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RESEARCH ARTICLE

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"Dark" (compacted) neurons may not die through the necrotic pathway

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Abstract "Dark" neurons were produced in the cortex of the rat brain by hypoglycemic convulsions. In the somatodendritic domain of each affected neuron, the ultrastructural elements, except for disturbed mitochondria, were remarkably preserved during the acute stage, but the distances between them were reduced dramatically (ultrastructural compaction). Following a 1-min convulsion period, only a few neurons were involved and their environment appeared undamaged. In contrast, 1-h convulsions affected many neurons and caused swelling of astrocytic processes and neuronal dendrites (excitotoxic neuropil). A proportion of "dark" neurons recovered the normal structure in 2 days. The non-recovering "dark" neurons were removed from the brain cortex through two entirely different pathways. In the case of 1-h convulsions, their organelles swelled, then disintegrated and finally dispersed into the neuropil through large gaps in the plasma membrane (necrotic-like removal). Following a 1-min convulsion period, the non-recovering "dark" neurons fell apart into membrane-bound fragments that retained the compacted interior even after being engulfed by astrocytes or microglial cells (apoptotic-like removal). Consequently, in contrast to what is generally accepted, the "dark" neurons produced by 1-min hypoglycemic convulsions do not die as a consequence of necrosis. As regards the case of 1-h convulsions, it is assumed that a necrotic-like removal process is imposed, by an excitotoxic environment, on "dark" neurons that previously died through a non-necrotic pathway. Apoptotic neurons were produced in the hippocampal dentate gyrus by intraventricularly administered colchicine. After the biochemical processes had been completed and the chromatin condensation in the nucleus had reached an advanced phase, the ultrastructural elements in the somatodendritic cytoplasm of the affected cells became compacted. If present in an apparently undamaged environment such apoptotic neurons were removed from the dentate gyrus through the apoptotic sequence of morphological changes, whereas those present in an impaired environment were removed through a necrotic-like sequence of morphological changes. This suggests that the removal pathway may depend on the environment and not on the death pathway, as also assumed in the case of the "dark" neurons produced by hypoglycemic convulsions.

Keywords Hypoglycemic convulsions · "Dark" neurons · Cell death · Silver staining · Electron microscopy

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Introduction

It has been generally assumed that the death of "dark" neurons is caused by necrosis, irrespective of the pathometabolic conditions initiating their formation [status epilepticus (Attilo et al. 1983, Kubova et al. 2001), ischemia (Petito and Pulsinelly 1984), hypoglycemia (Auer et al. 1985a, b), poisoning with *N*-methyl-D-aspartate (Dietrich et al. 1992) or ethanol (Obernier et al. 2002), etc.]. This assumption was based on the facts that the affected neurons did not display the morphological and/or biochemical characteristics of apoptosis, but their organelles swelled, disintegrated, and dispersed into the excitotoxic environment through large gaps in the plasma membrane.

On the other hand, the "dark" (compacted) neurons that do not recover in an apparently undamaged environment following an electric shock or a non-contusing concussive mechanical injury fall apart into membrane-bound fragments that retain the compacted ultrastructure even after

being engulfed by phagocytotic cells (Csordás et al. 2003). The explanation of why this finding is at variance with those mentioned above may lie in either of the following possibilities: in this study, "dark" neurons were produced by momentary physical insults (not by lasting pathometabolic conditions) or their environment was apparently undamaged (not excitotoxic).

In order to decide between these possibilities, the present paper deals with the fates of "dark" neurons produced by hypoglycemic convulsions in an environment that is either apparently undamaged or excitotoxic. For comparison, the ultrastructural sequence of the removal of apoptotic granule neurons from morphologically undamaged versus impaired hippocampal dentate gyri is demonstrated.

Materials and methods

Animal experiments

In order to minimize suffering, animal care and handling were carried out throughout the experiments in accordance with order 243/1998 of the Hungarian Government, which is an adaptation of directive 86/609/EGK of the European Committee Council.

Induction of hypoglycemic convulsions by insulin administration

Under deep anesthesia (50 mg/kg sodium pentobarbital, i.p.), 37 Wistar rats weighing between 200 and 220 g were fasted for 1 day and then received 25 IU/kg insulin (a 1:1 mixture of Humulin-R and Humulin-U, HUMAN RT, Gödöllő, Hungary) i.p. About 2 h later they became unresponsive and next underwent a short period of convulsions. At 1 min after the onset of the convulsions, 3 rats were killed by transcardial perfusion fixation, while 9 rats received 20% glucose solution i.p. (2.5 ml as the first dose, and 0.5 ml doses every half an hour thereafter) until the blood glucose level had been stabilized within the normal range. They were killed 1, 2, or 6 days thereafter (3 rats at each time). The remaining 25 rats suffered several shorter or longer periods of convulsions during the 1-h period following the onset of the first attack. Thirteen of them died during this period and were excluded from further investigation. Three of the surviving 12 rats were perfusion-fixed at the end of this period, whereas the remaining 9 received 20% glucose solution i.p. in the above doses; 6 of them were killed 1 or 2 days later (3 rats at each time), whereas the 3 rats reserved for the 6-day survival were also excluded from further investigation since they died "spontaneously" in the meantime and could not be perfusion-fixed.

Induction of apoptosis by colchicine administration

Under deep anesthesia (50 mg/kg sodium pentobarbital, i.p.), each of 9 Wistar rats weighing between 200 and 220 g was fixed in a stereotaxic head holder. The scalp was incised, stretched apart and then a hole was drilled through the calvaria on the right side, 1 mm caudal to bregma and 1.5 mm lateral to the midline. By means of a stereotaxic micromanipulator, the needle of a Hamilton syringe was inserted vertically into the brain, with its tip 3.5 mm deep to the cortical surface. Subsequently, 5 μg colchicine dissolved in 0.5 μl distilled water was injected into the lateral ventricle. The wound was sutured and the rats were allowed to recover. They were perfusion-fixed 1, 2, or 6 days later (3 at each time).

Control animals

Three rats were fasted for 1 day then fixed by transcardial perfusion. Three other rats received 0.5 μl physiological saline intraventricularly and were perfusion-fixed 2 days later.

Fixation, tissue processing, and staining

If necessary, the rats were re-anesthetized before perfusion fixation. The fixative was prepared by mixing 500 ml 0.2 M sodium cacodylate, 100 ml 20% paraformaldehyde, 100 ml 25% glutaraldehyde, 50 ml 0.1 M calcium chloride, and 250 ml 10% polyvinylpyrrolidone K25, followed by adjustment of the mixture to pH 7.5 with a few drops of 0.1 M hydrochloric acid. Before fixation, the vascular system was rinsed with physiological saline for 30 s. After fixation, the rats were left undisturbed at room temperature for 24 h before removal of the brain from the skull (Cammermeyer 1961). The caudal half of the cerebrum was vibratome-sectioned coronally at 150 μm. Every tenth section was stained by a special silver method that selectively demonstrates both the "dark" and the apoptotic neurons together with their dendrites (Gallyas et al. 1990) in a reproducible manner (Newman and Jasani 1998). For silver staining, sections were incubated for 48 h at 56°C in 1-propanol containing 0.8% sulfuric acid and 2% water (esterification), then washed with distilled water for 5 min and finally immersed in a special physical developer until the background had become light brown when examined under a low power lens.

Adjacent vibratome sections were flat-embedded in paraffin, cut at 10 μm, mounted on Vectabond-coated microscopic slides, and then stained with a fluorescent TUNEL kit (Upstate Biotechnology, Lake Placid, NY; catalogue #17141), with its "detection procedure" being strictly observed.

From other vibratome sections, 2×2-mm^2 areas of the neocortex adjacent to the areas that contained numerous silver-stained neurons were dissected out, postfixed with a 1:1 mixture of 2% osmium tetroxide and 3% potassium

ferrocyanide for 1 h at room temperature, and then flat-embedded in Durcupan ACM. Thin sections were cut at 40 nm and stained with uranyl acetate and lead citrate in the usual manner.

Durcupan-embedded semithin sections were cut at 0.5 μm and air-dried onto microscopic slides previously coated with Vectabond adhesive. They were stained in a solution containing 0.05% toluidine blue, 0.05% sodium tetraborate, and 0.1% saccharose (pH 9.5) for 1 min at 90° C, or in a solution containing 0.1% acid fuchsin, 10% acetic acid, and 50% 1-propanol for 1 min at 90°C. From several slides, toluidine blue was removed by washing with 1-propanol containing 0.5% sulfuric acid before staining with acid fuchsin. A green color filter was used for the microscopic examination and microphotography of acid fuchsin-stained sections. Microphotographs of such sections were contrasted by means of an electronic program (Adobe Photoshop 5.0).

Results

General information

The observations described below are confined to the morphological features pertinent to the formation, recovery, or death of "dark" neurons produced by hypoglycemic convulsions in the brain cortex of the rat, and also to the removal from the hippocampal dentate gyri of the granule neurons killed by intraventricularly administered colchicine. Other aspects of the hypoglycemic (Auer et al. 1985a, b; Kalimo et al. 1985) and the colchicine-induced (Goldschmidt and Steward 1980, 1982) brain damage have been dealt with adequately in previous papers.

Irrespective of the length of hypoglycemic convulsion period the dead "dark" neurons did not show TUNEL positivity. None of the morphological abnormalities described below were observed in any of the control rats.

Hypoglycemia, 1-min convulsion period, light microscopy

Immediate killing

Several neurons in the superficial cortical layers (layers I–III) were markedly shrunken, hyperbasophilic (capable of binding a striking excess of positively charged dye molecules such as toluidine blue) and stainable homogeneously with the silver method used (Figs. 1a, 2a). They were randomly scattered among normal-looking neurons in an apparently undamaged environment. The incidence of the affected neurons was estimated to be lower than a few per cent, even in the areas where they appeared to form small groups in thick vibratome sections. Virtually no such neurons were observed in the deep cortical layers (layers V and VI).

One-day survival

In the superficial cortical layers, neurons containing mitochondrion-sized silver-stained dots were intermingled with shrunken and homogenously silver-stained neurons (Fig. 1b). In a few instances, the distal part of a main dendrite was homogenously silver-stained, whereas both the proximal part and the parent soma contained silver-stained dots (Fig. 1c). In semithin sections, shrunken neurons exhibited convoluted profiles with sharp outlines, and were both hyperbasophilic (Fig. 2c) and acidophilic (stainable with acid fuchsin). In other respects, the parenchyma appeared undamaged.

Two-day and 6-day survival

Neurons containing silver-stained dots were not found. Shrunken neurons were both acidophilic and hyperbasophilic.

Hypoglycemia, 1-h convulsion period, light microscopy

Immediate killing

As compared with the findings in the rats with a 1-min convulsion period: (1) the number of affected neurons was much higher in the superficial cortical layers (Fig. 1d), (2) both their shrinkage and hyperbasophilia displayed more than one degree (Fig. 2b), (3) neurons in the deep cortical layers were also affected either in small groups (Fig. 1d) or solitarily, and (4) the neuropil was spongy (Fig. 2b).

One-day survival

In the deep cortical layers, most of the affected neurons contained silver-stained dots (Fig. 1e, f, h), whereas homogenously silver-stained neurons predominated in the superficial cortical layers (Fig. 1g). Their dendrites had separated into fragments that were much larger than mitochondria. In semithin sections, the affected neurons displayed faded outlines and an inhomogeneous interior (Fig. 2d), and were both acidophilic and hyperbasophilic (Fig. 2e, f).

Two-day survival

In the superficial cortical layers, numerous neuronal somata remained shrunken, acidophilic, hyperbasophilic, and stainable with silver (dead "dark" neurons). Their dendrites were completely fragmented (Fig. 1i). Neurons containing silver-stained dots were not found. With the exception of solitary dead "dark" neurons, the deep cortical layers exhibited no abnormality.

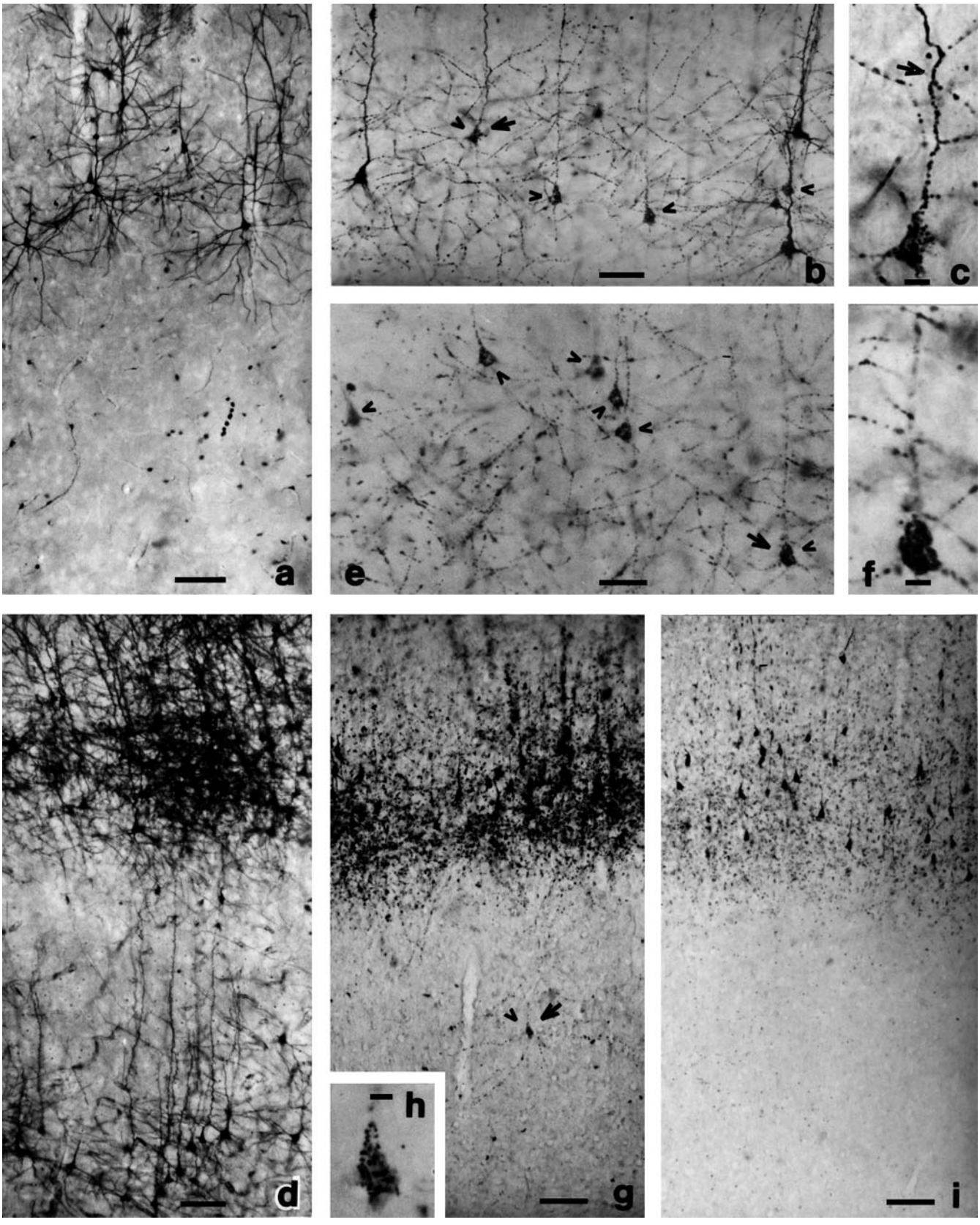


Fig. 1a–i Silver-stained "dark" neurons in 150-μm-thick vibratome sections cut from the frontoparietal cortex of rats exposed to hypoglycemia with a 1-min (**a–c**) or a 1-h (**d–i**) convulsion period. Survival times: **a**, **d** <1 min; **b**, **c**, **e–h** 1 day; **i** 2 days. *Arrows* in **b**, **e**, and **g** point to neurons depicted in **c**, **f**, and **h**, respectively. An *arrow* in **c** points to a dendritic site where the silver staining turns from dotted to homogenous. *Arrowheads* in **b** and **e** point to neurons containing silver-stained dots. *Scale bars* **a**, **d**, **g**, **i** 500 μm; **b**, **e** 100 μm; **c**, **f**, **h** 50 μm

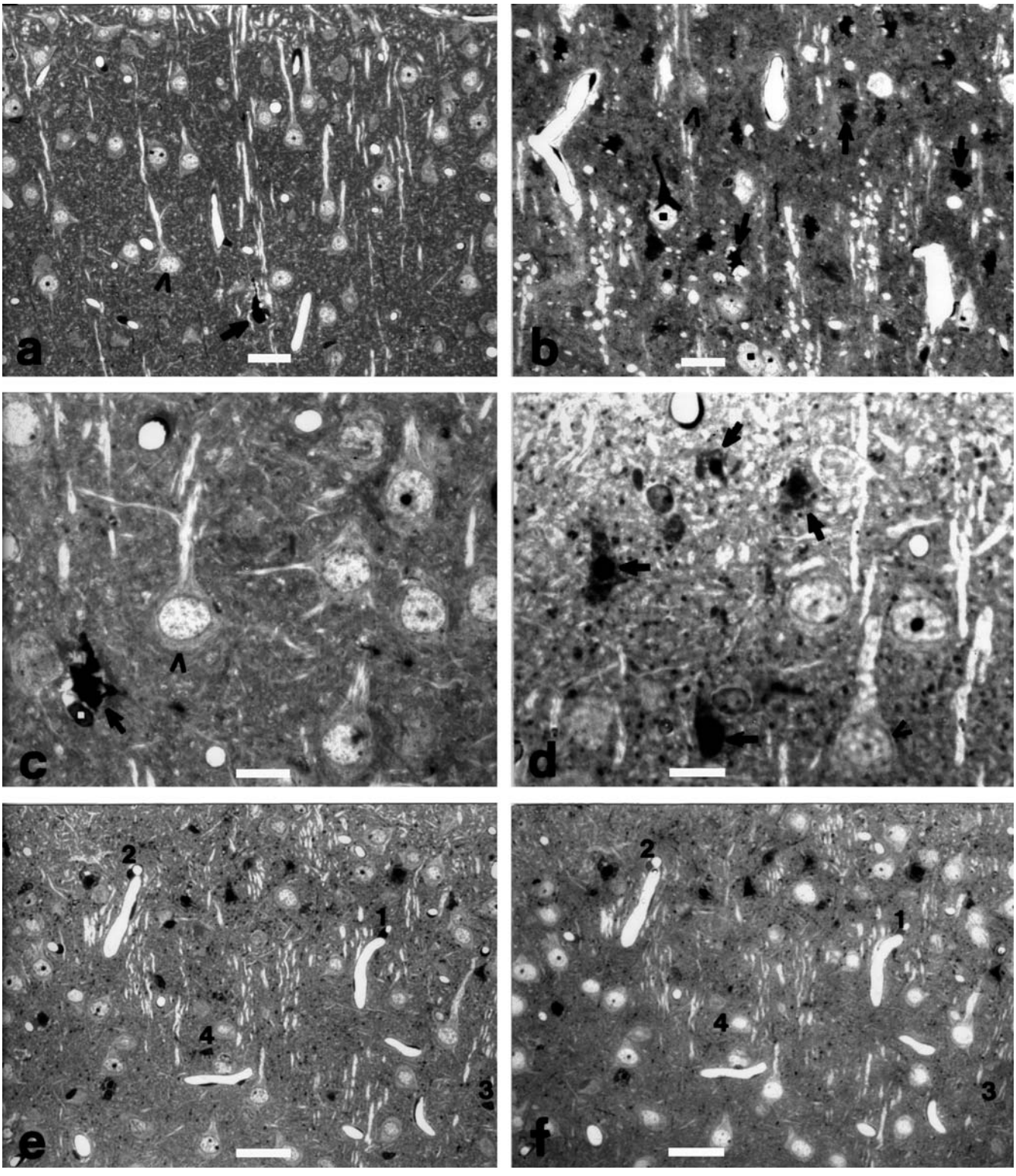


Fig. 2a–f "Dark" and normal neurons in the superficial pyramidal layers of the frontoparietal cortex of rats exposed to hypoglycemia with a 1-min (**a**, **c**) or a 1-h (**b**, **d–f**) convulsion period. Survival times: **a**, **b** <1 min; **c–f** 1 day. **a–e** Stained with toluidine blue; **f** stained with acid fuchsin after the removal of toluidine blue from the section depicted in **e** (photographed using a green color filter, contrasted electronically). In addition to "dark" neurons, nuclei of pericytes (*1*, *2* in **e**, **f**), oligodendrocytes, and microglial cells (*3*, *4* in **e**, **f**) are also hyperbasophilic, but hardly acidophilic. Minute black dots represent dendritic fragments of the "dark" neurons. In **a–d**, *arrows* point to "dark" neurons and *arrowheads* to normal neurons. In **b**, *small black squares* denote "light" neurons. In **c**, nucleus of a microglial cell is marked with a *small white square*. *Scale bars* **a**, **b**, **e**, **f** 100 μm; **c**, **d** 200 μm

Hypoglycemia, 1-min convulsion period, electron microscopy

Immediate killing

In the "dark" neurons, with the exception of a small proportion of mitochondria with disturbed interior, the ultrastructural elements were apparently undamaged, but the distances between them were strikingly reduced (ultrastructural compaction) and their electron density was markedly increased (Fig. 3a). The endoplasmic reticulum cisternae were contracted, whereas the Golgi cisternae were dilated. The extent of the ultrastructural compaction appeared to be commensurate throughout each somal or dendritic profile, whereas the pertinent axon remained unaffected. A large volume of fluid escaped from the affected neuronal somatodendritic domains through the apparently undamaged plasma membrane and had been taken up by adjacent astrocytic profiles. "Light" neurons were not present. In a few neurons with seemingly normal morphology, a small proportion of mitochondria was slightly swollen. In other respects, the neuropil in the environment of the "dark" neurons appeared undamaged.

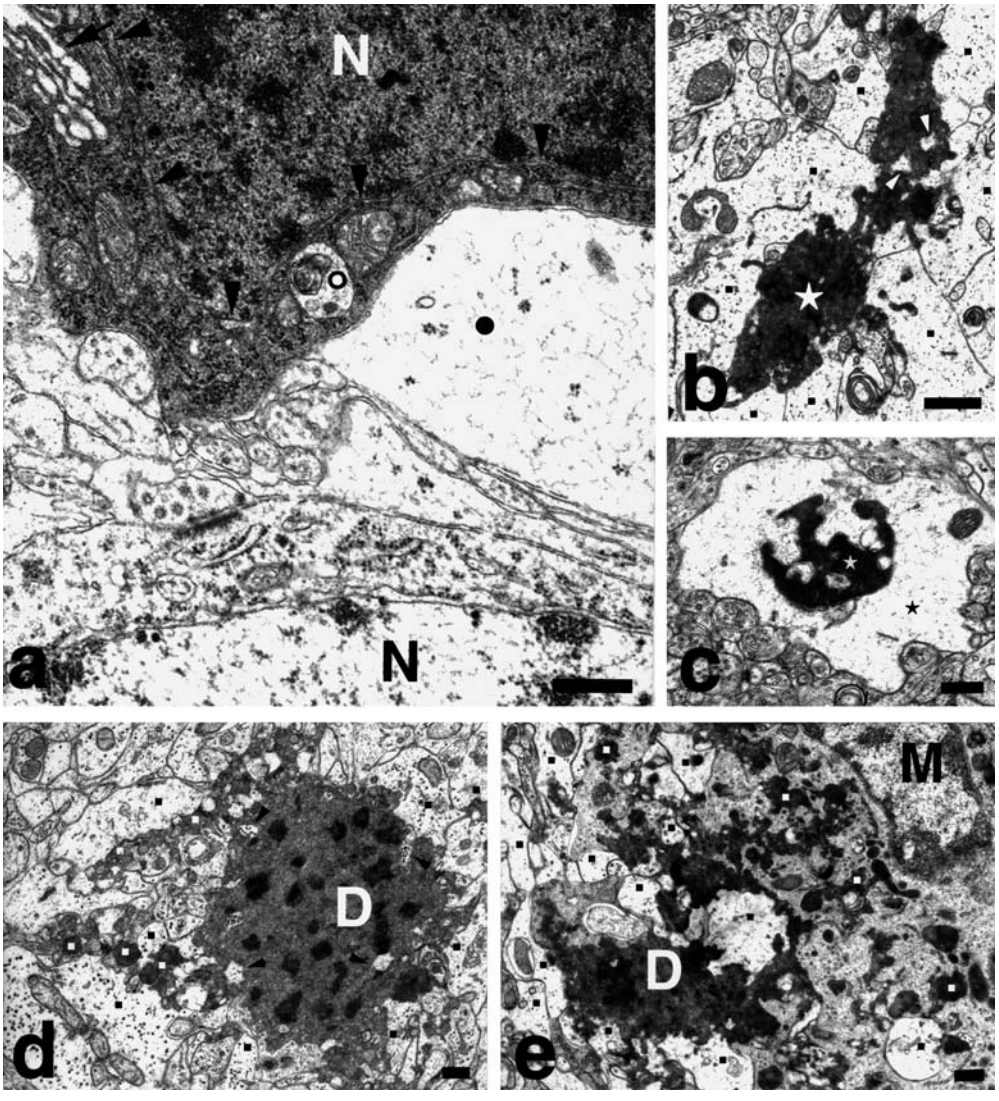


Fig. 3a–e Normal (*black N*) and freshly produced "dark" (*white N*) neurons (**a**), as well as, dead "dark" neurons in different stages of the apoptotic-like removal from the tissue (**b–e**) in the superficial layers of the frontoparietal brain cortex after hypoglycemia with a 1-min convulsion period. Survival times: **a** <1 min; **b**, **d** 1 day; **c**, **e** 6 days. **a** In a freshly produced "dark" neuron, a *black arrow* points to dilated Golgi cisternae and *black arrowheads* to the nuclear membrane; an *open circle* denotes a disrupted mitochondrion in the "dark" neuron and a *closed circle* an extremely swollen astrocytic process. **b**, **d** The main dendrite (*white asterisk*) and the soma (*D*) of a dead "dark" neuron exhibiting membrane-bound protrusions, invaginations, and glycogen-containing, electron-lucent cavities (*white or black arrowheads*). *Small white squares* denote sharply outlined quasi-homogeneous and dense fragments of the convoluted cell body, and *small black squares* slightly swollen, glycogen-containing astrocytic processes. **c** A sharply delineated dense fragment of a dead "dark" neuron (*white asterisk*) in a swollen astrocytic process (*black asterisk*). **e** A dead "dark" neuron (*D*) and its fragments (*small white squares*) engulfed by a microglial cell (*M*). *Black squares* denote swollen astrocytic processes. *Scale bars* **a**, **c–e** 500 nm; **b** 1 μm

One-day survival

In the soma and dendrites of the neurons that corresponded to those which displayed hyperbasophilia and acidophilia in the semithin sections, the ultrastructure was severely compacted (Fig. 3b, d). Individual organelles could hardly be discerned in them. Membrane-bound protrusions, invaginations, and glycogen-containing, electron-lucent

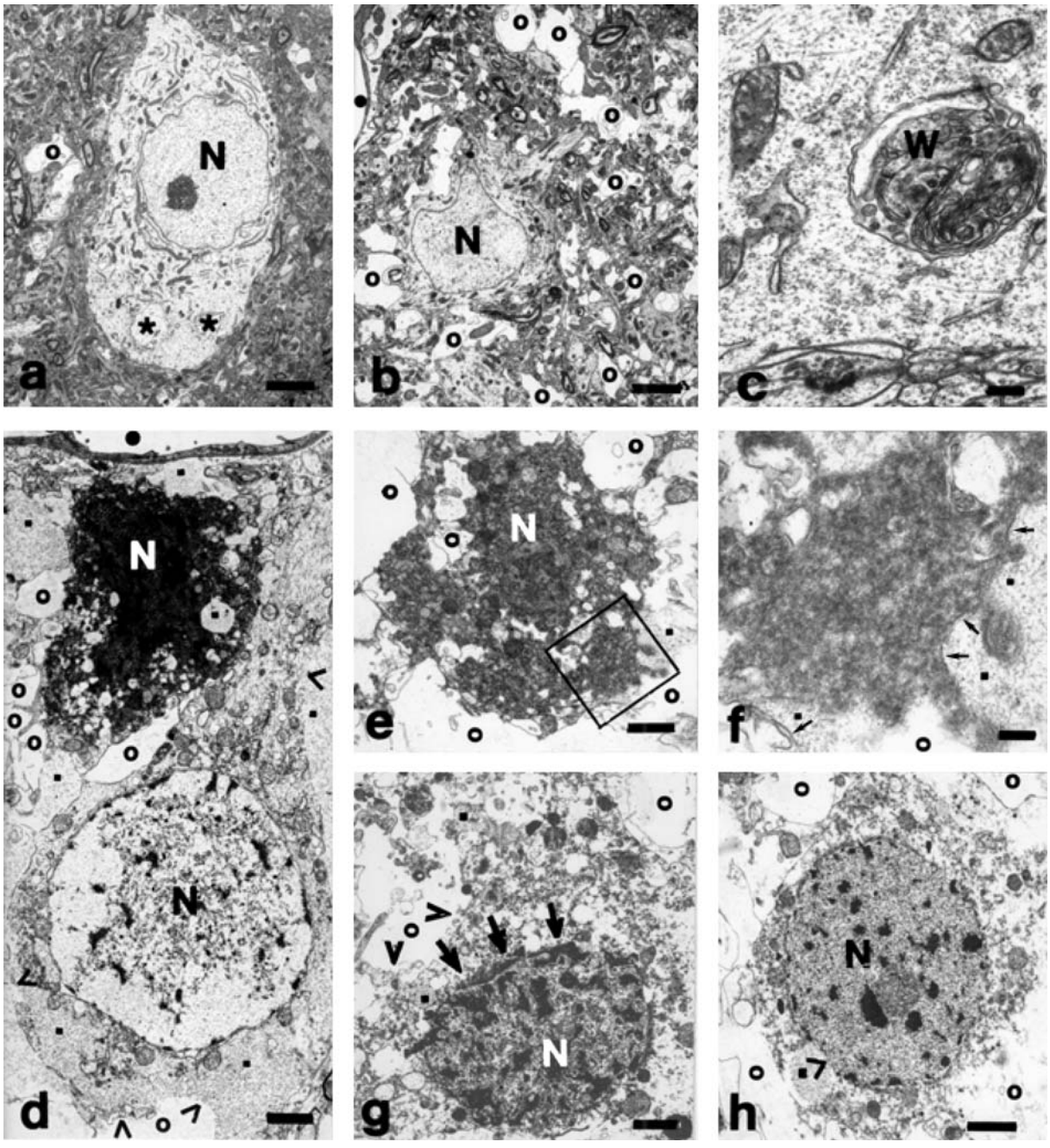


Fig. 4a–h "Light" (**a**), moderately damaged (**b**, **c**), necrotic (*bottom part* of **d**), and freshly produced "dark" (*upper part* of **d**) neurons, as well as, dead "dark" neurons in different stages of the necrotic-like removal from the tissue (**e–h**) in the superficial layers of the frontoparietal brain cortex after hypoglycemia with a 1-h convulsion period. Survival times: **a**, **b**, **d** <1 min; **c**, **e–h** 1 day. **a** A "light" neuron in slightly spongy surroundings. *Black asterisks* denote disintegrated cytoplasmic areas. **b** A slightly damaged neuron in very spongy surroundings. **c** A mitochondrion-derived membranous whorl (*W*) in a seemingly normal neuron. **d** A severely compacted ("dark") neuron containing several vacuoles representing disrupted mitochondria (*upper part*), and a severely swollen (necrotic) neuron with seriously disintegrated ultrastructure and partly missing plasma membrane (*bottom part*). **e**, **g**, **h** "Dark" neurons in intermediate stages of the necrotic-like removal from the tissue. **f** Medium enlargement of the area *boxed* in **e**. *Arrows* point to remnants of the plasma membrane. In **a–h**, *white* or *black N*s denote neuronal nucleus, *open circles* fluid-filled spaces of unknown origin, and *black squares* spaces filled with flocculent remnants of disintegrated tissue elements; *arrowheads* point to sites where either the plasma membrane or the nuclear membrane is missing. *Arrows* in **g** point to remnants of the compacted cytoplasm. *Closed circles* in **b** and **d** denote blood vessels. *Scale bars* **a**, **b** 5 μm; **d**, **e**, **g**, **h** 1 μm; **c**, **f** 200 nm

cavities gave the somata of such neurons a convoluted profile. In their close vicinity, several sharply outlined quasi-homogeneous and dense profiles of various shapes and sizes were present, giving the impression that the latter were separated fragments of the convoluted cell body. All these electron-dense profiles were isolated from the undamaged neuropil by slightly swollen, glycogen-containing astrocytic processes. Mitochondrion-sized membranous whorls (similar to those demonstrated in Fig. 4c) were present in a few neurons with an otherwise normal ultrastructure.

Two-day survival

The somata and dendrites of the dead "dark" neurons displayed a more advanced stage of fragmentation. The fragments were membrane-bound and displayed a quasi-homogeneous, dense interior. Most fragments were completely separated from their surroundings by slightly swollen, glycogen-containing astrocytic cytoplasm. The neuropil was otherwise undamaged.

Six-day survival

A few microglial cells contained dense bodies of various shapes and sizes reminiscent of fragments of dead "dark" neurons (Fig. 3e). Rarely, sharply outlined and dense neuronal fragments were present in normal-looking astrocytic processes (Fig. 3c). No other abnormality was observed.

Hypoglycemia, 1-h convulsion period, electron microscopy

Immediate killing

In the superficial cortical layers, in addition to the ultrastructural features observed in the rats that had suffered 1-min convulsions, were: (1) swollen mitochondria with disrupted cristae, frequent in the "dark" (severely compacted) neurons (upper part of Fig. 4d), (2) swollen neurons with a slightly damaged ultrastructure, but an apparently undamaged plasma membrane ("light" neurons; Fig. 4a), (3) markedly swollen neurons with a severely disintegrated ultrastructure and a partly missing plasma membrane (necrotic neurons, bottom part of Fig. 4d), and (4) the neuropil became spongy, mainly because of the swelling of neuronal dendrites and astrocytic processes (excitotoxic neuropil; Fig. 4b). In the deep cortical layers, the ultrastructural changes were commensurate with those found in the superficial cortical layers of the rats that had suffered a 1-min convulsion period.

One-day survival

In the superficial cortical layers, a few damaged neurons displayed the same ultrastructural features as the "dark" neurons in the rats killed immediately (severely compacted nucleus and cytoplasm, many disrupted mitochondria; like the neuron in the upper part of Fig. 4d). The astrocytic processes around such neurons were markedly swollen and did not contain glycogen particles. Some other damaged neurons displayed gross swelling, disintegration of ultrastructural elements into small flakes, and partial loss of the plasma and the nuclear membranes (advanced stage of necrosis; like the neuron in the bottom part of Fig. 4d). In addition to the above pathomorphological types, the ultrastructural features of many damaged neurons gave the impression that the neurons with necrotic morphology had derived from the "dark" neurons. Specifically, in the first type of such intermediate morphological forms, both the nucleus and the soma were moderately compact and moderately electron dense (Fig. 4e), and the cytoplasm had begun to disintegrate into small flakes at sites where the plasma membrane was missing (Fig. 4f). In another intermediate form, the nucleus was even less compact and less electron dense, while most parts of the soma had a "moth-eaten" appearance and large parts of the plasma membrane disappeared (Fig. 4g). However, in a thin zone just around the nucleus, the cytoplasm was still moderately compact and moderately electron dense. In a third intermediate form, most of the intracellular organelles had disintegrated and dispersed into the surroundings, whereas the nucleus was still slightly compact, although parts of the nuclear membrane appeared to be missing (Fig. 4h). In the deep cortical layers, none of the above pathological forms of damaged neurons were encountered. On the other hand, in both the deep and the superficial cortical layers, solitary mitochondrion-sized membranous whorls were detected (Fig. 4c).

Two-day survival

In the superficial cortical layers, severely compacted neurons were not present. In addition to the intermediate forms described in the previous paragraph, damaged neurons displayed the ultrastructural features characteristic of advanced necrosis. The deep cortical layers did not contain damaged neurons.

Colchicine-induced apoptosis

One-day survival

Apoptotic condensation of the nuclear chromatin into large clumps with rounded outlines was observed in a number of randomly distributed granule neurons in the hippocampal dentate gyri (Figs. 5b, 6a). Such neurons displayed TUNEL positivity, widening of the nuclear envelope,

dilated Golgi cisternae, and dispersed ribosomes. The two latter abnormalities were observed even in a proportion of the granule neurons without apoptotic chromatin condensation in the nucleus (Fig. 6b). In the somata and dendrites of most apoptotic granule neurons, all cytoplasmic structural elements visible in the electron microscope were markedly compacted (Fig. 6c). Such apoptotic neurons displayed increased electron density, hyperbasophilia, and stainability with the silver method used (Gallyas et al. 1990; Liposits et al. 1997). In other respects, the tissue structure appeared undamaged.

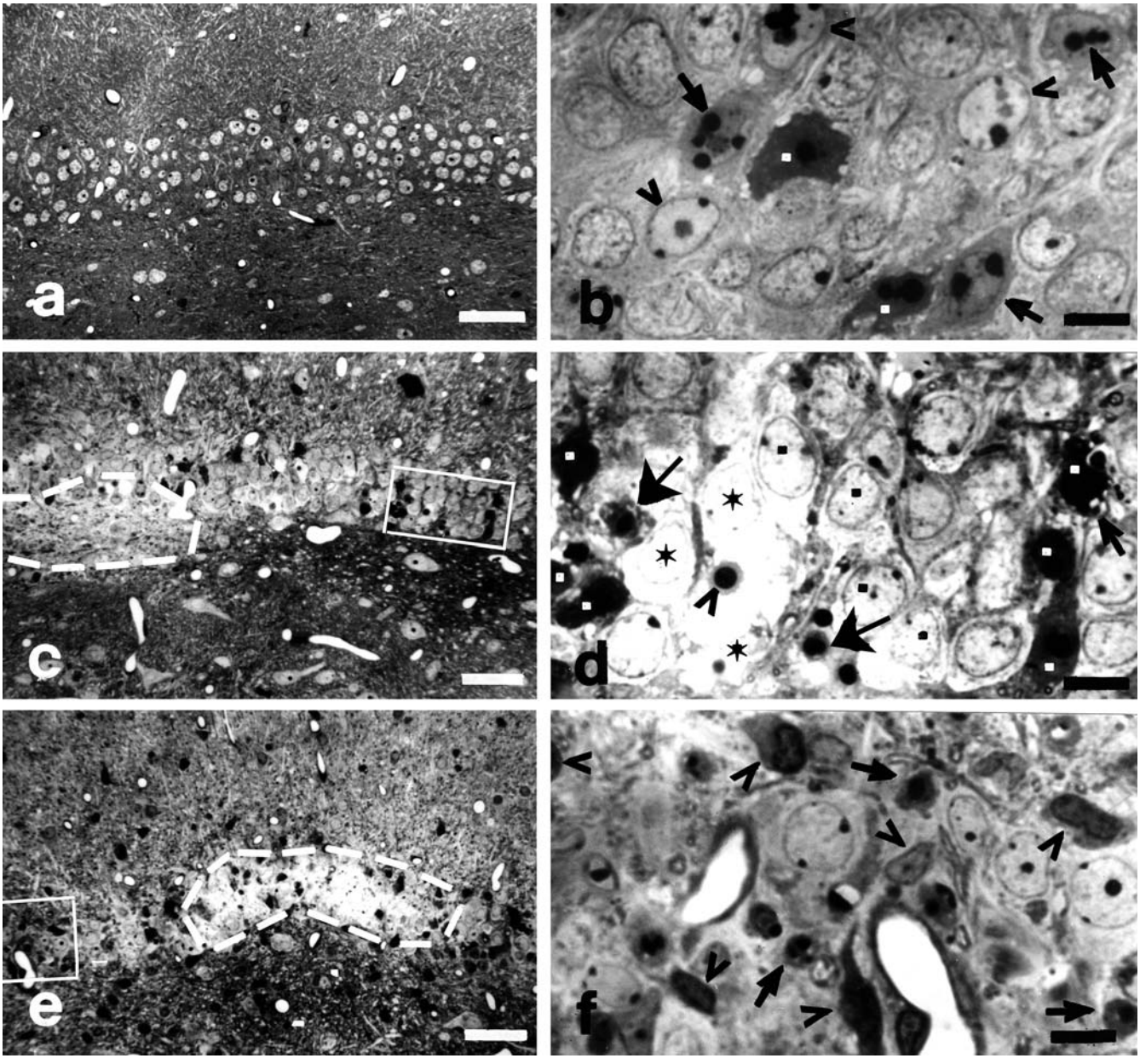
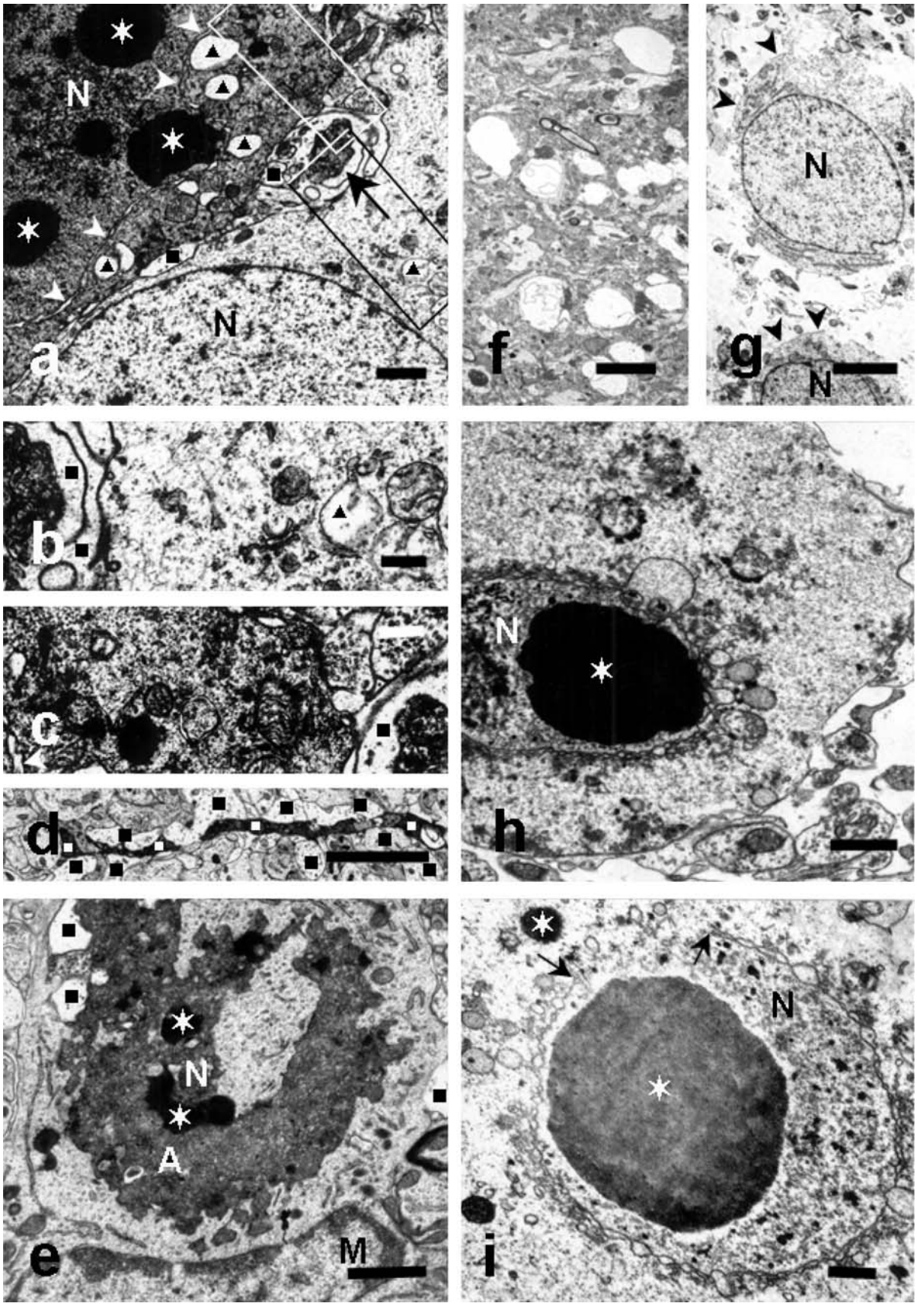


Fig. 5a–f Toluidine blue-stained pictures of the Durcupan-embedded granule neuron layer in the hippocampal dentate gyrus of a control rat (**a**) and of rats that survived for 1 day (**b**), 2 days (**c**, **d**), or 6 days (**e**, **f**) following the intraventricular administration of colchicine. In **b**, *arrowheads* point to granule neuron nuclei in an early stage of apoptotic chromatin condensation and *arrows* to apoptotic granule neurons with moderate shrinkage of the soma; *small white squares* denote apoptotic granule neurons with intermediate somatic shrinkage. In **c** and **e** swollen areas are surrounded by *white dashed lines*; the *boxed areas* are enlarged in **d** and **f**, respectively. In **d**, *small black squares* denote nuclei of "light" granule neurons, *black asterisks* neurons in an advanced stage of necrosis, and *small white squares* severely shrunken apoptotic granule neurons; a *thin arrow* points to an apoptotic granule neuron in an early stage of fragmentation, *large arrows* to apoptotic granule neurons in an early stage of the necrotic-like removal, and a *small arrowhead* to an apoptotic granule neuron in the last stage of the necrotic-like removal from the tissue. In **f**, *arrowheads* point to nuclei of phagocytotic cells and *arrows* to severely shrunken apoptotic granule neurons. *Scale bars* **a**, **c**, **e** 50 μm; **b**, **d**, **f** 10 μm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◀ **Fig. 6a–i** Electron microscopic pictures of granule neurons in the hippocampal dentate gyrus of rats that survived for 1 day (**a–c**), 2 days (**d**, **f–h**), or 6 days (**e**, **i**) following the intraventricular administration of colchicine. **a** An apoptotic granule neuron with compacted cytoplasm in juxtaposition to a non-apoptotic granule neuron with dilated Golgi cisternae and many dispersed ribosomes. *White arrowheads* point to the nuclear envelope and a *long arrow* to the cross-section of a compacted dendrite; *small black triangles* denote dilated Golgi cisternae and *small black squares* swollen astrocytic processes. **b**, **d** Enlargements of the areas *boxed* in **a**. **d** Fragments of a compacted dendrite (*small white squares*) surrounded by glycogen-containing astrocytic processes (*small black squares*). **e** A compacted apoptotic granule neuron with convoluted outlines (*A*) engulfed by a microglial cell (*M*). *Small black squares* denote engulfed astrocytic processes. **f** Extremely swollen dendritic or astrocytic profiles (*open squares*) in the border zone of a swollen area in the molecular layer. **g** Two non-apoptotic granule neurons with disintegrated cytoplasm and partly missing plasma membrane (*arrowheads* point to its remnants) in an area of serious extracellular edema. **h** An apoptotic granule neuron with partly disintegrated cytoplasm, surrounded by extracellular edema. **i** An apoptotic granule neuron in an advanced stage of the necrotic-like removal from the tissue. Between the *two arrows* the nuclear membrane is missing. In **a–i**, *black* or *white N*s denote granule cell nuclei, *white asterisks* apoptotic chromatin clumps. *Scale bars* **a**, **d–h** 1 μm; **b**, **c**, **i** 500 nm

Two-day survival

All granule neurons with apoptotic chromatin condensation in the nucleus and compacted somatodendritic domain exhibited two types of morphological changes additional to those found 1 day after the colchicine administration. Type I changes consisted of the homogenization of the shrunken cytoplasm accompanied by an additional increase in electron density, and also by the development of cytoplasmic protrusions and invaginations of various shapes and sizes (Fig. 5c, d). Occasionally, membrane-bound fragments of the convoluted cytoplasm were observed in the vicinity of such neurons. Dendrites of these neurons also became homogenized, more electron dense, and fragmented. The neuropil around such dendrites (Fig. 6d) and cell bodies was apparently undamaged, with the exception of slightly swollen, glycogen-containing astrocytic processes.

Type II changes consisted of a decrease in the nuclear and cytoplasmic shrinkage, which was accompanied by a fall in electron density and basophilia, and also by the partial disintegration of a proportion of the ultrastructural elements (Figs. 5d, 6h). Each of such neurons was present in swollen areas, the sizes of which ranged from that shown in Fig. 5d to a few times larger than that shown in Fig. 5c. The small swollen areas did not extend either into the molecular layer or into the hilus of the dentate gyrus (Fig. 5d). The larger swollen areas also involved an adjacent zone of the hilus and the molecular layer (Fig. 5c). In the border zones of the swollen areas, markedly dilated dendritic or astrocytic profiles were present (Fig. 6f), whereas extracellular edema predominated in their inner part, where several granule neurons without apoptotic chromatin condensation in the nucleus exhibited disintegrated cytoplasmic structures, and partially missing plasma membranes (Fig. 6d).

Six-day survival

In the non-swollen areas of the granule cell layer, apoptotic granule neurons with type I additional morphological changes were engulfed partly or completely by phagocytotic cells (Fig. 6e). In the swollen areas of the dentate gyri, most ultrastructural elements of the apoptotic granule neurons with type II additional morphological change were disintegrated into small flakes, and their plasma and nuclear membranes were partly (Fig. 6i) or completely missing. However, the chromatin clumps did not disintegrate, but swam into the dispersed cytoplasm after dissolution of the nuclear membrane, and remained TUNEL positive.

Discussion

Methodological comment

Acid fuchsin is an established stain for the demonstration of moribund or dead "dark" neurons; in contrast, their stainability with toluidine blue in an acidic milieu disappears gradually (Auer et al. 1985a; Kiernan et al. 1998). However, they do bind toluidine blue strongly from an alkaline solution in osmicated and Durcupan-embedded semithin sections (Fig. 2e, f), which simplifies their observation and microphotography.

Formation of the "dark" neurons produced by hypoglycemic convulsions

Despite their century-long history, the nature of "dark" neurons has not been cleared. Based on certain circumstances of formation (for example, in vivo versus postmortem), on the presence versus absence of swollen mitochondria, dispersed ribosomes or other pathomorphological features, and also on the stainability with acidic versus basic dyes, at least four types of "dark" neurons are nowadays accepted: irreversible excitotoxic, irreversible neostriatal, reversible, and artifactual (Graeber et al. 2002). However, there is a common morphological feature in each of these types: a dramatic compaction of all ultrastructural elements in the somatodendritic domain. According to a recent hypothesis (Gallyas et al. 2004), the formation of "dark" neurons consists of an initiating and an executive process. While the initiating process shows wide diversity (various pathobiochemical cascades or momentary physical insults, for example, ischemia or head injury) the executing process is the same physical event (gel-to-gel phase transition). If initiated somehow at any point of the hypothesized intracellular gel structure, which fills the space that is not occupied by the known ultrastructural elements, the gel-to-gel phase transition together with the concomitant ultrastructural compaction proceeds throughout the whole somatodendritic domain at the expense of stored non-covalent free energy. Simultaneously, the somatodendritic domain acquires excess

basophilia and type III argyrophilia. Under favorable conditions a considerable proportion of the compacted neurons recover, whereas another proportion dies. During the dying process the compacted neurons gradually lose basophilia and acquire acidophilia. Since the ultrastructural compaction is a physical process, it can be initiated by mechanical insults, even under postmortem conditions unfavorable for enzyme-mediated processes (artifactual "dark" neurons). When the ultrastructural elements are intact at the "start" of the compaction, they remain intact even for several minutes after its end (see the case of 1-min hypoglycemic convulsions). Swollen mitochondria (see the case of 1-h hypoglycemic convulsions), or the other distinctive pathomorphological features such as ribosomal dispersion, can only be observed when they were present at the "start" of the ultrastructural compaction.

If the above train of thought is true, the ultrastructural compaction is completed in a few minutes even in the case of hypoglycemic convulsions. Consequently, it is probably the time elapsing between the administration of insulin and the "start" of the ultrastructural compaction that varies widely (in the range of a few hours) from one affected neuron to the other and also from one affected area to the other. As regards the incidence of the "dark" neurons in the neocortex, many more of them were present at the end of a 1-h than a 1-min convulsion period. As regards the affected areas, the formation of "dark" neurons in the deep cortical layers ensued with a considerable delay as compared with the formation of those in the superficial cortical layers.

Recovery versus death of the "dark" neurons produced by hypoglycemic convulsions

The idea that "dark" neurons are capable of recovery (regeneration) was initially proposed by Auer et al. (1985a). Using a scrupulously controlled rat hypoglycemia paradigm, they observed numerous "dark" neurons in both the superficial and the deep cortical layers in the acute phase of hypoglycemia. In contrast, they detected hardly any dead "dark" neurons in the deep cortical layers of the surviving animals, whereas dead "dark" neurons were abundant in the superficial cortical layers. The above idea was supported in our experiments by revealing a much higher incidence of "dark" neurons in the deep cortical layers at the end of a 1-h convulsion period than 1-day later.

In our experiments, the ability to recover from the "dark" morphological state is not confined to the neurons in the deep cortical layers. This can be concluded from the occurrence of mitochondrion-sized silver-stained dots and mitochondrion-derived membranous whorls in neuronal somata and dendrites in the superficial cortical layers of the rats that survived for 1 day following hypoglycemic convulsions. As demonstrated earlier, these morphological features are characteristic of recovering "dark" neurons (Csordás et al. 2003). It should be mentioned that not only hypoglycemic "dark" neurons are capable of recovery, but also those produced by many other pathometabolic conditions such as ibotenic acid poisoning and stressful exercise (Ishida et al. 2004), or physical insults (reviewed by Csordás et al. 2003).

The existence of dendrites with dotted proximal and homogeneous distal segments in silver-stained hypoglycemic "dark" neurons (Fig. 1c) suggests that the process of morphological recovery spreads from the soma to the dendrites. It should be emphasized that a similar phenomenon was also observed in electrically produced "dark" neurons (our unpublished observation).

The time required for the recovering "dark" neurons to emerge from the stage detectable by dotted silver staining was longer than 1 day after hypoglycemic convulsions (Fig. 1b–f, h), but shorter than 4 h after momentary physical insults (Csordás et al. 2003). This difference suggests that pathometabolic conditions, such as those caused by hypoglycemia, are able to slow down the recovery.

The death rate of "dark" neurons in the superficial cortical layers appeared to be much higher following a 1-h than a 1-min convulsion period. This can be explained by assuming that the "dark" neurons in the former case were exposed to the hypoglycemia-induced pathobiochemical processes for a longer time than in the latter case, which blocked self-healing biochemical process.

Removal from the neocortex of the non-recovering "dark" neurons produced by hypoglycemic convulsions

In the present experiments, the non-recovering "dark" neurons were removed through two entirely different sequences of ultrastructural changes.

Necrotic-like removal sequence

In the excitotoxic environment, the sequential ultrastructural changes involved in the removal of non-recovering "dark" neurons (Fig. 4) resembled those generally accepted as characteristic of necrosis (see Kerr and Harmon 1991), except for a dramatic ultrastructural compaction preceding them. A similar removal sequence has been reported in previous papers for hypoglycemic "dark" neurons that had died in an excitotoxic parenchymal environment (Auer et al. 1985a, b; Kalimo et al. 1985).

Apoptotic-like removal sequence

In an undamaged environment, the sequential ultrastructural changes involved in the removal of non-recovering "dark" neurons (Fig. 4) resembled those generally accepted as characteristic of apoptotic cell death (see Kerr and Harmon 1991), except for the pattern of the

preceding chromatin condensation in the nucleus. A similar removal sequence has recently been described for the "dark" neurons that died in an apparently undamaged parenchymal environment following an electric shock or a non-contusing concussive head injury (Csordás et al. 2003).

Removal of the colchicine-produced apoptotic granule neurons from the hippocampal dentate gyrus

It is long known that, following intracerebral administration of colchicine to adult rats, a proportion of the granule neurons in the hippocampal dentate gyrus die through the apoptotic pathway (Ceccatelli et al. 1997; Pozas et al. 1999). The present study contributes to the knowledge in this field of research with two new observations. First, in a few, randomly distributed, small patches in the granule cell layer of the dentate gyrus, necrotic neuronal death is also induced. Concerning other noxious agents, a similar ability (induction of both apoptosis and necrosis) has long been known (see, for example, Ferrer et al. 1995). Second, the removal of granule neurons displaying the characteristic morphological features of apoptosis can follow both an apoptotic-like (compare Fig. 6d, e with Fig. 3a, d, e) and a necrotic-like (compare Fig. 6h, i with Fig. 4d–g) sequence of ultrastructural changes (see the previous section), depending on the absence or presence of morphological damage in their environment.

Cellular necrosis is assumed to occur in response to toxic agents taken up from impaired surroundings (Kerr and Harmon 1991). Analogously, toxic agents taken up from impaired surroundings may impose a necrotic-like ultrastructural sequence of removal even upon neurons that have previously died through the apoptotic pathway. Consequently, one ought to be cautious in concluding from the morphological sequence of removal to the type of cell death (compare with the relevant suggestion in the next paragraph).

"Dark" (compacted) neurons of whatever origin may die through a non-necrotic and non-apoptotic pathway

Our observations on "dark" neurons produced by 1-min convulsions (this paper), an electric shock, or a non-contusing concussive head injury (Csordás et al. 2003) in an apparently undamaged (non-excitotoxic, non-necrotic, non-edematous) environment preclude the necrotic pathway as the cause of death in their case. This fact suggests the possibility that even the non-recovering "dark" neurons produced by a 1-h convulsion period in the present study, and also by other pathological conditions in the studies that regard the death of "dark" neurons as caused by necrosis, also died through some non-necrotic pathway, but a necrotic-like ultrastructural sequence of removal was imposed on them by their excitotoxic environment, as in the case of colchicine-induced apoptosis.

Not one of the papers that regard necrosis as the death type of "dark" neurons (for example, those referred to in the Introduction) raised the question, why the generally accepted necrotic morphological sequence is preceded by a dramatic cytoplasmic compaction. This resembles preferably an apoptotic morphological event in the cytoplasm, which follows the apoptotic biochemical events and the chromatin condensation in the nucleus. Specifically, the ultrastructural compaction in both the "dark" and the apoptotic neurons is accompanied by the acquirement of hyperbasophilia and type III argyrophilia (Gallyas et al. 1990, 2004; Liposits et al. 1997; Csordás et al. 2003), and is followed by the same sequence of removal from a non-impaired environment. These similarities raise the possibility that, at the level of molecular cell biology, the mechanism of compaction in the apoptotic neurons is the same as in the "dark" neurons.

Despite the above similarities, apoptosis (or aponecrosis; see, for example, Formingli et al. 2000) should also be discounted as the cause of death of the "dark" neurons produced by "mild" hypoglycemia (this paper), an electric shock, or a non-contusing concussive head injury (Csordás et al. 2003) in an apparently undamaged environment since: (1) their nucleus displays non-apoptotic chromatin condensation and TUNEL negativity, (2) after the stage of cytoplasmic shrinkage, apoptosis is incompatible with recovery, (3) in the event of momentary physical insults, the time elapsing between their infliction and the completion of compaction (less than 1 min; Csordás et al. 2003) is too short for the apoptotic biochemical reaction cascades to take place, and (4) by means of momentary mechanical insults, the ultrastructural compaction can also be induced under conditions that are extremely unfavorable for any enzyme-mediated process (Gallyas et al. 1992, 2004; Tóth et al. 1997).

With regard to all these points, we propose to revive a disregarded idea raised by Harmon (1987) and Wyllie (1987) that the "dark" morphological state of numerous cell types in various tissues may relate to a special pathway for the death of single cells, and this pathway exhibits similarities to, but differs from apoptosis. The processes involved in this special death pathway at the level of molecular cell biology remain to be determined in further studies.

It should be noted that the type of neuronal death caused by oxidative stress in an immortalized mouse hippocampal cell line (Tan et al. 1998) and also by spontaneous neurodegeneration in a transgenic mouse model of Huntington's disease (Turmaine et al. 2000) was suggested to be distinct from either necrosis or apoptosis. Interestingly, the moribund neurons in these models suffered marked cytoplasmic and nuclear shrinkage.

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