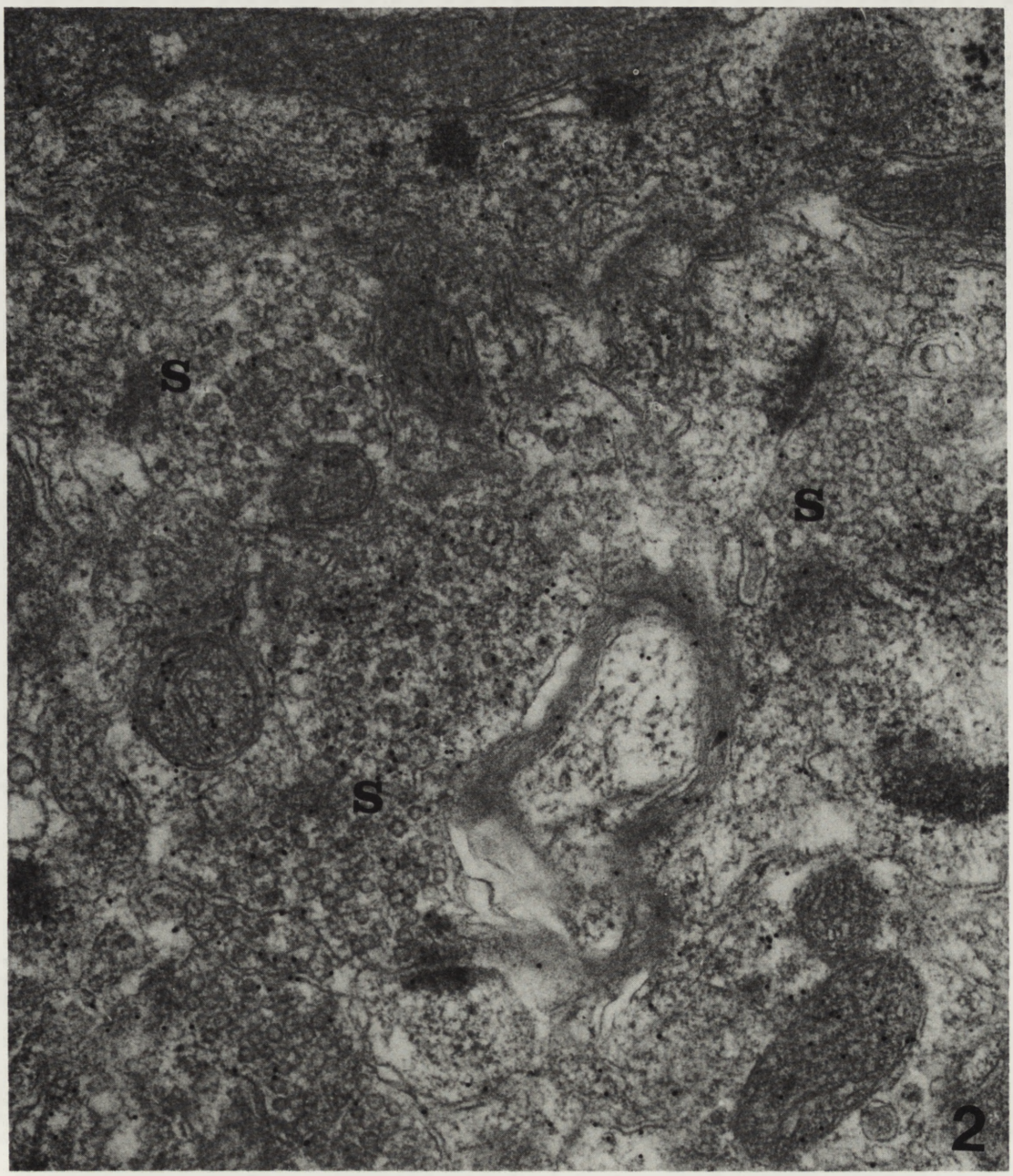

Fig. 2. Experimental animal: 6 h after ischemia. In some asymmetric synapses (S) glutamate-like immunoreactivity higher than in others. Mitochondria are strongly labelled. × 15 000

similar to that seen in the control animals, but its concentration varied in all the tissue profiles.

The labelling intensity in asymmetric contacts of nerve terminals was slightly higher than that in control animals (Fig. 2). On the contrary the immunoreactivity of glutamate in the astrocytic processes was clearly increased after ischemia (Fig. 3). The increase of gold particle density over mitochondria, microtubules in dendrites and profiles of myelinated axons was also observed (Fig. 4).

Some nerve terminals forming asymmetric junction in *stratum radiatum* of CA1 sector seemed to be slightly swollen and less intensely labelled. Gold particles were usually closely related to synaptic vesicles, although they were seen also over areas of cytoplasm devoided of synaptic vesicles (Fig. 5). Twelve and 24 hours after ischemia the immunoreactivity in asymmetric terminals and in other neuronal intracellular compartments was similar to that seen in the control animals. The labelling intensity

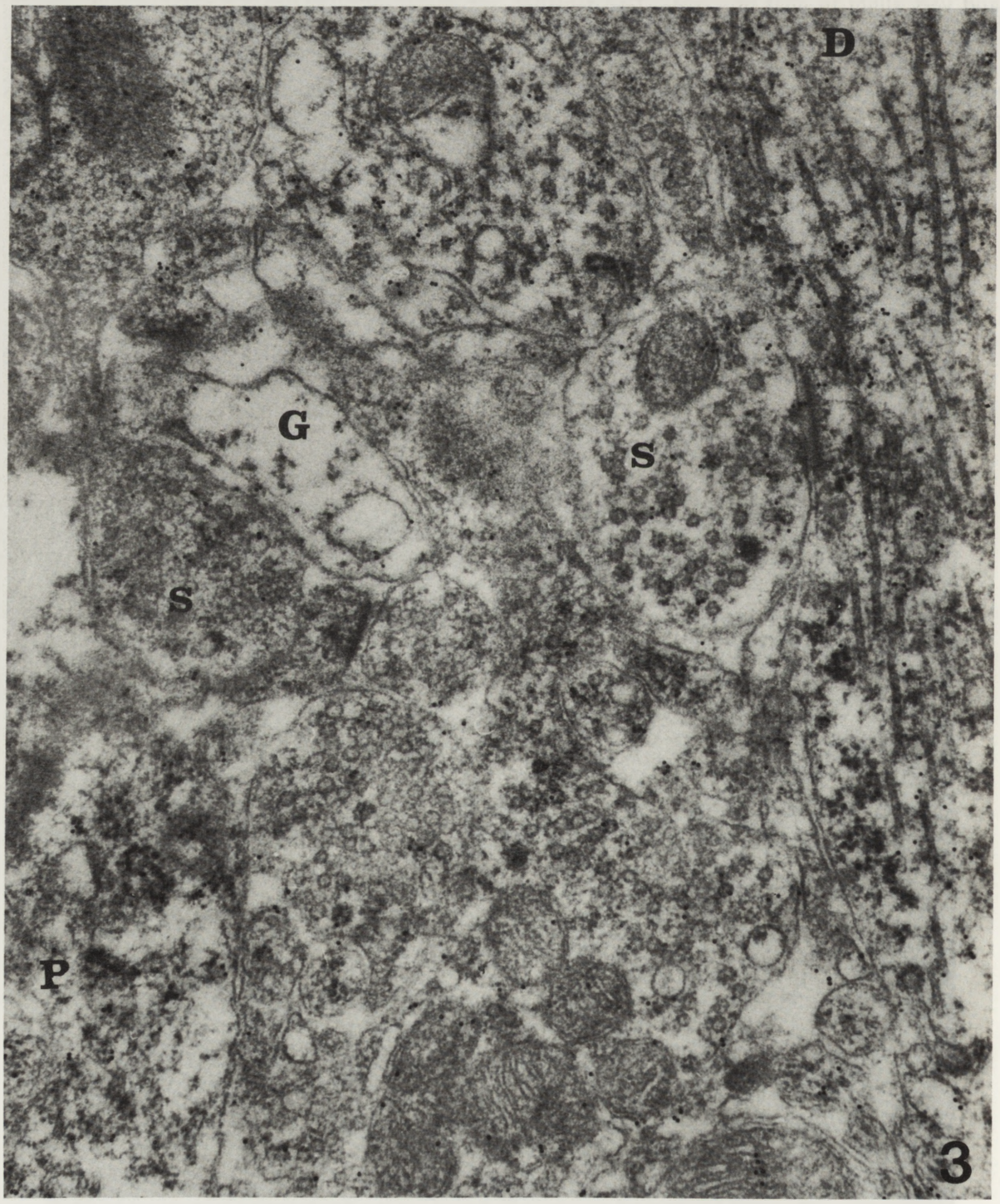

Fig. 3. Experimental animal: 6 h after ischemia. The gold particles indicative of glutamate immunoreactivity seen over some clear synaptic vesicles and mitochondria in asymmetric terminals (S). Note high density of gold particles in the perikarya (P) and process of glial cells (G) and over neurotubules in dendrite (D). × 15000

of glial cell bodies and processes was higher than in the controls, but some swollen astrocytes displayed a variable reduction of gold particle density. The distinct feature was an intense accumulation of gold particles over mitochondrial profiles (Figs 6 and 7).

II. Gamma-aminobutyric acid

Control animals. Colloidal gold particles indicating GABA-immunoreactivity were found mainly in axon terminals and less commonly in axonal, dendritic and somatic profiles throughout the CA1 sector of hippocampus. In axon terminals they were associated with small clear, round or oval vesicles (30-60 nm in diameter) and at times over pre- and postsynaptic densities (Figs 8 and 9). Within somatic, dendritic and axonal, both myelinated and unmyelinated, profiles the gold particles were seen over the cytoplasm in a close association with neurotubules and over mitochondria. Labelled nerve terminal often formed symmetrical synapses on dendritic and somatic profiles,

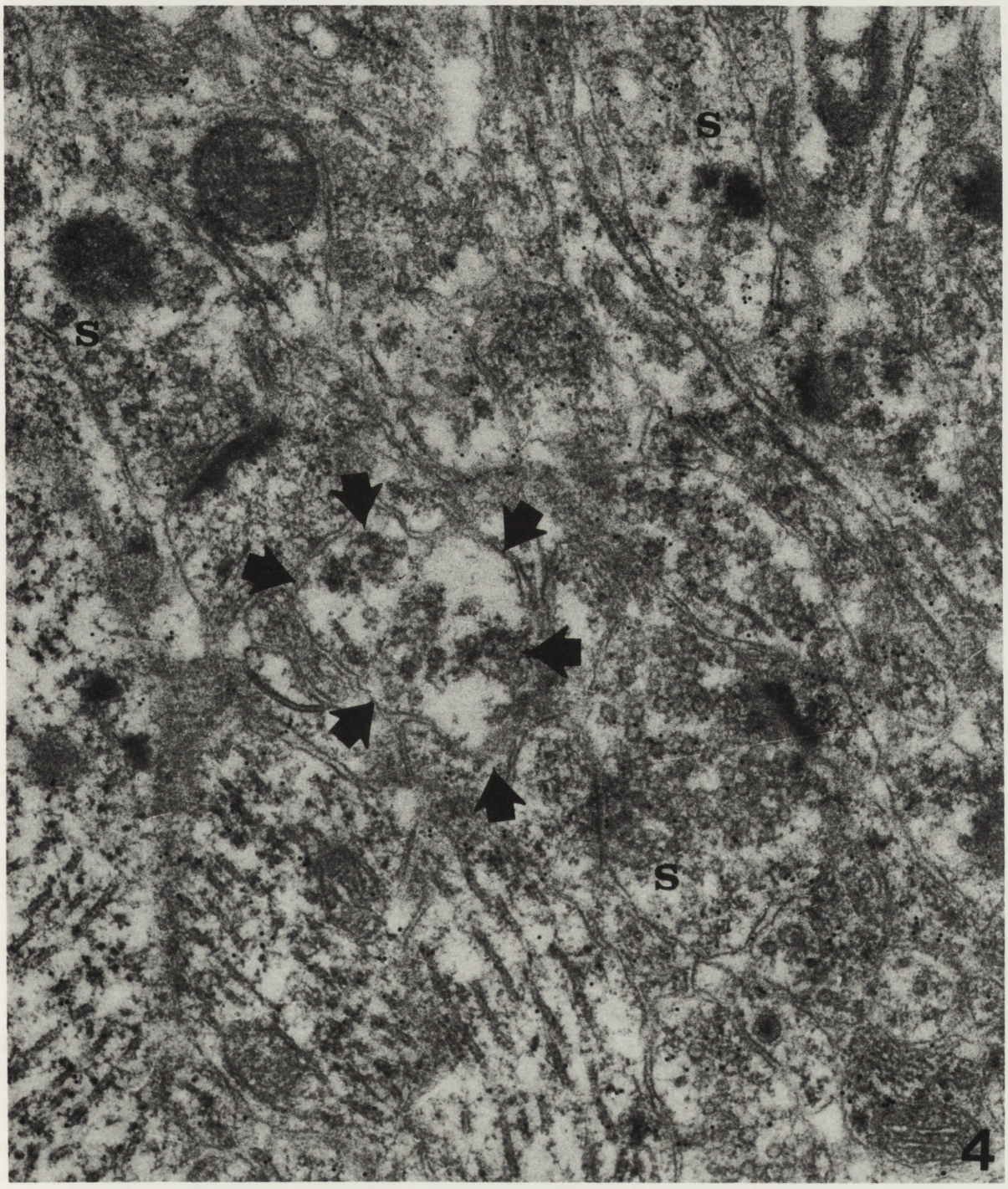

Fig. 4. Experimental animal: 6 h after ischemia. Immunoreactivity present in pre- and postsynaptic parts of asymmetric synapses (S). Note swollen terminal forming symmetric synapse (believed to be GABAergic terminal) devoided of immunoreactivity (arrowheads). × 25 000

sporadically containing dense core vesicles. Very low immunoreactivity was observed in glial processes. The terminals forming asymmetric synapses, considered to be glutamatergic ones, showed no immunolabelling.

Experimental animals. Six hours after ischemia GABA-like immunoreactivity was observed in numerous swollen neurons and nerve terminals (Figs. 10 and 11). Gold particles were located over pleomorphic synaptic vesicles as well as over areas devoided of synaptic vesicles and small mitochondria. The terminals forming symmetric contacts showed clearly lowered labelling intensity as compared with pictures observed in control animals. At the same time some ultrastructurally unchanged nerve terminals were even slightly enriched in gold particles, indicating GABA-like immunoreactivity. Numerous swollen astroglial processes showed increased number of gold particles in the cytoplasm as compared with the control animals.

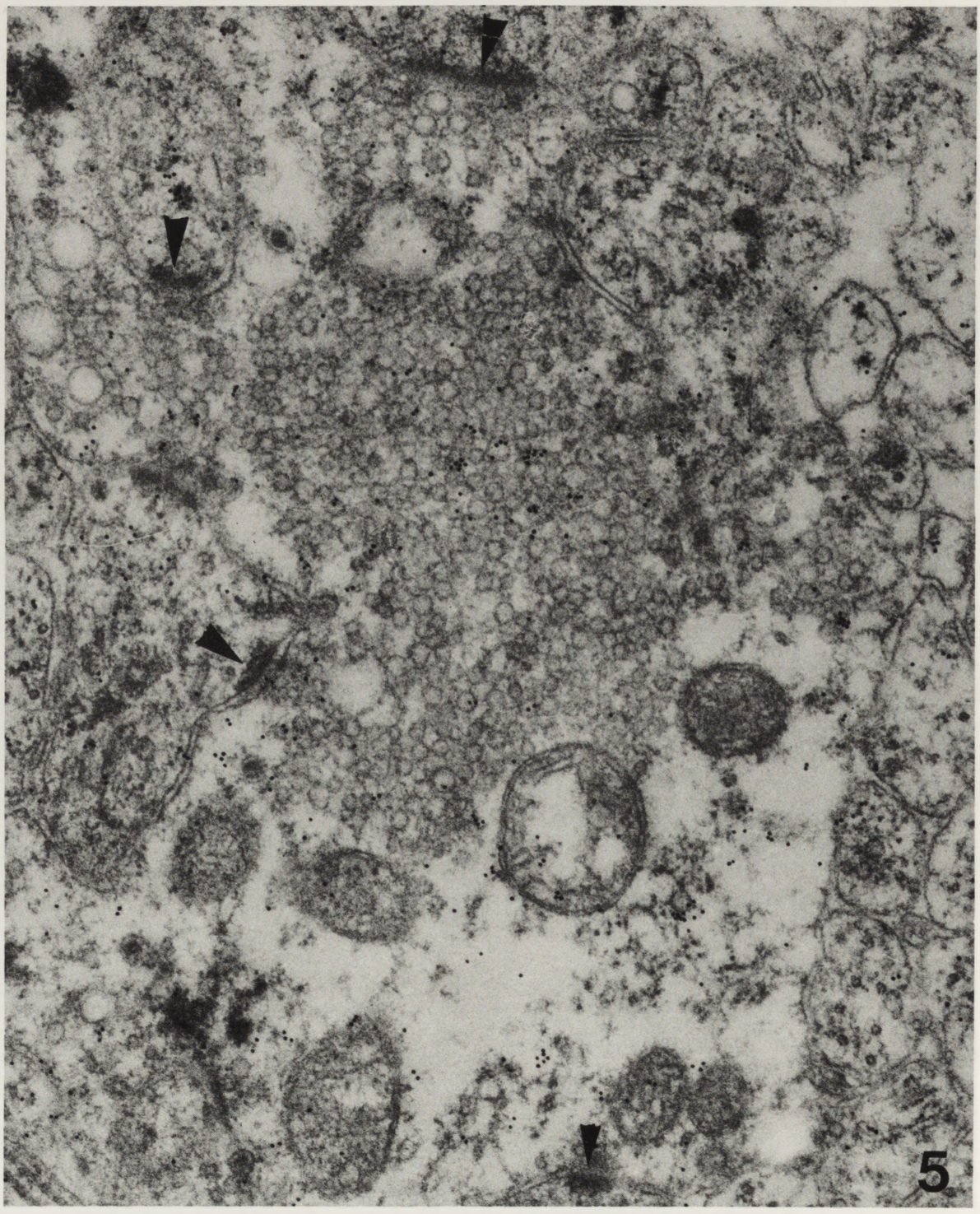

Fig. 5. Experimental animal: 12 h after ischemia. Large scalloped glutamate-like terminal making asymmetric contacts (arrowheads). Gold particles are located over synaptic vesicles. Immunostaining of post-synaptic parts is moderate. Axonal terminals at the periphery are not stained. × 30 000

Twelve and 24 hours after ischemia swollen neurons and nerve terminals were observed only sporadically in a strong contrast to the glial processes, most of which were markedly swollen (Figs 12, 13). The majority of GABA-ergic nerve terminals were ultrastructurally unchanged. Immunolabelling was scattered and associated with pleomorphic synaptic vesicles in the axon terminals of the symmetric synapses. The intensity of immunoreactivity in these terminals was similar to that in the control animals. The mitochondrial profiles were strikingly enriched in gold particles.

Discussion

The results of our studies performed with application of antibodies against both amino acid transmitters indicate that the postembeding immunogold cytochemistry may give reliable information about the distribution of glutamate and GABA in both cellular and subcellular compartments. Good correlation between the density of gold particles and the content of a given neurotransmitter in particular cellular structures indicate that the data of this type of immunocytochemistry can serve as a marker for relative neurotransmitter concentration. The axon terminals forming asymmetric contacts in *stratum radiatum* and in *stratum pyramidale* of the CA1 sector of the hippocampus are particularly rich in glutamate-like immunoreactivity. These terminals belonging to Schaffer's collaterals, which originate from neurons of CA3 hippocampal sector as well as to commissural fiber from the identical hippocampal areas of the contralateral hemisphere are thought to

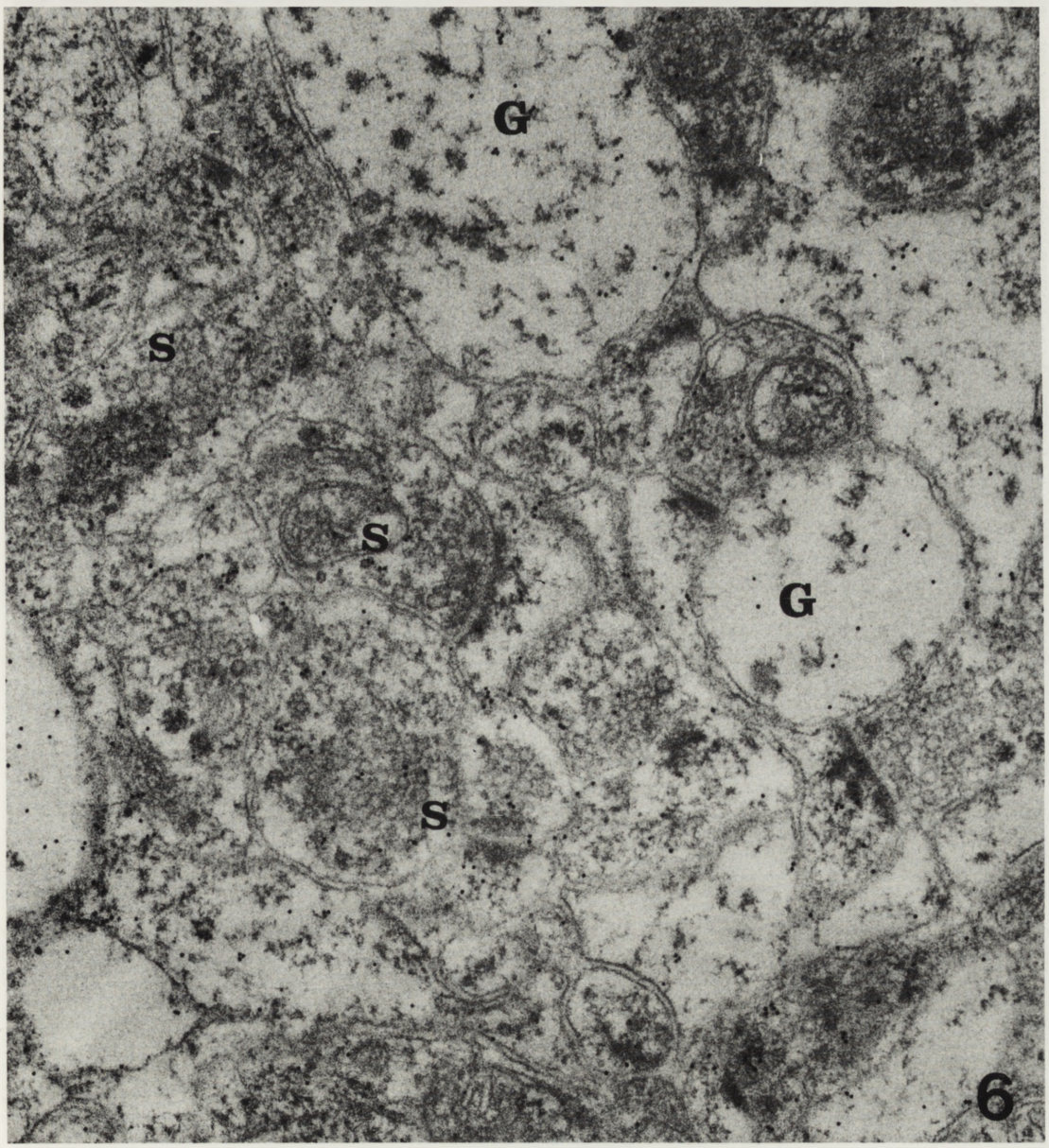

Fig. 6. Experimental animal: 12 h after ischemia. Strong labelling in ultrastructurally unchanged synapses forming asymmetrical contacts (S), and in the cytoplasm of swollen glial processes (G). × 15000

utilize glutamate as a neurotransmitter (Cotman et al. 1987; Ottersen, Storm-Mathisen, 1989). The fact that these particular terminals are displaying the highest density of gold particles as compared with other tissue elements, proves the glutamate immunocytochemistry can be applied for identification of nerve terminals using glutamate as a synaptic transmitter (Liu et al. 1989).

The distinct differences in gold densities in various cell profiles showed its higher concentration in the putative neurotransmitter pool than in the non-neurotransmitter („metabolic") pool, GABA-precursor pool and in the glial pool. These differences observed by us in gerbil brain corresponded well with those described in rat hippocampal slices (Ottersen et al. 1990).

The short-term forebrain ischemia changed both intensity of the immunocytochemical reaction and its localization in gerbil hippocampus. Six hours after ischemic incident there was slight enhancement of glutamate-like immunoreactivity in both neurotransmitter and glial pools, the former being more evident. Twelve and 24 hours after ischemia glutamate-like immunoreactivity of axon terminals returned to the intensity typical for the control animals, remaining increased within the glial pool. Some questions arising from this observation require a short comment. The first is the reaction of the glutamatergic system

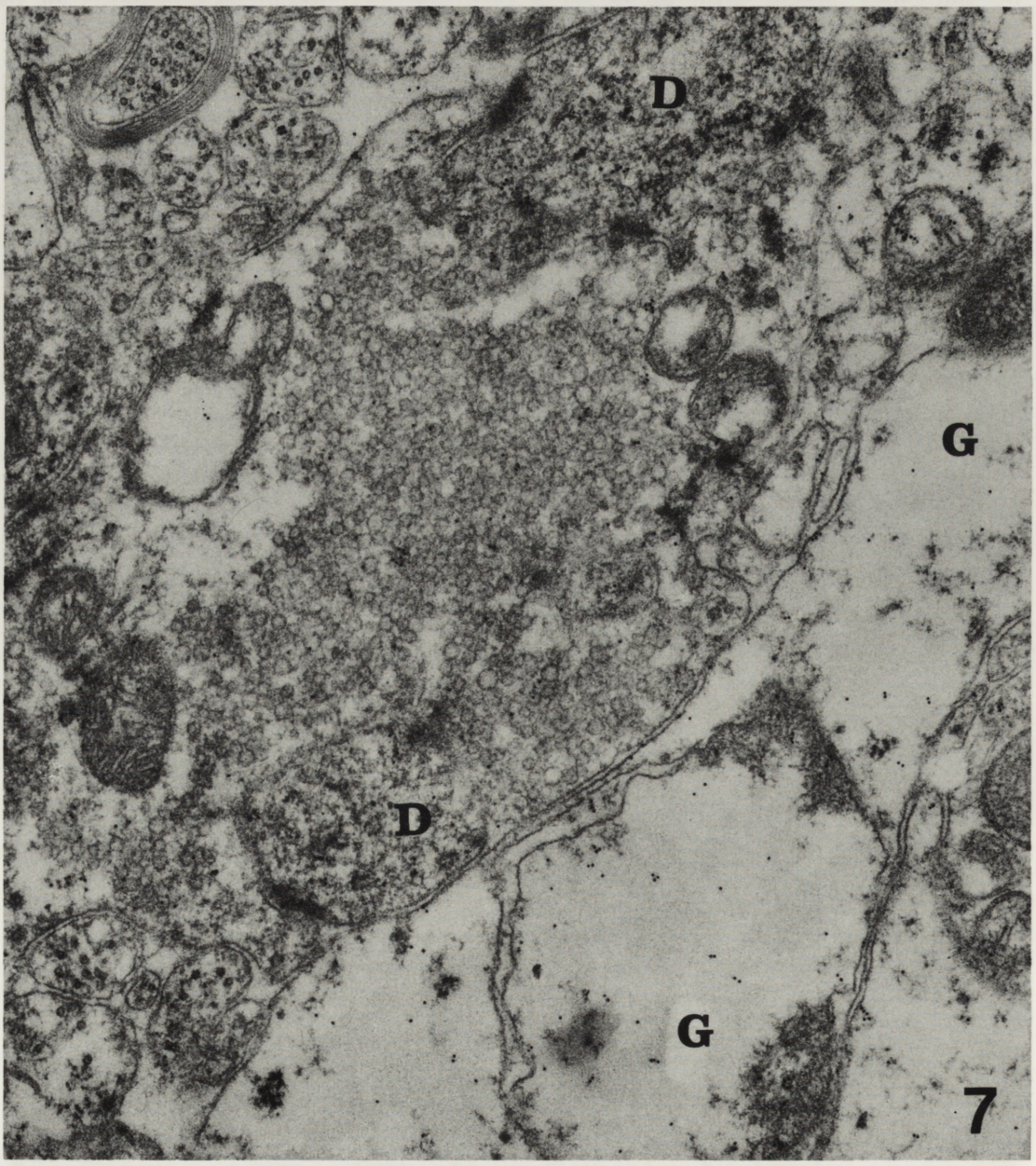

Fig. 7. Experimental animal: 24 h after ischemia. Labelling is seen over synaptic vesicles in large slightly swollen glial asymmetrical synapse, and in the cytoplasm of postsynaptic parts (D). Strong labelling in severely swollen processes (G). × 15 000

to the ischemic incident. In general our findings are in agreement with observation of Benveniste et al. (1984, 1989) and Busto et al. (1989), who showed that short-term ischemia resulted in a small but significant increase of glutamate release. However, according to their data, glutamate levels returned to normal values within one hour and a half after ischemia, while functional disturbances attributed to excess glutamate release were reported to appear several hours later (Suzuki et al. 1983; Andine et al. 1988). In our experiments increased gold particle densities in synaptic terminals, indicating enhanced neurotransmitter level, were present 6 hours after ischemic incident, being closer to physiological than biochemical observations mentioned above.

The second question concerns neuronal-glial interrelation. Relatively strong glutamate-like immunoreactivity within glial cell bodies and processes persisting during the whole observation period may suggest redistribution of glutamate from neurons to glia. The observation in normal conditions indicates that part of the released glutamate is being taken up into glia. Biochemical data suggest that the glial glutamate uptake is a first step in the glutamate carbon shuttle between neurons and glia that is aimed to replenish the releasable stores of glutamate (van den Bergh et al. 1975; Schousboe, Hertz 1983). The subsequent steps in this shuttle involve a transformation of glutamate to glutamine in the glial cells and then a transfer of glutamine to the neurons where it acts as a main precursor of transmitter glutamate. The elevated level of glutamate-like im-

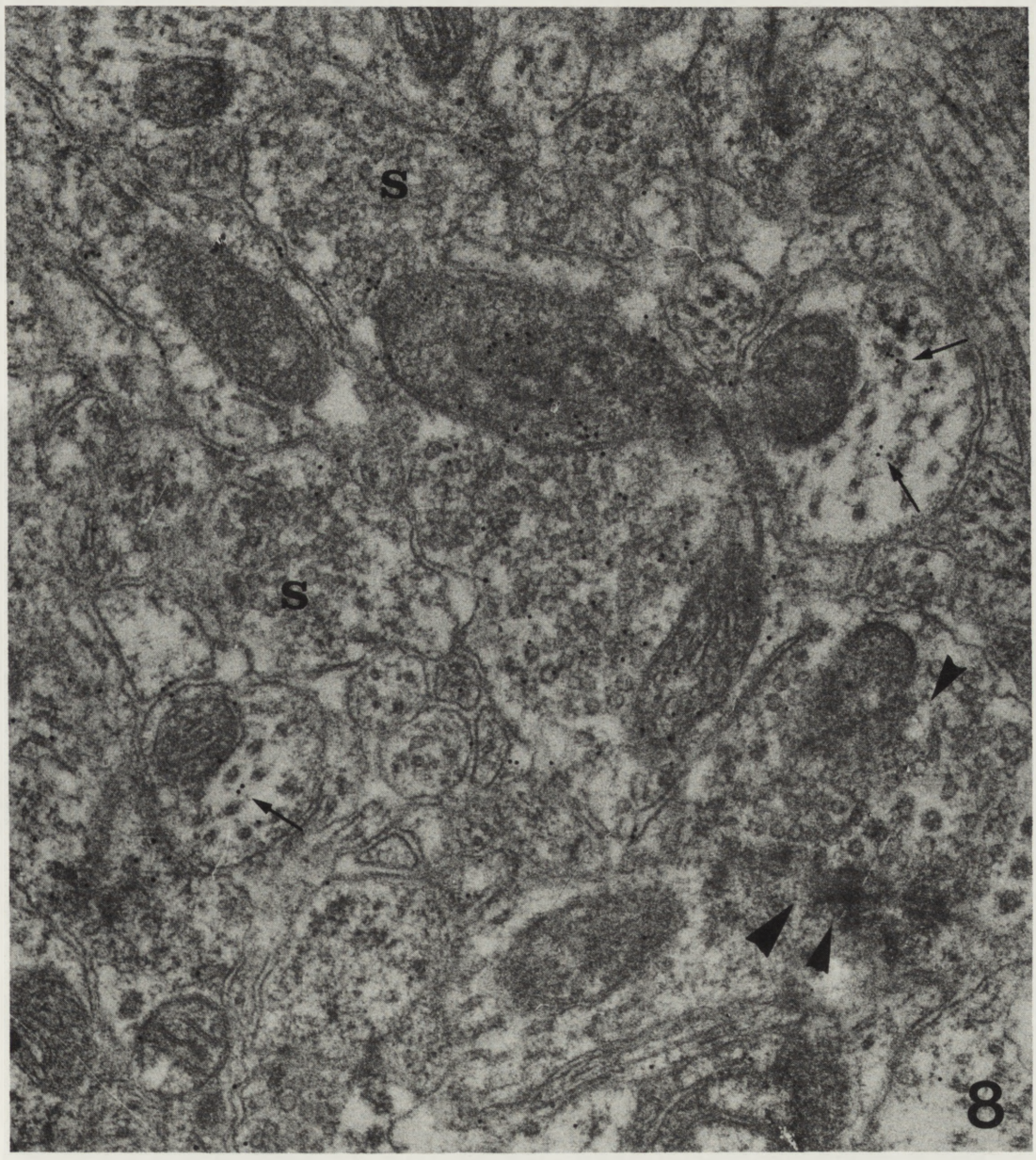

Fig. 8. Control animal. GABA-like immunoreactivity (gold particles) over synaptic vesicles in terminals forming symmetrical synapses (S) is present. Immunostaining of some dendrites and glial cells is moderate (arrows). Terminals forming asymmetric synapses believed to be glutamatergic ones devoid of immunostaining (arrowheads). × 15 000

munoreactivity in the swollen glial cells suggests that the ischemia results in increased content of glutamate within the glial cells. Our finding of a high concentration of gold particles over mitochondria probably reflects the mitochondrial localization of phosphate-activated glutaminase, which is one of the key enzymes in glutamine synthesis (Kvamme et al. 1988; Palaiologos et al. 1989). It is worth of stressing that the mitochondria were even more strongly labelled than the synaptic vesicles in spite

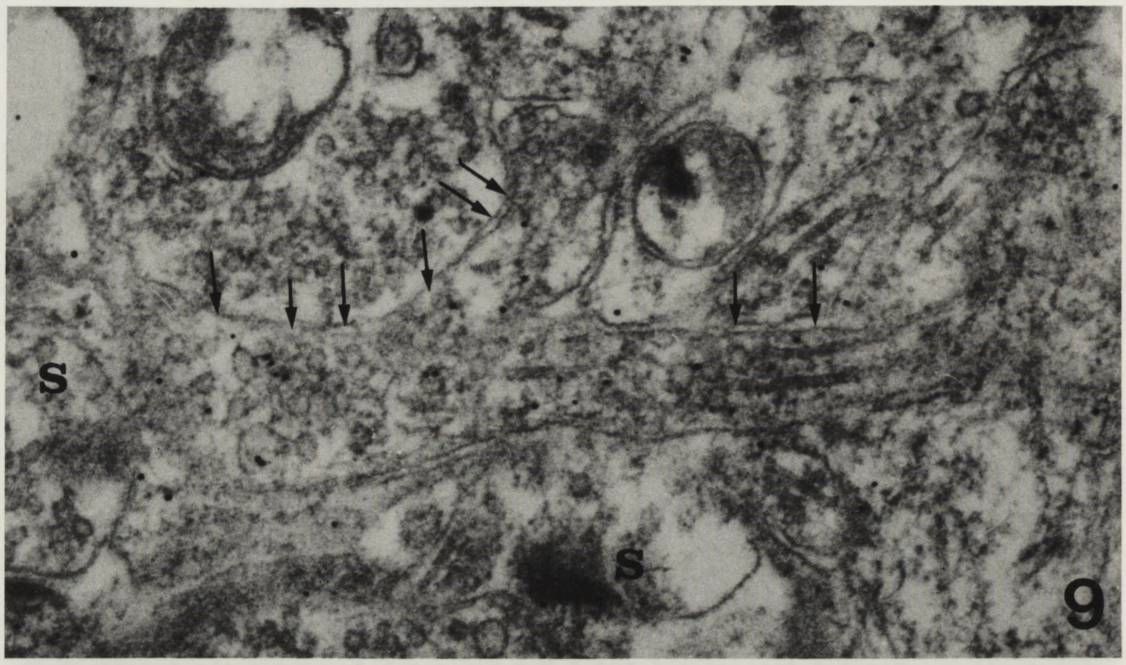

Fig. 9. Control animal. Transverse section of symmetric synapses with strongly labelled pleomorphic vesicles (arrows). Note terminals forming asymmetric synapses (S) devoided of immunolabelling. × 25 000

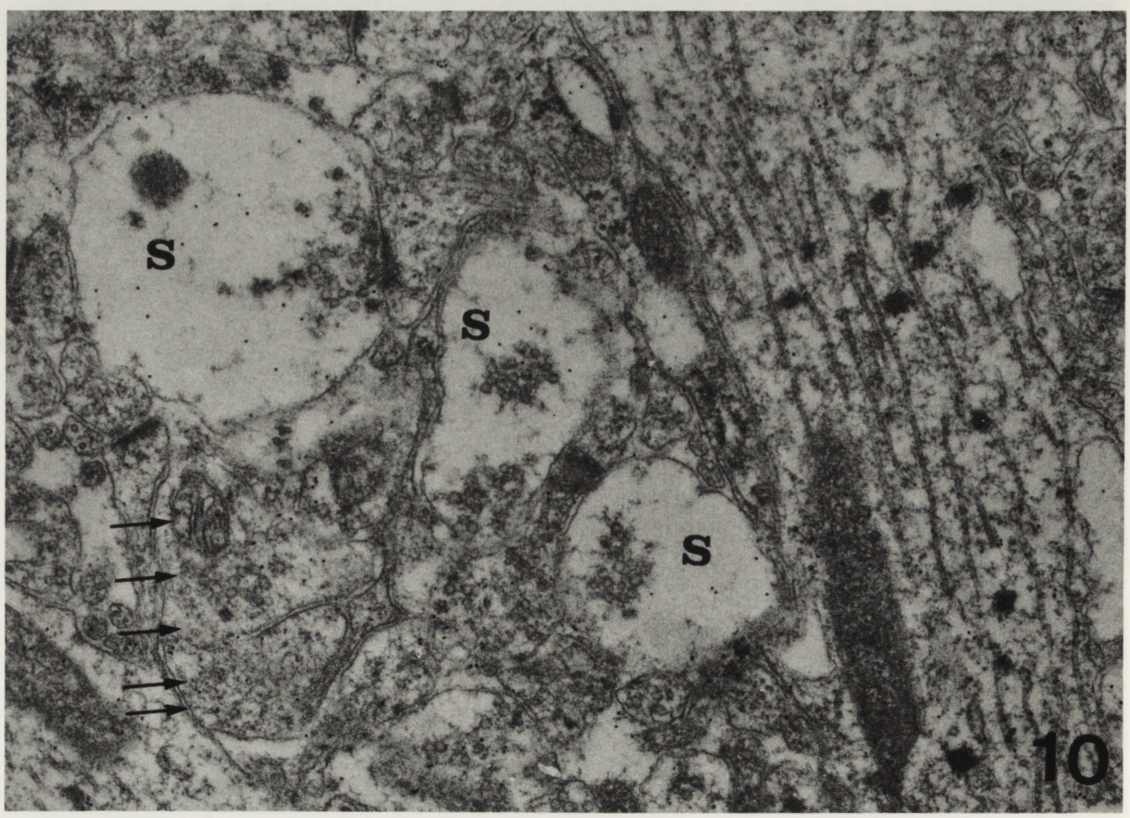

Fig. 10. Experimental animal: 6 h after ischemia. The gold particles indicative of GABA-immunoreactivity are seen in swollen symmetrical synapses (S). Ultrastructurally unchanged symmetrical synapses and dendrites exhibit low density of gold particles (arrows). × 15 000

of the evidence of their swelling. Therefore it seems likely that glial accumulation of glutamate during ischemia represents the combined effect of an increased supply and reduced ability of enzymatic conversion of glutamate. Therefore the insufficient capacity of the swollen glial cells to metabolize glutamate should be considered as a possible factor contributing to the development of intracellular glutamate overflow and neuronal injury during ischemia. Additionally, the glial cells reveal important functional capacities, easily disturbed during ischemia, such as buffering of K^+ ions and production of trophic factors for neurons that in normal conditions help in their survival (Gage et al. 1988; Hansson 1988; Walz 1989).

There are good grounds to believe that GABA immunolabelling pattern observed in immunogold preparations of the hippocampal CA1 sector reflects closely the *in vivo* distribution of free GABA. The distribution of gold particles suggest that most, if not all of hippocampal GABA belongs to the „transmitter pool". In contrast, as mentioned above,

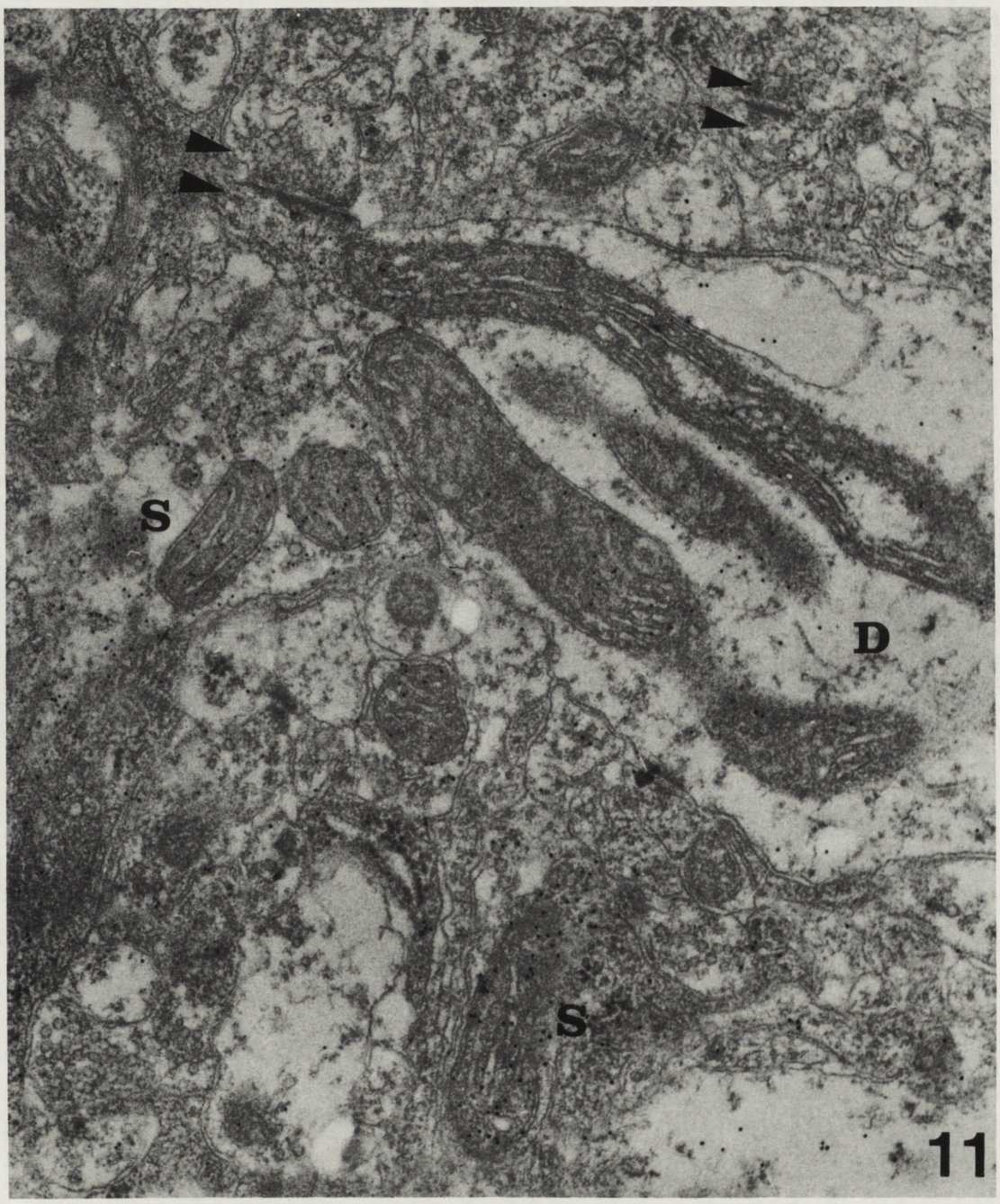

Fig. 11. Experimental animal: 6 h after ischemia. The density of gold particles is variable in GABA-ergic synapses (S). Note strongly labelled mitochondria and swollen GABA-ergic dendrites and unlabelled asymmetrical synapses (arrowheads). × 25 000

glutamate from the „metabolic" and glial pools may be larger than that from the „transmitter pool". We found two distinct classes of GABA stained terminals in *stratum radiatum* and *stratum pyramidale* of CA1 sector of hippocampus. The most immunoreactive profiles were axonal terminals containing small, clear, pleomorphic vesicles. They formed symmetric contacts on somatic and dendritic profiles. Some of them contained large, electron dense vesicles. We hypothetize that some GABA stained terminals are of local origin from GABAergic interneurons while the others come from „basket" interneurons (Gamrani et al. 1986). In addition, the presence of polymorphic vesicles in GABAergic terminals is closely associated with other transmitters which might be colocalized with GABA (Milner, Bacon 1989; Merighi et al. 1989). A weak GABA immunoreactivity was found in the cyto-

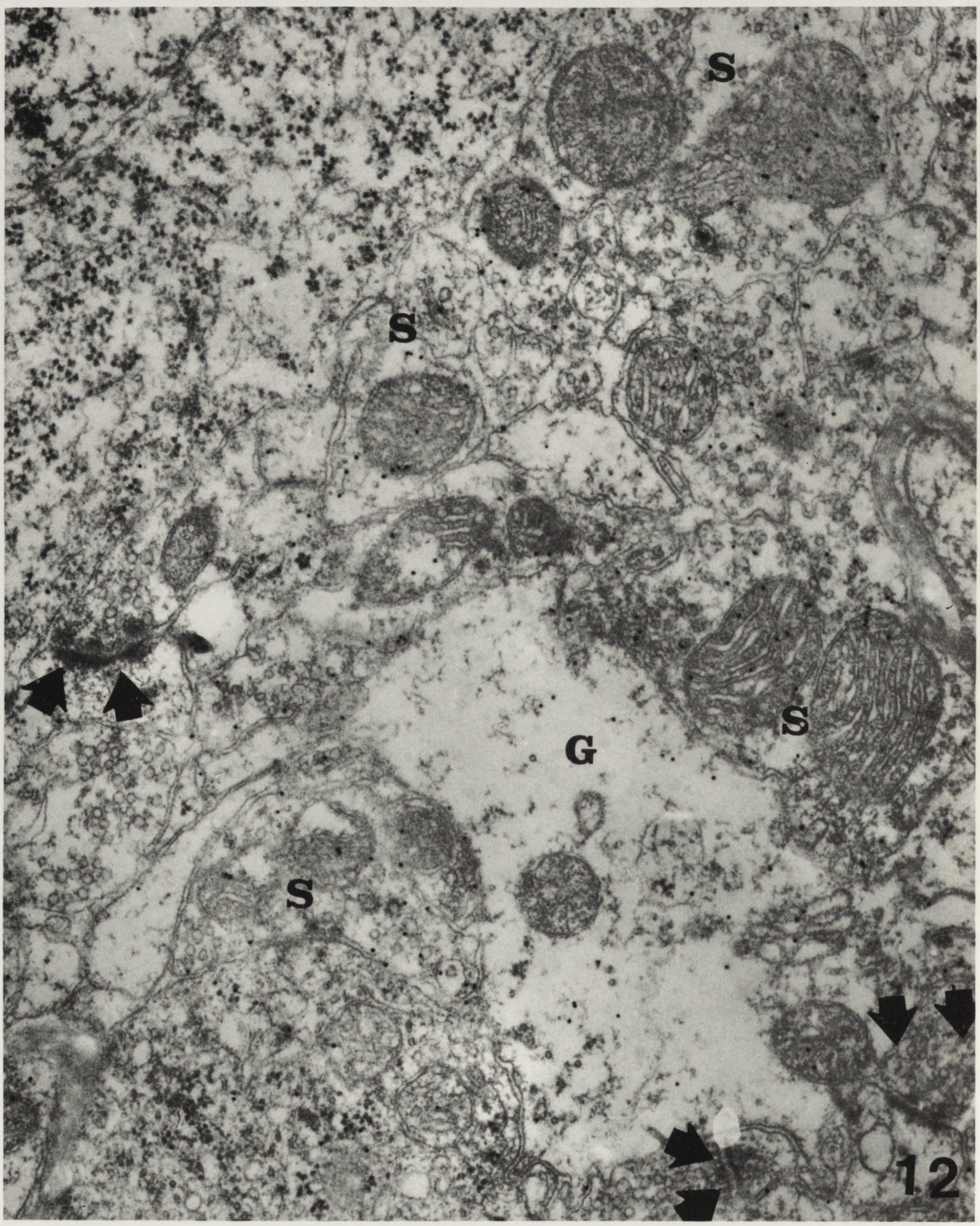

Fig. 12. Experimental animal: 12 h after ischemia. Symmetric synapses (S) with GABA-like immunoreactivity over synaptic vesicles and unlabelled asymmetric synapses (arrowheads). × 15000

plasm of the interneurons but high over the mitochondria. Thus it can be implied that mitochondria contain GABA as well. It is in agreement with the fact that the main biosynthetic pathway for GABA is that from glutamate by glutamic acid decarboxylase (Fonnum et al. 1978).

GABAergic interneurons play a crucial role in hippocampus by offsetting excitatory inputs to the pyramidal cells. Our previous electron microscopy study provided evidence that the imbalance between excitatory and inhibitory neurotransmitters in an early postischemic stage may be responsible for the irreversible damage of hippocampal pyramidal neurons (Gajkowska et al. 1989). In the present study we provide the direct proof that most immunoreactive GABAergic terminals were swollen 6 and 12 hours after ischemia and the labelling intensity of these symmetric terminals was lower than in the

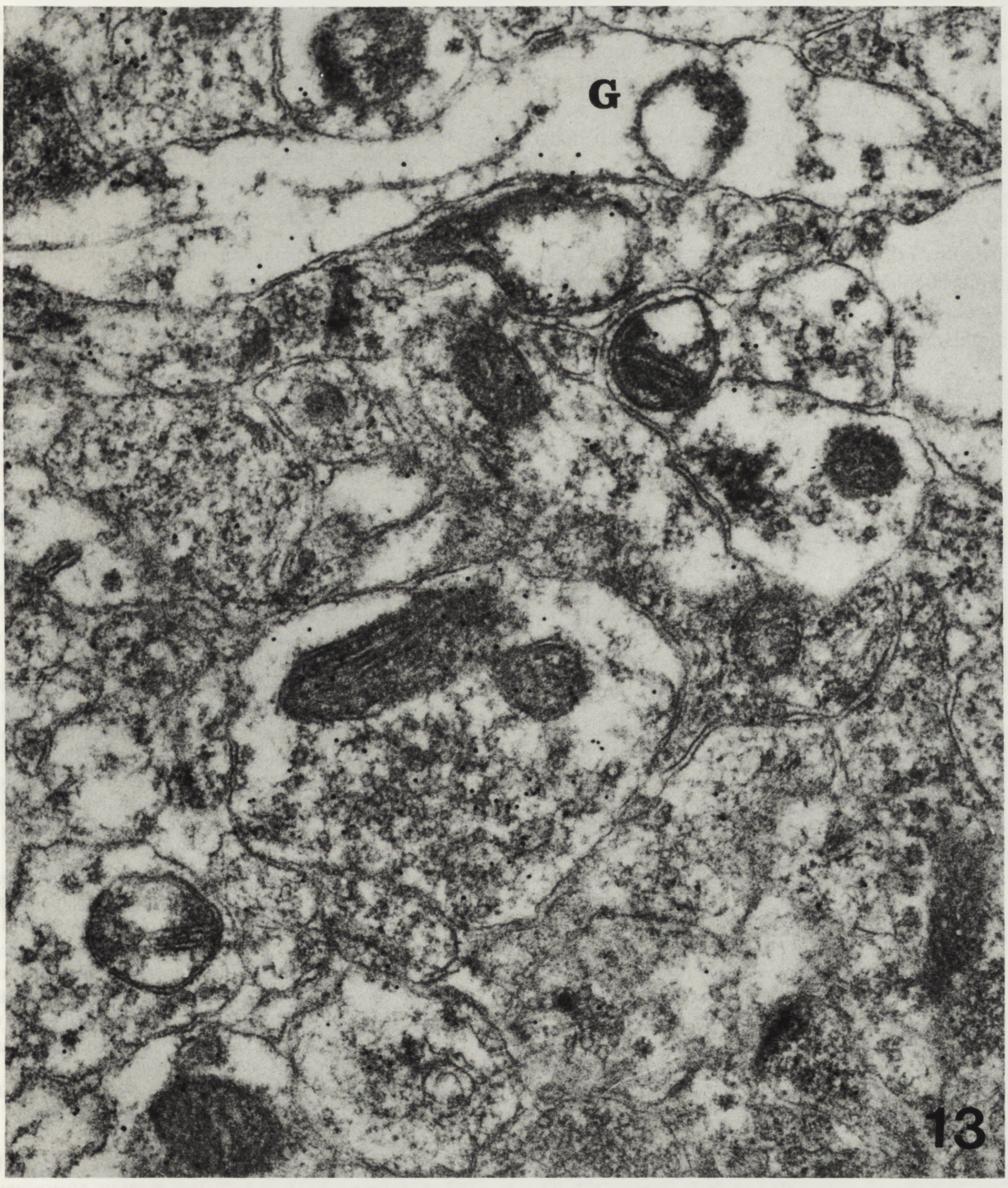

Fig. 13. Experimental animal: 24 h after ischemia. Strong labelling seen in GABA-ergic synapse. Glial processes (G) showing variable intensity of immunoreactivity. × 15000

same terminals of control animals. In contrast, after 24 hours the GABA labelling was significantly increased as compared with the control animals. Moreover, the symmetrical synapses were ultrastructurally unchanged. Therefore it seems reasonable to assume that the above presented data demonstrate temporal insufficiency of GABAergic interneurons after the ischemic incident.

In the literature we were able to find only one paper reporting a decrease of tissue GABA level after ischemia in the hippocampal CA1 sector and pointing out to the pathogenic role of disturbances in inhibiting synaptic transmission (Yasumoto et al. 1988). It seems that our present results are strongly suggestive that disturbances in the level of neurotransmitters and thus in the balance between neuronal excitation and inhibition can play the key role in the ischemic hippocampal injury.

Zachowanie Gaba- i glutaminergicznych synaps w hipokampie chomików mongolskich w następstwie niedokrwienia mózgu. Mikroskopowo-elektronowe badania immunocytochemiczne

Streszczenie

Scharakteryzowano subkomórkowe rozmieszczenie glutaminianu i kwasu gamma-aminomasłowego (GABA) w synapsach hipokampa przy zastosowaniu techniki immunocytochemicznej, dostosowanej do mikroskopii elektronowej. Wyznacznikiem immunologicznym były 10 nm cząsteczki koloidalnego złota, skonjugowane z przeciwciałami przeciwko glutaminianowi i GABA. Skupienia cząsteczek złota dostarczały przekonywającą informację o względnej koncentracji obu aminokwasowych neuroprzekaźników. Uzyskane wyniki wskazują, że niedokrwienie prowadziło do przejściowego obniżenia odczynu, stanowiącego wykładnik nagromadzenia GABA w symetrycznych synapsach hipokampa, przy niewielkim wzroście nasilenia odczynu ujawniającego obecność glutaminianu w synapsach asymetrycznych. Na podkreślenie zasługuje redystrybucja immunoreaktywności glutaminianu z neuronów do gleju. Sugeruje to zdolność gleju do metabolizowania nadmiaru glutaminianu po niedokrwieniu. Podkreślano zaburzenia zawartości neuroprzekaźników w następstwie niedokrwienia, jako możliwego czynnika odpowiedzialnego za uszkodzenia komórek nerwowych.

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