



# Evaluation of methods of modeling and formation of experimental allergic encephalomyelitis

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## Abstract

**Introduction:** Experimental autoimmune (allergic) encephalomyelitis (EAE) induced by intradermal injection of homogenate of the brain, spinal cord and peripheral nerve with Freund's stimulator, refers to a true autoimmune disease of the nervous system.

**Materials and methods:** Experimental studies were conducted on white nonlinear rats. To induce experimental allergic encephalomyelitis (EAE), homologous brain homogenates was used, which leads among other drugs (homologous, heterogeneous brain and spinal cord homogenates) by encephalitogenicity. The connective tissue of the animal's tail base was injected with a mixture of encephalitogenic suspension of 0.1 ml per 100 g of the body weight.

**Results and discussion:** According to the results, in the rats, there was weight loss, and the abnormal neurological symptoms were found on an average of 10–12<sup>th</sup> days. Our experimental studies on the formation of EAE were confirmed morphologically by electron microscopy.

**Conclusion:** Thus, the use of this technique allowed us to obtain a simulated pathologic condition of multiple sclerosis in the form of experimental allergic encephalomyelitis and can be used in future studies to identify appropriate laws, the extent and nature of changes in the immune and nervous systems of the body when inducing experimental pathological conditions.

## Keywords

multiple sclerosis, experimental allergic encephalomyelitis, experimental model of demyelinating state.

## Introduction

Despite many years of research on the pathogenesis of demyelinating diseases of the central nervous system, in particular, multiple sclerosis, their treatment remains a

rather acute problem of our time. Nevertheless, there are a number of outstanding achievements: the concept of heterogeneity of nosological forms of demyelinating diseases has been formed, new and atypical clinical forms in the central nervous system (CNS) and peripheral nervous

system (PNS) have been described, new antigens have been identified, new drugs have been tested, etc.

Due to the fact that considerable attention is paid to various methods of treating demyelinating pathology of the CNS, it becomes especially important to have a well-formed experimental pathological model that reproduces demyelination in the central nervous system, which is most similar to the manifestations of multiple sclerosis (MS) in humans (Browne et al. 2014; Pichkur et al. 2017; Burlaka et al. 2021).

Animal models, such as experimental autoimmune (allergic) encephalomyelitis (EAE), are widely used to investigate potential therapeutic agents for MS (Constantinescu et al. 2011; Dobson and Giovannoni 2019). While sensitization to autoantigens naturally occurs by unknown mechanisms, the EAE model requires external immunization (Gran et al. 2007; Belenichev et al. 2015; Burlaka et al. 2020). EAE is an organ-specific cell-mediated autoimmune demyelinating disease of the CNS in which macrophages and T-lymphocytes mediate damage to the myelin sheath. Due to multifactorial nature of the etiology of MS and the complexity of inflammatory and immunological processes, several protocols are used to induce EAE in mice and rats (Teixeira et al. 2005; Bert et al. 2011; Nefodov et al. 2020). There are some recognized MS antigens that act as targets for the B- and T-cell responses, such as **myelin oligodendrocyte glycoprotein (MOG)**, **myelin basic protein (MBP)**, **myelin-associated glycoprotein (MAG)**, and **proteolipid protein (PLP)** (Hollenbach and Oksenberg 2015; Harirchian et al. 2017; Glatigny and Bettelli 2018). These peptide antigens are emulsified in Freund's complete adjuvant (FCA), enhancing the immunogenicity of the autoantigens upon induction of EAE, and then administered by injection to induce demyelination and neurosis in animals. The use of these antigens for the induction of the development of a paralytic disease resembling multiple sclerosis was essential for establishing their encephalitogenic potential. The use of these myelin antigens, emulsified in Freund's complete adjuvant, has led to the development of various experimental EAE models. In addition, EAE can be actively induced by adoptive transfer of activated myelin-specific T-cells to naive recipient animals. The ability to induce EAE by transferring myelin-specific T-cells confirmed the idea that MS is an autoimmune disease mediated by T-cells.

Some examples of methods for forming EAE will be described in more detail below.

**MBP-induced EAE.** MBP makes up 30–40% of CNS myelin proteins and 5–15% of peripheral myelin proteins (Lemke 1988; Perchellet et al. 2004). They were among the first proteins to be purified from a spinal cord homogenate. They were shown to induce EAE. According to the distribution of antigens, MBP-specific T-cells also cause inflammation in the peripheral nerve roots during the development of EAE (Pender et al. 1995). Two families of proteins: Classic MBP and Golli-MBP – are transcribed from the MBP locus (Givogri et al. 2000). The first three exons at this locus are expressed only in the Golli-MBP

proteins. While Golli-MBP isoforms are expressed in the CNS, thymus, and peripheral lymphoid tissues, the promoters driving expression of Classic MBP isoforms are mainly active in the central and peripheral nervous systems (Goverman 2011). The course of the disease caused by MBP and its peptides is characterized by an acute paralytic episode, from which mice partially or completely recover (Zamvil et al. 1985; Cross and Naismith 2014; Glatigny and Bettelli 2018).

**PLP-induced EAE.** PLP is a major transmembrane protein in the CNS and an important component of CNS myelin densification (Ronchi et al. 2016). PLP was the first identified encephalitogenic myelin component (Waksman et al. 1954). Active EAE can be induced by PLP or its immunodominant epitopes in susceptible strains (McRae et al. 1995). Immunization of mice SJL/J by PLP 139–151 or by passive transfer obtained from SJL cluster-differentiation-4-positive (CD4+) PLP 139–151-specific T-cell line (Whitham et al. 1991) induces a recurrent course of remitting disease (McRae et al. 1992).

**MOG-induced EAE.** MOG is a very minor component (0.01–0.05% membrane protein) that is expressed on the outer surface of CNS myelin (Iglesias et al. 2001). MOG has become an important target in MS, as MOG-reactive T-cells are arguably easier to target in MS patients than PLP and MBP-responsive T-cells (Raddassi et al. 2011). MOG and its peptides have been shown to induce EAE in several mouse strains, including C57BL/6, C3H.SW, SJL/J, PL/J and non-obese and non-diabetic mice (NOD) (Encinas et al. 1999). In PL/J mice, MOG causes non-classical chronic recurrent EAE (Amor et al. 1994). Interestingly, NOD mice immunized with MOG35-55 develop an initial acute episode of EAE followed by a secondary progressive EAE, which may serve as a basis for modeling a secondary progressive phase of the disease, seen in some MS patients (Degenhardt et al. 2009). This model is becoming increasingly popular for studying the role of specific molecules in the development and regulation of EAE, since most gene-specific knockout (KO) mice were created C57BL/6.

**Spontaneous models EAE.** Actively induced EAE depends on the priming of myelin-specific T-cells with myelin antigen or peptide emulsified in FCA and, depending on the strain of mice, injection of whooping cough toxin. While this method of inducing EAE has been useful in identifying the mechanisms that regulate pathogenic T-cell responses and disease progression, they rely on artificial stimulation of T-cells and are not well suited to study the initial phases of autoimmune CNS development. The development of transgenic mice expressing myelin-specific T- or B-cell receptors solved some of these problems and provided models of spontaneous autoimmune development of the CNS with different frequencies, clinical and pathological patterns. The combination of these spontaneous EAE models and mice with conditional deletion or expression of specific genes in discrete cell types will undoubtedly further clarify the mechanisms of disease initiation and regulation.

Due to the importance of experimental allergic encephalomyelitis as a model of demyelinating pathology of the CNS, these diseases are widely studied by experimenters to obtain data and develop a number of theoretical statements regarding the pathogenesis of development and the biological role of neuroallergic reactions of various types associated with immunocompetent cells and humoral antibodies.

The administration of only one heterologous brain tissue causes in animals in a number of cases the development of neurological and morphological changes characteristic of demyelinating diseases of the nervous system. Rivers and Schwentker (1935) showed for the first time that repeated (from 14 to 93) introduction of heterologous brain tissue led to the development of lesions of the central nervous system in monkeys (Ghareghani et al. 2021).

Therefore, the experimental aim of our work was the most adequate modeling of multiple sclerosis in the form of autoimmune allergic encephalomyelitis for further use in the study of experimental equivalents of neurodegenerative conditions.

## Materials and methods

### Animals

The objects of the experimental study were white nonlinear rats. The Bioethics Commission of the State Institution Dnepropetrovsk Medical Academy of the Ministry of Health of the Ukraine (after 2021 Dnipro State Medical University) (Minutes № 2 of 13 February 2008) established that scientific research carried out on the experimental animals met the ethical requirements in accordance with the order of the Ministry of Health of the Ukraine №231 dated 01.11.2005. The studies were carried out in accordance with the principles of “the Declaration of Helsinki”, adopted by the General Assembly of the World Medical Association (2000), Council of Europe Convention on Human Rights and Biomedicine (1997), Relevant WHO Regulations, International Council of Medical Scientific Societies, International Code of Medical Ethics (1983), “General Ethical Principles of Experiments on Animals”, approved by the I National Congress of Bioethics (Kiev, 2001) in accordance with the provisions of the “European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Educational Purposes” (Strasbourg, March 18, 1986).

Before the start of the experiment on rats, all the animals were examined, weighed, taking into accounts their age, physical activity and skin condition. After external examination and culling, the experiment began simultaneously with the that in the control group. Before and during the experiment, the rats were kept in a vivarium at a temperature of 20–25 °C, a humidity of at least 50%, in ventilated rooms and a day/night light regime in standard plastic cages of no more than 5 animals each, on a standard diet. All the animals used in the experiment had a healthy appearance and were active (Zapadnyuk et al. 1983).

### Experiment scheme

To simulate experimental allergic encephalomyelitis, the most common method of introducing an encephalitogenic emulsion intradermally into the finger pads (Kozhemyakin et al. 2002), into the sternum or back of animals (Davydova 1969; Davydova and Markov 1975), and also into the base of the rat tail (Davydova and Markov 1970) was used.

For the formation of experimental allergic encephalomyelitis, homologous brain homogenates were used; in terms of encephalitogenicity, they rank first among other drugs (homologous, heterogeneous brain and spinal cord homogenates). An encephalitogenic mixture was injected into the connective tissue of the base of the animal's tail in a volume of 0.1 ml per 100 g of body weight. The obtained results were compared with those of the group of intact animals, which had been injected with physiological saline in the same volume. Also, for the formation of a pathological state, the encephalitogenic mixture was introduced into the paw pads of the experimental animals (white rats), but when compared with the introduction into the base of the tail, this method of administration did not cause reliably significant results.

Method for the preparation of Freund's complete adjuvant and the homogenate of the brain, spinal cord of rats was as follows: one rat was injected with Freund's complete adjuvant (FCA) + brain homogenate in the ratio (volume/volume) 1:1 at the base of the tail. Thus, for 20 rats weighing 200 g each, we used 2.0 ml of FCA and 2.0 ml of rat brain homogenate. But to obtain 4.0 ml of the finished solution, we took 2.5 ml each, based on the errors in preparation and introduction (in a pounder and residues in a syringe).

#### 1. Preparation of rat brain homogenate:

- 67 mg of brain per 1 rat (per 20 rats – 1.4 g);
- homogenization buffer 2.0 ml (0.175 M of KCl; 0.025 M of tris-HCl; pH=7.4).

1.4 g of rat brain is homogenized using a homogenizer in 2.0 ml of buffer solution (from a refrigerator) at room temperature for 3 minutes.

#### 2. Preparation of FCA:

- for 1 ml – 0.85 ml of vaseline oil; 0.15 ml used w/w lanolin and BCG;
- for 2.5 ml – 2.1 ml of vaseline oil; 0.4 ml of w/w lanolin and 1 mg of BCG (BCG at the rate of 0.025 mg per 100 g/rat).

In our study, we ground a sample of dry powder, when heated, *M. tuberculosis* or *M. butyricum* in a porcelain mortar in a small volume of vaseline oil (1.0 ml) to obtain a suspension with a final concentration of 1 mg/ml. Then, the remainder of 1.1 ml of vaseline oil was added to this

suspension and triturated with 0.4 ml of lanolin without aqueous and an aqueous solution of antigen (homogenate), usually the adjuvant: antigen ratio is 1:1.

The mixture was drawn up into a syringe with an adapter and the emulsion was “distilled” from one syringe to another until it “turned white” and thickened. The quality of the antigenic emulsion was monitored using a simple technique (Jurd 1987). A drop of emulsion was applied to the surface of ice-cold distilled water. A drop of a stable water-in-oil emulsion was immersed in water for a short time and then floated on the surface without spreading, but if the drop spread on the surface of the water, the emulsification had to be continued. Obtaining a stable antigenic emulsion is necessary to achieve an immunostimulating effect; unstable emulsion quickly disperses in animal tissues. A significant excess of detergents in the antigen preparation prevents the formation of a stable emulsion with CFA.

The results of modeling experimental multiple sclerosis were evaluated according to the following scale: 0 – no clinical manifestations, 1 – decreased tone of the tail, 2 – muscle weakness or mild paralysis of the forelimbs, 3 – severe paralysis of the hind limbs or all limbs, 4 – dying condition, 5 – death.

Electron microscopy was performed to assess a degree and confirm the formation of ultrastructural changes in the frontal cortex and hippocampus of rats under conditions of experimental allergic encephalomyelitis (EAE). For ultrastructural studies, samples of the hippocampus and cerebral cortex were fixed for 3–4 hours at +2 °C in a 2.5% glutaraldehyde solution in 0.2 M of phosphate buffer (pH 7.3). Further fixation was carried out in 1% buffered (pH 7.4) solution of osmium tetroxide (SPI, USA) for 1 hour. Dehydration of tissue samples was carried out in alcohols of increasing concentration and completed with three changes in propylene oxide. For the manufacture of epoxy blocks, the composition Epon-812 (SPI-Pon812 Epoxy Embedding Kit, USA) was used. Before the ultrathin sections were made, the semi-thin sections were analyzed by light microscopy. Ultrathin sections with a thickness of 60–80 nm were made with an ultramicrotome UMTF-6 M (SELMi, Ukraine), with their subsequent placement on the support grids (Mesh Regular Grid 200, SPI Supplies, USA). Double contrasting was carried out with 2% aqueous solution of uranyl acetate for 15 min at +37 °C followed by impregnation with a solution of lead citrate by the Reynolds method for 30 min (Mikhailov and Simirsky 1991). The studies were carried out using a transmission electron microscope TEM-100-01 (SELMi, Ukraine) at an acceleration voltage of 70–75 kV and primary increases from 4000 to 20000 times according to the standard scheme. Material preparation for ultrastructural analysis was carried out according to the generally accepted standards (Reynolds 1963; Sarkisov and Perov 1996). Ultrastructural changes were assessed by analyzing the features of changes in the neuronal, glial and components (hemocapillaries of the somatic type, in particular the state micropinocytic vesicles indicating the state of the

outer membranes of mitochondria, the state of the basement membrane of the endothelial cells themselves at the sites of interaction with protoplasmic astrocytes) of the microvasculature of the hippocampus and cerebral cortex.

## Statistical research methods

### *The choice of statistical procedures and analysis of the nature of the distribution of the studied quantities*

Analysis of the normality of distribution was evaluated according to the criteria of Kolmogorov-Smirnov (D) and Lilliefors, as well as Shapiro-Wilk (W), which was preferred. Also, as the criteria for agreement, the magnitude of the skewness and kurtosis of the data distribution was evaluated. When it was not possible to reject the null hypothesis of statistically significant differences, it is used from normal, non-parametric methods of data analysis were used, and in other cases, other parametric methods were used.

### *Verification of quantitative data*

If there were variants in the studied population that deviated sharply from the main number of observations, based on the properties of the standard normal distribution, they were excluded from further analysis if they were more or less than the absolute value of the critical value, calculated as the sum of the sample mean and the sample mathematical expectation of the triple value.

### *Data representation*

The data are presented as the mean and standard error of the representativeness of the sample mean.

### *Estimating the difference of unrelated samples*

In the cases when the distribution of the variables obeyed the normal law, taking into account that the number of the compared groups exceeded 2, to test the statistical hypothesis that the groups belonged to different general populations, the value of one-way analysis of variance was used, discarding the null hypothesis about the absence of a sample population disagreement at  $p < 0.05$ , comparing the calculated value of the F-criterion with the critical one; for the subsequent pairwise comparison of the groups, the Games-Howell criterion was used.

In the case of non-normal distribution or analysis of ordinal variables, the Mann-Whitney U-test was applied for 2 unrelated samples; for more samples, the Kruskal-Wallis H-test was used with further comparison by Games-Howell. If the number of groups was 2, the statistical significance of differences was assessed using the Gosset pooled variance U heteroscedastic t-test for unrelated groups with Bonferroni correction.

### *Evaluation of differences in sets of samples in parallel groups*

When analyzing the effect of treatment on the studied parameters in the case of a normal distribution of variables, one-way analysis of variance of repeated changes was

used, followed by the use of Newman-Keuls or Games-Howell tests, taking into account multiple comparisons.

In cases when the distribution of the studied variables did not correspond to the normal law, a nonparametric analogue of the analysis of variance of repeated changes was used – the Fridman criterion. In the case of 2 groups, comparisons were made using the Wilcoxon test.

### Chi-square

Comparison of groups on a qualitative basis, as well as in the study of the frequencies of occurrence of indicators, was carried out using the  $\chi^2$  criterion with the analysis of contingency tables.

### Multivariate analysis of variance repeated changes and analysis of covariance

Analyzing the influence of various factors on the studied parameters during therapy, the dynamics of indicators depending on the initial values, we used the procedure of multivariate analysis of variance of repeated changes and covariance analysis.

### Correlation analysis

The assessment of the degree of relationship between pairs of independent features expressed in a quantitative scale was carried out using the Pearson (r) or P. Spearman (R) rank correlation coefficient, depending on the nature of the distribution of variables. The reliability of the correlation coefficients was assessed by comparing the calculated coefficients with the critical ones (based on the properties of the correlation coefficients and degrees of freedom).

### Regression analysis

To determine the presence and nature of the relationship between numerical variables, a regression analysis procedure was used using linear, logarithmic, exponential, polynomial (second and third degree) models, achieving an independent (according to the Durbin-Watson criterion) normal distribution of residuals (in this case, the values of skewness and kurtosis were used as a criterion of agreement). The final selection of the regression equations was carried out using the generalized quality criterion, which was found as a weighted sum of the generalized accuracy criterion and the generalized adequacy criterion. Normalized values of the average relative approximation error were often used as criteria, and normalized values of the Durbin-Watson test were used as adequacy criteria. When analyzing the regression equations, the value of the multiple correlation coefficients, as well as the value of the coefficient of determination  $R^2$ , were taken into account.

The results of the study were processed using the statistical package of the licensed software package STATISTICA for Windows 6.0 (StatSoft Inc., № AXXR712D-833214FAN5), as well as SPSS 16.0 and Microsoft Excel 2003. Separate statistical procedures and algorithms were implemented in the form of specially written macros in the corresponding software. For all types of analysis, differences were considered statistically significant at  $p < 0.05$ .

## Results and discussion

According to the experimental data obtained, the rats of the experimental group lost body weight, had a number of pathological neurological symptoms, which appeared on average after 10–12<sup>th</sup> days. An increase in the symptoms continued for an average of 7 days and was not very stable. The experimental animals had pareses, manifestations of ataxia, walking disorders and urinary and fecal incontinence, lethargy, which corresponded to 1–2 degrees of the rating scale in 13 animals. In 7 rats, a paresis in the hind limbs and a loss of tail tone were expressed (grade 3). Most of the rats recovered spontaneously within 6–8 days after the onset of clinical manifestations (18–20 days after immunization). According to the requirements of the experiment, during the paralytic stage, the rats had free access to food and water (Davydova and Markov 1970).

Our research found that the simulated pathology led to damage to neurons in the sensorimotor cortex of the experimental animals. In the group of rats with EAE, a decrease in the density of neurons by 19% ( $p < 0.05$ ) was observed, which indicated cell death and an increase in their area by 10%, which confirmed the development of their edema. In addition, under EAE conditions, a decrease in transcripti- onal processes in neurons of the sensorimotor cortex was found, as evidenced by a decrease in the RNA level by 21% ( $p < 0.05$ ) (Table 1).

**Table 1.** Assessment of Morpho-functional Parameters of Neurons in the Sensorimotor Zone of the Brain of White Rats Under EAE Conditions,  $M \pm m$ ,  $n=10$

Experimental groups	Neuron density (neuron/mm <sup>2</sup> )	Area of neurons ( $\mu\text{m}^2$ )	RNA content ( $U_{\text{opt}}$ )
Intact ( $n=10$ )	1250.2 $\pm$ 25.5	83.0 $\pm$ 3.86	9.52 $\pm$ 0.33
EAE (control) ( $n=10$ )	1006.7 $\pm$ 10.7*	91.5 $\pm$ 3.93*	7.45 $\pm$ 0.62*

**Note:** \* –  $p \leq 0.05$  in relation to indicators in the intact group (according to Whitney-Mann), EAE – experimental autoimmune (allergic) encephalomyelitis,  $U_{\text{opt}}$  – optical density unit.

As evidenced by the results of the studies, the induction of EAE led to the activation of neuroapoptosis. Thus, in the sensorimotor cortex of the animals with experimental pathology, an increase in the density of apoptotic and destructive cells by 150% ( $p < 0.05$ ) was observed. At the same time, the proportion of apoptotic cells in the indicated structure of the brain increased almost 5 times ( $p < 0.05$ ) (Table 2).

**Table 2.** Assessment of Apoptosis Indices of Neurons in the Sensorimotor Zone of the Brain of White Rats Under EAE Conditions,  $M \pm m$ ,  $n=10$

Experimental groups	Density of apoptotic and destructively altered cells per 1 mm <sup>2</sup>	Part of apoptotic cells, %
Intact ( $n=10$ )	59.4 $\pm$ 6.89	3.4 $\pm$ 0.96
EAE (control) ( $n=10$ )	148.0 $\pm$ 16.4*	15.7 $\pm$ 1.7*

**Note:** \* –  $p \leq 0.05$  in relation to indicators in the intact group (according to Whitney-Mann), EAE – experimental autoimmune (allergic) encephalomyelitis.

To study the morphology of neurons on a rotary microtome, sections were made in the region of the V-VI layer of the sensorimotor cortex 5 microns thick after fixation in Bouin. Sections were dewaxed and stained for nucleic acid determination with Einarson's halocyanine chromium alum. Morphometric studies were performed using an Axioskop microscope (Zeiss, Germany), magnification  $\times 40$ . Images of neurons obtained on a microscope using a highly sensitive video camera COHU-4922 (COCHU Inc., USA) were entered into a computer hardware-software system for digital image analysis VIDAS. Image analysis was performed in a semi-automatic mode (Burlaka et al. 2021).

In addition, our experimental studies on the formation of EAE were confirmed morphologically, using electron microscopy.

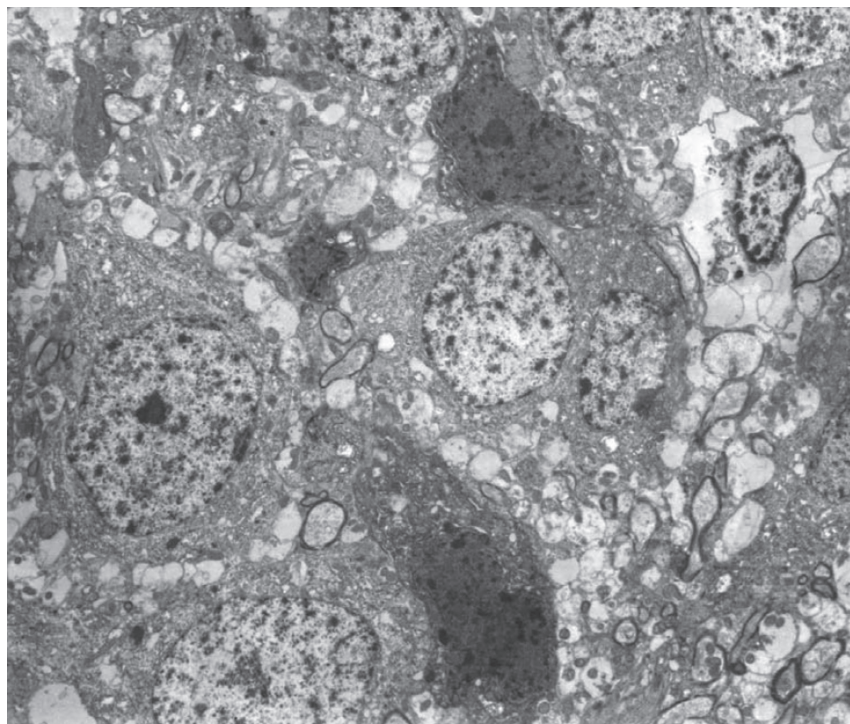
According to the analysis of the features of the ultrastructure of the frontal cortex and hippocampus of rats with simulated allergic encephalomyelitis, a number of changes in the neuronal and glial apparatus and components of the microvasculature of these brain formations were established. It was found that, under EAE conditions, the cytoarchitectonics of the frontal cortex was generally preserved. A moderate number of apoptotic altered neurons of the 2<sup>nd</sup> and 3<sup>rd</sup> layers of the cortex were observed. The nuclei are deformed in pyramidal neurons. The cytoplasm is saturated with the tubules and cisterns of the Golgi complex, in which there was a significant number of autophagolysosomes containing the inclusion of lipoproteins. The tubules of the rough endoplasmic reticulum (EPR) are disorganized and homogenized; the agranular tubules are dilated. Many mitochondria have blurred profiles and signs of cristolysis. There were fragmented processes of

neurons, around which there were recorded the areas of perineuronal edema of the frontal cortex, with a disseminated character. Disturbances in the structure of most axosomatic synapses were noted (Fig. 1).

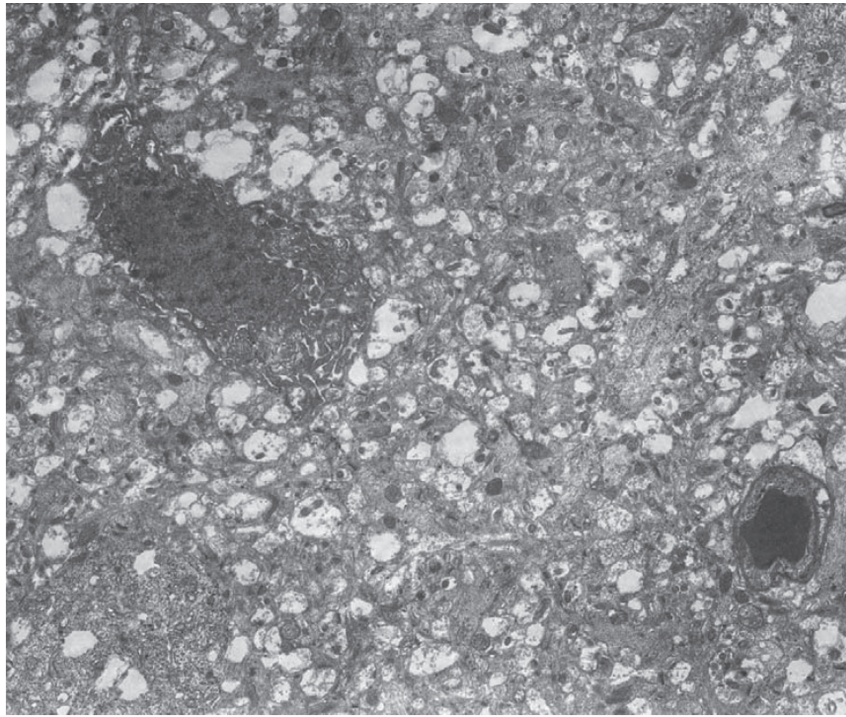
Our research found that the modeled pathology led to damage to neurons in the sensorimotor cortex of experimental animals.

The processes of astrocytes adjacent to the walls of the hemocapillaries extended profiles and partially lysed the cytoplasm. In some of them there was an accumulation of autophagolysosomes, fragments of damaged rough EPR. Often there were cell clumps of astrocytes with microglial cells. Microglial cells had an amoeba-like shape, with cytoplasm of increased electron density due to the hypertrophied Golgi complex, impressive sizes of autophagolysosomes and dense mitochondria. Hyaloplasm was saturated with ribosomes and polysomes expanded elements of agranular EPR. Nuclei had a high content of euchromatin. At a distance from the hemocapillaries, oligodendrocytes with partially lysed cytoplasm were usually found. In the composition of the nuclei, the peripheral regions of chromatin were indistinct and represented by homogeneous masses, indicating the initial stages of karyolysis. The processes of astrocytes and oligodendrocytes in their cytoplasm contained lipoprotein granules and had loosened or stratified plasma membranes (Fig. 2).

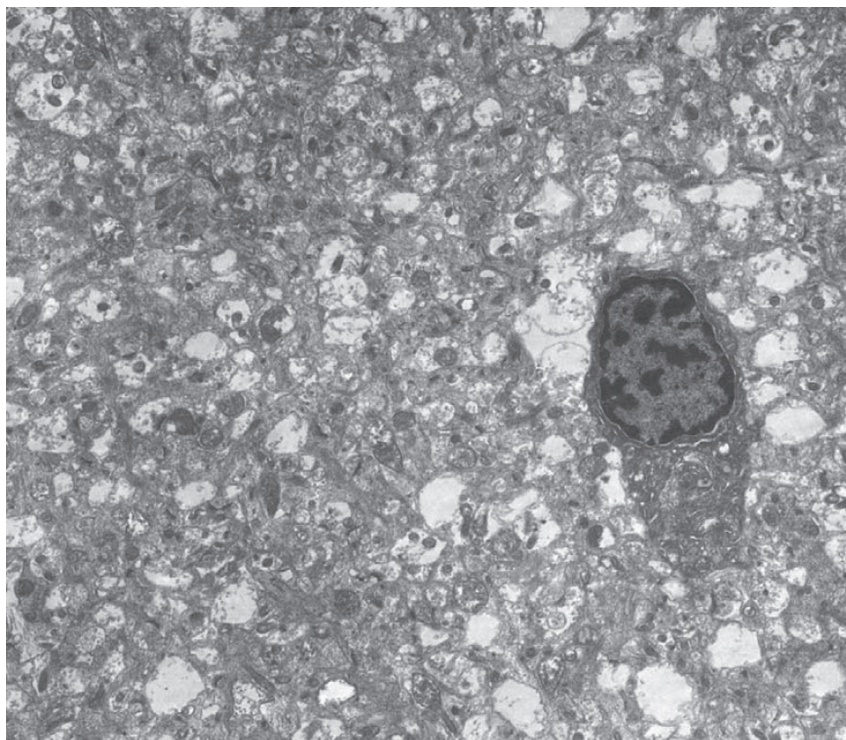
Disorganized in the overwhelming majority of cases were myelinated fibers. Their axoplasm contained coagulates, lysis sites, and mesaxon whorl. Many fibers are demyelinated with signs of neurofibril fragmentation. Most of the fiber demyelination loci were located near microvessels (Fig. 3).



**Figure 1.** Frontal cortex of a rat with experimental autoimmune encephalomyelitis . Electronogram.  $\times 4000$ .



**Figure 2.** Frontal cortex of rats with experimental autoimmune encephalomyelitis. Electronogram.  $\times 5000$ .



**Figure 3.** Frontal cortex of rats with experimental autoimmune encephalomyelitis. Electronogram.  $\times 6000$ .

Lumens of hemocapillaries of the cortex were narrowed and deformed, and contained disorganized erythrocytes. In most cases, the plasma membranes of erythrocytes were significantly damaged and loosened. Pericytes in the immediate vicinity of the described areas of endothelial cells had a reduced electron density and, by their damaged cortical layers, were combined with homogeneous masses of thickened and loosened basement membranes. The

cytoplasm of endothelial cells in the organelle zone retained its typical structure; however, most areas of the luminal and basal parts of the plasma membrane were loosened.

On the luminal surface of endotheliocytes, there was a wavy relief with significant invagination in the form of caveolae. The cytoplasm of the basal part of endothelial cells contained fuzzy profiles of single mitochondria and ribosomes. The nuclei of such endothelial cells were enlarged, filled

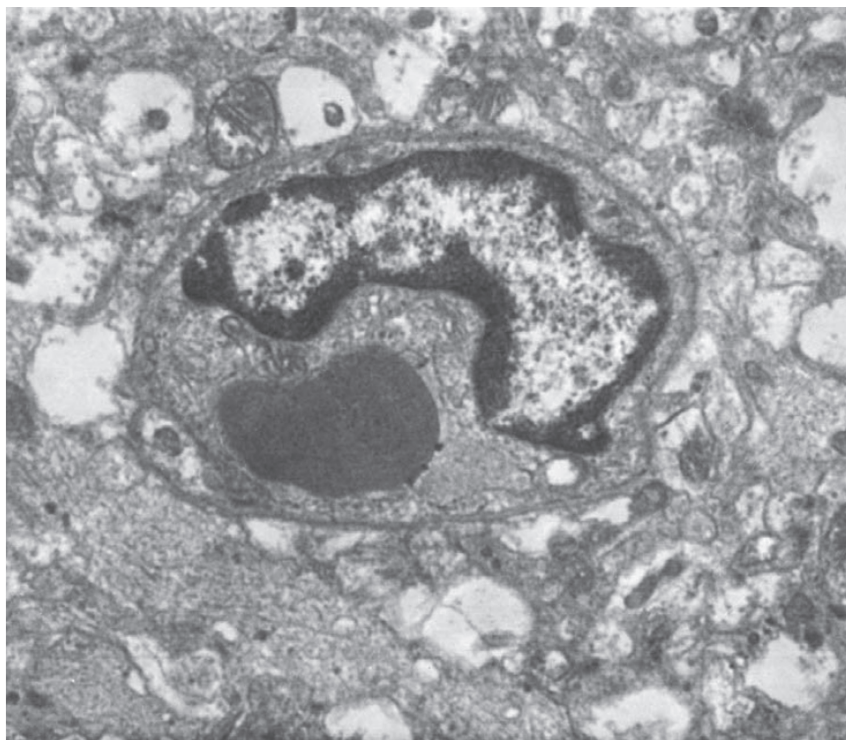
with a significant mass of heterochromatin, and contained a disorganized nucleolus. The membranes of the karyoteca along the perimeter of the nucleus had loci of loosening. Large zones of perivascular edema were observed around arterioles and venules. Lumens of postcapillary venules were unevenly dilated, full-blooded, with manifestations of stasis of formed elements, as well as signs of lymphoid perivascular infiltration. Small infiltrates were diffusely distributed in the cortical tissue and often contained microgliaocytes (Fig. 4).

It was found that the disturbances in the ultrastructure of hippocampal neurons were of a diffuse nature. Some neurons had signs of necrobiotic changes or were in a state of apoptosis. In neurons with the phenomena of chromatolysis, there was swelling and vacuolization of the cisterns of the lamellar apparatus, often with deformation of the cisterns and their fragmentation. Nearby, there were an increased number of lysosomes of varying degrees of maturity, multivesicular bodies and small osmiophilic inclusions. The structure of mitochondria in neurocytes with moderate damage was excited by the vacuolar lytic type. Most mitochondria were enlarged, had a fragmented outer membrane, and destroyed cristae and an electron-transparent matrix. An insignificant part of mitochondria had relatively preserved cristae, small in size. There was a sharp fragmentation of the neurofibrils. The cytoplasm and nuclei of astrocytes were of moderate electron density, with a decrease in the filling of the endoplasmic reticulum of the granular type, ribosomes, polysomes, mitochondria with signs of crystallization, and fine-grained hyaloplasm. The tubules of the agranular EPR are dilated and partially filled with electrically dense masses of heterogeneous consistency. The processes of astrocytes are filled with fine-grained hyaloplasm, ribosomes, and mitochondria. The

nuclei contained a moderate amount of euchromatin. In the majority of myelinated nerve fibers, there was no characteristic neurofilamentous organization of neuronal processes, and mitochondria were not identified. The axoplasm contained large coagulates and lysis sites. The axolemma was often fragmented, edematous, separated from the inner surface of the myelin sheath to a considerable extent, varying in width by slit-like spaces. Degenerative changes in myelinated processes were of mosaic character (Fig. 5).

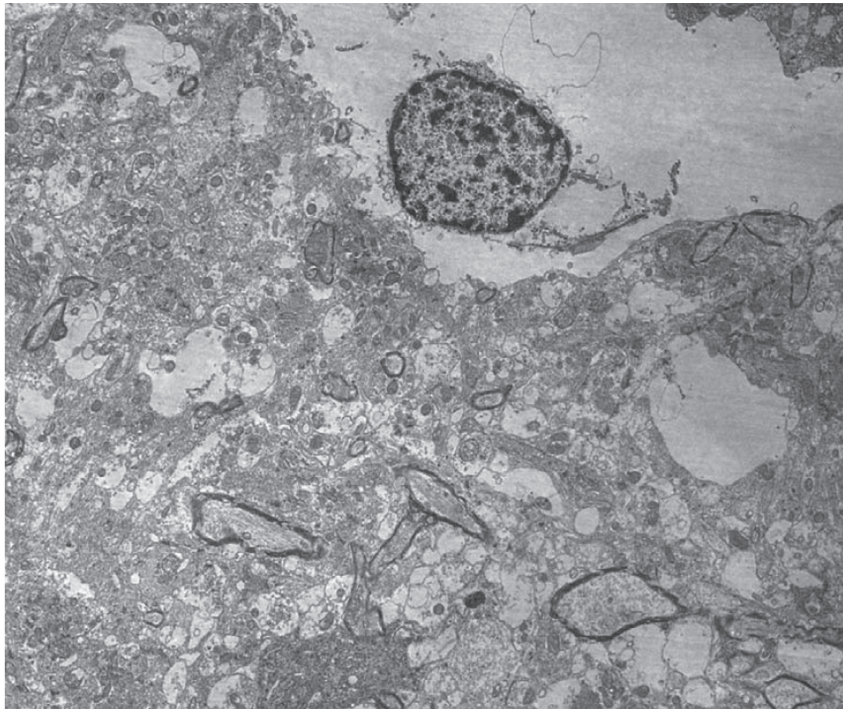
Lumens of hemocapillaries were narrowed; in blood plasma, there were diffusely located electronecapillary loose masses or deformed erythrocytes. The cytoplasm of endothelial cells had an average electron density. In the areas of the cytoplasm adjacent to the karyoteca, ribosomes, polysomes and mitochondria of very small sizes were found in significant quantities. Often mitochondrial membranes were without clear contours, loosened. The nuclei of arterioles and venules were irregular in shape, filled mainly with heterochromatin. The cytoplasm of endothelial cells in the basement membrane of microvessels contained a significant number of microvesicles, which were often in contact with the plasmolemma. Between clearly contoured hemocapillaries and areas of the plasma membrane of the basal part of the cytoplasm of endothelial cells, there was a narrow electron-light layer of the subendothelial layer. Pericytes in the walls of venules often showed signs of necrobiotic changes. Significant areas of perivascular edema were observed around the venules. Lumens were with manifestations of stasis; significant lymphoid perivascular infiltration was recorded, which was of diffuse nature (Fig. 6).

Thus, a single subcutaneous inoculation of an encephalitogenic mixture (EGM) in Freud's complete adjuvant led

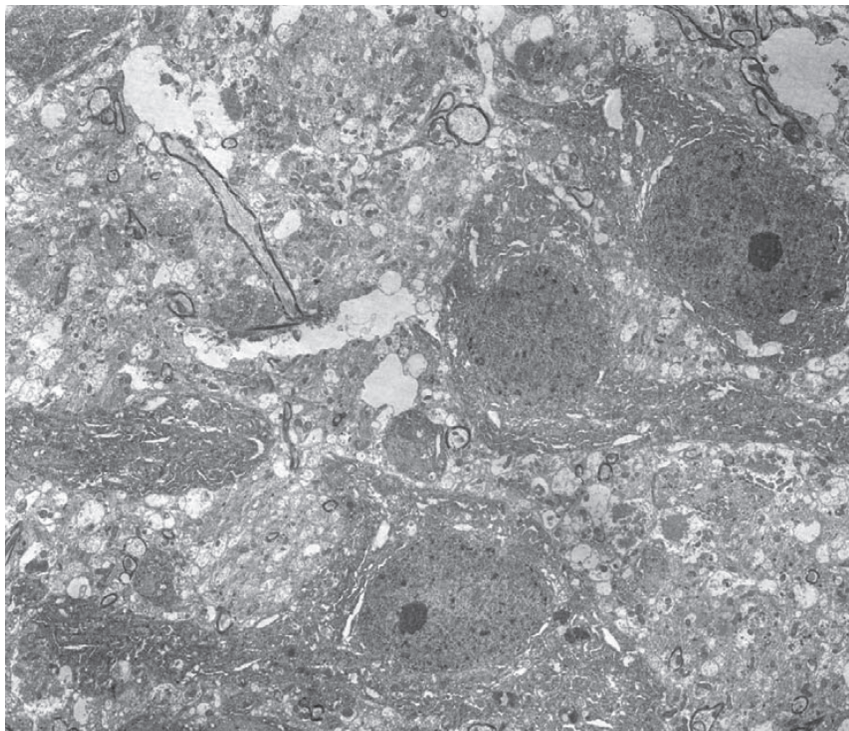


**Figure 4.** Frontal cortex of rats with experimental autoimmune encephalomyelitis. Electronogram.  $\times 8000$ .





**Figure 5.** Hippocampus of rat with experimental autoimmune encephalomyelitis. Electronogram×5000.



**Figure 6.** Hippocampus of rat with experimental autoimmune encephalomyelitis. Electronogram×3000.

to the development of multifocal demyelination and axonal degeneration in the hippocampus and frontal cortex of experimental animals. The results obtained are generally consistent with the data of our earlier morphometric analysis of hippocampal neurons, as well as the studies of markers of programmed cell death in patients with multiple sclerosis (Davydova and Markov 1970; Weakley et al. 1975; Zavalishina and Golovkina 2000).

## Conclusions

1. The use of this technique for the formation of EAE made it possible to obtain a simulated pathological state of multiple sclerosis in the form of experimental allergic encephalomyelitis and can be used in further studies to identify the corresponding patterns, the degree and nature of changes in the immune and

nervous systems of the body during the simulation of an experimental pathological state. The data obtained can be the basis for further research of drugs and their combinations with the aim of improving and rationalizing the pharmacotherapy of multiple sclerosis.

2. EAE causes apoptosis of neurons in the cerebral cortex, disseminated perineuronal edema of the medulla, disruption of the structure of most axosomatic synapses, demyelination of nerve conductors with signs of neurofibril fragmentation.
3. In the hippocampus, a single subcutaneous inoculation of EGM in Freud's complete adjuvant leads to the development of necrobiotic changes in neurons with disruption of the mitochondrial structure (increase in size, fragmentation of the outer membrane, destruction of cristae), disruption of the neurofilament organization of myelin nerve fibers, changes in the pericytes of the venule walls, and edema of the perivascular.

This model allows for preclinical studies to assess the specific activity of primary and secondary neuroprotective agents and nootropics. Modern neuroprotective agents do not always show therapeutic efficacy in a clinic, have a number of side effects with prolonged use, and due to the lack of a reliable therapeutic effect, their use in the clinic is limited. Currently, there is a search for newer neuroprotectors among various aza-heterocyclic systems, natural compounds, neuropeptides, etc. (Belenichev et al. 2015). The reliability and validity of the results obtained in the preclinical evaluation of the potential drugs investigated as promising neuroprotective agents are largely due to the extent to which the experimental models are adequate to the clinical manifestations of MS. In particular, these models are capable of reproducing the main mechanisms of neurodegeneration – an increase in the expression of pro-inflammatory cytokines, a disturbance in the NO system (an increase in the expression of nNOS, iNOS, an overproduction of cytotoxic forms of nitrogen monoxide), energy deficiency, glutamate-calcium excitotoxicity, oxidative stress, expression of early response genes, initiation of neuropapoptosis, etc. An important

point in assessing the efficacy of the neuroprotective action of drugs is the ability of the experimental model to simulate the clinical picture of multiple sclerosis: impaired motor activity, signs of neurological deficits (pareses, paralysis, etc.), impaired attention, learning and memory. It is also important to choose biochemical, molecular and cellular markers that allow assessing the picture of nerve tissue damage in the experiment and characterizing the potential neuroprotector under study. It is recommended to use Ceraxon, Cerebrocurin, Cortexin alone or together with hormonal therapy as reference preparations for pre-clinical studies.

In conclusion, it should be noted that the pathophysiology of multiple sclerosis is complex and includes various cell types, myelin-associated autoantigens, and modified by genetic and environmental factors. Therefore, it is unrealistic to expect that one animal model simulates all the characteristics of the pathophysiology of MS and covers the genetic diversity of the patient population and a number of environmental elements that may affect the onset and progression of the disease. Instead, each EAE model simulates a specific aspect of multiple sclerosis, and the variety of models provides much greater scope for researchers, thereby significantly accelerating the process of identifying the mechanisms underlying the development, maintenance, and regulation of pathogenic immune responses during CNS autoimmunity.

### Prospects for further research

Modeling experimental allergic encephalomyelitis is an important aspect for animal studies of multiple sclerosis. An urgent issue is the search for new pharmacological drugs and combinations of classical drugs on an experimental model for further implementation in clinical trials and medical practice of pharmacotherapy for multiple sclerosis.

## Conflict of interests

The authors declare no conflict of interests.

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