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ULTRASTRUCTURAL CHANGES OF THE PINEAL GLAND IN EPILEPSY*

T. A. Litovchenko[1], A. E. Dubenko[2], S. O. Sazonov[2], V. A. Florikian[1], O. P. Zavalna[1], O. Y. Sukhonosova[1]

[1] Kharkiv National Medical University, Kharkiv, Ukraine;
[2] SI «Institute of Neurology, Psychiatry and Narcology of the National Academy of Medical Sciences», Kharkiv, Ukraine
t.litovchenko@yahoo.com

Epilepsy as a global health problem affects about 1% of the population. The occurrence of epilepsy appears to be due to the presence of various factors, such as neurological, perceptual, psychological, and social, that can either cause or aggravate the disease [1].

There is no doubt about the pathogenetic role of neurotransmitter systems' disorders in the pathogenesis of epilepsy. Monoaminergic systems deserve special attention in the context of the problem of formation of epileptic activity.

The prerequisite for this is that monoaminergic neural systems form pathways to the neocortical parts, limbic system, cerebellum, hypothalamus; have an activating or inhibitory effect on the structures of the intermediate, anterior and neocortical parts of the brain; have not only neurotransmitter, but also neuromodulatory properties; participate in the regulation of brain homeostasis; are determined in structures with low convulsive thresholds; in the process of metabolism in the brain, they form a large number of products characterized by neurotropic activity.

The circadian rhythm of epileptic seizures was described more than 2,000 years ago, and the first modern scientific research was conducted in the late nineteenth century. In these early studies, approximately two-thirds of people with epilepsy showed circadian patterns of epileptic seizures (day, night). Daytime seizures are known to cluster during wakefulness or late afternoon, while nighttime seizures occur frequently before falling asleep, sleeping and early in the morning before waking up [2]. This circadian pattern of seizures is usually well preserved. The circadian rhythm of epileptic seizures has a huge impact on sleep (and vice versa), and the link between epilepsy and the sleep-wake cycle is being actively studied. Clinical and experimental observations have shown that sleep deprivation can trigger seizures [3, 4].

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A lot of studies have shown that epilepsy is closely related to circadian rhythms [5]. For example, Quigg (2000) reported an association with the circadian rhythm of some types of epileptic seizures in both humans and experiments [6]. In another study, Durazzo et al. (2008) found that most epileptic seizures that occur in the occipital and temporal lobes occur during the day, while seizures that occur in the frontal and parietal lobes usually occur at night during sleep. Thus, endogenous circadian rhythm differently affects the manifestations of epilepsy in different areas of the brain [7, 8]. Temporal-partial seizures are characterized mainly by the spread of adhesions during the phase of slow sleep [9]. Temporal lobe epilepsy is often pharmacologically resistant and the most common type of symptomatic epilepsy involving the hippocampus, entorhinal cortex, and amygdala [10].

Circadian rhythms are endogenously controlled, which are almost 24-hour cycles of behavioral and physiological processes, such as the sleep-wake cycle, hormone production, regulation of body temperature and blood pressure, and a number of other physiological indicators [11].

The excitability of neurons is homeostatically regulated by excitatory and inhibitory influences in the nervous system. Hyperexcitability, which is caused by a violation of this delicate balance, causes hypersynchronous electrical discharges of brain neurons, which can clinically manifest as epileptic seizures. It was shown that neurotransmitter receptors and ion channels are rhythmic expression and activity in the circadian regulation. Analysis of the binding of radioactive ligand by several neurotransmitter receptors in the rat brain showed that the cerebral cortex has the highest variations and the cerebellum the lowest. The hippocampus has circadian patterns of ligand-binding activity of α1-adrenergic and benzodiazepine receptors. Changes in the rhythm of membrane excitability and electrical activity of neurons are provided mainly by changes in potassium conductivity [12, 13]. The expression of pyridoxalkinase, an enzyme involved in the metabolism of pyridoxal phosphate and neurotransmitters (e.g., serotonin and dopamine), is regulated by circadian transcription factors bZIP PAR [14]. Thus, circadian mechanisms modulate neuronal excitability at several levels, and their destruction can cause excessive uncontrolled excitability. Further studies have confirmed that the main circadian rhythm genes, BMAL1 and CLOCK, affect both the seizure threshold and excitability [15].

Melatonin is an amine hormone synthesized and secreted by the pineal gland that regulates circadian rhythm and seasonal responses. Melatonin is the main hormone secreted by the pineal gland, which is synthesized from tryptophan and serotonin. Previous studies have shown that epilepsy is also associated with melatonin levels. For example, administration of exogenous melatonin can reduce the number of seizures, extend the latent period before the seizure, and reduce the severity of epilepsy [16, 17]. In a study by Mosi´nska et al. (2018) suggested that melatonin may control the frequency and threshold of epileptic seizures, which regulates the expression of circadian rhythm genes. However, the exact mechanism underlying this protective effect and whether melatonin can restore circadian rhythm gene expression in epilepsy has not been fully studied.

Circadian rhythm is associated with epilepsy through two main mechanisms. First, it is known that circadian rhythm genes (BMAL1 and CLOCK) and their complex of transcription factors, BMAL1-CLOCK, affect the expression of other genes causally involved in epilepsy (for example, PAR DBP, TEF, and HLF). It was reported that disabling CLOCK in excitatory pyramidal neurons in rats led to a decrease in the seizure threshold and an increase in the number of attacks during sleep. This was combined with a decrease in the formation of «dendritic spines» and changes in the electrophysiological characteristics of neural networks containing excitatory pyramidal cells. The same phenomenon was observed in mice with BMAL1 disabled [18, 19].

The second mechanism is related to the mTOR pathway, which is known to be regulated by circadian rhythm factors [20]. It has been shown that mutations in the genes of mTOR inhibitors, TSC1 and TSC2, and other regulatory proteins can lead to disinhibition of the mTOR pathway, which leads to the development of seizures [21, 22].

Despite a significant body of research on the role of melatonin in the development of epileptic seizures, the cause of changes in the level of this hormone in patients with epilepsy remains unknown, as well as whether these changes are persistent or a dynamic condition associated with epileptic seizures as such.

Given that almost all melatonin synthesis occurs in the pineal gland and structural changes in this gland are an indicator of persistent (possibly irreversible) violation of its secretory function, the aim of the study was to identify ultrastructural changes in the pineal gland in an experimental model of epilepsy.

MATERIALS AND METHODS

Histological examinations were performed on Wistar rats, aged 5–6 months and weighing an average of 200 g. The animals were kept in standard vivarium conditions on a normal diet. The 36 rats were used in the experiment, the control group consisted of 10 similar animals.

Epileptic seizures were simulated by repeated action of a sinusoidal amplitude-modulated electric current with a carrier frequency of 5000 Hz, a modulation frequency of 150 Hz, a modulation depth of 100 %, a strength of 50–60 mA, exposure 3c through cutaneous electrodes superimposed on the rat neck. For each animal, the threshold current strength was determined — the current strength that causes an initial epileptic reaction. Stimulation was started with a current of 35 mA, gradually increasing by 10 mA until the appearance of tonic extension of the hind limbs. The rats developed spontaneous generalized seizures after 9–14 stimulations. Material for histological examinations was taken after stable formation (for > 1 month) of repeated seizures. Ultrathin sections, after contrast with Reynolds lead citrate, were studied under an electron microscope at an accelerating voltage of 75 kV. The increase was selected adequate to the study and ranged from 20,000 to 60,000 times. The control tissue

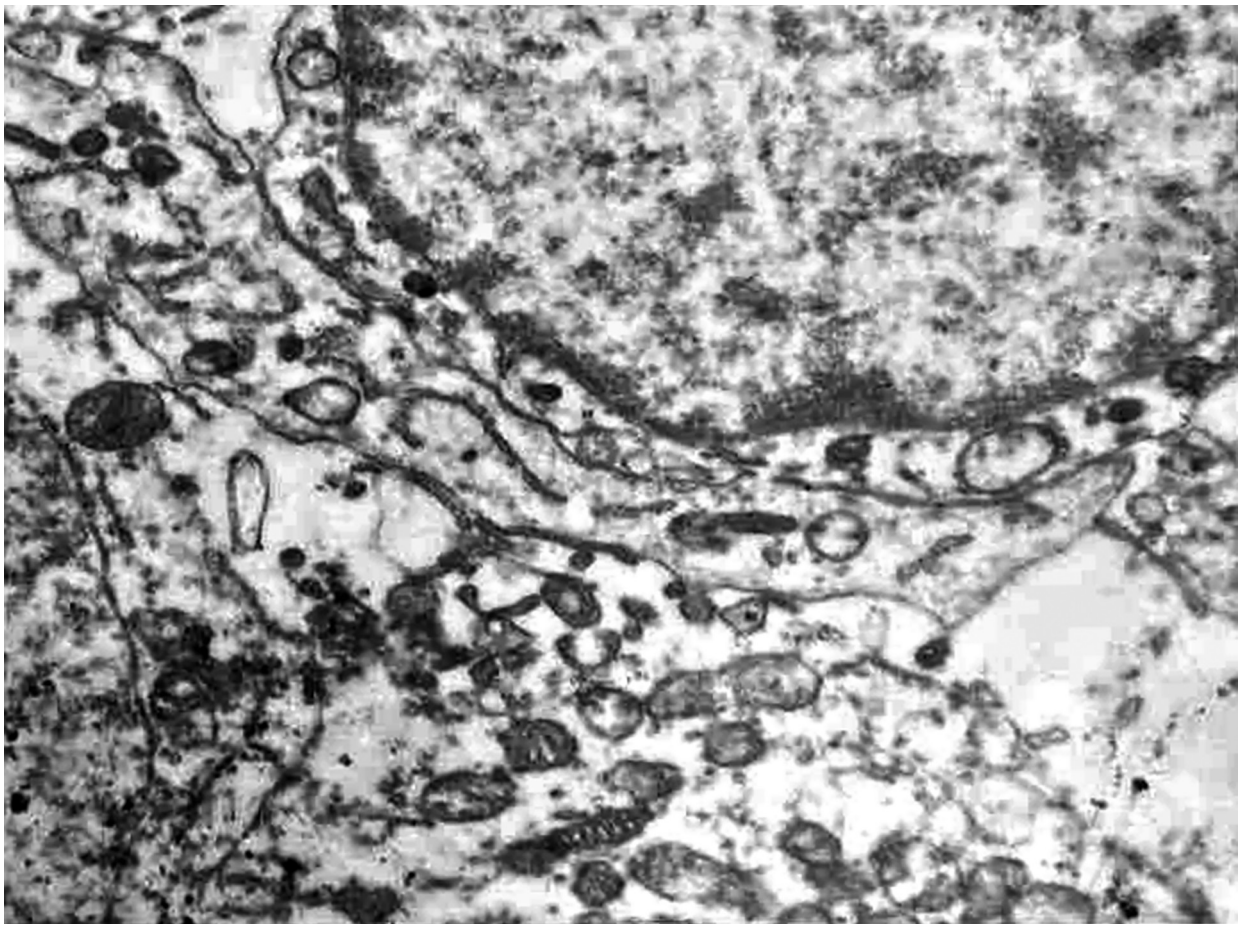

Fig. 1. Ultrastructure of pinealocytes (pinealocyte nuclei) of the epiphysis of rats with experimental epilepsy. The increase is 31000. Contrasted with lead citrate.

was taken from intact animals under similar conditions. Electron microscopic examination of the pineal gland of rats with experimental epilepsy in comparison with the control group of animals revealed dystrophic and destructive changes of organelles and membranes.

Experiments on animals in this research are approved by authors' affiliations institutions. When working with animals, we followed international and Ukrainian recommendations for working with experimental animals (Counsil for International Organizations of Medical Sciences (CIOMS) (1985); Guide for the care and use of laboratory animals 1996) [23, 24].

RESULTS AND THEIR DISCUSSION

The nuclei of pinealocytes (Fig. 1) were large with evenly distributed chromatin granules. They contain rather large nucleoli. The matrix of nuclei was significantly enlightened. However, the nuclear membrane was clearly outlined and had small areas of destruction. It formed shallow intussusception. In the perinuclear zone of the cytoplasm, the number of organelles decreased, and this zone acquired electronic transparency. A significant number of pinealocytes contained nuclei, the nuclear shells of which formed very deep intussusception (Fig. 2). Chromatin in such nuclei was located in the form of accumulations of granules along the nuclear membrane. The nuclear membrane was not subject to significant changes.

The cytoplasm of individual pinealocytes, the number of which is small, had a significant dilution (Fig. 3). The perinuclear zone of the cytoplasm had practically no organelles. The number of mitochondria and cisterns of the endoplasmic reticulum is significantly reduced. Free ribosomes and polysomes are absent.

Hyaloplasm had a low electron density. Few mitochondria contained a small number of crystals. The mitochondrial matrix had a coarse

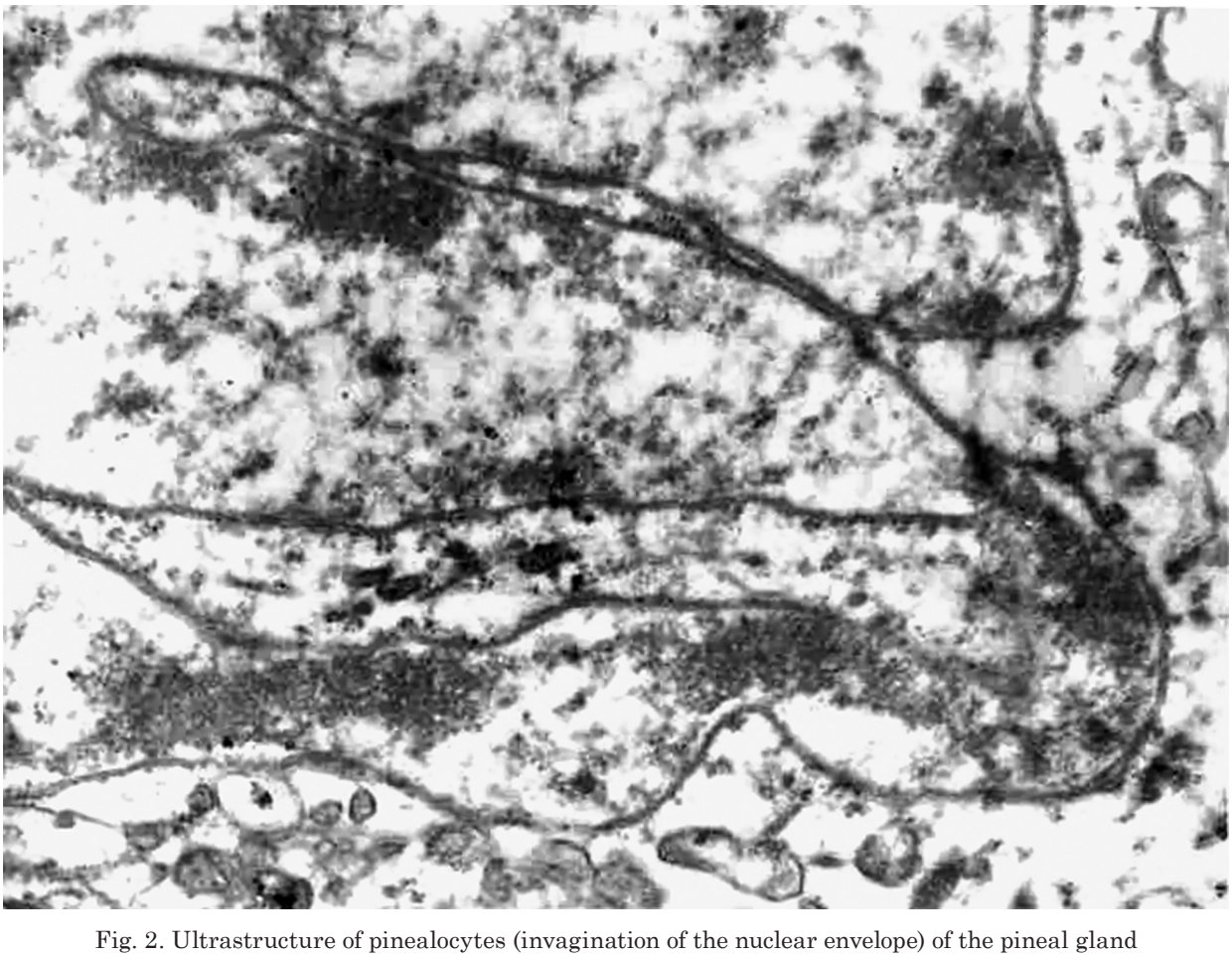

Fig. 2. Ultrastructure of pinealocytes (invagination of the nuclear envelope) of the pineal gland of rats with experimental epilepsy. The increase is 30,000. Contrasted with lead citrate.

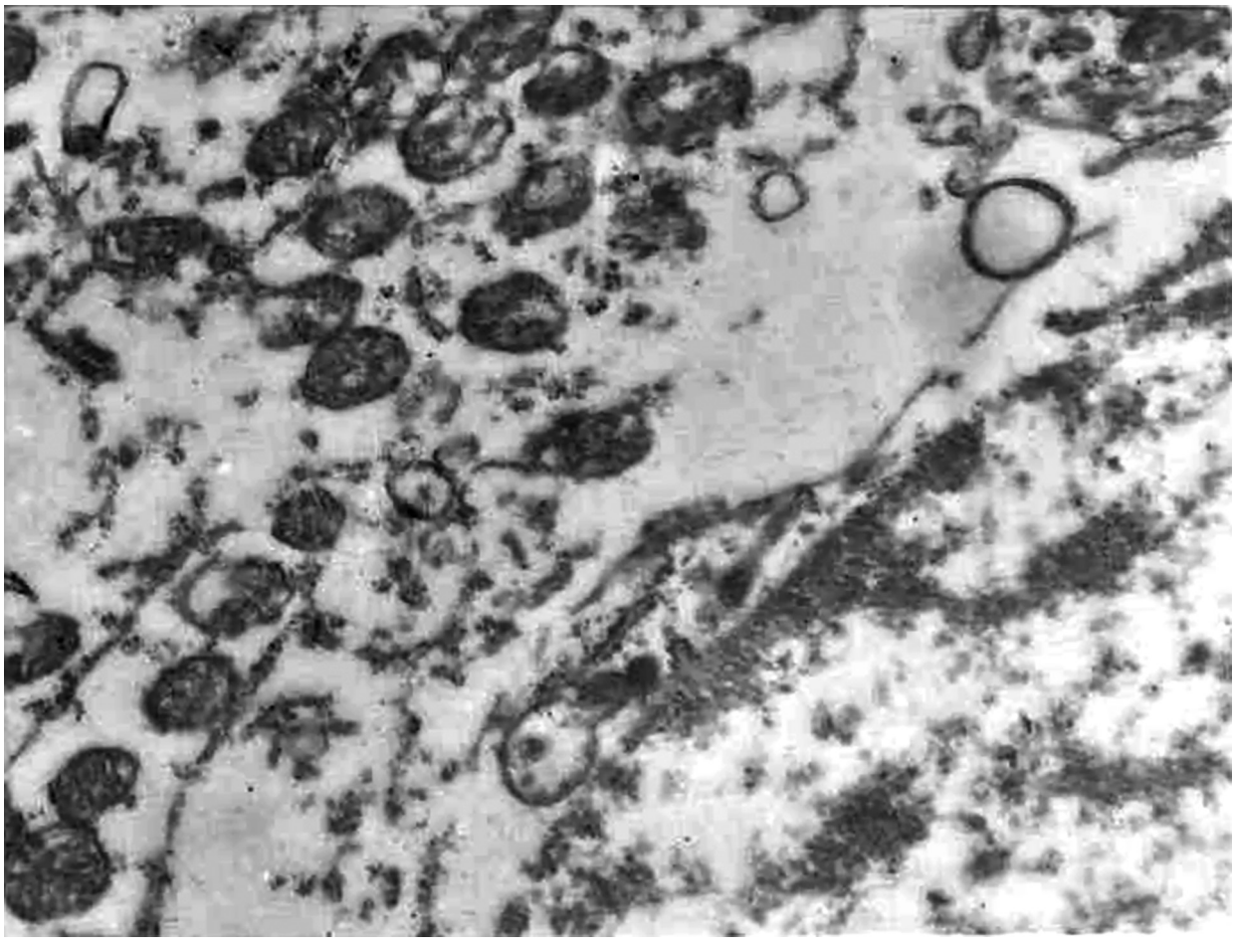

Fig. 3. Ultrastructure of pinealocytes (cytoplasmic dilution) of the epiphysis of rats with experimental epilepsy. The increase is 26000. Contrasted with lead citrate.

fibre structure. Focal lysis of membrane structures was very often observed.

A significant number of pinealocytes contained a large number of mitochondria, with a fine-grained matrix, the size of which varied widely (Fig. 4).

In general, mitochondria had a rounded shape, but there were also rod-shaped. At the same time, sometimes, electronically transparent zones were found in the matrix. Some mitochondria had almost no cristae. The agranular endoplasmic reticulum was moderately vacuolated, and its tanks contained a substance of very low electron density. The reduction in the number of ribosomes and polysomes in the cytoplasm is noteworthy.

Sometimes there are pinealocytes that have mitochondria of various shapes and sizes, the main feature of which is the location of crysts along the axis of the organelle, as well as the appearance of electron-transparent vacuoles in the mitochondrial matrix (Fig. 5).

The lamellar cytoplasmic Golgi complex (Fig. 6) was significantly hypertrophied, the parallel arrangement of its smooth membranes is broken. The vacuoles of the Golgi complex became very large. Autophagosomes were almost always present in the area of localization of the latter.

The cytoplasmic membrane of pinealocytes was very loose and lost the typical structure of the elementary membrane. There is a significant increase in its osmophilicity.

In addition, individual apoptotically altered pinealocytes were found in the epiphysis tissue in experimental rats with epilepsy (Fig. 7). The cytoplasm of such pinealocytes has a fairly high electron density. The nuclei contain electron-dense chromatin and are significantly reduced in size. Numerous ribosomes and polysomes are present in the cytoplasm. It is characterized by a significant polymorphism in the size and shape of numerous mitochondria, which contain a large number of clearly

contoured crystals and have a well-contoured outer membrane. There is a gradual absorption of cellular organelles by a large number of autophagosomes. The cytoplasmic membrane retains its integrity, but significantly changes its configuration and has deep intussusception. Thus, the cell in the apoptosis stage retains functionally active organelles, which ensure the process of utilization of cytoplasmic structures.

Thus, under the conditions of modeling epilepsy in experimental rats, dystrophic changes in pinealocytes were detected, which are structurally manifested in sharp mitochondrial edema with a decrease in the number of crysts, partial destruction of external membranes and crysts of mitochondria, vacuolization of cisterns of the agranular endoplasmic reticulum, a decrease in the number of ribosomes, as well as enlightenment of the matrix of the nucleus. These changes indicate a violation of the synthetic function of pinealocytes and energy processes. Violation of the protein-synthesizing function of pinealocytes is confirmed by the detected reduction of the lamellar cytoplasmic Golgi complex. As a result, catabolic processes develop at the intracellular level, the structural manifestation of which is the presence of autophagosym and lipid inclusions in the cytoplasm. A small number of cells had apoptotic changes with intact membranes and functionally active organelles that provided the processes of utilization of cytoplasmic structures.

The study of the control group's material did not reveal such changes.

Experimental studies have confirmed the presence of persistent morphological changes in the pineal gland in epilepsy, which is the reason for the decrease in its functional activity.

In recent decades, a number of systemic functions of melatonin have been established, largely determining the vital functions of the body. These aspects of melatonin are usually well known [25].

Despite sufficient work on the role of melatonin in epileptogenesis, the results are often

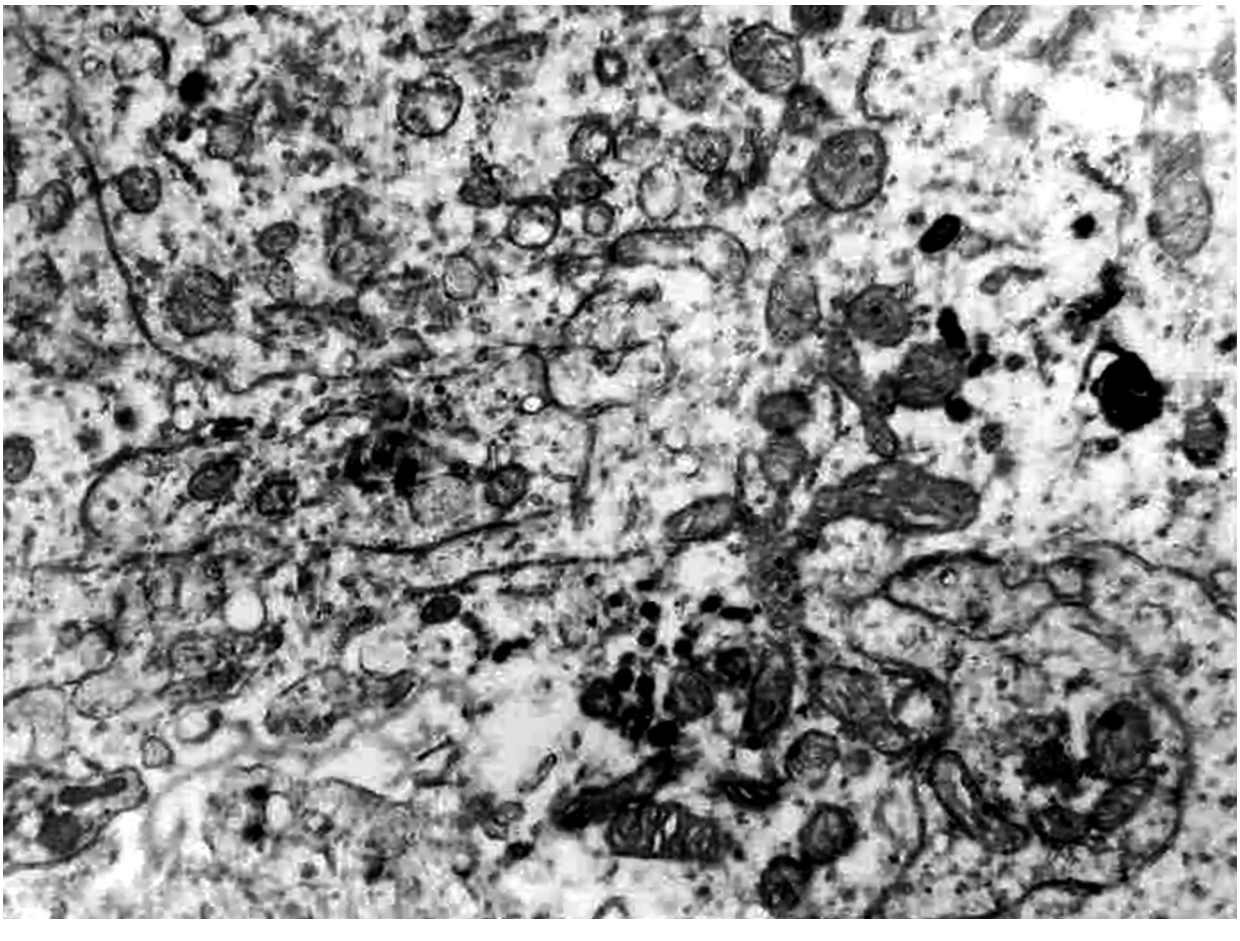

Fig. 4. Ultrastructure of pinealocytes (mitochondria) of the epiphysis of rats with experimental epilepsy. The increase is 32000. Contrasted with lead citrate.

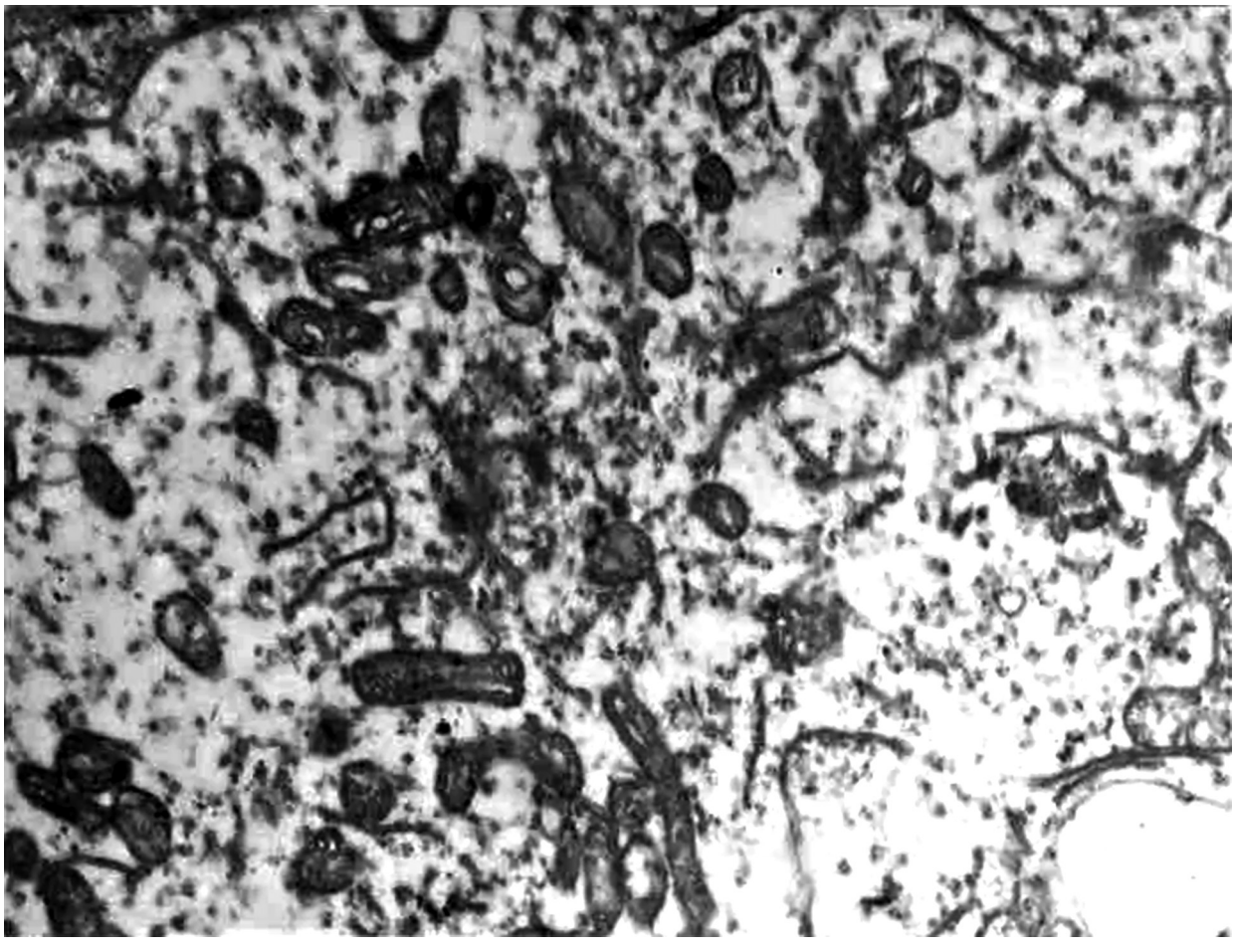

Fig. 5. Ultrastructure of pinealocytes (structure of mitochondria) of the pineal gland of rats with experimental epilepsy. The increase is 38000. Contrasted with lead citrate.

divergent, obtained on different clinical and experimental material and are not subject to systematic analysis and comparison, although the participation of melatonin in the formation of epileptic activity is not in doubt. Our experimental data on the morphological changes of the pineal gland confirm the conclusion about a stable, possibly irreversible, decrease in gland function in epilepsy due to damage and disruption of the structure of its cells.

Opinions about the anticonvulsant properties of melatonin are contradictory. Guo et al. (2009) reported that serum melatonin levels were reduced in children with epilepsy and febrile seizures and recommended the use of melatonin as adjunctive therapy [26]. Another study presented data from patients with resistant temporal lobe epilepsy, which showed a decrease in melatonin in the interictal period and its increase by 3 times after seizures. The authors believe that melatonin has anticonvulsant properties [27] and such an increase is a compensatory response. There is evidence of decreased melatonin levels in patients with nocturnal and diurnal focal seizures. Molina-Carballo et al. in their study showed that the level of melatonin in the serum increased during seizures and returned to normal values after 1 hour and concluded that such an increase in melatonin secretion may represent the body's response to seizures and aimed at achieving homeostasis [28].

Schapel et al. (1995) concluded that melatonin production increases in untreated patients with active epilepsy and has a circadian model with phase differences compared to controls [29].

But there is the opinion of a number of authors who deny the significant role of changes in melatonin levels in the development and relief of epileptic seizures [30]. Other studies have shown sufficient efficacy of exogenous melatonin as adjunctive therapy in incurable epilepsy [31, 32].

Our studies of the ultrastructure of the pineal gland confirm a steady decrease in its functional activity in epilepsy. Most likely, this is due to the depletion of compensatory mechanisms in the conditions of epileptic brain. This opinion is indirectly confirmed by the role of melatonin in the physiology of mitochondria as a protector of neuronal mitochondrial oxidative phosphorylation under toxic effects and under conditions of hypoxia-reoxygenation [33]. In addition, melatonin is a unique regulator of apoptosis and neuroplasticity [34]. This is especially important given the widespread presence of melatonin receptors in the CNS and, especially, in the hippocampus (CA3 field, traditionally plays a leading role in epileptogenesis) [35]. Melatonin plays a role in Ca^{2+} homeostasis, and it inhibits the increase in Ca^{2+} caused by acidification and prevents the increase in Ca^{2+} caused by glutamate in the cerebral cortex of rats and acts as an antiexatotoxic and anti-inflammatory molecule in neurons [36, 37]. Melatonin also increases the neuroplasticity of the hippocampus [38].

CONCLUSIONS

The established morphological changes of the pineal gland indicate stable and possibly irreversible changes in the gland, which is accompanied by a significant decrease in melatonin production. Given the polyfunctionality of this hormone, its deficiency can be one of the causes of a number of metabolic disorders, changes in neural networks, impaired permeability of synaptic membranes, and as a consequence, increased epileptic activity of neurons. Further activation of free radical processes, energy deficiency, hemodynamic disorders in epilepsy contribute to an increase in melatonin deficiency, depletion of the functional reserves of the pineal gland and the death of melanocytes.

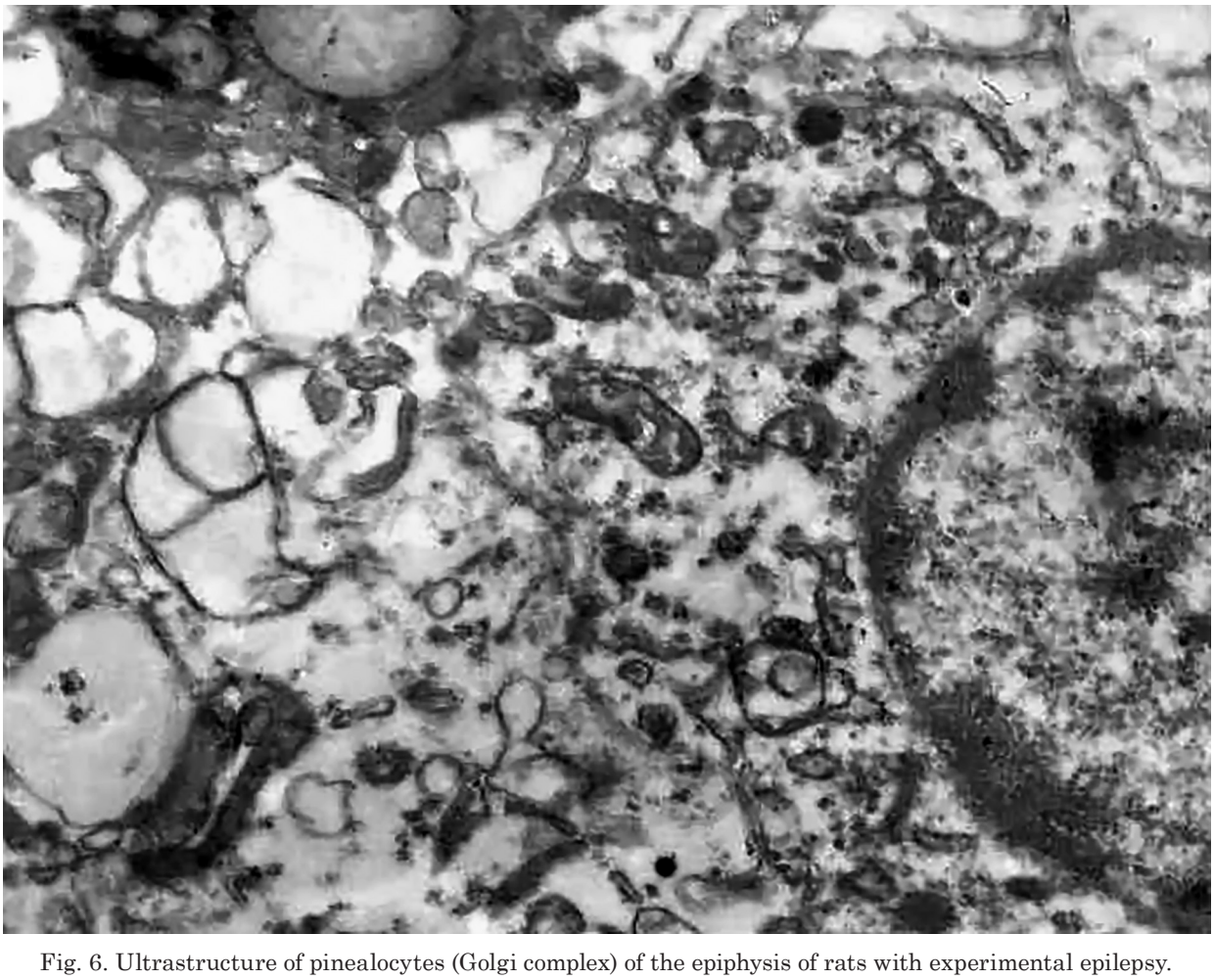

Fig. 6. Ultrastructure of pinealocytes (Golgi complex) of the epiphysis of rats with experimental epilepsy. The increase is 37000. Contrasted with lead citrate.

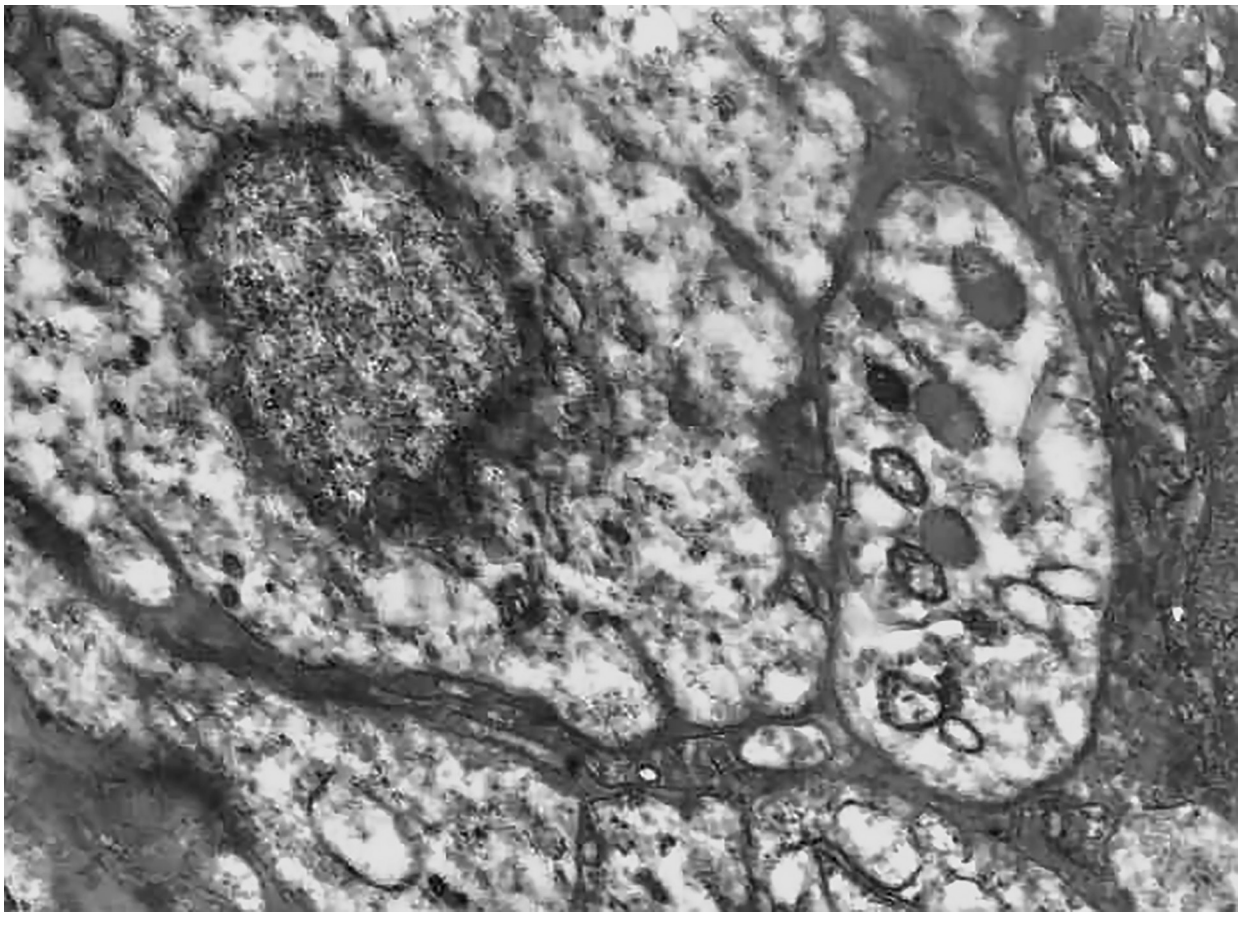

Fig. 7. Ultrastructure of pineal pinealocyte rats with experimental epilepsy.
The increase is 29000. Contrasted with lead citrate.

The epileptic seizures, from a neurophysiological point of view, can be considered as a violation of the physiological bioelectrical activity of neurons, their rhythm. Rhythmic neuronal activity is registered in the form of EEG and its changes are the main diagnostic criterion of epilepsy. This suggests a significant role of pineal gland dysfunction in the formation of epileptic activity.

Clinical Implications. Our studies confirm the decline in pineal function (often irreversible) in epilepsy and may be the basis for the use of melatonin in the treatment of resistant epilepsy in patients.

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[1] Kharkiv National Medical University, Kharkiv, Ukraine;
[2] SI «Institute of Neurology, Psychiatry and Narcology of the National Academy of Medical Sciences», Kharkiv, Ukraine
t.litovchenko@yahoo.com

Circadian mechanisms modulate neuronal excitability at several levels, and their destruction can cause excessive uncontrolled excitability. Previous studies have shown that epilepsy is associated with melatonin levels. Melatonin is a powerful chronobiotic secreted from the pineal gland; helps to maintain normal circadian rhythms and is used to treat some neurological and psychiatric disorders. Circadian rhythms and sleep/wake mechanisms play role in the epilepsy. Melatonin deficiency can decrease seizure threshold, and hence could increase seizure activity. The aim of the study was to identify ultrastructural changes in the pineal gland in an experimental model of epilepsy.

Materials and methods. The experiment was carried out on Wistar rats, 5–6 months of age, weight of about 200 g, 36 experimental and 10 control animals. Electrically provoked experimental epileptic seizures model in rats was reproduced. The rats developed spontaneous generalized seizures after 9–14 stimulations. Material for histological examinations was taken after stable formation (for > 1 month) of repeated seizures.

Ultrathin sections, after contrast with Reynolds lead citrate, were studied under an electron microscope at an accelerating voltage of 75 kV. The increase was selected adequate to the study and ranged from 20,000 to 60,000 times.

Results. Experimental studies have confirmed the presence of stable dystrophic, up to apoptosis of some cells, morphological changes of the pineal gland in the experimental model of epilepsy in rats, which is the reason for the decrease in its functional activity.

Conclusions. Our experimental data on the morphological changes of the pineal gland confirm the conclusion about a stable, possibly irreversible, decrease in gland function in epilepsy due to damage and disruption of the structure of its cells. Given the polyfunctionality of melatonin, its deficiency can be one of the causes of changes in neural networks, impaired permeability of synaptic membranes, and as a consequence, increased epileptic activity of neurons.

Keywords: epilepsy, experimental epileptic seizures, pineal gland, melatonin.

УЛЬТРАСТРУКТУРНІ ЗМІНИ ШИШКОПОДІБНОЇ ЗАЛОЗИ ПРИ ЕПІЛЕПСІЇ

Літовченко Т. А.[1], Дубенко А. Є.[2], Сазонов С. О.[2], Флорікян В. А.[1], Завальна О. П.[1], Сухоносова О. Ю.[1]

[1] Харківський національний медичний університет, м. Харків, Україна;
[2] ДУ «Інститут неврології, психіатрії та наркології Національної академії медичних наук України», м. Харків, Україна
t.litovchenko@yahoo.com

Циркадні механізми модулюють нейрональну збудливість на кількох рівнях, їх руйнування може викликати надмірну неконтрольовану збудливість. Попередні дослідження показали, що епілепсія пов'язана з рівнем мелатоніну. Мелатонін є потужним хронобіотиком, що виділяється шишкоподібною залозою; допомагає підтримувати нормальний циркадний ритм і використовується для лікування деяких неврологічних і психіатричних розладів. Циркадні ритми та механізми сну/неспання відіграють роль у розвитку епілепсії. Дефіцит мелатоніну може знизити судомний поріг і, отже, збільшити судомну активність. **Метою** дослідження було виявлення ультраструктурних змін епіфіза на експериментальній моделі епілепсії.

Матеріали та методи. Експеримент проводили на щурах лінії Вістар віком 5–6 місяців, масою близько 200 г, 36 піддослідних і 10 контрольних тварин. Було відтворено модель експериментальних епілептичних нападів, спровокованих електричним струмом, на щурах. Після 9–14 стимуляцій у щурів розвивалися спонтанні генералізовані напади. Матеріал для гістологічного дослідження брали після стійкого формування (протягом > 1 місяця) повторних нападів. Ультратонкі зрізи після контрастування цитратом свинцю за Рейнольдсом досліджували під електронним мікроскопом при прискорювальній напрузі 75 кВ. Збільшення було обрано адекватним дослідженню і коливалося від 20 000 до 60 000 разів.

Результати. Експериментальними дослідженнями підтверджено наявність стійких дистрофічних, аж до апоптозу деяких клітин, морфологічних змін епіфіза при експериментальній моделі епілепсії у щурів, що є причиною зниження його функціональної активності.

Висновки. Отримані експериментальні дані про морфологічні зміни шишкоподібної залози підтверджують висновок про стійке, можливо незворотне, зниження функції залози при епілепсії внаслідок пошкодження і порушення структури її клітин. Враховуючи поліфункціональність мелатоніну, його дефіцит може бути однією з причин змін нейронних мереж, порушення проникності синаптичних мембран і, як наслідок, підвищення епілептичної активності нейронів.

Ключові слова: епілепсія, експериментальні епілептичні напади, шишкоподібна залоза, мелатонін.