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Ultrastructural changes in the hippocampal CA1 region following transient cerebral ischemia: evidence against programmed cell death

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Summary. The ultrastructural changes in the pyramidal neurons of the CA1 region of the hippocampus were studied 6 h, 24 h, 48 h, and 72 h following a transient 10 min period of cerebral ischemia induced by common carotid occlusion combined with hypotension. The pyramidal neurons showed delayed neuronal death (DND), i.e. at 24 h and 48 h postischemia few structural alterations were noted in the light microscope, while at 72 h extensive neuronal degeneration was apparent. The most prominent early ultrastructural changes were polysome disaggregation, and the appearance of electron-dense fluffy dark material associated with tubular saccules. Mitochondria and nuclear elements appeared intact until frank neuronal degeneration. The dark material accumulated with extended periods of recirculation in soma and in the main trunks of proximal dendrites, often beneath the plasma membrane, less frequently in the distal dendrites and seldom in spines. Protein synthesis inhibitors (anisomycin, cycloheximide) and an RNA synthesis inhibitor (actinomycin D), administered by intrahippocampal injections or subcutanously, did not mitigate neuronal damage. Therefore, DND is probably not apoptosis or a form of programmed cell death. We propose that the dark material accumulating in the postischemic period represents protein complexes, possibly aggregates of proteins or internalized plasma membrane fragments, which may disrupt vital cellular structure and functions, leading to cell death.

Key words: Cerebral ischemia – Glutamate – Heat shock protein – Hippocampus – Programmed cell death – Rat – Ubiquitin

Abbreviations. DND – delayed neuronal death; ER – endoplasmic reticulum; GA – Golgi apparatus; HSP – heat shock protein; IR – immunoreactivity; PSD – postsynaptic density; RNA – ribonucleic acid; SER – smooth endoplasmic reticulum; UIR – ubiquitin immunoreactivity

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Introduction

Transient cerebral ischemia, if severe enough to cause neuronal damage, leads to selective necrosis of neurons in specific brain regions. The hippocampal subfield CA1 is a brain region particularly sensitive to cerebral ischemia (Ito et al. 1975; Brierley 1980; Pulsinelli et al. 1982). After a transient (10–15 min) period of 2-vessel occlusion (2–VO) ischemia followed by one week of recovery, practically all neurons in the CA1 region are degenerated (Smith et al. 1984A) Cell death in the CA1 region usually develops over several days (Ito et al. 1975; Pulsinelli et al. 1982; Petito et al. 1987), and has been termed the "maturation phenomenon" by Ito et al. (1975) and "delayed neuronal death" (DND) by Kirino (1982). In other brain regions, for example in the striatum, the hilus of the dentate gyrus or the reticular nucleus of the thalamus, neuronal necrosis is usually complete within hours after the ischemic insult (McGee-Russell et al. 1970; Brown and Brierley 1972; Petito and Pulsinelli 1984A). Ultrastructurally, this acute type of neuronal degeneration is characterized by mitochondrial swelling with the formation of flocculent densities, nuclear pyknosis and karyohexis, plasma membrane disruption and complete cellular disintegration (Brown and Brierley 1972; Garcia et al. 1978). In contrast, DND is characterized by an early disaggregation of polyribosomes, proliferation of endoplasmic reticulum (ER) and Golgi apparatus (GA) and accumulation of dense material in the cytoplasm. This is followed by condensation of cytoplasm and finally disintegration which appears as dark, shrunken or watery, swollen neurons (Pulsinelli et al. 1982; Kirino and Sano 1984; Kirino et al. 1984). One ultrastructural feature mentioned but not commented upon in several investigations of DND is the appearance of electron dense fluffy material in dendrites and soma (Bubits et al. 1976; Brown et al. 1979; Petito and Pulsinelli 1984A; Kirino et al. 1984).

In the present investigation we describe the development of neuronal degeneration in the hippocampal CA1

Table 1. The effects of protein synthesis inhibitors and an RNA synthesis inhibitor on neuronal necrosis in the hippocampal CA1 region of rats after 10 min ischemia and 3–7 days recovery

Experimental group	n	dose[a]	neuronal necrosis (% of control)		
			ipsilateral side		contralateral side
Actinomycin-D	3	0.5 μg i.c.	100		100
Anisomycin	4	0.3 μg i.c.	66±11		71±12
Cycloheximide	4	30 μg i.c.	90±22		96± 2
Saline	4	i.c.	93±13		97± 2
Anisomycin	6	25 mg · kg^{-1} s.c.		88±15[b]	
Saline	6	s.c.		85±19[b]	
Anisomycin	4	25 mg · kg^{-1} × 3 s.c.	99± 1		99± 0.5
Saline	4	s.c.	97± 2		99± 0.5

Values are means ± SEM

[a] The inhibitors were administered intracerebrally (i.c.) and subcutaneously (s.c.) prior to induction of ischemia

[b] Mean of both brain hemispheres

region following 10 min of 2–VO ischemia in the rat, and discuss the changes during DND in view of some current hypotheses of ischemic cell death. We particularly emphasize the temporal and topographical formation of the dark cytoplasmic material, in relation to the proposal that ischemic cell death is a form of programmed cell death, apoptosis (Shigeno et al. 1990).

Material and methods

Male Wistar-SPF rats were obtained from Moellegaard's Avls-laboratorium (Copenhagen, DK). The ubiquitin-conjugate antibody was a generous gift from Dr A Haas (University of Wisconsin, Milwaukke), and the HSP 70 antibody was purchased from Amersham, UK. Anisomycin and actinomycin D were purchased from Sigma (St Louis, MO), and cycloheximide from Boehringer Mannheim, Germany.

Experimental groups

This investigation is based on the results obtained from 67 animals divided into the following experimental groups: light and electron microscopy (n = 18); calcium histochemistry (n = 6); immunohistochemistry (n = 8); effect of protein synthesis inhibitors (n = 35).

Induction of ischemia

The model of cerebral ischemia used in these studies has been described in detail by Smith et al. (1984B). Briefly, anesthesia was induced with 3.5% halothane in N_2O:O_2 (2:1). During ischemia halothane was maintained at 0.7% in N_2O:O_2 (2:1). Muscle relaxation was achieved with suxamethonium (Celocurin, Vitrum AB, Stockholm, Sweden; 4 mg · kg^{-1}). Hypotension was induced with trimethaphan (Arfonad; Hoffman-LaRoche, Basel, Switzerland; 4–5 mg · kg^{-1}) and withdrawal of blood. When the mean arterial blood pressure reached 50 mmHg, vascular clamps were placed on both common carotid arteries. At the end of ischemia the blood was reinfused, the clamps removed and 75 mg · kg^{-1} $NaHCO_3$ administered. The EEG was monitored prior to ischemia, during the ischemic period and early recirculation. Blood gases and pH were monitored throughout the experiment and were kept in the following ranges: PaO_2 100–120 mmHg; $PaCO_2$ 35–40 mmHg; pH 7.35–7.42. The blood glucose levels varied between 4.5–7.7 mmol · l^{-1}. The body and skull temperatures were kept at 37° C ± 0.5.

In one set of experiments the rats were injected with protein synthesis and RNA synthesis inhibitors. Those animals were anesthetized with isoflurane instead of halothane, recieved vercurone bromide (Norcurone, Organon, The Netherlands; 10 mg · kg^{-1} · h^{-1}) as muscle relaxant (Gustafson et al. 1989), and hypotension was achieved by withdrawal of blood with no administration of trimethaphan bromide.

Rats were injected intracerebrally (i.c) into the hippocampus or subcutaneously (s.c) with either actinomycin D, anisomycin or cycloheximide (see Table 1). The i.c. injections were given 45–50 min prior to the onset of ischemia. The coordinates were 3.0–4.0 mm caudally, 2.0–4.0 mm laterally and 3.0–4.0 mm ventrally (Paxinos and Watson, 1982). Following injection the needle remained in the brain for a total of 5 min. Cycloheximide (10 mg · ml^{-1}) was injected in a volume of 3 μl over 3 min. Actinomycin D (0.5 mg · ml^{-1}) was injected in a volume of 1 μl over 1 min. Anisomycin (3 mg · ml^{-1} dissolved in equimolar amount of 1M NaOH to pH 7.0, and then diluted with destilled water) was administered i.c. in a volume of 1 μl. When anisomycin was given subcutaneously (25 mg · kg^{-1}), the first injection was administered 30 min before the onset of ischemia and then either at 24 h and 48 h postischemia or at 2, 4, and 6 h postischemia. Rats treated with anisomycin recovered for 4–7 days before sacrified, actinomycin D recovered for 3 days while animals from all other experimental groups recovered for 7 days.

Electron- and light-microscopy

After the predetermined recovery, the animals were reanesthetized and sacrificed by perfusion fixation with 3% glutaraldehyde in 0.1 mol · l^{-1} phosphate buffer (pH 7.4, 37° C and at a pressure of 180 cm H_2O) preceded by a short rinse with saline. The brains were allowed to stabilize within the cranium for 24 hours, whereafter they were removed and stored in the same fixative until processed further. Slices of 500 μm thickness were sectioned from both hemispheres of the sagitally divided brain at the level of anterior hippocampus. The hippocampus was dissected free from these slices (at 4 levels) and divided into lateral and medial halves. The slices were postosmicated, dehydrated in ethanol and embedded flat in epon. Semithin sections stained with toluidine blue were used for high resolution light microscopy and selection of areas for thin sectioning. Thin sections were cut with an LKB Ultrotome, stained with LKB Ultrostainer and examined with a Philips 400 or JEM 100C electron microscope.

Immunocytochemistry

The immunocytochemical procedure included transcardial perfusion of the rats with saline (37°C) followed by ice-cold 2% paraformaldehyde in 0.1 M sodium acetate buffer, pH 6.5, and ice-cold 2% paraformaldehyde/0.01% glutaraldehyde in 0.1 M sodium borate buffer, pH 8.5. The brains were removed and placed in ice-cold perfusion solution pH 8.5 at 4°C overnight and cut with a vibratome Micro-cut H 1200 (Bio Rad Polaron Division, Richmond, CA) in 50 μm sections. The sections were washed and incubated overnight in a 1 $\mu g \cdot ml^{-1}$ solution of an affinity purified polyclonal antibody directed against ubiquitin and ubiquitin conjugates, and transferred to a solution of biotinylated protein A before incubation in avidin-biotin-horseradish peroxidase complex (Vector Labs, Burlingame, CA). The specificity of the ubiquitin antibody has been described (Haas and Bright 1985). For heatshock-protein 70 (HSP 70) immunocytochemistry the sections were incubated in a 1/200 dilution of a monoclonal HSP 70 antibody solution (Amersham, UK), and then transferred to a secondary biotinylated goat antimouse antibody solution (Dakopatts A/S, DK). The sections were then treated with 3,3′-diaminobenzidine (Sigma, St. Louis, MO) until desired staining was obtained. When normal rabbit serum was used no staining was seen.

Oxalate-pyroantimonate staining

The oxalate pyroantimonate staining was performed according to Van Reempts and Borgers (1985). The brain tissue was fixed by 5 min of intracardiac perfusion with 2% formaldehyde + 2.8% glutaraldehyde containing 0.09 $mmol \cdot l^{-1}$ potassium oxalate at +4° C, then immersed in situ overnight. Thick sections (100 μm) of the brain were rinsed in 0.09 $mmol \cdot l^{-1}$ potassium oxalate in 7.5% sucrose for 15 min. Postfixation was done in 1% osmium tetroxide + 2% potassium pyroantimonate for 2 h. The sections were then rinsed for 5 min with distilled water and buffered to pH 10 using 0.1 $mmol \cdot l^{-1}$ KOH. Dehydration was done in a 70%–90%–100%–100% alcohol series for 5 min. After 5 min in propylene oxide, the sections remained overnight in a mixture of propylene oxide and epon 1:1. All reactions were carried out at +4° C. The next day the sections were embedded in epon and studied in the electron microscope as above.

Histopathology

Animals treated with protein synthesis and RNA synthesis inhibitors were reanesthetized and perfusion-fixed three to seven days after the onset of ischemia. After rinsing with saline, the rats were perfusion-fixed with phosphate-buffered 4% formaldehyde. The brains were removed, dehydrated, paraffin embedded and sectioned coronally at 8 μm. The sections were then stained with celestine blue and acid fuchsin according to Auer et al. (1984).

Quantification of brain damage was accomplished by visual counting of neurons. Necrotic neurons staining bright red with the acid fuchsin dye (Auer et al. 1984) were distinguished from intact violet-stained cells. Spared neurons in the hippocampal CA1 region were counted at a magnification of 400×. Neuronal necrosis is presented as percent damage of hippocampal CA1 neurons ± SEM.

Results

Light microscopy

In control animals, the hippocampal CA1 region displays a compact cell layer of the pyramidal neurons (data not shown). The cell bodies have large round nuclei and prominent nucleoli. The neuropil is characterized by scattered cell bodies of interneurons and compact dendritic shafts. Fortyeight hours following 10 min of 2–VO ischemia most of the CA1 neurons still appear normal (Fig. 1A). The cytoplasm is darker than normal, and the nuclei have a slightly irregular shape but are still large with prominent nucleoli. A few condensed neurons are seen. The dendrites in the *stratum radiatum* are not different from those in control animals. Seventytwo hours after ischemia extensive neuronal disintegration is noted (Fig. 1B). In the cell body layer extensive vacuolization is seen and several neurons are condensed. One week following ischemia more than 80% of the neurons in the CA1 region have degenerated (data not shown).

Ultrastructural changes in the hippocampal CA1 region

Control group. In control animals the nuclei of neurons in the CA1 region are rounded with few discontinuities of the nucleolemma (Fig. 2). The chromatin is evenly dispersed and the *pars fibrosa* and *pars granulosa* of the nucleoli readily discernable. The organelles of the cytoplasm appear normal with polyribosomes formed in characteristic rosettes, or bound to the endoplasmic reticulum (ER). The ER is often arranged in parallel stacks, and the Golgi apparatus (GA) is large and numerous in the perikarya and also commonly found in the proximal dendrites. The postsynaptic densities (PSDs) in the *stratum radiatum* are prominent and the mitochondria compact.

The major structural features of the dendrites are microtubuli, mitochondria and smooth endoplasmic reticulum (SER) with some associated ribosomes aligned parallel with the axis of the dendrites. The neuropil between the perikarya and the large proximal dendrites is compact with structurally normal neuronal and astrocytic processes intermingled with each other.

10 min ischemia + 6 h recirculation. Despite the normal appearance of neurons in the light microscope (data not shown), some significant ultrastructural changes are noted in this group. The ribosomes have lost their orientation in rosettes and assume pinpoint appearance (Fig. 3A). Appreciable amount of fluffy dark material appears close to the slightly dilated GA and ER. The material is not surrounded by a membrane but appear associated with a tubular structure, similar to the SER (Peters et al. 1976). In the soma the dark material is found centrally in the cytoplasm as well as close to the plasmalemma. The nucleus, nucleolus and mitochondria are unchanged and not different from control.

In the dendrites, mitochondria and microtubules appear normal (Fig. 3B). The dark material is observed mainly in the major dendritic trunks, either free or associated with SER. It appears often close to synaptic structures and resemble PSDs. However, in the neuropil the PSDs appear intact, with no dark material present. At this recovery time the dark material is preferentially seen

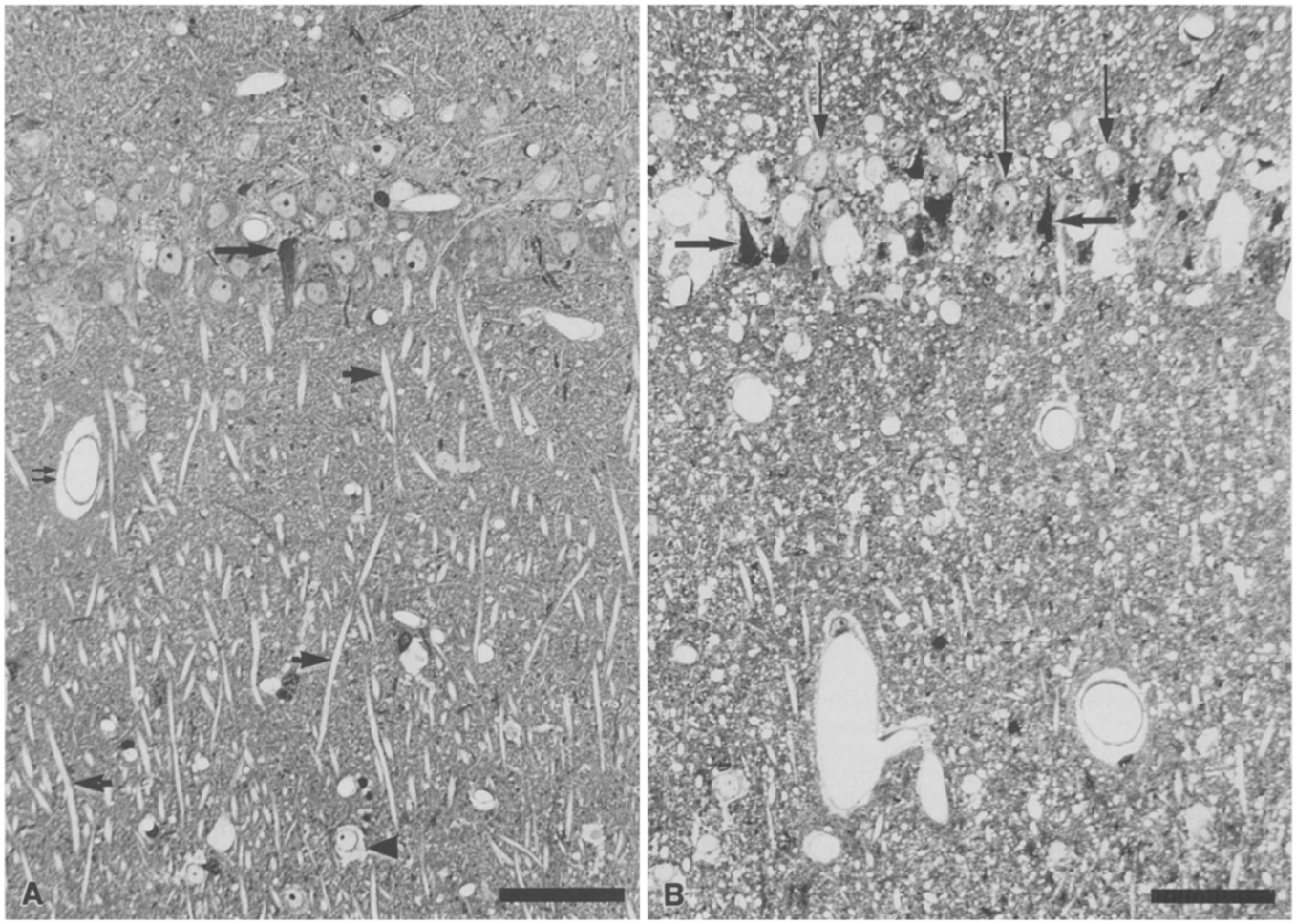

Fig. 1. A *10 min ischemia + 48 h recirculation.* In the pyramidal cell layer of the hippocampal CA1 region, one dark and condensed neuron is seen (long arrow) amongst seemingly normal neurons. The *stratum radiatum* is compact with regular pale dendrites which are not swollen (short arrow). Some cells have a pale swollen appearance (arrowhead) and perivascular edema is observed (small arrows). **B** *10 min ischemia + 72 h recirculation.* Marked destruction of the CA1 pyramidal cell layer is evident. Many disintegrating dark condensed neurons are seen (thick arrows), with a few well preserved intact neurons (thin arrows). The *stratum radiatum* is spongy with plenty of small vacuoles. Epon + toluidine blue. Bars 50 μm

close to the plasma membrane and less frequently in the centre of the dendrites.

10 min ischemia + 24 h recirculation. In general, the alterations of the cytoplasmic organelles of the 24 h group are similar to those in the 6 h animals. In the soma the nuclear membrane is irregularly shaped, but the chromatin is dispersed and the nucleoli unremarked. The mitochondria are normal but the orientation of the microtubuli is less ordered, and the GA and ER are slightly dilated. The plasma membrane is unremarked with no protrusions or bleb formation. At this time, the dark material accumulate in large amounts in the soma. The dendritic diameter is not different from control animals and the surrounding neuropil is compact except for slight dilatation of astrocytic processes. Frequent clusters of dilated SER containing dark material, and sometimes associated with mitochondria, are seen in the centre of the dendrites (Fig. 4).

10 min ischemia + 48 h recirculation. The changes seen at 48 h of recirculation are more marked than at 24 h (Fig. 5). The nuclear membrane is highly crenated, and the chromatin seems slightly condensed and marginated in loosely textured aggregates. In the cytoplasm there is an increased amount of dark material as well as increased loss of microtubular orientation, giving the soma a darker appearance than in control neurons (Fig. 1A). In some neurons ribosomal rosettes reappear. The density of the dark material is highest in the soma, decreasing along the apical dendrites, and rarely seen in the spines. In the proximal dendrites the dark material is found close to the plasma membrane. In addition there is an increasing number of clusters in the centre consisting of SER which include dark material, and occasionally, multivesicular bodies. In the distal parts the dark material is mainly found close to the plasma membrane.

A few neurons are markedly condensed with convoluted nuclear membrane, and the GA is extensively

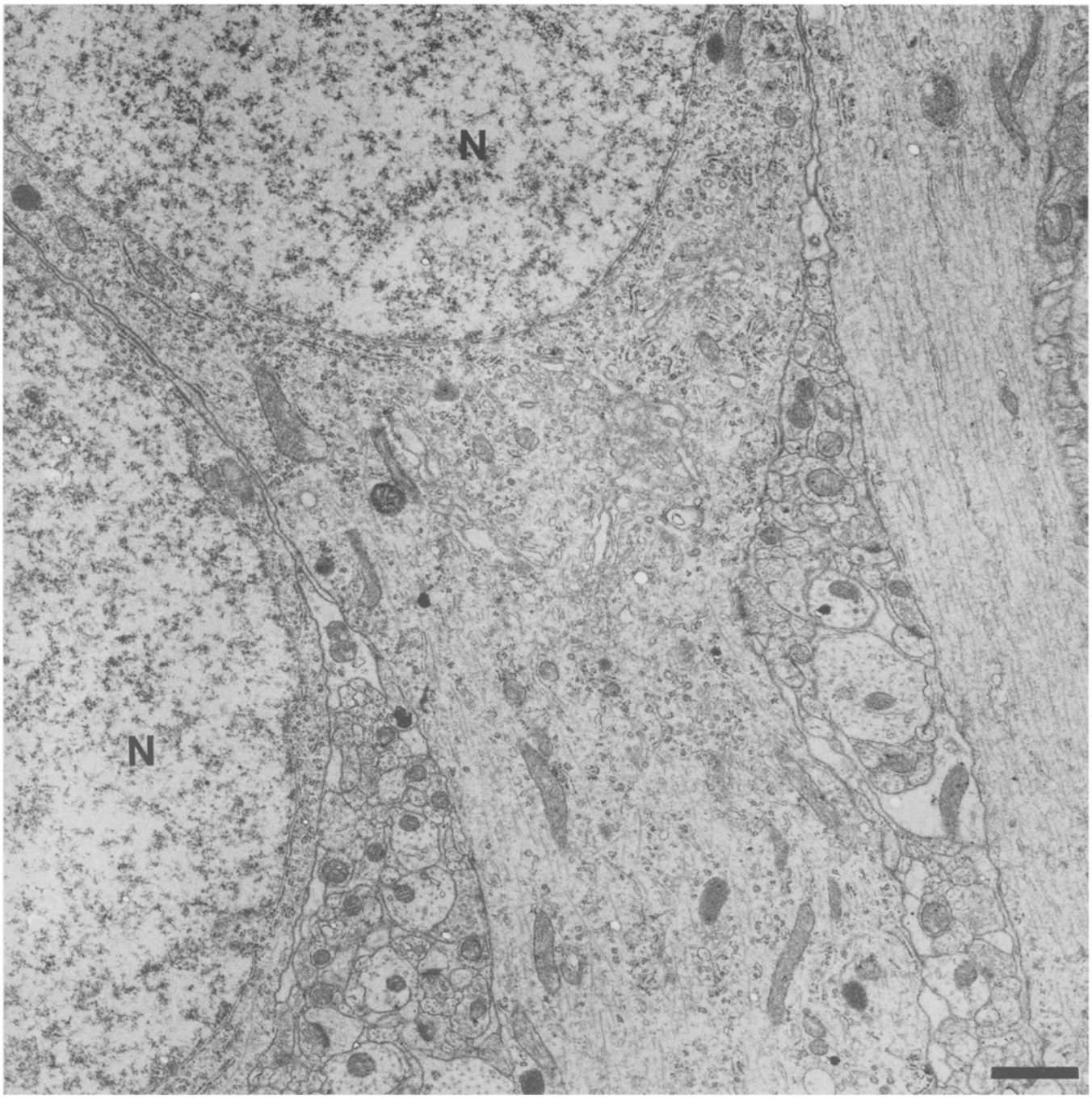

Fig. 2. *Control.* The nuclear chromatin (N) of the CA1 neurons is evenly dispersed and the cytoplasmic organelles appear normal. Ribosomes both in rosettes and close to endoplasmic reticulum cisternae are of normal size and orientation. The neuropil between the perikarya and the large proximal dendrites is compact with structurally normal neuronal and astrocytic processes. Bar 1 μm

dilated (Fig. 6). Some dendrites are dark and condensed with normal-appearing nerve endings abutting (Fig. 7). Astrocytes and their processes are somewhat swollen and contain watery cytoplasm rich in glycogen (Fig. 6).

10 min ischemia + 72 h of recirculation. At this time of recirculation the majority of the neurons display severe signs of degeneration although a few neurons are remarkably intact (Fig. 8). In the degenerated neurons the nucleus is disintegrated with clumping of chromatin and rupture of nuclear membranes concomitant with destruction of other organelles. In some neurons the cytoplasm contain membraneous whorls, vacuolar structures and translucent mitochondria. No dilatation of dendrites is evident in the *stratum radiatum*. In some of the neurons the dark material has disappeared from the cytosol. Instead, patches of tubular membrane structures with homogenous dark material is seen. The nuclei in these neu-

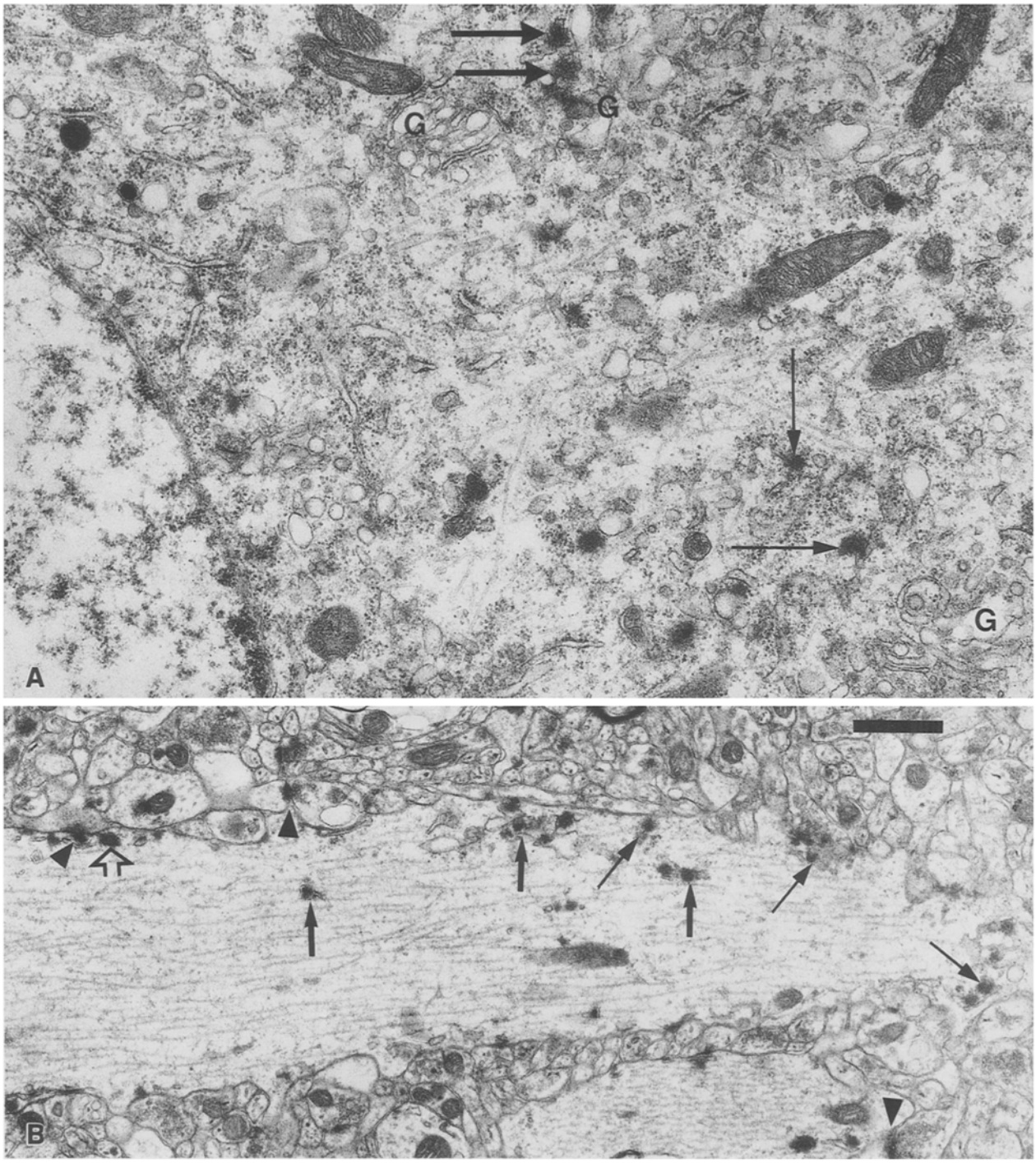

Fig. 3. A *10 min ischemia+6 h recirculation.* The nucleus and mitochondria look normal, but the ribosomes have become smaller and have lost their orientation in rosettes. Dark, fluffy material appears close to a Golgi apparatus (thick arrows) and to cisternae of endoplasmic reticulum (thin arrows). **B** A dendrite from the *stratum radiatum* of the CA1 region. Dark material is present in the dendroplasm, both attached to (thin arrows), or included into tubular saccules (thick arrows). Dark material is found close to synaptic structures (open arrow). The neuropil appears normal and the PSD's intact (arrowheads). No swollen dendrites are observed. Bar 0.5 μm

rons are still irregularly shaped but the chromatin dispersed. The mitochondria appear intact but not as compact as in the earlier recirculation periods. These features are particularly marked in one animal with only a few degenerated neurons in CA1 (Fig. 9). This particular animal display extensive damage in the hilus of the dentate gyrus, an area where necrosis develops rapidly (Smith et al. 1984A), showing that the hippocampus in this animal has been exposed to an ischemic insult.

Ultrastructural changes in other hippocampal subfields

In the neurons of the CA3 subfield and dentate gyrus no dark material is observed in any of the recovery groups.

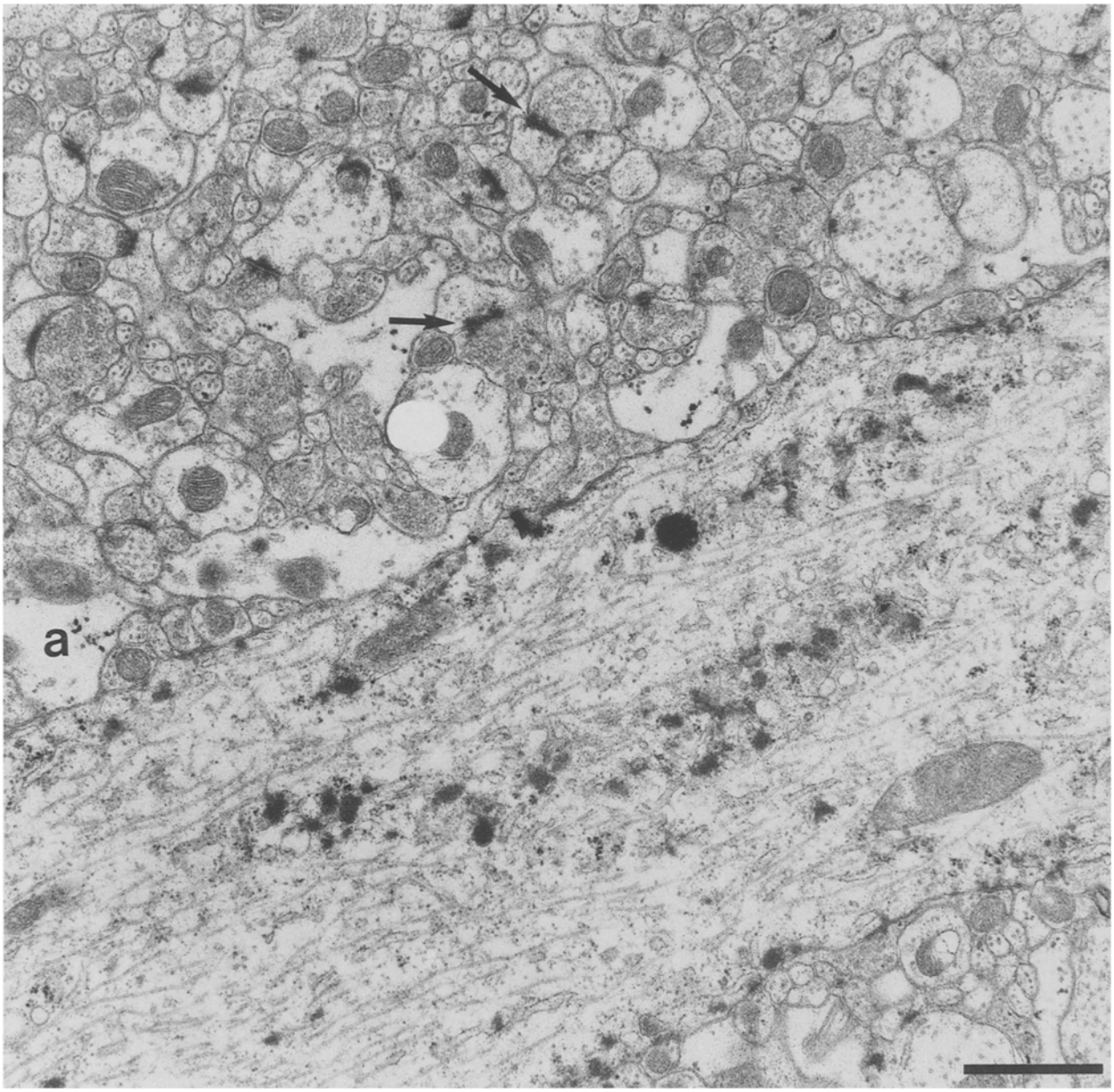

Fig. 4. *10 min ischemia + 24 h recirculation.* Dark material is forming clusters bound to saccules and is seen in the centre and periphery of the dendrites. The postsynaptic densities (arrows) of spines are preserved. Astrocytic processes are swollen with visible glycogen particles (a). Bar 0.5 μm

Calcium oxalate-pyroantimonate precipitation

In order to investigate whether the dark material was precipitable with oxalate-pyroantimonate (van Reempts and Borgers 1985), and thus potentially calcium precipitates, rats were subjected to 10 min of ischemia and 24 h or 48 h of recirculation. Figure 10 shows a section of CA1 pyramidal cell soma 48 h following 10 min ischemia. Precipitates are found in synaptic vesicles as previously reported (van Reempts and Borgers 1985). Precipitates are also found in cell nuclei, in dilated ER and in some mitochondria. In none of the sections studied does the intracellular location of calcium precipitates coincide with the dark material.

Heat-shock protein 70 and ubiquitin-conjugate immunoreactivity

Heat-shock protein 70 immunoreactivity (HSP 70–IR) and ubiquitin-protein conjugate immunoreactivity (UIR) were studied in rats subjected to 10 min ischemia and 24 h, 48 h or 72 h of recirculation. In control animals, UIR is seen in soma and dendrites of pyramidal CA1 neurons, similar to our earlier findings (Magnusson and Wieloch 1989) (Fig. 11A), while HSP 70–IR is not significantly increased compared with background (Fig. 11B). After 24 h and 48 h of recirculation, UIR disappears in all cells but the interneurons (Fig. 11C) while HSP 70–IR is markedly expressed (Fig. 11D). At 72 h postischemia

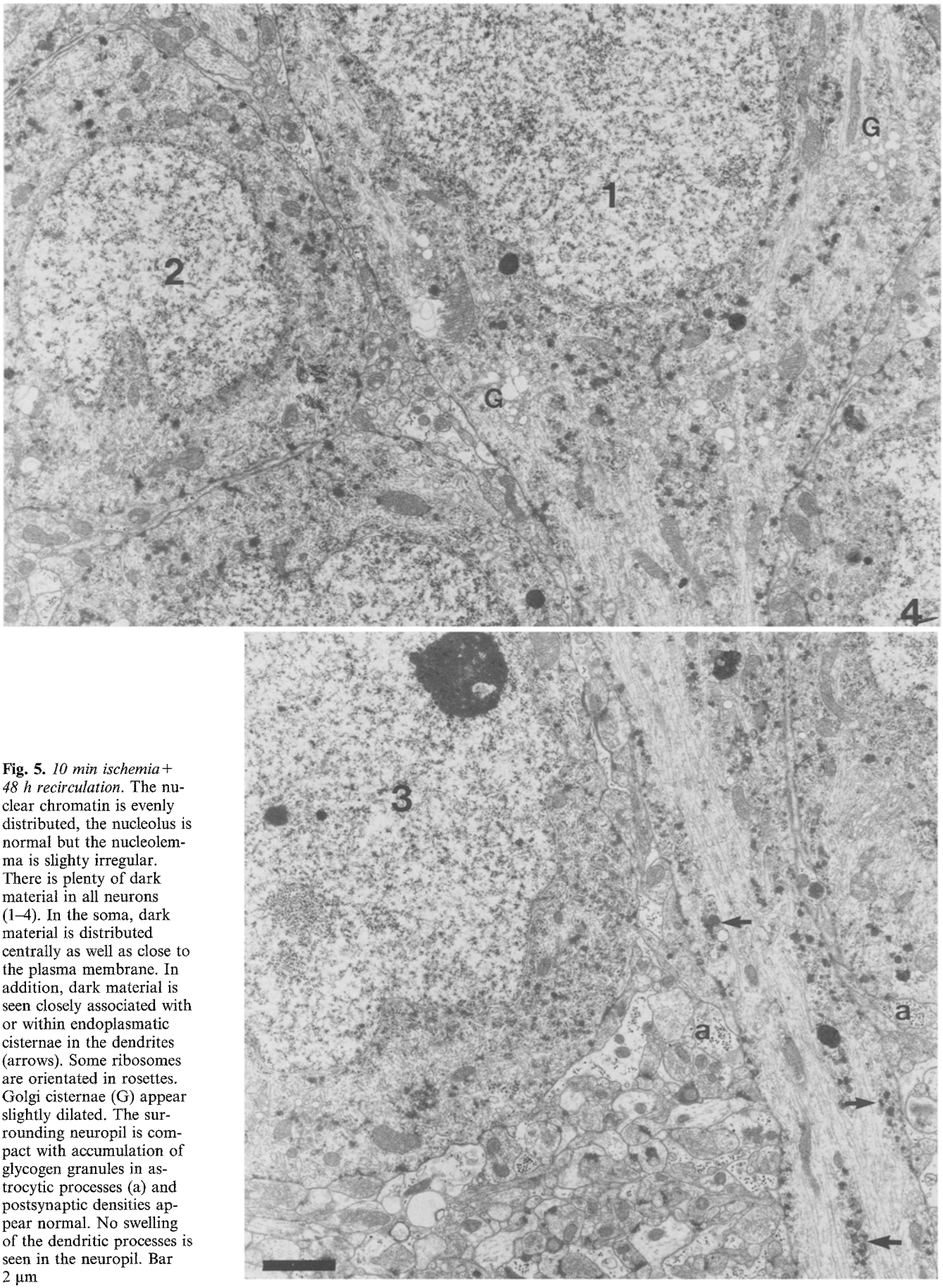

Fig. 5. *10 min ischemia + 48 h recirculation.* The nuclear chromatin is evenly distributed, the nucleolus is normal but the nucleolemma is slighty irregular. There is plenty of dark material in all neurons (1–4). In the soma, dark material is distributed centrally as well as close to the plasma membrane. In addition, dark material is seen closely associated with or within endoplasmatic cisternae in the dendrites (arrows). Some ribosomes are orientated in rosettes. Golgi cisternae (G) appear slightly dilated. The surrounding neuropil is compact with accumulation of glycogen granules in astrocytic processes (a) and postsynaptic densities appear normal. No swelling of the dendritic processes is seen in the neuropil. Bar 2 μm

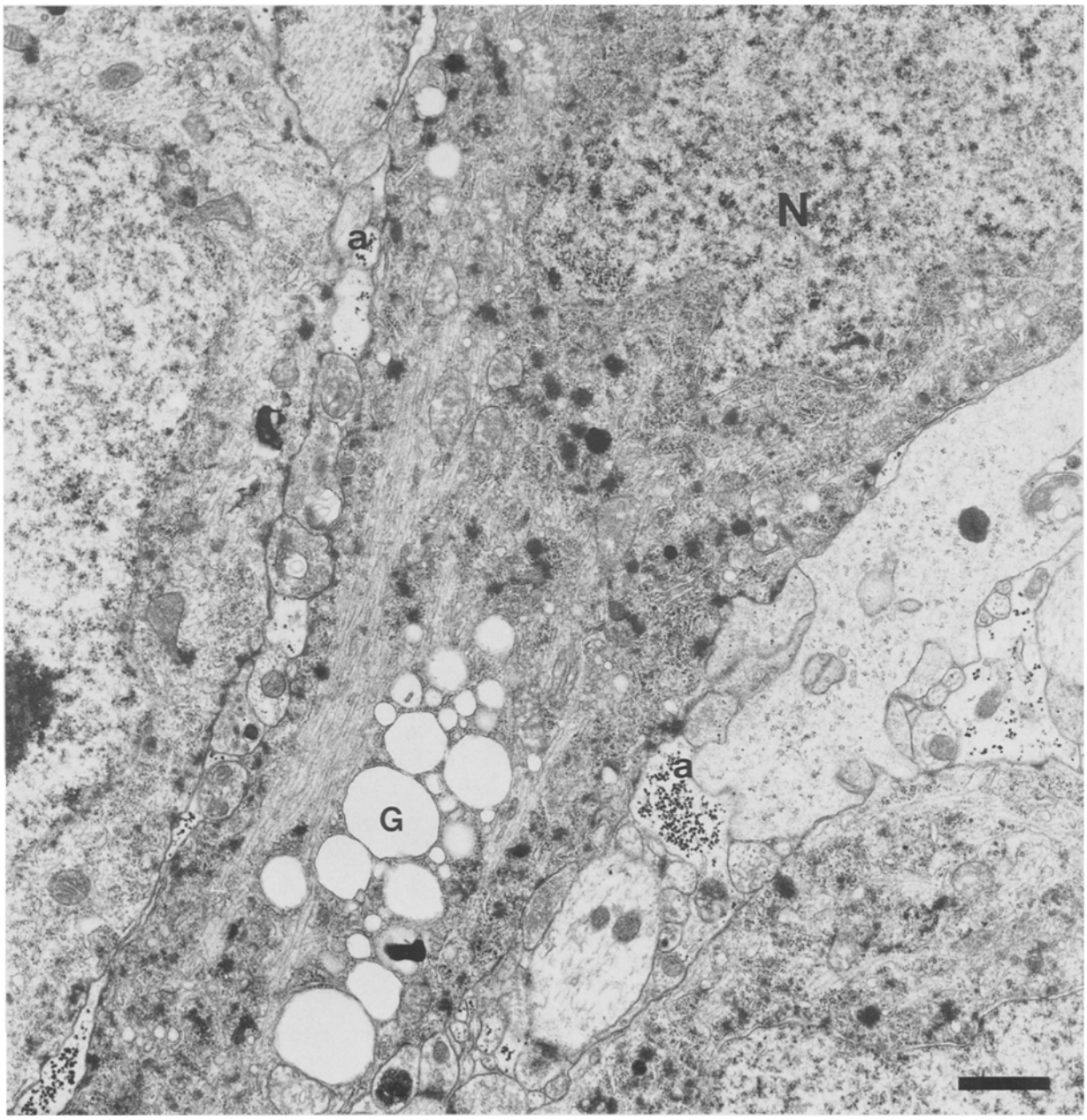

Fig. 6. *10 min ischemia + 48 h recirculation.* This neuron is condensed with dark patchy nuclear chromatin in the crenated nucleus (N) and increased stainability of the cytoplasm where the organelles appear crowded because of the condensation of the cytoplasm. Aggregates of nonmembrane bound dark material are often in close proximity to endoplasmic reticulum cisternae. The Golgi apparatus (G) is markedly swollen. There is an increased amount of glycogen granules in the perineuronal astrocytic processes (a). Bar 1 μm

when extensive neuronal degeneration is evident, UIR recovers in a few cells (Fig. 11E), whereas HSP 70–IR disappears in all but a few cells (Fig. 11F).

Effects of protein synthesis inhibitors

Table 1 shows the extent of neuronal necrosis in the CA1 region of animals exposed to 10 min of 2–VO ischemia treated with saline, protein synthesis inhibitors or an RNA synthesis inhibitor. In intrahippocampally saline-injected animals $95 \pm 2\%$ neuronal damage is observed. Neither cycloheximide, anisomycin nor actinomycin D decreases neuronal necrosis. When rats were subcutaneously injected with a single dose of anisomycin (25 $mg \cdot kg^{-1}$) 30 min prior to ischemia, $88 \pm 15\%$ neuronal damage in the CA1 region was observed. The neuronal damage found in the saline-injected control group was $85 \pm 19\%$. When anisomycin was given in multiple doses (30 min prior to ischemia and then 3 times every 2 h postischemia), all ($n = 4$) animals died within 48 h. In a paradigm where anisomycin was given in 3 injections (25 $mg \cdot kg^{-1} \times 3$ with 24 h intervals), the animals survived but the treatment did not improve the survival the CA1 neurons.

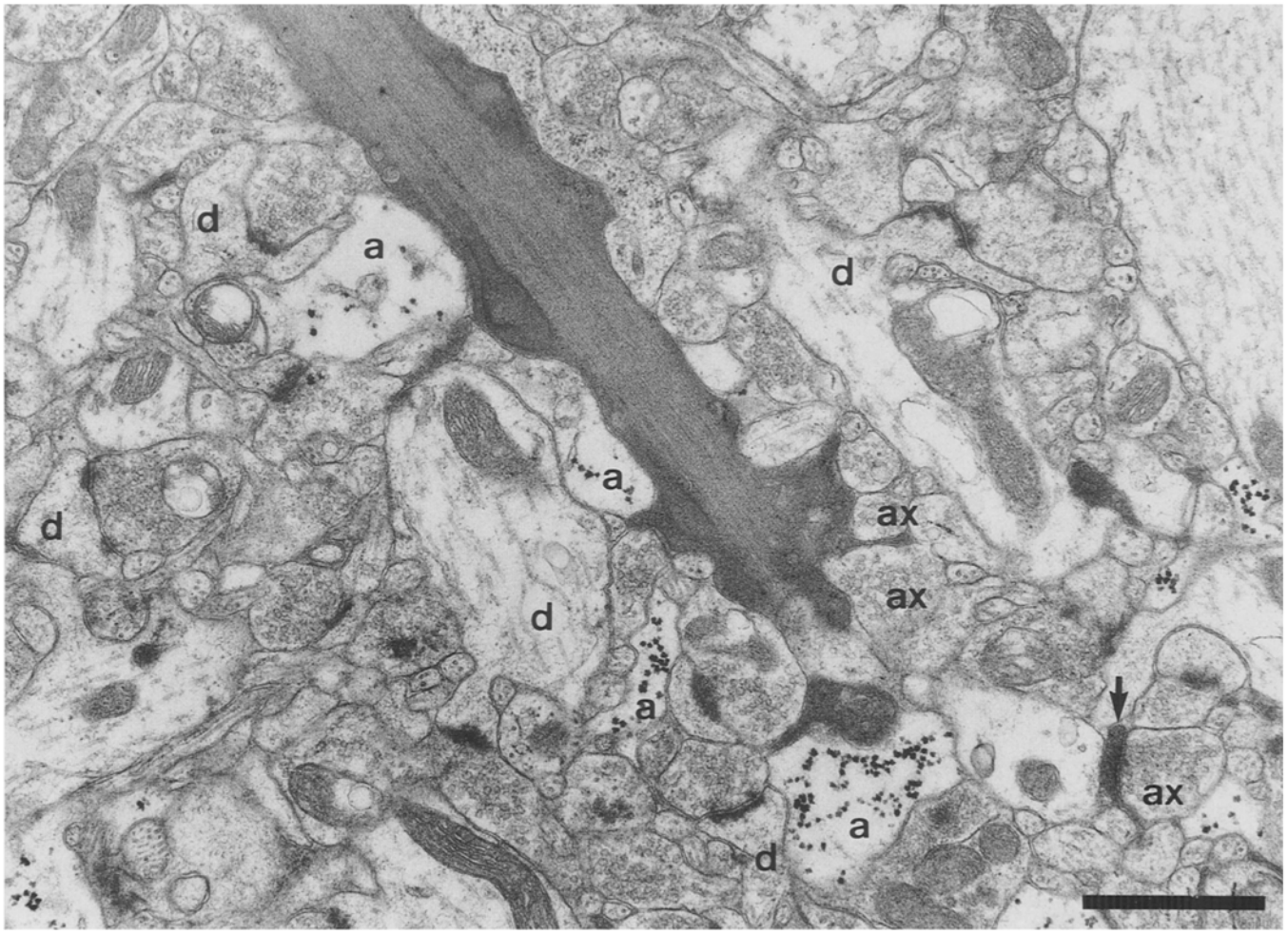

Fig. 7. *10 min ischemia+48 h recirculation.* A condensed dendrite in the CA1 *stratum radiatum,* with normal appearing axonal nerve endings (ax) abutting on it, and another nerve ending on a dark condensed profile (arrow) in the neuropil. No marked swelling of the dendrites (d) are observed. The astrocytic processes (a) are pale with abundant glycogen. Bar 1 μm

Discussion

During recent years, several pathophysiological mechanisms of delayed neuronal death have been proposed. The most extensively discussed are those related to free radical formation and lactic acidosis (Siesjö 1985), calcium toxicity (Choi 1987; Siesjö and Bengtsson 1989), release of neurotransmitters (Wieloch 1985; Choi 1988), and inhibition of protein synthesis (Widmann et al. 1991). The importance of these mechanisms in DND is still unclear. In previous discussions, the ultrastructural changes occurring during ischemia have often been neglected. In the presentation below we focus on the postischemic ultrastructural changes in the CA1 pyramidal neurons in relation to some current hypotheses of cell death.

Mitochondria and energy metabolism

One ultrastructural feature of the irreversibly injured CA1 neurons is the preservation of the mitochondria up to the moment of neuronal disintegration. These findings correlate with those observed in the gerbil (Kirino and Sano 1984) and in the rat following 4–VO ischemia (Petito and Pulsinelli 1984B), and are in accord with the revival of the mitochondrial function demonstrated by the rapid recovery and preservation of the ATP production in the reperfusion phase (Pulsinelli and Duffy 1983; Blomqvist et al. 1985). Although energy depletion *during* ischemia triggers processes leading to neuronal necrosis, the postischemic events leading to DND appear not to be due to energy failure during reperfusion.

Cell structure and glutamate neurotoxicity

Glutamate is an excitatory transmitter and an important factor mediating neuronal death in the CA1 region during the postischemic phase. For example lesions of the glutamatergic excitatory pathways to the hippocampus (Wieloch et al. 1985; Onodera et al. 1986; Jörgensen et al. 1987), pharmacological blockade of two glutamate receptor subtypes, the N-methyl-D-aspartate (NMDA)

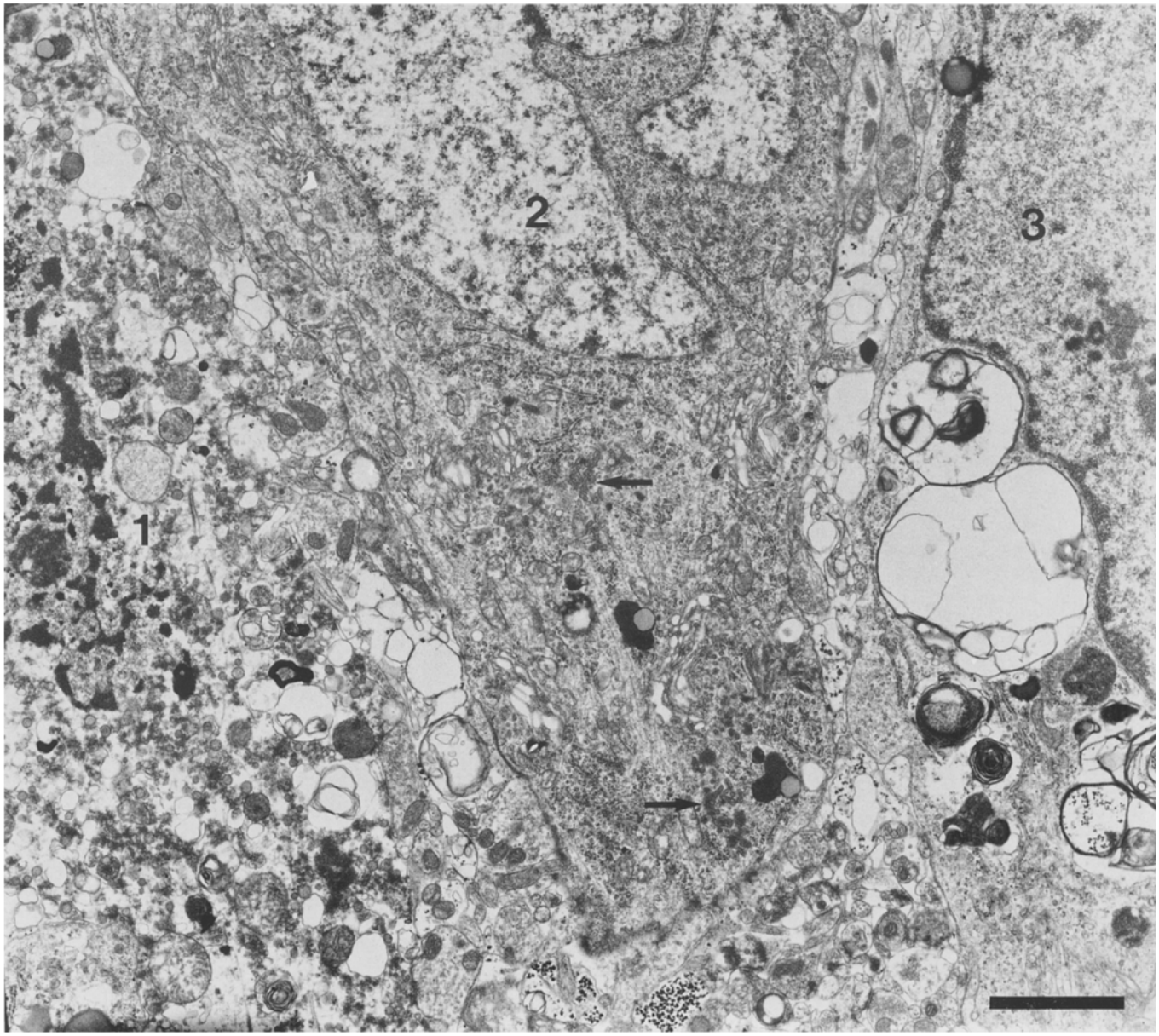

Fig. 8. *10 min ischemia + 72 h recirculation.* One neuron (1) is completely disintegrated with dark nuclear clumps within cytoplasmic debris. The plasma membrane can no longer be discerned. Another neuron (2) is well preserved, with ribosomes in rosettes. Note the dark material within small membrane bound vesicles (arrows) in the vicinity of Golgi apparatuses, and the transluscent mitochondria. In a third neuron (3) membraneous whorls are seen. Bar 2 μm

(Meldrum and Swan 1989) and the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) receptors (Sheardown et al. 1990; Nellgård and Wieloch 1992), decrease neuronal damage in the CA1 region. Two mechanisms of glutamate toxicity during and following ischemia have been proposed. One suggests that glutamate activates postsynaptic receptors, increasing the influx of sodium across the plasma membrane (Rothman 1984), accompanied by passive influx of chloride ions and water, leading to cell swelling and mechanical cytolysis. Dendritic swelling is not observed in the present investigation, therefore DND is not likely to be caused by glutamate-induced osmotic cytolysis. In fact, neurons appear to preferentially degenerate by a process preceded by neuronal condensation (Fig. 8). The other mechanism of glutamate neurotoxicity during ischemia was proposed by Choi (1988), who suggested that the NMDA receptor mediated calcium influx activates degenerative processes, leading to cell death. In the model employed in this study a severe ischemic insult is induced, and blockade of the NMDA receptors by antagonists given pre- or postischemia does not decrease neuronal damage (Nellgård et al. 1991). However AMPA receptor blockade by NBQX (2-3 dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline) markedly decreases cell death (Nellgård and Wieloch 1992). The mechanism behind AMPA receptor toxicity following ischemia is not understood. However, the highest density of AMPA receptors

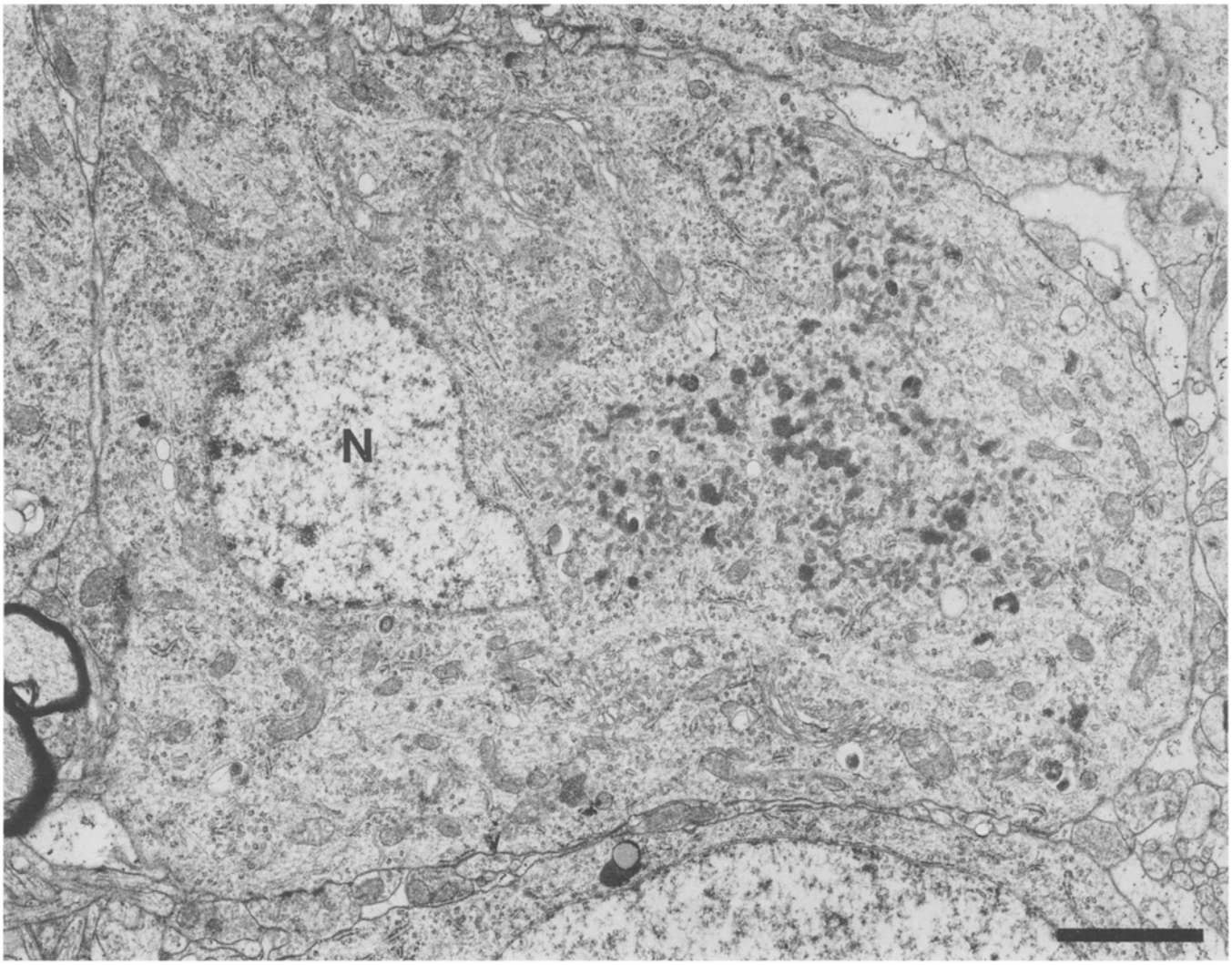

Fig. 9. *10 min ischemia + 72 recirculation.* A CA1 neuron after 72 h recirculation. This animal exhibited minor damage in the entire CA1 region. In the cytoplasm of this otherwise normally appearing neuron, dark material is seen within small vesicles and tubular structures. N = nucleus. Bar 2 μm

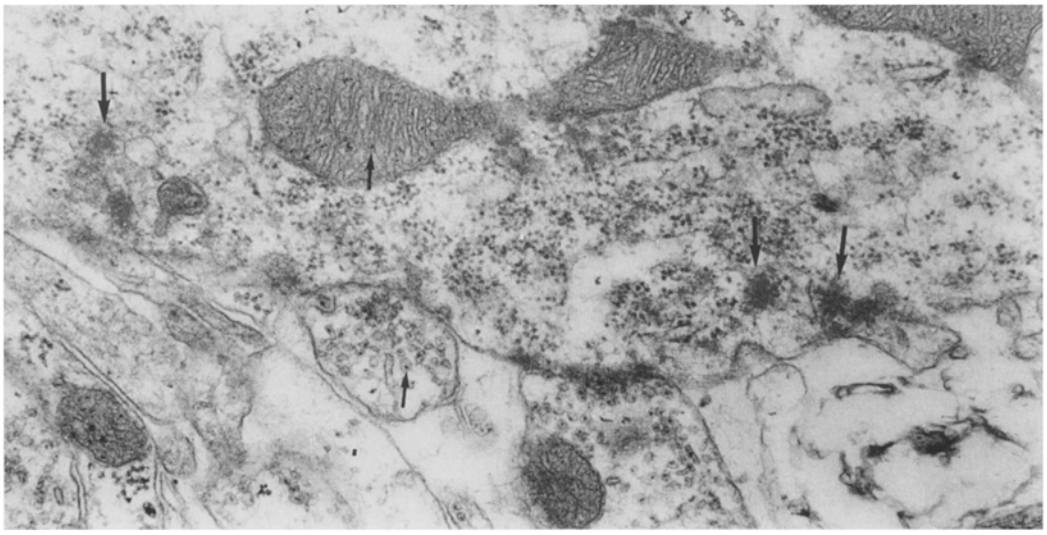

Fig. 10. *10 min ischemia + 6 h recirculation.* The section was stained using the oxalate pyroantimonate technique. Calcium precipitates are found in synaptic vesicles and mitochondria (small arrows). No precipitate coincides with the dark material (larger arrows)

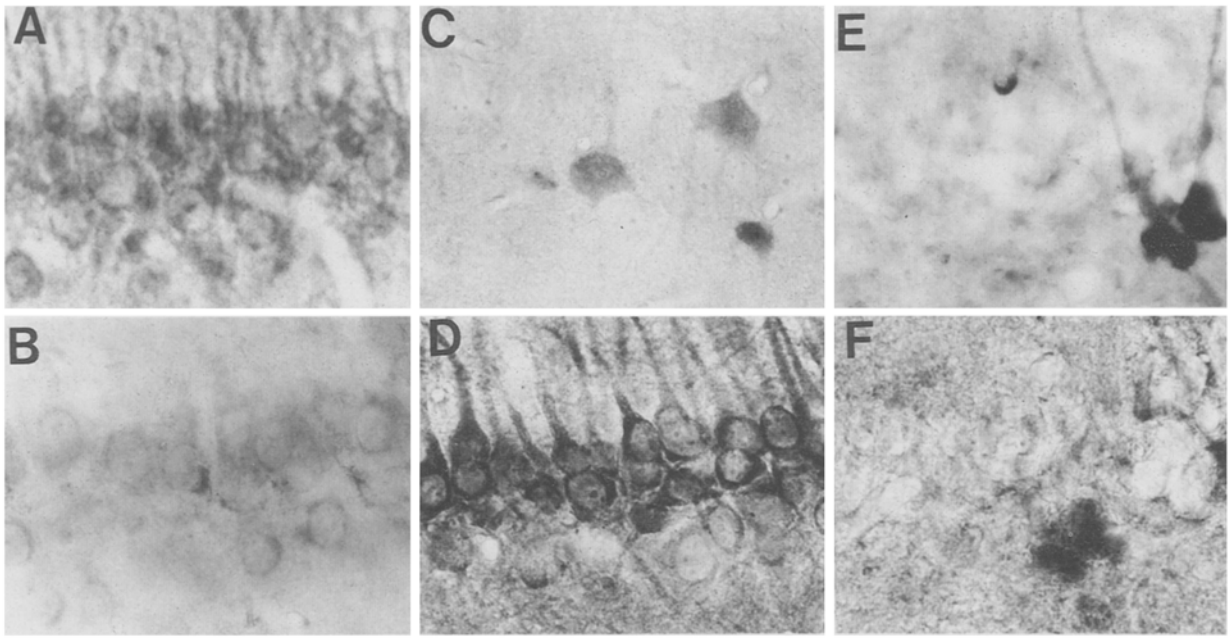

Fig. 11A–F. Photomicrographs of ubiquitin immunoreactivity (UIR) and heat shock protein 70 immunoreactivity (HSP–70 IR) in the hippocampal CA1 region. In control animals ubiquitin immunoreactivity is seen in all neurons (**A**) while HSP–70 IR is weak (**B**). Fortyeight hours following 10 min ischemia UIR is lost from all but a few neurons (**C**). In contrast a marked HSP–70 IR can be seen in the cytoplasm of the CA1 pyramidal cells (**D**). Seventytwo hours postischemia no UIR (**E**) and HSP–70 IR (**F**) is seen in but some scattered cells

is found in the pyramidal cell layer of the CA1 region (Westerberg et al. 1989; Monaghan et al. 1990) correlating with the site of accumulation of the dark material. This implies that the formation of the dark material may be stimulated by AMPA receptor activation and be a part of a process leading to cell death.

Protein synthesis and delayed neuronal death

It is very likely that the dark material is constituted of protein aggregates and that these could be formed as a result of changes in gene expression and/or protein or membrane turnover. In the present study we demonstrate that HSP–70IR, not found in control brains, is present in the CA1 region at 24 h of recirculation, and remains elevated until neuronal degeneration. So, although general protein synthesis is severely depressed (Widmann et al. 1991), the CA1 pyramidal neurons still have a capacity to synthesize certain classes of proteins. One attractive possibility is therefore that DND is an active protein synthesis-dependent process leading to cell death similar to apoptosis (Wyllie et al. 1984; Martin et al. 1988). Apoptosis is characterized by early extensive chromatin condensation and margination, dispersion of the nucleolus, dilatation of ER, concomitant with a preservation of mitochondrial structure. In addition, the nucleus is often displaced to one pole of the cell, and filamental bundles may appear in conjunction with plasma membrane protrusions called blebs. In the final stages of cellular dissolution, apoptotic bodies may be formed by cellular fragmentation into spheric vesicles (Wyllie et al. 1984.) However, DND is not accompanied by these ultrastructural changes, and is therefore not likely to be a form of apoptosis. Another form of protein synthesis-dependent neuronal death is observed when sympathetic neurons in vivo and in vitro are deprived of nerve growth factor (NGF) (Levi-Montalcini et al. 1969; Angletti et al. 1971; Martin et al. 1988). Protein synthesis and RNA synthesis inhibitors prevent neuronal death induced by depleting cell cultures of NGF, and it has been suggested that NGF normally depresses the formation of proteins that, if expressed, induce neuronal degeneration. Protein synthesis inhibitors and RNA synthesis inhibitors also prevent apoptosis (Pratt and Greene 1976). However, as demonstrated in the present investigation neither systemically administered nor locally applied protein synthesis or RNA synthesis inhibitors mitigated neuronal damage in the CA1 region following 10 min of 2–VO ischemia in the rat. It is thus less likely that DND is a form of programmed cell death. This is in contrast to the findings in gerbils by Shigeno et al. (1990) where subcutaneous administration of a protein synthesis inhibitor was protective after 5 min ischemia. This protective effect was abolished when body temperature was controlled (Kiessling et al. 1991). Our results do not exclude the possibility that deficiencies in growth factor linked processes, important for cell survival (Mattson et al. 1989), others than those stated above, also may be important in DND.

Possible identity of dark material

The possibility that the dark material is a lysosomal structure is discounted by the fact that it is not surrounded by a membrane (Peters et al. 1976) at early recirculation periods (Fig. 8). It is not a calcium precipitate resulting from intracellular calcium overload since the dark material or the associated tubular structures do not contain precipitated calcium, in accord with earlier findings (van Reempts et al. 1985).

The dark material is often associated with a tubular structure similar to SER in the soma, and the hypolemma in the dendrites (Peters et al. 1976). It is similar in size and structure to the postsynaptic filamentous material (Kadota and Kadota 1979; Tatsuoka 1986), PSDs (Peters et al. 1976) or internalized receptors (Aoki et al. 1987). It is indeed tempting to conclude that the dark material is a similar structure dissociated from the plasma membrane due to for example proteolysis (Siman et al. 1989; Seubert et al. 1989), or overstimulation of receptors by increased extracellular ligand concentrations (Hagberg et al. 1986). Disturbances in the protein turnover due to a defect in the ubiquitin and ATP-dependent proteolysis system may lead to the precipitation of large protein aggregates (Finley and Varshavsky 1985; Reichenstein 1987; Parag et al. 1987). The absence of UIR in the CA1 (Fig. 11) demonstrates that the requisite ubiquitin conjugation and ATP dependent protein degradation is interrupted postischemia. The stalled ubiquitin dependent proteolysis may contribute to the accumulation of the dark material, disrupting cell homeostasis and architecture, leading to cell death (Finley and Varshavsky 1985; Reichenstein 1987). On the other hand, in cells that survive an ischemic insult (Fig. 9) the accumulated proteins (dark material) may be processed in the lysosomal pathway of protein degradation (Mayer et al. 1991).

Conclusion

Our results show that delayed neuronal death in the hippocampus is not a form of programmed cell death or apoptosis, since the ultrastructural changes during DND are different from those observed during apoptosis and since protein synthesis and RNA synthesis inhibitors do not prevent neuronal degeneration. Delayed neuronal death is accompanied by an increased accumulation of dark cytoplasmic material, preferentially located in the soma and proximal dendrites, both centrally and close to the plasma membrane. The dark material, present in areas with high AMPA receptor density, may be formed due to an aberrant turnover of cellular proteins induced by glutamate receptor stimulation, leading to cell death.

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