available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

The mode of death of epilepsy-induced "dark" neurons is neither necrosis nor apoptosis: An electron-microscopic study

Ferenc Gallyas[a,*], *Viola Kiglics*[b], *Péter Baracskay*[b], *Gábor Juhász*[b], *András Czurkó*[b]

[a]*Department of Neurosurgery, Faculty of Medicine, University of Pécs, H-7623 Pécs, Rét utca 2, Hungary*
[b]*Laboratory of Proteomics, Institute of Biology, Faculty of Natural Sciences, Eötvös Loránd University, H-1117 Budapest, Hungary*

ARTICLE INFO

Article history:
Accepted 25 August 2008
Available online 4 September 2008

Keywords:
Rat
Brain cortex
4-aminopyridine
Silver staining
Electron microscopy

ABSTRACT

Morphological aspects of the formation and fate of neurons that underwent dramatic ultrastructural compaction ("dark" neurons) induced by 4-aminopyridine epilepsy were compared in an excitotoxic and a neighboring normal-looking area of the rat brain cortex. In the excitotoxic area, the later the ultrastructural compaction began after the outset of epilepsy, the higher the degree of mitochondrial swelling and ribosomal sequestration were; a low proportion of the affected neurons recovered in 1 day; the others were removed from the tissue through a necrotic-like sequence of ultrastructural changes (swelling of the cell, gradual disintegration of the intracellular organelles and dispersion of their remnants into the surroundings through large gaps in the plasma and nuclear membranes). In the normal-looking area, the ultrastructural elements in the freshly-formed "dark" neurons were apparently normal; most of them recovered in 1 day; the others were removed from the tissue through an apoptotic-like sequence of ultrastructural changes (the formation of membrane-bound, electrondense, compact cytoplasmic protrusions, and their braking up into membrane-bound, electrondense, compact fragments, which were swallowed by phagocytotic cells). Since these ultrastructural features differ fundamentally from those characteristic of necrosis, it seems logical that, in stark contrast with the prevailing conception, the cause of death of the epilepsy-induced "dark" neurons in the normal-looking cortical area cannot be necrosis. An apoptotic origin can also be precluded by virtue of the absence of its characteristics. As regards the excitotoxic environment, it is assumed that pathobiochemical processes in it superimpose a necrotic-like removal process on already dead "dark" neurons.

1. Introduction

Ever since the publication of the relevant pioneering paper (Söderfeldt et al., 1983), the "dark" neurons induced by epilepsy have been unanimously believed (Thom et al., 2008) to die through the necrotic pathway. The same has been stated (Auer et al., 2008) for the "dark" neurons induced by ischemia (Smith et al., 1984) or hypoglycemia (Auer et al., 1985). This assumption was based on light- and electron-microscopic observations suggesting that, from necrotic, excitotoxic or contused brain areas, the "dark" neurons produced by these noxae are removed via a necrotic-like sequence of morphological changes.

In contrast, our recent electron-microscopic observations suggested that the mode of death of traumatic (Csordás et al.,

* *Corresponding author*. Fax: +36 72 535931.
E-mail address: ferenc.gallyas.sen@aok.pte.hu (F. Gallyas).

0006-8993/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2008.08.069

2003), electric (Csordás et al., 2003), hypoglycemic (Gallyas et al., 2005) and ischemic (Kövesdi et al., 2007) "dark" neurons is neither necrosis nor apoptosis. This assumption was based on electron-microscopic observations proving that a number of "dark" neurons are produced by these noxae even in non-necrotic, non-excitotoxic or non-contused (apparently normal) tissue areas, from which they are removed via an apoptotic-like sequence of morphological changes.

In the present paper, we investigate whether or not this assumption also applies to "dark" neurons induced by epilepsy. Since there are biochemical differences between the pathological circumstances at issue (Auer and Siesjö, 1988; Liou et al., 2003), it is not evident that the epilepsy-produced "dark" neurons are removed from apparently normal (non-excitotoxic) brain areas via the apoptotic-like sequence of ultrastructural changes, which could support the non-necrotic and non-apoptotic nature of their death.

For the induction of epilepsy, a 4-aminopyridine paradigm was used which produces a relatively large number of "dark" neurons in an apparently normal cortical area not far from a large excitotoxic area (Baracskay et al., in press).

2. Results

The observations presented below are confined to the morphological features pertinent to the nature and fate of "dark" neurons induced by placing a 4-aminopyridine crystal on the exposed cortical surface of the rat (Baracskay et al., in press). Other aspects of epilepsy-induced morphological brain damage have been demonstrated and discussed appropriately in previous papers (Söderfeldt et al., 1983; Ingvar et al., 1988; Covolan and Mello, 2000; Pena and Tapia, 2000; Baracskay et al., in press). With the epilepsy paradigm utilized here, a special silver method allows the division of the cortical areas in the hemisphere of 4-aminopyridine application into a pan-necrotic (all ultrastructural elements become fatally damaged), an excitotoxic (numerous dendritic segments become extremely swollen), a normal-looking and an intermediate area, as demonstrated in Fig. 1a. Of these, only the excitotoxic and the intermediate areas are dealt with here. It should be stressed that the individual differences in the extensions of these areas and the numbers of damaged neurons contained in them did not influence the statements and interpretations detailed in the next paragraphs.

In the control rats, neuromorphological changes were discerned only under the site of removal of the dura (a few mechanically produced "dark" neurons were seen, similar to those depicted in Figs. 7a,b in Gallyas et al., 1990). In these rats, no behavioral anomaly was observed. In contrast, within 30 min after termination of the Halothane anesthesia, the rats treated with 4-aminopyridine displayed generalized clonic seizures, which continued for at least 3 h and abated to head nodding and/or myoclonic jerks during the next few hours in the rats that survived for 1 day or 3 days.

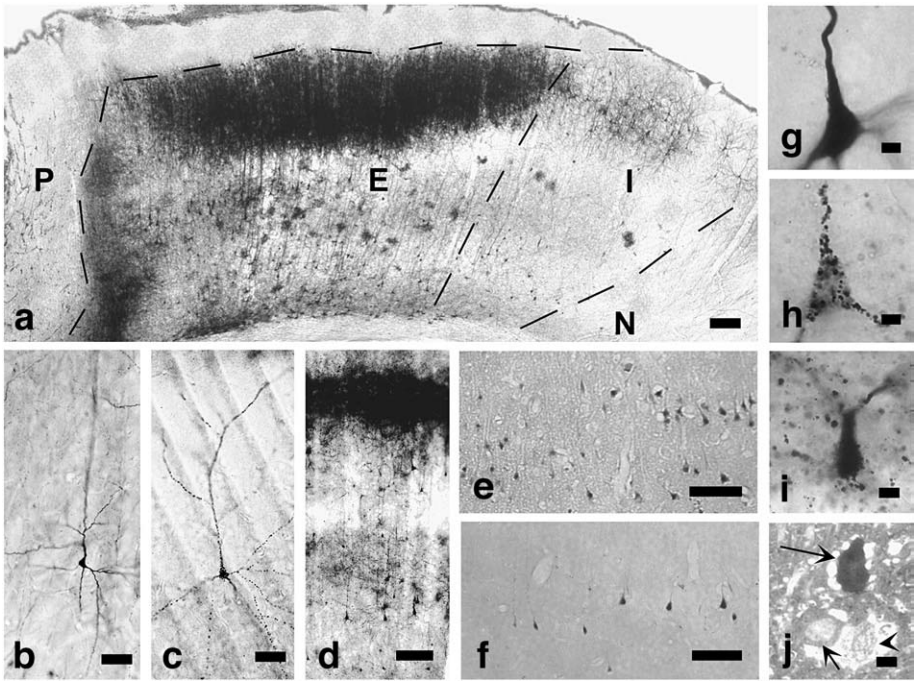

Fig. 1 – Light-microscopic images of epilepsy-induced "dark" neurons in 150-μm vibratome sections (a–d and g–i), 10-μm paraffin-embedded sections (e and f) and a 1-μm osmicated and Durcupan-embedded section (j) of the rat cortex, stained with silver (a–d and g–i), acid fuchsin (e and f) or toluidine blue (j). Rats were sacrificed 1 h (b and g), 3 h (a, c, h and j) or 1 day (d–f and i) after the end of 4-aminopyridine crystal application. In panel a, the dashed lines border the pan-necrotic (P), excitotoxic (E), intermediate (I) and normal-looking (N) areas of the cortex. In panel j, a long arrow points to a "dark" neuron, a short arrow to a slightly damaged neuron and an arrowhead to an extremely swollen neuron. Scale bars: a and d–f ~200 nm, b and c ~50 nm, g–j ~10 nm.

2.1. *Light-microscopic findings*

2.1.1. *Thick vibratome sections of glutaraldehyde-fixed brains: silver staining*

In the rats sacrificed 1 h after removal of the 4-aminopyridine crystal, a few homogenously stained neuronal soma-dendrite domains were present in layers II and III of the intermediate area (Figs. 1b,g), while there were many such neurons in the excitotoxic area. These were scattered among many more unstained neurons. Two hours later, the homogenously silver-stained neurons were more numerous in both the intermediate and the excitotoxic area (Fig. 1a). Additionally, the soma-dendrite domains of several other neurons were outlined with mitochondrion-sized silver-stained dots in both the intermediate (Figs. 1c,h) and the excitotoxic area. In the rats that survived for 1 day, besides numerous dotted and a few homogenously stained neurons, a few homogenously silver-stained neurons displayed fragmented dendrites and somatic protrusions (Fig. 1i). In thick vibratome sections, individual somata or dendrites of these types of silver-stained neurons could hardly be distinguished at low magnifications, because of their superimposition (Fig. 1d). Observations under a high-magnification microscopic lens demonstrated that the dotted neurons were more numerous in layers V and VI than in layers II and III of the excitotoxic cortical area. Two days later, a confused mass of silver-stained dots of various sizes was observed there.

2.1.2. *Paraffin-embedded sections of formaldehyde-fixed brains*

Acid fuchsin homogenously stained many shrunken neurons in the excitotoxic area (Fig. 1e) and a few in the intermediate area (Fig. 1f). As regards the survival period between 3 h and 1 day, their numbers in the excitotoxic area appeared commensurable, whereas in the intermediate area they decreased considerably. TUNEL-positive cells were not observed during this survival period.

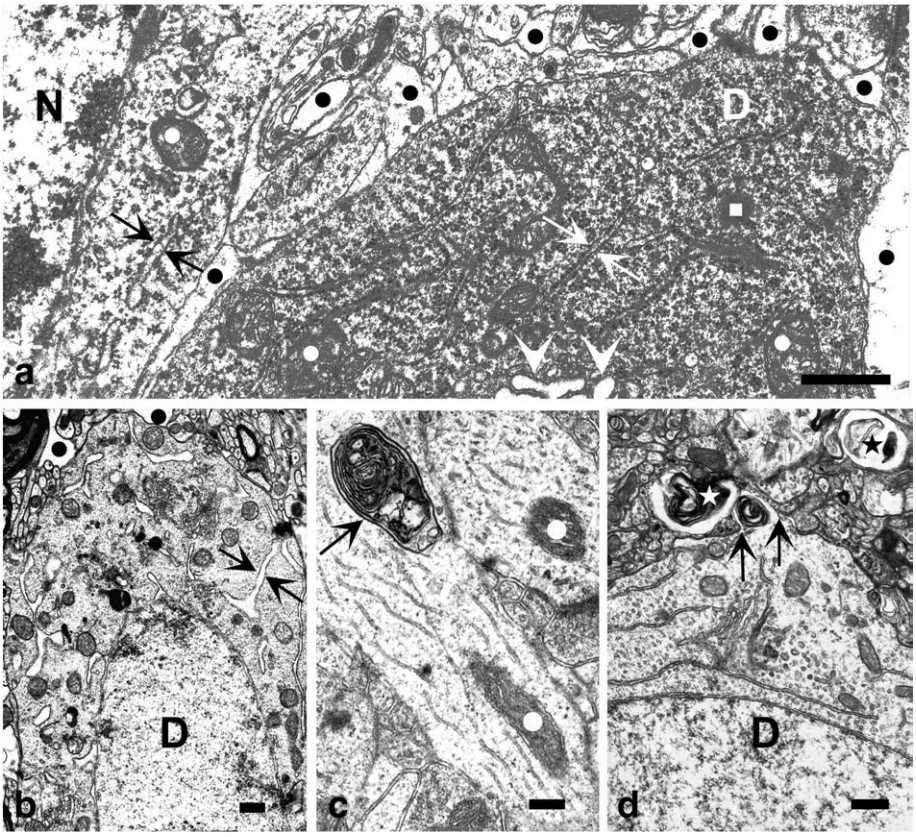

Fig. 2 – Recovery of epilepsy-induced "dark" neurons in the intermediate area of the rat cortex: (a) the cytoplasm of a "dark" neuron shortly after its production; (b) the early, (c) the advanced and (d) the final stages of recovery. Rats were sacrificed 1 h (a), 3 h (b and c) or 1 day (d) after the end of 4-aminopyridine crystal application. In panel a, N denotes the nucleus of a normal neuron, and D the perikaryon of a freshly-formed "dark" neuron. Black or white arrows point to endoplasmic reticulum cisternae, and white arrowheads to Golgi cisternae. White closed circles indicate mitochondria, and black closed circles dilated astrocytic processes. In panel b, arrows point to dilated endoplasmic reticulum cisternae; D denotes the nucleus of a recovering "dark" neuron; black closed circles indicate swollen astrocytic processes. In panel c, an arrow points to a mitochondrion-sized membranous whorl in a dendrite; white closed circles indicate mitochondria. In panel d, D denotes the nucleus of a recovered "dark" neuron; white or black star indicates large membranous whorls in swollen astrocytic process; arrows point to the edges of an opening in the plasma membrane through which a mitochondrion-sized membranous whorl appears to leave the neuron. Scale bars: a, b and d ~500 nm, c ~200 nm.

2.1.3. *Osmicated and Durcupan-embedded sections of glutaraldehyde-fixed brains*

Independently of the survival times tested, toluidine blue revealed both intensely-stained and pale neurons besides apparently normal neurons in the excitotoxic areas, frequently adjacent to each other (Fig. 1j), but there were only intensely-stained and apparently normal neurons in the intermediate area throughout the survival period tested.

2.2. *Electron-microscopic findings in the intermediate cortical area*

2.2.1. *1-h survival*

A few neuronal somata, together with their dendrites, displayed a dramatic volume decrease and a considerable increase in electron density. High-magnification pictures revealed markedly reduced distances between any two neighboring parts of apparently intact ultrastructural elements (compaction), including mitochondria, lysosomes, plasma and nuclear membranes, ribosome rosettes, components of the filamentous cytoskeleton and the exterior of the endoplasmic reticulum and Golgi cisternae; although the interior of the endoplasmic reticulum cisternae was contracted, that of the Golgi cisternae was dilated (Fig. 2a). The nuclear chromatin aggregated to numerous small clumps with irregular outlines and a myriad of minute granules. The degree of ultrastructural compaction appeared to be similar throughout the affected somatic and dendritic profiles encountered. Interestingly, around the affected somata and dendrites, the extracellular spaces were not dilated, whereas the astrocytic processes were considerably swollen. Such neurons were thinly scattered among apparently intact neurons in an otherwise normal-looking environment.

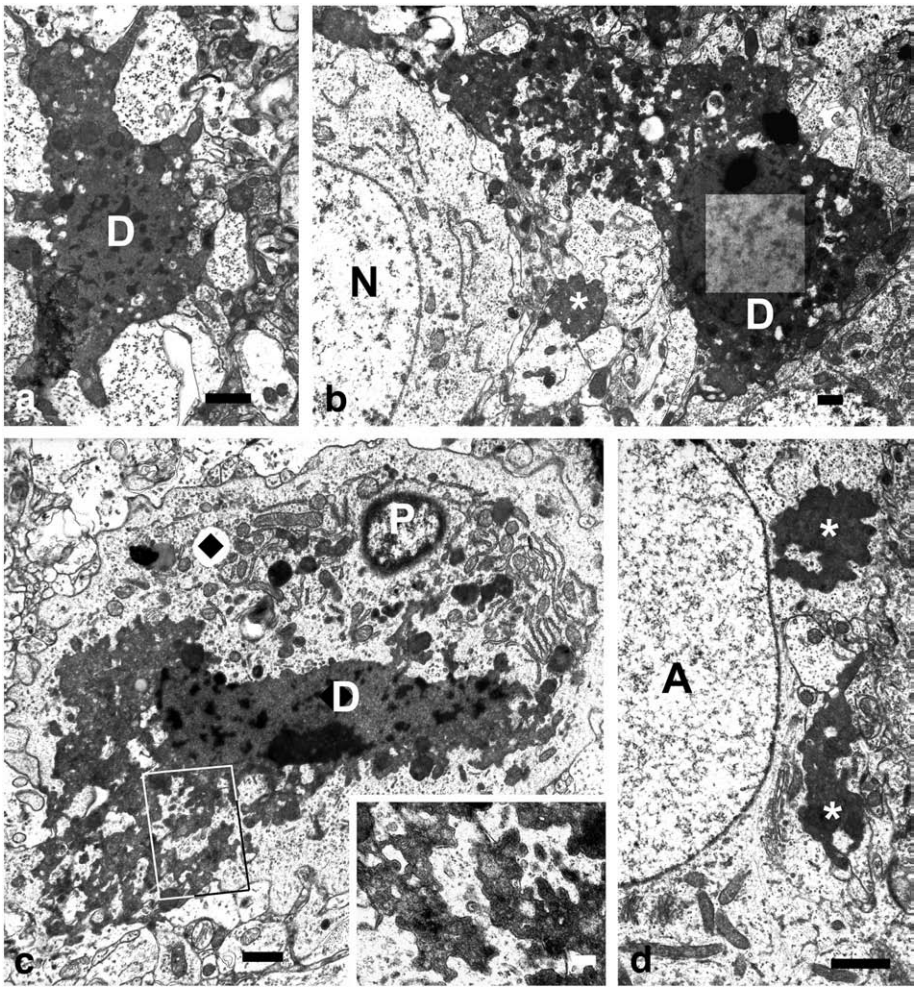

Fig. 3 – Apoptotic-like removal of epilepsy-induced "dark" neurons from the intermediate area of the rat cortex: (a) a "dark" neuron displaying several protrusions; (b) a "dark" neuron displaying a complicated system of protrusions from which fragments appear to have separated; (c) a partly fragmented "dark" neuron engulfed by a phagocytotic cell; (d) two large fragments engulfed by an astrocyte. The insert in panel c is a magnification of the boxed area. Rats were sacrificed 1 day (a) or 3 days (b–d) after the end of 4-aminopyridine crystal application. In panels a–d, nuclei of "dark" neurons, an apparently normal neuron, a phagocytotic cell and an astrocyte are denoted D, N, P and A, respectively. Fragments of "dark" neurons are indicated with white asterisks. Light profiles containing many small dots around the protrusions and fragments are glycogen-containing astrocytic processes. In panel b, a square part of the nucleus has been made lighter electronically. Scale bars: a and d ~1 μm, b and c ~2 μm, insert in c ~200 nm.

2.2.2. 3-h survival

The number of compacted neurons had increased considerably. In addition, a few slightly shrunken neurons contained dilated endoplasmic reticulum cisternae (Fig. 2b), and a few apparently normal neuronal dendrites (Fig. 2c) and somata contained mitochondrion-sized membranous whorls.

2.2.3. 1-day survival

Neurons with dilated endoplasmic reticulum cisternae were not observed. The membranous whorls had become larger; several of them had left the neuronal cell body through a breach in the plasma membrane (Fig. 2d). The compacted neurons had decreased considerably in number, but were more compact and more electrondense, so that individual ultrastructural elements could not be distinguished in them even at high magnification. Most such neurons exhibited membrane-bound protrusions and were surrounded by somewhat swollen, glycogen-containing astrocytic processes (Fig. 3a).

2.2.4. 3-day survival

The remaining compact, electrondense neurons displayed complicated systems of protrusions, from which smaller or larger fragments appeared to have separated (Fig. 3b). Both the protrusions and their fragments were membrane-bound and were surrounded by slightly swollen, glycogen-containing astrocytic processes. Such fragments or even large parts of such neurons, frequently together with the surrounding glycogen-containing astrocytic processes, were found inside phagocytotic cells (Fig. 3c) or astrocytes (Fig. 3d).

2.3. Electron-microscopic findings in the excitotoxic cortical area

2.3.1. 1-h survival

Slightly damaged, compacted and considerably swollen neurons were observed in an environment that contained numerous extremely swollen dendrites (Fig. 4a). In the slightly damaged neurons among apparently intact ultrastructural elements, a proportion of polyribosomes were sequestered and several mitochondria were swollen to various degrees. In large cytoplasmic areas of the swollen neurons, most ultrastructural elements were disintegrated. The ultrastructure of a few compacted neurons was similar to that of those found in the intermediate area; only a few of their mitochondria were somewhat dilated (Fig. 4a). Other compacted neurons displayed a more increased electron density and many mitochondrion-sized vacuoles, and it was difficult to distinguish

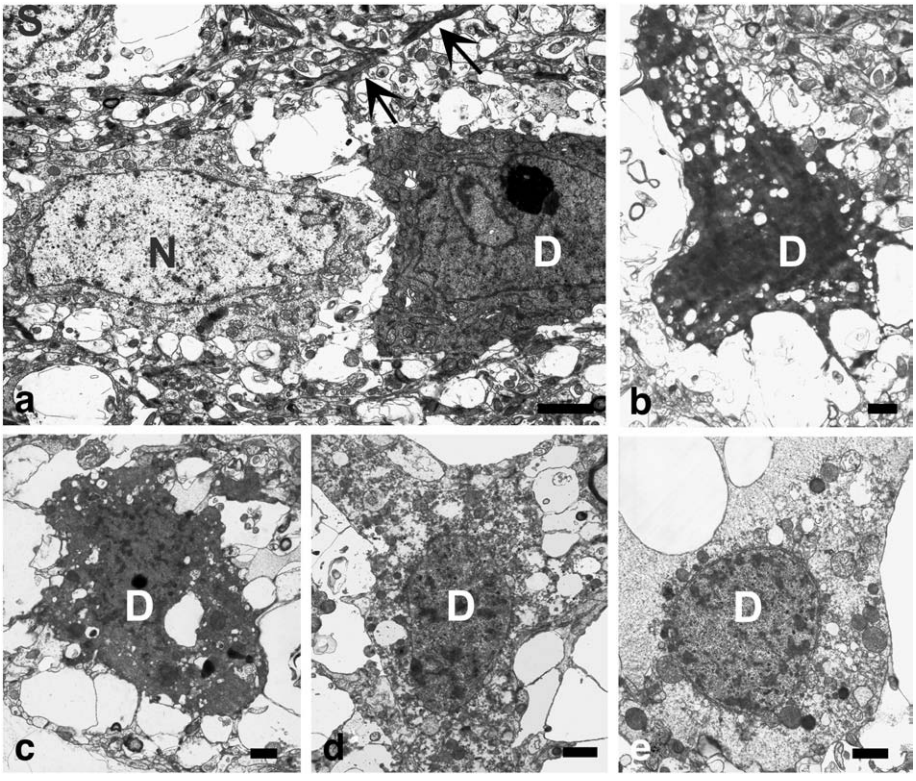

Fig. 4 – Necrotic-like removal of epilepsy-induced "dark" neurons from the excitotoxic area of the rat cortex: (a) a slightly damaged, a swollen and a "dark" neuron formed in an early stage of epilepsy; (b) a "dark" neuron formed in an advanced stage of epilepsy; (c–e) progression of the necrotic-like disintegration of "dark" neurons. Rats were sacrificed 1 h (a), 3 h (b and c) or 1 day (d and e) after the end of 4-aminopyridine crystal application. In panel (a), nuclei of a slightly damaged, a swollen and a "dark" neuron are denoted N, S and D, respectively. Round empty spaces in the neuropil are extremely swollen dendrites. Arrows point to a "dark" dendritic profile. In panels b–e, nuclei of disintegrating "dark" neurons are denoted D. Membrane-bound large empty spaces just around these neurons are markedly swollen astrocytic processes that do not contain glycogen particles. Scale bars: a ~2 μm, (b–e) ~1 μm.

individual ultrastructural elements in them; they were surrounded by extremely dilated astrocytic processes, which did not contain glycogen particles (Fig. 4b).

2.3.2. *3-h survival*

Numerous slightly damaged neurons were still present. A small number of considerably swollen neurons were also observed in which, except for a few mitochondria with a flocculent interior, most ultrastructural elements were disintegrated, the plasma membrane displayed large breaks, and short sections of the nuclear membrane were missing. Several somewhat shrunken neurons exhibited dilated endoplasmic reticulum cisternae, similar to those to be seen in Fig. 2b. As regards the compacted neurons, a few were similar to that depicted in Fig. 4b, while many others were less electrondense and crumb-like chromatin aggregates could be discerned in the nucleus (Fig. 4c).

2.3.3. *1-day survival*

Each of the above forms of damaged neurons was observed. Additionally, the cytoplasm of some neurons with a medium electrondense nucleus demonstrated various degrees of swelling and disintegration, which gave the impression that the neurons with a necrotic-like ultrastructure (Fig. 4e) may originate from the gradual disintegration and swelling of earlier-compacted neurons (Figs. 4b–e). The astrocytic processes around such neurons were extremely dilated and did not contain glycogen particles.

2.3.4. *3-day survival*

Many apparently normal neurons were still present. Most cytoplasmic elements in numerous neurons with a medium electrondense nucleus and an incomplete nuclear membrane were dispersed and merged with the disintegrated surroundings. No neurons similar to those depicted in Figs. 2a–d and 3a–d were encountered.

3. Discussion

3.1. *Formation of "dark" neurons*

In neuropathology, at least three types of "dark" neurons are generally accepted: reversible, irreversible and artifactous (Graeber et al., 2002). In connection with *in-vivo* or *post-mortem* head injuries (Csordás et al., 2003; Gallyas et al., 2004), *in-vivo* or *post-mortem* electric shocks (Csordás et al., 2003; Kellermayer et al., 2006), hypoglycemia (Gallyas et al., 2005) and ischemia (Kövesdi et al., 2007), we have demonstrated that the process of formation of "dark" neurons induced by these noxae display a common essential feature: a dramatic compaction of the very ultrastructural elements that are present at the moment of its outset. In the cases involving momentary physical noxae, all of the compacted ultrastructural elements were apparently intact; in those involving pathobiochemical noxae, the later the compaction began, the more abnormal the affected ultrastructural elements were. Following compaction, such neurons underwent additional morphological changes, these depending on the circumstances in their environment (Csordás et al., 2003; Gallyas et al., 2005; Kövesdi et al., 2007). All these factors are involved in the ensuing morphological differences between "dark" neurons of various origins, ages and fates, differences which obscure the common nature of their formation.

The 4-aminopyridine paradigm used here is advantageous for study of the epilepsy-induced formation of "dark" neurons because a proportion of them are present in a seriously damaged (excitotoxic) environment, whereas others are in an otherwise normal-looking environment. In the latter, the freshly-formed "dark" neurons contained apparently normal ultrastructural elements. In the excitotoxic environment, the ultrastructural elements in the "dark" neurons formed in the first hour of epilepsy revealed little damage (only a slight swelling of a few mitochondria), whereas during the next few hours, the freshly-formed "dark" neurons displayed various degrees of mitochondrial disintegration and ribosomal sequestration. These observations are in accordance to those mentioned in the previous paragraph, supporting thereby the theorem that "dark" neurons of various origins have a common mechanism of formation (for details of this mechanism see Gallyas et al., 2004; Gallyas, 2007; Kovács et al., 2007).

3.2. *Recovery of "dark" neurons*

Microscopic observations in animal experiments led to the assumption that "dark" neurons induced by epilepsy (Attilio et al., 1983) or a considerable number of metabolic or physical noxae (reviewed by Csordás et al., 2003) are capable of recovery. This assumption was supported by a quantitative experiment (Csordás et al., 2003): in the otherwise undamaged hippocampal dentate gyri, an electric-shock paradigm simultaneously initiated compaction in about 10% of the granule neurons whose dendrites pointed toward the negative electrode. About 99% of the affected neurons regained the normal volume within a few hours, indicating the high potential of "dark" neurons for recovery. In the early stage of recovery, the affected neurons were outlined with mitochondrion-sized dots in silver-stained sections, and were seen in the electron microscope to contain dilated endoplasmic reticulum cisternae in the electron microscope. In the late phase of recovery, these morphological signs disappeared, but mitochondrion-sized membranous whorls appeared in both the karyoplasm and the dendrites of the affected neurons. Finally, these whorls were delivered to astrocytic processes through transient gaps in the plasma membrane.

In the present study, all these morphological signs of recovery from the "dark" state were observed not only in the intermediate, but also in the excitotoxic areas. While the proportion of recovering "dark" neurons appeared to be relatively high in the intermediate area, it was low in cortical layers II and III and moderate in cortical layers V and VI of the excitotoxic area. Consequently, several "dark" neurons retained the capability of recovery even in an excitotoxic environment. These observations support earlier findings (Gallyas et al., 2006) suggesting that the high potential of "dark" neurons for recovery can be suppressed by pathobiochemical processes in their vicinity.

3.3. *The mode of death of "dark" neurons in the intermediate cortical area cannot be either necrosis or apoptosis*

In this area, the non-recovering epilepsy-induced "dark" neurons underwent the following sequence of ultrastructural changes: (i) a further increase in compaction and electron density, (ii) the formation of membrane-bound, electrondense, compact cytoplasmic protrusions, (iii) the breaking up of the latter into membrane-bound, electrondense, compact fragments, and (iv) the swallowing up of all these by phagocytotic cells. Since these ultrastructural features fundamentally differ from those characteristic of necrosis (swelling of the cell, gradual disintegration of the intracellular organelles and dispersion of their remnants into the surroundings through large gaps in the plasma and nuclear membranes; Wyllie et al., 1980; Kerr and Harmon, 1991), it can be stated that, in stark contrast with the prevailing conception (Thom et al., 2008), the cause of death of the epileptic "dark" neurons in the intermediate cortical area cannot be necrosis. The presence of acidophilic neurons in this area, widely accepted as a sign of selective neuronal necrosis, is not in contradiction with this statement, since it has been demonstrated indisputably that, in the early phase of their non-necrotic removal from an otherwise normal environment, the non-recovering "dark" neurons are also acidophilic (Zsombok et al., 2005).

On the other hand, the absence of large chromatin clumps with rounded outlines in the nucleus, the negative results of TUNEL staining and the capability of the epileptic "dark" neurons for recovery are incompatible with the apoptotic nature of their death. However, there is a certain relationship between "dark" neurons and apoptotic neurons. Specifically, after completion of the decisive biochemical processes and condensation of the nuclear chromatin into large clumps with rounded outlines, the perikarya and dendrites of apoptotic neurons undergo ultrastructural compaction like the "dark" neurons, and are removed from an otherwise normal-looking environment through the same sequence of morphological changes as those for "dark" neurons (Gallyas et al., 2005; Kövesdi et al., 2007). In view of these and some other similarities, the formation of "dark" neurons was assumed to consist in the non-apoptotic initiation of ultrastructural compaction, the mechanism of which is programmed in the neuron as the first step of the morphological execution of ontogenetic apoptosis.

With regard to similarities to and differences from the apoptotic and the necrotic morphologies, various kinds of "dark" cells in certain non-nervous tissues were assumed to have a death pathway different from either necrosis or apoptosis (Harmon, 1987; Wyllie, 1987). In several instances, the death of neurons has been reported to fall into neither of these categories (Graeber and Moran, 2002), and we have made similar suggestions for traumatic (Csordás et al., 2003), electric (Csordás et al., 2003), hypoglycemic (Gallyas et al., 2005) and ischemic (Kövesdi et al., 2007) "dark" neurons.

Dead "dark" neurons displaying the morphological changes depicted in Fig. 3 in the present paper must surely have been encountered in the periphery of excitotoxic brain areas by a number of earlier authors. Nevertheless, we have found only one paper reporting such a "dark" neuron (see Fig. 6 in Ingvar et al., 1988). This was interpreted as a "persisting dark neuron" that would undergo necrosis at a later time. However, such a fragmented neuron ought to be taken as already dead.

3.4. *The mode of death of "dark" neurons in the excitotoxic cortical areas may not be necrosis*

The widely-held assumption that "dark" neurons die through the necrotic pathway stems from observations similar to those demonstrated in the present paper. Specifically, in excitotoxic, necrotic or contused brain areas, the "dark" neurons begin to swell, and thereafter their organelles gradually disintegrate and finally disperse into their surroundings through large gaps in their plasma and nuclear membranes, or are engulfed by phagocytotic cells. As a considerable proportion of neurons and non-neuronal cells survive the injurious circumstances existing in an excitotoxic environment, the above sequence of ultrastructural changes in "dark" neurons has been designated selective necrosis (Auer et al., 2008). This term comes from the era when, solely on the basis of morphological features, only two kinds of cell death were recognized: apoptosis and necrosis. Unfortunately, the third ("dark"-cell) pathway assumed at that time by Harmon (1987) and Wyllie (1987) has subsequently been totally neglected.

In our view, it is improbable that "dark" neurons possess two death mechanisms, one occurring in an otherwise undamaged environment and another in excitotoxic, pannecrotic or contused environments. It is more probable that in these environments pathobiochemical processes superimpose a necrotic-like morphological removal process on already dead "dark" neurons. This idea is supported by the fact that, after chromatin condensation and cytoplasmic compaction (i.e. after death), even apoptotic neurons undergo the same necrotic-like removal process (Gallyas et al., 2005; Kövesdi et al., 2007).

Besides those described above, there is a further noteworthy difference in ultrastructural features between the apoptotic-like and the necrotic-like removal processes: during the former, the astrocytic processes around the affected "dark" or apoptotic neurons abundantly contain glycogen particles, whereas during the latter they are glycogen-depleted. Since an excess or the absence of glycogen granules in astrocytes indicates an environment rich or poor, respectively, in metabolic energy (Castejon et al., 2002), it may be assumed that the type of the removal process (apoptotic-like or necrotic-like) of both the "dark" and the apoptotic neurons depends on the metabolic energy content of their environment.

3.5. *Concluding remark*

The main purpose of this paper is eventually to bring the phenomenon at issue, for a "heretical" explanation of which is presented here, to the attention of researchers who are in the position of investigating its validity by means of experimental paradigms other than those we used.

4. Experimental procedures

4.1. *Animal experiments*

A total of 18 randomly elected male Sprague–Dawley rats weighing between 300 and 500 g were anesthetized with a 1.5:98.5 v/v mixture of Halothane and air. A hole of 1.5 mm in diameter was drilled into the exposed calvaria, above the right brain cortex, 6.2 mm caudal to the bregma and 2.5 mm lateral from the midline. In each rat, the dura mater was carefully removed and a 0.5 mg/kg 4-aminopyridine crystal was placed onto the cortical surface for 40 min. Thereafter, the crystal was washed out with physiological saline, the hole was covered with bone wax and the anesthesia was discontinued. A further 6 rats, which underwent the same operation procedure but without 4-aminopyridine crystal application, served as control. The rats were sacrificed under deep urethane (2 g/kg i.p.) anesthesia by transcardial perfusion for 30 min with 500 ml of either cacodylate-buffered glutaraldehyde (Gallyas et al., 1990) or cacodylate-buffered formaldehyde (Gallyas et al., 1993), preceded by a short rinsing with physiological saline. The 12 glutaraldehyde-fixed, 4-aminopyridine-treated rats were allowed to survive for 1 h, 3 h, 1 day or 3 days, while the 6 control rats and the 6 formaldehyde-fixed, 4-aminopyridine-treated rats survived for 3 h or 1 day, with 3 rats at each time point. These survival times were found in our relevant papers (Csordás et al., 2003; Gallyas et al., 2005; Kövesdi et al., 2007; Baracskay et al., in press) to be appropriate for the demonstration of the morphological changes characteristic of both the recovery and the death of "dark" neurons.

Animal care and experiments were performed in compliance with order 243/1988 of the Hungarian Government, which is an adaptation of directive 86/609/EGK of the European Committee Council.

4.2. *Tissue processing and staining*

Brains were removed from the skull with a 1-day delay in order to prevent the artifactual (non-epileptic) formation of "dark" neurons (Cammermeyer, 1960). From the caudal two-thirds of the brains fixed with glutaraldehyde, 150-μm coronal vibratome sections were cut. Every fifth section (40 sections from each rat) was stained by a silver technique (Gallyas et al., 1990) that is specific (Gallyas et al., 2002) and reproducible (Newman and Jasani, 1998) for "dark" neurons. Briefly, following dehydration with graded 1-propanol, sections were incubated for 16 h at 56 °C in 1-propanol containing 0.8% sulfuric acid and 2% water, rehydrated with graded 1-propanol, treated with 1% acetic acid for 10 min and then immersed in a special physical developer until the background had become light-brown. For electron microscopy, the cortical areas that corresponded to those demonstrated in Fig. 1a were cut from the vibratome sections neighboring those which contained "dark" neurons in non-excitotoxic environment according to the silver staining (10 specimens from each rat). These were post-fixed with a 1:1 mixture of 2% osmium tetroxide and 3% potassium hexacyanoferrate (II) for 1 h at room temperature, and flat-embedded in Durcupan ACM. Semithin (1-μm) sections 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. Thin (50-nm) sections were contrasted with 5% uranyl acetate in 50% methanol for 2 min and then with 0.5% lead citrate for 1 min. Ultrastructural investigations were carried out with a Jeol JEM 1200EX transmission electron microscope.

The caudal two-thirds of the brains fixed with formaldehyde were embedded into paraffin and cut at 10 μm. For the demonstration of dead (acidophilic) neurons, every tenth section (40 sections from each rat) was stained with 1% acid fuchsin dissolved in 0.01% acetic acid. For the demonstration of apoptotic cells, 10 neighboring sections from each rat were processed through the steps of an *in-situ* cell death detection (TUNEL) kit (Roche, Cat. No. 11684817910), with strict adherence to the instructions provided in the kit manual.

Acknowledgments

The authors thank Andok Csabáné, Nyirádi József and Dr. Nádor Andrásné for their valuable help in the light-microscopic, electron-microscopic and photographic work, respectively. This study was supported by Hungarian research grants ETT-176/2006 and Regional Center of Excellence DNT RET and CellKom RET.

REFERENCES

Attilio, A., Söderfeldt, B., Kalimo, H., Olsson, Y., Siesjö, B.K., 1983. Pathogenesis of brain lesions caused by experimental epilepsy. Light and electron-microscopic changes in the rat hippocampus following bicucculine-induced status epilepticus. Acta Neuropathol. 59, 11–24.

Auer, R.N., Siesjö, B.K., 1988. Biological differences between ischemia, hypoglycemia and epilepsy. Ann. Neurol. 24, 699–707.

Auer, R.N., Kalimo, H., Olsson, Y., Siesjö, B.K., 1985. The temporal evolution of hypoglycemic brain damage. I. Light- and electron-microscopic findings in the rat cerebral cortex. Acta Neuropathol. 67, 13–24.

Auer, R.N., Dunn, J.F., Sutherland, G.R., 2008. Hypoxia and related conditions. In: Love, S., Louis, D.N., Ellison, D.W. (Eds.), Greenfield's Neuropathology. Arnold, London, pp. 63–105.

Baracskay, P., Szepesy, Z.S., Orbán, G., Juhász, G., Czurkó, A., in press. Gneralization of seizures parallels the formation of "dark" neurons in the hippocampus and pontine reticular formation after focal-cortical application of 4-aminopyridine (4-AP) in the rat. Brain Res.

Cammermeyer, J., 1960. The post mortem origin and mechanism of neuronal hyperchromatosis and nuclear pycnosis. Exp. Neurol. 2, 399–405.

Castejon, O.J., Diaz, M., Castejon, H.V., Castellano, A., 2002. Glycogen-rich and glycogen-depleted astrocytes in the oedematous human cerebral cortex associated with brain trauma, tumours and congenital malformations: an electron microscopy study. Brain Inj. 16, 109–132.

Covolan, L., Mello, L.E., 2000. Temporal profile of neuronal injury following pilocarpine or kainic acid-induced status epilepticus. Epilepsy Res. 39, 133–152.

Csordás, A., Mázló, M., Gallyas, F., 2003. Recovery versus death of "dark" (compacted) neurons in non-impaired parenchymal environment. Acta Neuropathol. 106, 37–49.

Gallyas, F., 2007. Novel cell-biological ideas deducible from morphological observations on "dark" neurons revisited. Ideggyogy. Szle. 60, 212–222.

Gallyas, F., Güldner, F.H., Zoltay, G., Wolff, J.R., 1990. Golgi-like domonstration of "dark" neurons with an argyrophil III method for experimental neuropathology. Acta Neuropathol. 79, 620–628.

Gallyas, F., Hsu, M., Buzsáki, G., 1993. Four modified silver methods for thick sections of formaldehyde-fixed mammalian central nervous tissue: "dark" neurons, perikarya of all neurons, microglial cells and capillaries. J. Neurosci. Methods 50, 159–164.

Gallyas, F., Farkas, O., Mázló, M., 2002. Traumatic compaction of the axonal cytoskeleton indiuces argyrophilia: histological and theoretical importance. Acta Neuropathol. 103, 36–42.

Gallyas, F., Farkas, O., Mázló, M., 2004. Gel-to-gel phase transition may occur in mammalian cells: mechanism of formation of "dark" (compacted) neurons. Biol. Cell 96, 313–324.

Gallyas, F., Csordás, A., Schwarcz, A., Mázló, M., 2005. "Dark" (compacted) neurons may not die through the necrotic pathway. Exp. Brain Res. 160, 473–486.

Gallyas, F., Gasz, B., Sziget, A., Mázló, M., 2006. Pathological circumstances impair the ability of "dark" neurons to undergo spontaneous recovery. Brain Res. 1110, 211–220.

Graeber, M.B., Moran, L.B., 2002. Mechanism of cell death in neurodegenerative diseases: fashion, fiction and facts. Brain Pathol. 12, 385–390.

Graeber, M.B., Blakemore, W.F., Kreutzberg, G.W., 2002. Cellular pathology of the central nervous system. In: Graham, D.I., Lantos, P.L. (Eds.), Greenfield's Neuropathology. Arnold, London, pp. 123–191.

Harmon, B.V., 1987. An ultrastructural study of spontaneous cell death in mouse mastocytoma with particular reference to dark cells. J. Pathol. 153, 345–355.

Ingvar, M., Morgan, P.F., Auer, R.N., 1988. The nature and timing of excitotoxic neuronal injury in the cerebral cortex, hippocampus, thalamus due to flurothyl-induced status epilepticus. Acta Neuropathol. 75, 362–369.

Kerr, J.F.R., Harmon, B.V., 1991. Definition and incidence of apoptosis: an historical perspective. In: Tomel, L.D., Cope, F.O. (Eds.), Apoptosis, the Molecular Basis of Cell Death. Cold Spring Harbor Laboratory Press, pp. 5–29.

Kellermayer, R., Zsombok, A., Auer, T., Gallyas, F., 2006. Electrically induced gel-to-gel phase-transition in neurons. Cell Biol. Intern. 30, 175–182.

Kovács, B., Bukovics, P., Gallyas, F., 2007. Morphological effects of transcardially perfused SDS on the rat brain. Biol. Cell 99, 425–432.

Kövesdi, E., Pál, J., Gallyas, F., 2007. The fate of "dark" neurons induced by transient focal cerebral ischemia in a non-necrotic and non-excitotoxic environment: neurobiological aspects. Brain Res. 1147, 472–483.

Liou, A.K., Clark, L.S., Henshall, D.C., Yin, X.M., Chen, J., 2003. To die or not to die for neurons in ischemia, traumatic brain injury and epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways. Prog. Neurobiol. 69, 103–142.

Newman, G.R., Jasani, A.B., 1998. Silver development in microscopy and bioanalysis: past and present. Review article. J. Pathol. 186, 119–125.

Pena, F., Tapia, R., 2000. Seizures and neurodegeneration induced by 4-aminopyridine in rat hippocampus in vivo: role of glutamate- and GABA-mediated neurotransmission and ion channels. Neuroscience 101, 547–561.

Smith, M.L., Auer, R.N., Siesjö, B.K., 1984. The density and distribution of ischemic brain injury in the rat following 2–10 min of forebrain ischemia. Acta Neuropathol. 64, 319–332.

Söderfeldt, B., Kalimo, H., Olsson, Y., Siesjö, B.K., 1983. Bicucculine-induced epileptic brain injury. Transient and persistent cell changes in rat cerebral cortex in the early recovery period. Acta Neuropathol. 62, 87–95.

Thom, M., Sisodiya, S., Najm, I., 2008. Neuropathology of epilepsy. In: Love, S., Louis, D.N., Ellison, D.W. (Eds.), Arnold, London, pp. 833–877.

Wyllie, A.H., 1987. Apoptosis: cell death in tissue relations. J. Pathol. 153, 313–316.

Wyllie, A.H., Kerr, J.R., Currie, A.E., 1980. Cell death: the significance of apoptosis. Int. Rev. Cytol. 68, 251–306.

Zsombok, A., Tóth, Z.S., Gallyas, F., 2005. Basophilia, acidophilia and argyrophilia of "dark" (compacted) neurons in the course of their formation, recovery or death in an otherwise undamaged environment. J. Neurosci. Methods 142, 145–152.