

Dark Neurons of the Brain

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The structure and functional characteristics of dark hyperchromic and hyperchromic shrunken neurons in the brain have been studied at the light and electron microscopic levels in health and various pathologies. Hyperchromic dark neurons are cells with active protein synthesis which, however, die by apoptosis as a result of prolonged and intense exposure to unfavorable factors or because of genetic abnormalities.

Keywords: hyperchromic neurons, brain.

All investigators find dark (hyperchromic) neurons in histological sections, sometimes also encountering dark shrunken neurons. Dark neurons in the brain have always been subject to discussion in clinical and experimental neuromorphology. It remains unclear whether they are an artifact or constitute evidence of pathological changes. What is the origin, nature (morphofunctional features), and fate of these neurons?

Morphofunctional Characteristics and Frequency of Dark Neurons. Dark nonshrunken brain neurons are cells with hyperchromic cytoplasm, whose sizes are no different from those of normochromic neurons, while shrunken hyperchromic cells are small, extended, narrow, and deformed, and sometimes have corkscrewing processes with pyknotic nuclei [4, 5].

In specimens which have been collected accurately and fixed rapidly in Carnoy fluid, the proportion of hyperchromic neurons in the frontal cortex of intact adult rats is 2%, compared with 4% in the cerebellum and 1% in nucleus E2 of the hypothalamus. Dark shrunken neurons are rarer, accounting for fewer than 1% of cells [4]. During postnatal ontogeny, 8% of all neurons on Nissl-stained sections of the neocortex of intact rats collected on postnatal day 2 are dark, compared with 11% on day 45 and 9% on day 90. Hyperchromic shrunken neurons are seen only from postnatal day 20 (2%), accounting for 3% of neurons on day 45 and being virtually absent on day 90 [8, 9].

The numbers of dark neurons can increase significantly in experimental treatments and pathological states. On modeling of subhepatic cholestasis in rats, the neocortex showed increases in the numbers of dark and dark shrunken neurons to 6%, with increases to 16% and 25%, respectively, in the cerebellar cortex [4]. Collection of all bile for five days in rats led to a sharp increase in the numbers of dark neurons in the cerebral and cerebellar cortex. Their nuclei repeated the shape of the cell and the nuclear rim formed pleats; the number of nuclear pores increased, and there was massive release of ribonucleoprotein granules into the cytoplasm. The rough endoplasmic reticulum (RER) had dilated cisterns and decreased numbers of ribosomes. However, large numbers of ribosomes were present in the cytoplasm, forming free polyribosomes. Mitochondrial cristae were degraded or completely absent and Golgi complex cisterns were dilated. Shrunken neurons had areas of cytoplasmic degeneration with vacuoles [4, 5].

Treatment with aminazine at a dose of 1 mg/kg induced a significant increase in the number of hyperchromic neurons in the somatosensory cortex of rats, to a level of about 30% of all cells counted. Changes in neurons were accompanied by decreases in the numbers of synaptic terminals, swelling, and depletion [14, 27]. Epileptic convulsions induced by application of crystals of the calcium channel blocker 4-aminopyridine to the parietal cortex of anesthetized rats was accompanied by the appearance of dark neurons in the hippocampus and reticular formation of the brain [34].

In rats, antenatal alcoholization led to the appearance of increased numbers of dark pyramidal neurons in the cere-

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bral cortex at all postnatal time points. The extents of these changes are different at different time points of postnatal development. Thus, on day 45, the number of hyperchromic shrunken neurons was increased by 66% compared with controls. Hyperchromic shrunken neurons almost disappeared from control animals on days 45 and 90, while in rats subjected to antenatal alcoholization, conversely, the number of these cells increased sharply [8, 9].

The cytoplasm of nonshrunken dark neurons in the cerebral cortex of rats subjected to antenatal alcoholization showed significantly smaller numbers of mitochondria per unit area of cytoplasm on postnatal days 20 and 45 than that in controls. Mitochondria became more spherical and less extended, and the number and lengths of cristae per μm^2 in these mitochondria were lower on postnatal day 20 and especially day 45. The total number of ribosomes per unit area and the ribonucleoprotein content in hyperchromic neurons in the cytoplasm in these animals were significantly greater than in normochromic neurons. The relative number of free ribosomes in these cells increased progressively during postnatal ontogeny, while the relative number of bound ribosomes decreased. There was a significant reduction in the extent of RER cisterns per unit area of cytoplasm, along with dilation of cisterns, especially on day 45 of postnatal development [11, 12].

Dark shrunken neurons on postnatal development days 20 and 45 in rats after antenatal alcoholization had dark cytoplasm and folded nuclei. The cytoplasm showed disorganization and destruction of organelles. RER canals bore few ribosomes, Golgi cisterns were dilated, and mitochondria were swollen, lacked cristae, and had an osmiophilic matrix. Free ribosomes forming extensive groups dominated. The cytoplasm contained hyperosmiophilic homogeneous areas, determining the dark coloration of these neurons on electron microscopy photographs [11, 12].

The sensorimotor cortex of the offspring of rats with different pathological states showed, at the electron microscopy level, three types of dark neuron. Type 1 hyperchromic cells contained nuclei which were less osmiophilic than the cytoplasm, while the cytoplasm contained dilated endoplasmic reticulum cisterns, Golgi complex cisterns broken up into vacuoles, and mitochondria with degraded cristae. Type 2 hyperchromic cells had increased cytoplasmic osmiophilia, due to the accumulation of microgranular material; flattened nuclei acquired irregular outlines. Type 3 hyperchromic neurons had dark, irregularly shaped nuclei; their cytoplasm showed cleftlike densities and damaged organelles [19, 20, 26].

After chronic alcohol consumption by rats at a dose of 3.5 g/(kg·day), the proportion of hyperchromic neurons in histaminergic hypothalamic nucleus E2 increased from 1% to 10%. The cytoplasm of these neurons underwent changes evidencing activation of the nuclear apparatus (hypertrophy and displacement of nucleoli towards the nuclear envelope, condensation of ribosomal subunits close to the inner mem-

brane, dilation of the perinuclear space, and increases in the folding of the nuclear envelope) and destruction and hypertrophy of various organelles (endoplasmic reticulum, Golgi complex, lysosomes, and mitochondria), reflecting adaptive changes to the neurons under study [13].

Dark neurons are typical of cerebral hypoxia and are evaluated as ischemically altered shrunken cells [32]. Occlusion of the middle cerebral artery in rats led to detection of hyperchromic neurons in the neocortex, hippocampus, lateral striatum, thalamic nuclei, and amygdalar body [35]. After 30-min subtotal cerebral ischemia by bilateral ligation of the common carotid arteries, the numbers of dark and dark shrunken neurons in the frontal cortex of the brain increased significantly [29]. Hyperchromic neurons made up the greater part of all neurons in histaminergic nucleus E2. The nuclei of hyperchromic neurons are angular/oval, with convoluted envelopes. The nuclear perimeter in these neurons was 45% greater and sphericity was 10% less than in controls. The number of ribosomes per μm of the outer membrane of the nuclear envelope was 60% greater. The integrity of this membrane is often impaired in hyperchromic neurons. Chromatin in these cells is microgranular and uniformly distributed, and only small regions of heterochromatin are found; nucleolar area was 44% greater than in controls. Displacement of the nucleoli to the periphery and massive release of ribonucleoprotein granules from them occur. The number of free ribosomes per μm^2 of the cytoplasm of hyperchromic neurons in histaminergic nucleus E2 was 46% greater than in controls. Mitochondria in these neurons were distributed irregularly through the cytoplasm; their cristae and inner and outer membranes often showed destruction. The total number of lysosomes in these neurons was 25% greater than in animals of the control group [23]. Analogous ultrastructural changes also developed in the cerebral cortex in hypoxia [20, 32].

The Origin of Dark Neurons. The question of the origin of dark neurons is controversial. Do they constitute an artifact or a pathological state? Increases in the time intervals from collecting a brain specimen to fixation have been shown to lead to increases in the number of dark neurons: while the numbers showed no significant increases at 10 and 30 min, all neurons became dark by 12–24 h [37].

Even after intracardiac perfusion with glutaraldehyde solution, transient mechanical damage to the brain using a rod induced the appearance of large numbers of dark neurons in the surrounding zone. Analogous damage produced by microwave heating of the brain was not accompanied by the appearance of dark neurons [40]. Dark and shrunken neurons were seen in areas of mechanical damage to the cortex in rats when collecting samples for study. Rough collection of brain samples could later lead to artifactual formation of dark neurons. This artifact can be avoided by careful collection of study material.

Light and electron microscopic data from animal experiments led to the suggestion that dark neurons appear as a re-

sult of impairment to the functioning of energy-storing gel-like structures occupying the spaces between ultrastructural elements (*the author is probably referring to hyaloplasm*). The mechanism of formation of dark neurons consists of unprogrammed initiation of a phase transition between these structures (*perhaps the transition of hyaloplasm from a sol to a gel*), which may be reversible (in some physiological states) or irreversible, leading to cell death by a route differing from necrosis and apoptosis [37, 38].

Administration of aminazine at a dose of 5 mg/kg into the cerebral cortex and reticular formation led to a predominance of the appearance of hyperchromic staining of neurons due to accumulation of RNA and proteins, with a simultaneous decrease in acid phosphatase activity, which is regarded as an expression of decreased functional activity in cortical cells. A dose of 20 mg/kg induced the appearance of sharply hyperchromic neurons and occasional shrunken examples [1]. Prolonged (30 days) administration of aminazine increased neuron hyperchromia and swelling [14].

It was suggested that preceding (in life) pathological changes only increase the sensitivity of neurons to artificial ischemia and the development of dark neurons [37].

The formation of dark (squeezed, shrunken) neurons has been suggested to be accompanied by large-scale excretion of water, which occurs in many neurological diseases such as ischemia, via nonenzymatic mechanisms [39]. Dark shrunken neurons are thought to arise as a result of the rapid loss of more than half the volume of cytoplasmic fluid (water, inorganic ions, and metabolites), which over a number of minutes leave the cell by passive diffusion, without any expenditure of energy. This process is strengthened by dehydration of the body (using diuretics) [38].

The Morphofunctional State of Dark Neurons. Einarsen and Krogh [36] defined cellular hyperchromatophilia as a state of inhibition with temporary termination of activity. The accumulation of basophilic substance in neurons and their hyperchromic staining are regarded as morphological expressions of the process of protective inhibition [24, 27]. Klosovskii and Kosmarskaya [21] identified several stages in changes in cerebral cortex neurons in the inhibited state from initial swelling of perikarya and hyperchromia to hyperchromic coloration. Hyperchromic staining, with the exception of shrinkage, are related to functional changes. Hyperchromia sometimes affects only a proportion of neurons, leading to the thought that individual zones of a cell can be in different functional states [27]. Confirmation of the low functional activity of dark shrunken neurons is provided by results from electron microscopic and autoradiographic studies demonstrating decreases in the rate of release of newly synthesized RNA from the nucleus into the cytoplasm [28]. In addition, there are data indicating that dark unshrunken neurons are cells with intense protein synthesis due to overexpression of amplified genes [16].

Kalinichenko and Matveeva regard hyperchromic shrunken cells as cells in the state of pyknosis and atrophy with

signs of coagulative necrosis [17]. However, Yarygin and Yarygin [33] excluded hyperchromic staining of cells with signs of shrinkage from the category of dystrophic changes and regarded these changes as functionally adaptive.

Hyperchromatophilia of neurons can be characterized by a predominance of protein synthesis over degradation [26], while shrinkage is associated with dehydration of the cytoplasm, possibly in association with impairment to the water-salt balance of neurons.

Our data indicate that hyperchromic neurons in the cerebral cortex have a number of characteristic features in terms of the development of organelles during postnatal ontogeny. Thus, there are reductions in the relative number of mitochondria and the number and lengths of their cristae, which are accompanied by decreases in the activities of mitochondrial marker enzymes succinate dehydrogenase and NADH dehydrogenase in the cytoplasm of these neurons [10]. This is evidence of decreases in the functional activity of mitochondria and the provision of energy to neurons. As compared with normochromic neurons, hyperchromic neurons have many more bound and particularly free ribosomes, ensuring their hyperchromic Nissl staining. Decreases in the quantity of RER-bound ribosomes and increases in the number of free ribosomes provide evidence of switching of protein biosynthesis to the neuron's own needs, which is required for their survival in unfavorable conditions. However, because of the decrease in the synthesis of proteins for export to terminals, the involvement of these neurons in cerebral cortical activity will evidently be decreased. Shrinkage of a proportion of hyperchromic neurons can probably be regarded as a failure of adaptation leading to cell death [8].

Shrinkage of hyperchromic neurons was regarded by Snesev [31] as an expression of degenerative atrophy, the biological value of which consisted of adaptation to long-term conservation in unfavorable conditions [25]. Korzhevskii regarded shrunken neurons as a pathological state preceding cell death [22]. Shrunken neurons are characterized by pyknosis of the cell nucleus, reflecting the occurrence of necrobiotic processes in the cell, providing evidence of structures in the state of physiological degeneration [2, 6, 15].

The Fate of Dark Unshrunken and Shrunken Neurons. Data on the fate of dark neurons are very contradictory. In the early period following experimental craniocerebral trauma (the first seven days), rats display hyperchromic shrunken neurons with poorly discriminable nuclei and nucleoli, with tortuous, deformed processes, some of which then regained their structure [18].

After ischemia, during the first hour after restoration of the circulation, hyperchromic neurons showed an increase in organelle density, though their ultrastructure recovered only during the first day. Those neurons in which recovery of processed did not occur died and were eliminated by phagocytosis [43].

Nissl staining of dark neurons in the neocortex was studied in lateral hydraulic impact injury to the brain in rats (a model of craniocerebral trauma). In the neocortex, the number of dead neurons at 24 h after injury was less than the number of dark neurons at the earlier period. The number of dead neurons in the hippocampus was equal to the number of dark neurons [41, 44].

The morphological features and the subsequent presence of hyperchromic neurons in the cerebral cortex, hippocampus, and reticular formation were studied after induction of epilepsy. Damaged cells showed swelling of the mitochondria, and some neurons were edematous, undergoing gradual degradation of organelles and release of residues into the surrounding medium through large breaks in the plasmalemma and by phagocytosis [34, 39].

Anodic depolarization induces the appearance of dark neurons in the neocortex independently of the intensity and duration of the polarizing currents. These neurons are barely encountered in control animals. Dark neurons were most widespread in layers II and IV of the frontal cortex 24 h after the last depolarization. After this, almost all neurons gradually returned to the normal morphofunctional state within one month of the last polarization [42]. Hyperchromia and shrinkage of cerebral cortex neurons was assessed as degenerative, but the possibility of reverse changes persisted [14].

During the hour after restoration of blood flow following 1-h ligation of the middle cerebral artery, the bodies and dendrites of dark neurons showed hyperbasophilia, argyrophilia, osmophilia, and sharp electron microscopic thickening of structures. Between one hour and one day after ligation, the extent of ultrastructural thickening decreased and spiral layered structures arising from mitochondria appeared. The cytoplasm of some neurons then showed apoptotic condensation of nuclear chromatin as in newly appeared "dark" neurons. Apoptotic neurons were then converted into membrane-bound, dense, and electron-dense fragments which were taken up by phagocytes [43].

Published data indicate that the life cycle of hyperchromic neurons can be divided into three periods. The first period is characterized by a paranecrotic state. In some cases, dark cells exit this state and are converted into normochromic cells. In other cases, the manifestations characterizing the first period progress, such that dark cells degenerate and disappear. Hyperchromic cells actively synthesizing protein show activation of the genetic apparatus, comparable with stress situations at the genome level. In these conditions, there is a high probability of failure in the mechanisms regulating gene activity. The result of this failure can be "chaotic" expression, leading to transformation of cells and programmed cell death, i.e., apoptosis [3, 7, 16, 30]. The mechanisms of formation of "dark" neurons may be reversible (in some physiological states) or irreversible, leading to cell death by a pathway different from necrosis and apoptosis [37].

In cholestasis or loss of bile in rats, dark shrunken brain neurons in the cerebral and cerebellar cortex were also hy-

perfunctional, which characterizes them as dying neurons [45]. In addition, the cerebral cortex of animals subjected to antenatal alcoholization showed significant increases in the number of dark shrunken neurons from postnatal day 20, not accompanied by further losses in the total number of neurons in investigations performed on days 45 and 90 [8, 9].

The fate of "dark" neurons (recovery or death) depends on the ongoing conditions. Restoration of the volume of shrunken neurons requires energy, as blockade of mitochondrial enzymes inhibits this recovery and leads to the death of these neurons [38].

Conclusions. In normal conditions, with appropriate preparation of samples, the brains of animals and humans contain only occasional dark (hyperchromic) neurons and even rarer dark shrunken neurons. The numbers of these cells can increase significantly in areas of mechanical brain damage when samples are collected for investigation or when fixation is delayed (in these cases they should be regarded as artifacts). In experimental treatments and pathological states, the number of dark neurons can undergo significant increases. Dark unshrunken neurons have damaged mitochondria and increased numbers of free ribosomes, determining hyperchromatosis of the cytoplasm and increased protein synthesis for the cell's own needs. These latter can be regarded as an adaptive reaction directed to promoting neuron survival in unfavorable conditions. However, the ability of these neurons to carry out their usual functions in the brain remains unclear.

Shrunken neurons probably arise as a result of sharp disturbances to their water-salt exchange and the rapid loss of significant quantities of water. The volume of the cell cytoplasm decreases, which leads to an increase in the density of their ribosomes and hyperchromatosis, in addition, fragmentary thickening and increases in the density of the hyaloplasm of these neurons occur, which is visible on electron microscope images as homogeneous areas of hyperosmophilia. Shrinkage of neurons should be regarded as a severe pathological change, sometimes irreversible and leading to cell death. In addition, questions of the functional state and fate of dark brain neurons remain unresolved and require further study.

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