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Basophilia, acidophilia and argyrophilia of "dark" (compacted) neurons during their formation, recovery or death in an otherwise undamaged environment

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Abstract

By means of a condenser discharge electric shock paradigm, "dark" (compacted) granule neurons were momentarily produced in the hippocampal dentate gyri of rats, which were sacrificed either immediately or following survival periods ranging from 1 h to 30 days. Except for the morphological changes related to the formation, recovery or death of the "dark" neurons, the affected brain areas remained undamaged. Vibratome, frozen, cryostat and paraffin sections were stained with Mayer's hematoxylin, acid fuchsin, or Fluoro-Jade and by three silver methods widely used for the demonstration of damaged neurons; with or without previous removal of nucleic acids, partial digestion of proteins or blockade of the negatively charged side-groups of nucleic acids and proteins. The results allowed the following conclusions: (i) "Dark" neurons acquire argyrophilia and excess basophilia simultaneously with their momentary formation. (ii) Negatively charged protein molecules are responsible for these processes. (iii) From the recovering "dark" neurons, the acquired basophilia and argyrophilia disappear within a few hours post-insult. (iv) From the moribund or dead "dark" neurons, the acquired basophilia disappears in the same period of time while the acquired argyrophilia in a few days. (vi) Freshly-produced or recovering "dark" neurons are slightly acidophilic, whereas the moribund or dead ones display intense acidophilia.
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1. Introduction

A common morphopathologic response to various injuries of individual neurons scattered among apparently intact ones is a momentary, dramatic compaction of undamaged ultrastructural elements in both the cytoplasm and the nucleus. The affected neurons are traditionally referred to as "dark". The formation of "dark" neurons can be initiated by physical forces, such as head injuries (Gallyas et al., 1992) or an electric shock (Gallyas et al., 1993) or by pathometabolic conditions, including hypoglycemia (Auer et al., 1985), status epilepticus (Igvar et al., 1988) and ischemia (Petito and Pulsinelli, 1984). Independently of the nature of the initiating condition, a proportion of the "dark" neurons recover, while the others die (Auer et al., 1985; Csordás et al., 2003).

Post-mortem mechanical damage to the unfixed or improperly fixed brain (e.g. removal of such brains from the

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skull) may also initiate the formation of "dark" neurons. In experimental neuropathology, this unwanted process can be prevented by a 24 h delay of the brain autopsy following transcardial perfusion fixation (Cammermeyer, 1961).

Since no specific immunocytochemical method is available, light-microscopic identification of the "dark" neurons is commonly based on their markedly increased stainability with cationic dyes (such as Mayer's hematoxylin or toluidine blue), and stainability with anionic dyes (such as acid fuchsin or eosin). During the past decade, a fluorescent anionic dye (Fluoro-Jade; Schmued et al., 1997) and its improved variant (Fluoro-Jade B, Schmued and Hopkins, 2000) has been gaining ground.

It is long known that argyrophilia is also acquired by "dark" neurons (Cammermeyer, 1979). This property has been made use of for their demonstration by three quite different silver staining methods (amino-cupric-silver method of de Olmos, 1969; chemical-development-silver method of Gallyas et al., 1980; physical-development-silver method of Gallyas et al., 1990). Silver staining with the FD NeuroSilver^{TM} Kit, which has been also widely used since a few years ago, is a commercial variant of the above chemical-development-silver method (Kuhlmann and Guilarte, 2000).

Most information on the basophilia, acidophilia and argyrophilia of "dark" neurons has been obtained by means of experimental paradigms that initiate the formation of individual "dark" neurons at markedly different time points. Consequently, at any given time point following the onset of the initiating condition, "dark" neurons in different stages of recovery or death may be present. Additionally, most relevant experimental paradigms produce "dark" neurons in a damaged (necrotic, contused, excitotoxic, etc.) environment, which may influence their staining properties considerably.

In a recent study, a condenser discharge electric-shock paradigm was morphologically characterized (Csordás et al., 2003) that appears suitable for investigation of the time course of staining properties in the "dark" neurons without any exogenous influence. This study demonstrated that, in the hippocampal dentate gyri of the rat, the electric-shock paradigm (i) momentarily caused ultrastructural compaction (striking shrinkage of the cell body) in a large number of granule neurons whose dendrites pointed towards the negative electrode (henceforth: freshly-produced "dark" neurons), (ii) the compacted granule neurons were randomly distributed among morphologically intact granule neurons (henceforth: normal neurons), (iii) in a majority of the "dark" granule neurons, the degree of compaction gradually decreased during the first few hours of survival (henceforth: recovering "dark" neurons) and the normal morphological features were regained within 24 h (henceforth: recovered "dark" neurons), and (iv) a small proportion of the "dark" neurons retained the compacted morphology during the 1st survival day (henceforth: moribund "dark" neurons), thereafter underwent a week-long process of breaking up into membrane-bound, electron dense fragments (henceforth: dead "dark" neurons), which were engulfed by glycogen-containing astrocytes in an otherwise intact environment.

This paradigm is used in the present paper to examine the stainability of "dark" granule neurons in the course of their formation, recovery or death, with Mayer's hematoxylin, acid fuchsin and Fluoro-Jade, and also by the silver methods mentioned. In order to increase the specificity of staining with these techniques, nucleic acids and easily digestible proteins were removed, and the negatively charged side-groups of macromolecules were blocked in several sections. In order to increase the number of "dark" granule neurons that die, 10 condenser discharges were delivered consecutively in the present study.

2. Materials and methods

2.1. Animal experiments

A total of 14 Wistar rats each weighing about 200 g were anesthetized with a mixture of 4% isoflurane, 29% oxygen and 67% nitrous oxide, using a SurgiVet respirator (Waukesha, WI). Each of the rats was subjected to 10 condenser discharges (250 V and 500 μF), administered at intervals of 1 min by a laboratory-built apparatus (Gallyas et al., 1993) through surface electrodes (4 mm × 4 mm) pressed by hand bilaterally onto the temporal muscles of the exposed calvaria. With the exception of two rats that were perfusion-fixed immediately after the last condenser discharge, the respiration was continued for an additional 30 min, and the rats were then allowed to recover. Following a survival period lasting 1 or 4 h or 1, 2, 6 or 30 days (two rats at each time), the rats were sacrificed by perfusion fixation under deep anesthesia (100 mg/kg sodium pentobarbital, i.p.). Two other rats that had not received electric shocks served as controls. The fixative was prepared by mixing 500 ml of 0.2 mol/l sodium cacodylate, 200 ml of 20% paraformaldehyde, 50 ml of 0.1 mol/l calcium chloride and 250 ml of 10% polyvinylpyrrolidone K25 (all as aqueous solutions), followed by adjustment of the mixture to pH 7.5 with a few drops of 0.1 mol/l hydrochloric acid. Before fixation, the vascular system was rinsed with physiological saline for 30 s. After fixation, the rats were left untouched at room temperature for 24 h before removal of the brain from the skull (Cammermeyer, 1961). Thereafter, the cerebellum was cut off, and the two hemispheres were separated.

Animal care and handling were carried out in accordance with order 243/1998 of the Hungarian Government, which is an adaptation of directive 86/609/EGK of the European Committee Council.

2.2. Tissue processing and staining

The left hemispheres were vibratome-cut at 100 μm up to the coronal plane about 5.8 mm caudal to the bregma and

then frozen-cut at 30 μm. Sections were preserved in the fixative. Several vibratome sections were embedded in Tissue-Tec (Miles Inc. Elkhart, USA), cut at 8 μm and air-dried onto microscopic slides using a cryostat microtome. After embedding into paraffin, the corresponding blocks of the right hemispheres were cut at 8 μm and the sections were mounted on microscopic slides previously coated with Vectabond adhesive.

2.2.1. Light-microscopic demonstration of basophilia

Sections were treated for 10 min at room temperature with 0.1% Mayer's hematoxylin (Fluka 51257), and then washed with tap water for 10 min. Thereafter, filter paper was pressed on the paraffin and the cryostat sections to remove the bulk of the water, complete dehydration was achieved with three changes of clove oil, and finally the sections were covered with Canada balsam; the vibratome sections were dehydrated with isopropanol, cleared with xylene and finally covered with Canada balsam.

2.2.2. Light-microscopic demonstration of acidophilia

Sections were treated for 10 min at room temperature in 0.01% acid fuchsin dissolved in 1% acetic acid, and then rinsed with distilled water for 1 min. Thereafter, they were dehydrated and covered as described above.

2.2.3. Fluorescent-microscopic demonstration of acidophilia with Fluoro-Jade

The staining protocol described in Schmued et al. (1997) was strictly observed.

2.2.4. Demonstration of argyrophilia by the amino-cupric-silver method of de Olmos (1969)

The staining protocol described in Carlsen and de Olmos (1981) was strictly observed.

2.2.5. Demonstration of argyrophilia by the chemical-development-silver method of Gallyas et al. (1980)

The staining protocol described in Nadler and Evenson (1983) was strictly observed.

2.2.6. Demonstration of argyrophilia by the physical-development-silver method of Gallyas et al. (1990)

The staining protocol described in Gallyas et al. (1994) was strictly observed.

2.2.7. Removal of DNA and RNA

Sections were incubated in a solution containing 2 mg of deoxyribonuclease (Sigma D-4527), 1 mg of ribonuclease (Sigma R-7003) and 1 mg of $MgCl_2$ in 10 ml of neutral PBS buffer for 4 h at 37 °C and then washed with 1% acetic acid for three 1 min periods (Barka and Anderson, 1963).

2.2.8. Partial removal of proteins

Sections were incubated in a solution containing 10 mg of proteinase-K (Sigma P-6556) in 10 ml of neutral PBS buffer for 1 h at 37 °C, and then washed with 1% acetic acid for three 1 min periods.

2.2.9. Blockade of negatively charged side-groups of macromolecules

Sections were dehydrated with graded methanol, incubated with 0.1 mol/l hydrochloric acid in methanol at 56 °C for 16 h (esterification; Barka and Anderson, 1963), rehydrated with graded methanol, and then washed with 1% acetic acid for 1 min.

3. Results

3.1. Paraffin and cryostat sections

The following description of the findings summarized in Table 1 refers to the results obtained with formaldehyde-fixed paraffin sections. However, the very same staining features were observed for formaldehyde-fixed 8 μm cryostat sections. Of the silver methods tested, only the physical-development-silver method proved suitable on paraffin and thin cryostat sections. Although the other two silver methods worked well in thick non-embedded sections (see later), the thin cryostat sections were loosened from the microscopic slides and then broken into small parts in the alkaline solutions utilized in them.

In the hippocampal dentate gyrus, the normal granule neurons did not stain with acid fuchsin or Fluoro-Jade, or by the physical-development-silver method. In contrast, their nucleoli and nuclear margins were stained with Mayer's hematoxylin (henceforth, normal basophilia; Fig. 1a).

Both the nucleus and the cytoplasm of the freshly-produced "dark" neurons were stained strongly and homogeneously with Mayer's hematoxylin (henceforth: acquired basophilia; Fig. 1a), but barely detectable with acid fuchsin or Fluoro-Jade. On the other hand, their whole soma–dendrite domain became stainable homogeneously by the silver method (henceforth: acquired argyrophilia; Fig. 2a).

The recovering "dark" neurons gradually lost the acquired argyrophilia and the acquired basophilia, while their normal basophilia gradually reappeared.

In the moribund "dark" neurons, both the normal and the acquired basophilia disappeared within a few hours. On the other hand, the acquired argyrophilia persisted for 48 h, but disappeared completely by the sixth day. Acidophilia became easily observable by the fourth hour of survival, reached a maximum by the end of the first survival day and retained this level until the dead "dark" neurons were removed from the tissue. In the 24–48 h period, the silver method labeled the same neurons as did Fluoro-Jade, while only the latter method stained the dead

Table 1
Intensity of staining in 8 μm paraffin sections of the normal (N) and the electric shock-produced "dark" (D) neurons as a function of the survival time

Staining method	Pretreatment	Survival times													
		<1 min		1 h		4 h		1 day		2 days		6 days		30 days	
		N	D	N	D	N	D	N	D	N	D	N	D	N	D
Hematoxylin		○	□	○	+	○	±	○	−	○	−	○	−	○	−
Hematoxylin	Acidic methanol	±	±	±	±	±	−	±	−	±	−	±	−	±	−
Hematoxylin	Nucleases	±	□	±	+	±	±	±	−	±	−	±	−	±	−
Hematoxylin	Proteinase-K	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Acid fuchsin		−	±	−	±	−	+	−	□	−	□	−	+	−	−
Acid fuchsin	Acidic methanol	−	+	−	+	−	+	−	□	−	□	−	+	−	−
Acid fuchsin	Nucleases	−	±	−	±	−	+	−	□	−	□	−	+	−	−
Acid fuchsin	Proteinase-K	−	−	−	−	−	+	−	□	−	□	−	+	−	−
Fluoro-Jade		−	±	−	±	−	+	−	■	−	■	−	▢	−	−
Fluoro-Jade	Acidic methanol	−	+	−	+	−	+	−	■	−	■	−	▢	−	−
Fluoro-Jade	Nucleases	−	±	−	±	−	+	−	■	−	■	−	▢	−	−
Fluoro-Jade	Proteinase-K	−	−	−	−	−	+	−	■	−	■	−	▢	−	−
Silver method[a]		−	■	−	■	−	■	−	■	−	▢	−	±	−	−
Silver method[a]	Acidic methanol	−	■	−	■	−	■	−	■	−	▢	−	−	−	−
Silver method[a]	Nucleases	−	▢	−	▢	−	▢	−	▢	−	▢	−	−	−	−
Silver method[a]	Proteinase-K	−	−	−	−	−	−	−	−	−	−	−	−	−	−

(−) Neither the somata and dendrites nor the nucleoli and nuclear margins are distinguishable; (○) good staining of the nucleoli and nuclear margins; (±) the somata are barely detectable; (+) the somata are easily detectable; (□) intense, homogenous staining of the somata, but not the dendrites; (■) intense, homogeneous staining of both the somata and dendrites; (▢) intense, homogeneous staining of the somata, but incomplete staining of the dendrites.

[a] The physical-development-silver method.

(but not phagocytosed) "dark" neurons on the sixth day of survival.

Enzymatic digestion of the nucleic acids removed the normal basophilia nearly completely, whereas it insignificantly decreased the acquired basophilia (Fig. 1b), the acquired argyrophilia (Fig. 2b) and the acquired acidophilia of the "dark" neurons. The acidic methanol pretreatment caused both the normal and the acquired basophilia to disappear nearly completely (Fig. 1c) but did not influence the acquired argyrophilia (Fig. 2c). Furthermore, it induced mild acidophilia in the freshly-produced and the recovering "dark" neurons. The proteinase-K pretreatment completely removed the acquired argyrophilia (Fig. 2d) and the acquired basophilia (Fig. 1d), whereas it insignificantly decreased the normal basophilia of neurons and the acquired acidophilia of the moribund or dead "dark" neurons.

3.2. Vibratome and frozen sections

The following description of the findings summarized in Table 2 refers to the results obtained with formaldehyde-fixed vibratome sections. However, the very same staining features were observed for frozen sections.

Mayer's hematoxylin displayed the same staining features in "dark" neurons of various fates, as it did in paraffin sections. Acid fuchsin proved unsuitable due to low contrast and intense background staining. Fluoro-Jade excellently stained the moribund and dead "dark" neurons but in a barely detectable manner the freshly produced ones. Both the somata and dendrites of the latter were homogeneously stained by the amino-cupric-silver method and the physical-development-silver method, whereas the chemical-development-silver method outlined them with mitochondrion-sized puncta. The moribund or dead "dark" neurons were stained by each of the silver methods tested (Fig. 3d–f), but for a shorter time by the physical-development-silver method than by the other two silver methods, or with Fluoro-Jade.

3.3. Other important particulars

Mayer's hematoxylin and acid fuchsin are simple, reliable and compatible with each other and with immunohistochemical techniques. On the other hand, sporadically occurring "dark" neurons are difficult to detect in thick non-embedded preparations due to overlapping of cells.

Fluoro-Jade is also simple, reliable and compatible with other fluorescent techniques including immunohistochemical methods. Furthermore, it makes possible the identification of the phenotype of the acidophilic neurons by demonstrating their dendritic tree. On the other hand, a fluorescent microscope must be available.

The silver staining methods demonstrate both the somata and dendrites of the "dark" neurons with high contrast, even in 100 μm sections. Regarding the physical-development silver method, the background staining in vibratome or frozen sections was lighter than in paraffin sections. The silver-stained preparations can be examined un-

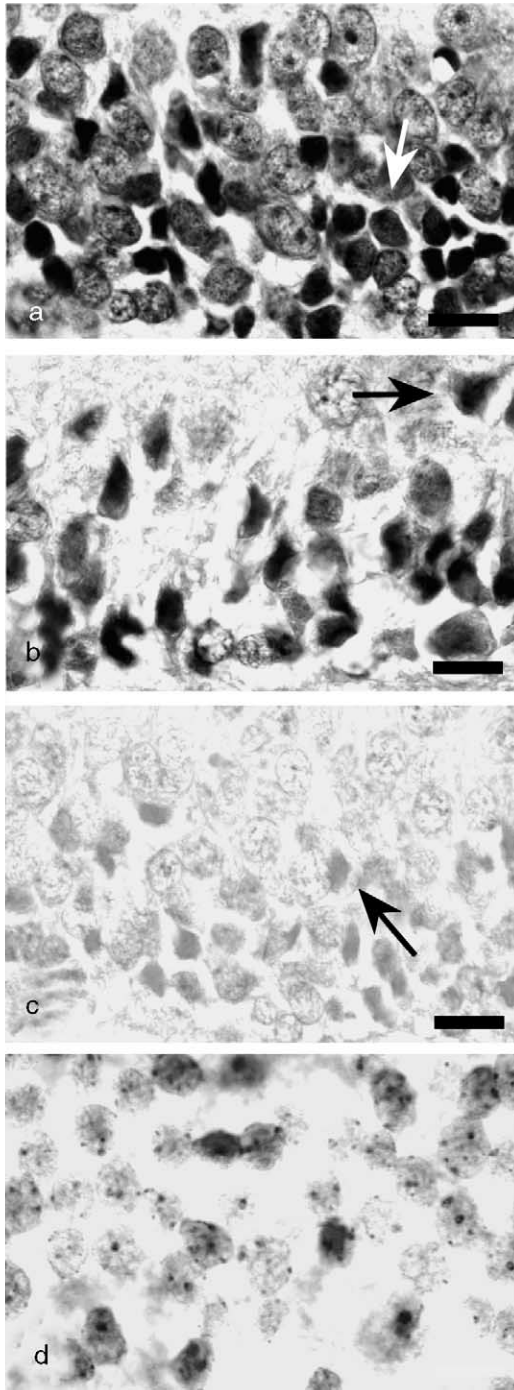

Fig. 1. Demonstration of basophilia with Mayer's hematoxylin in normal and freshly-produced "dark" granule neurons of the rat hippocampal dentate gyrus, without (a) and following enzymatic removal of nucleic acids (b), blockade of the negatively charged groups of macromolecules (c) and proteinase-K digestion (d). Arrows point to "dark" neurons. Scale bars: (a–d) 75 μm.

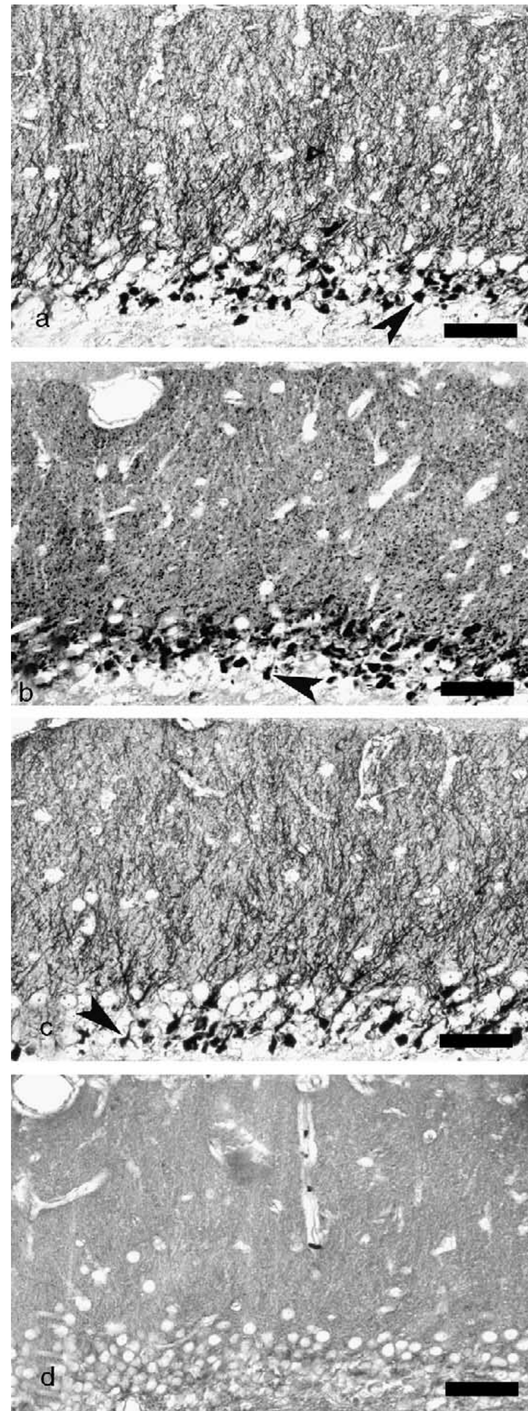

Fig. 2. Demonstration of argyrophilia by the physical-development-silver staining method in freshly-produced "dark" granule neurons of the rat hippocampal dentate gyrus, without (a) and following enzymatic removal of nucleic acids (b), blockade of the negatively charged groups of macromolecules (c) and proteinase-K digestion (d). Arrowheads point to "dark" neurons. Scale bars: (a–d) 500 μm.

der the microscope for an unlimited time and can be stored for years. On the other hand, the silver techniques are laborious and need much experience to bring good results, especially the amino-cupric-silver method, and they are incompatible with other staining techniques. Furthermore, distinguishing the freshly-produced or recovering "dark" neurons from the moribund or dead ones needs expertise (see Section 4).

Table 2
Intensity of staining in 100 μm vibratome sections of the normal (N) and the electric shock-produced “dark” (D) neurons as a function of the survival time

Staining method	Survival times													
	<1 min		1 h		4 h		1 day		2 days		6 days		30 days	
	N	D	N	D	N	D	N	D	N	D	N	D	N	D
Hematoxylin	○	□	○	±	○	±	○	–	○	–	○	–	○	–
Acid fuchsin	–	–	–	–	–	–	–	±	–	±	–	–	–	–
Fluoro-Jade	–	±	–	±	–	+	–	■	–	■	–	◘	–	–
Cupric-silver method	–	■	–	■	–	■	–	■	–	◘	–	±	–	–
Chemical-development method	○	●	○	●	○	●	○	●	○	●	○	±	○	–
Physical-development method	–	■	–	■	–	■	–	■	–	◘	–	±	–	–

(–) Neither the somata and dendrites nor the nucleoli and nuclear margins are distinguishable; (○) good staining of the nucleoli and nuclear margins; ± the somata are barely detectable; (+) the somata are easily detectable; (□) intense, homogenous staining of the somata, but not the dendrites; (●) intense, punctuate staining of both the somata and dendrites; (■) intense, homogeneous staining of both the somata and dendrites; (◘) intense, homogeneous staining of the somata, but incomplete staining of the dendrites.

4. Discussion

4.1. Acquired basophilia and acidophilia

It has long been known that both the nucleus and the cytoplasm of the “dark” neurons of various origins are strongly and homogenously basophilic in the early phase of their existence; they then lose this property during either recovery or death, and become acidophilic when they die (Auer et al., 1985; Kiernan et al., 1998). It was recently demonstrated that the excess basophilia is acquired simultaneously with the momentary ultrastructural compaction of the whole soma–dendrite domain, which is the electron-microscopic equivalent of the formation of “dark” neurons (Csordás et al., 2003; Gallyas et al., 2004). The present paper contributes to knowledge in this field of research with the following findings and conclusions. (i) In an intact environment, the acquired basophilia disappears from the recovering “dark” neurons in a few hours post-insult. (ii) In an intact environment, the change from basophilia to acidophilia in the moribund “dark” neurons occurs at about the fourth hour post-insult. (iii) The acidophilia in the dead “dark” neurons persists during the first week post-insult (probably until they have been phagocytosed). (iv) The sudden increase of stainability with basic dyes in the “dark” neurons cannot be the simple consequence of condensation of the ultrastructural elements responsible for the basophilia of normal neurons, since the acquired basophilia could not be eliminated by enzymatic digestion of the nucleic acids. On the other hand, its disappearance following proteinase-K pretreatment or esterification suggests that negatively charged protein molecules play the principal role in binding the excess dye molecules. It appears improbable that negatively charged protein molecules can be newly synthesized and distributed throughout the whole cell by any cascade of enzymatic processes during the momentary formation of the “dark” neurons. If a recent hypothesis of their formation (Gallyas et al., 2004) is true, the sudden increase in negative charges can be the result of a cooperative conformational change in numerous cytoplasmic protein molecules forming the matrix of a gel that ubiquitously fills the intracellular space non-occupied by cytoskeletal elements. Thereby, pre-existing anionic groups of the matrix proteins can become detached from loosely-bound cationic groups of the gel structure, escape from a hydrophobic environment or be rendered more electronegative. This hypothesis brings to a “common denominator” why the morphological appearance and staining properties of the “dark” neurons are independent of the nature (enzyme-mediated or physical) of the initiating circumstances and also of their in-vivo and post-mortem formation. (v) As regards the slow acquirement of acidophilia, Tanaka and Simon (1994) assumed that it is caused by the separation of the nucleic acids from the positively charged nucleoproteins. Based on histochemical investigations, Kiernan et al. (1998) demonstrated this assumption to be improbable and proposed that proteins rich in arginine (mainly in the nucleus) and lysine (mainly in the cytoplasm) are responsible for the increase in acidophilia. They supposed that these protein molecules are newly synthesized. However, the stainability of the dendritic tree of the moribund or dead “dark” neurons with Flouro-Jade, which indicates that not only their somata or nuclei but also their dendrites become positively charged, does not support this supposition. Namely, it is improbable that newly synthesized protein molecules are transported even into distant dendrites and become homogeneously dispersed in the whole soma–dendrite domain in moribund neurons. According to a recent paper, certain polyamines formed during neuronal degeneration might account for the binding of anionic dyes, such as Fluoro-Jade B (Schmued and Hopkins, 2000).

4.2. Acquired argyrophilia

Argyrophilia is a generic term that means the ability of tissue elements to form (when incubated in a solution containing silver ions, some buffer material and complexing agent) submicroscopic grains of metallic silver (crystallization nuclei). These can be enlarged to the level of microscopic visibility by means of extraneous reducing substances (physical or chemical developers). Argyrophilia has at least three main types, depending on the mechanism of formation of the crystallization

nuclei (Gallyas, 1980,1982a–c). Further, for each type, the argyrophilia additionally depends on factors, such as the pH and the compositions of the solutions involved, or on previous chemical manipulations. Consequently, without specifying the silver method used, argyrophilia is not clearly defined. With this in mind, the differences in staining features between the chemical-development-silver method and the other silver staining methods tested (punctate versus homogeneous staining of the freshly-produced "dark" neurons) is an indication that they visualize different kinds of argyrophilia.

Several of the publications utilizing any of the silver staining methods tested here assumed the possibility that, in addition to moribund and dead neurons, temporarily damaged (recovering) neurons were also demonstrated (e.g. Ishida et al., 1999; Poirier et al., 2000; Baram et al., 2002). On the contrary, other authors viewed all silver-stained neurons as moribund or dead (e.g. Du et al., 1998; Kubová et al., 2001; Bianco et al., 2002). Our present findings render the first-mentioned assumption indisputable.

The physical-development-silver method visualizes type III argyrophilia, where the formation of silver grains is mediated by tissue-related catalytic sites. The latter are assumed to consist of a few side-groups of macromolecules in a favorable spatial arrangement (Gallyas, 1982). One kind of such side groups can be negatively charged, to which alcohol molecules must be bound (esterification) by the silver staining method to form catalytic sites (Gallyas, 1981). Bearing it in mind, the disappearance of argyrophilia following proteinase-K pretreatment suggests that negatively charged protein molecules play the principal role in its acquirement. It appears improbable that such protein molecules can be newly synthesized and distributed throughout the whole cell during the momentary formation of the "dark" neurons. On the other hand, catalytic sites may result from a compaction-derived conformational change in the matrix proteins of the ubiquitous intracellular gel hypothesized by us (Gallyas et al., 2004), which leads to a favorable change in the spatial arrangement of their preexisting side-groups, forming catalytic sites after esterification.

4.3. Supplementary remarks

On the basis of our previous papers, the present paper's findings can be completed with the next remarks, which help in selecting the method(s) to be used for the demonstration of basophilia, acidophilia or argyrophilia in an individual case. (i) In contrast with the chemical-development-silver methods, the physical-development-silver method used here works well in glutaraldehyde-fixed sections. (ii) The apoptotic neurons display the same staining properties as described here for the moribund and the dead "dark" neurons (Liposits et al., 1997). (iii) In 1 μm sections cut from aldehyde-fixed, osmicated and Durcupan-embedded brains, alkaline solutions of basic dyes stain intensely and homogeneously the somata and main dendrites of both the freshly-produced and the moribund or dead "dark" neurons, while acid fuchsin stains only the moribund or dead "dark" neurons (Csordás et al., 2003). (iv) The silver techniques are commonly believed to be capricious. However, the recent ones that utilize physical developers instead of the traditional chemical developers do give reproducible results (Newman and Jasani, 1988). One of the silver methods tested here employs a time-honored physical developer (Gallyas, 1971), which renders it as reliable as the common histological methods in which a dye is used.

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