THE DELAYED EFFECT OF THE NEUROPROTECTOR FABOMOTIZOLE ON THE BRAIN PROTEOME IN RATS WITH THE ROTENONE MODEL OF PARKINSONISM

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Fabomotizole is an original anxiolytic agent developed at the Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies that acts on a number of important receptor systems of the brain. In a model of Parkinson's disease induced in rats by a course of rotenone administration, fabomotizole attenuated manifestations of behavioral impairments and influenced the profile and relative content of brain proteins. Five days after the last administration of rotenone, the fabomotizole effect on the behavioral reactions of rats persisted. According to the proteomic study, the profile of brain proteins and changes in their relative content differed significantly from the results obtained immediately after the last administration of rotenone, as well as rotenone in combination with fabomotizole. Changes in the relative content of almost all proteins detected immediately after the last administration of rotenone or rotenone with fabomotizole were not detectable five days later. However, at this time point, there were changes in the relative content of other proteins associated with neurodegeneration in Parkinson's and Alzheimer's diseases. Such dynamics suggests a wave-like change in the content of pathogenetically important brain proteins involved in the mechanisms of neurodegeneration and neuroprotection.

Keywords: fabomotizole; neuroprotector; brain proteins; Parkinson's disease; rotenone; neurodegeneration; proteomic profiling

DOI: 10.18097/PBMCR1586

INTRODUCTION

Novel developments in diagnostics and therapy of Parkinson's disease (PD), a common neurodegenerative disorder characterized by damage to the nigrostriatal dopaminergic (DA) system, require the creation of experimental models of PD *in vivo*. One of the most adequate and widely used models of PD in rats is the so-called rotenone Parkinsonism induced by the introduction of the neurotoxin rotenone. By inhibiting complex I of the mitochondrial respiratory chain, rotenone causes degeneration of DA system cells and the development of motor disorders and other changes similar to symptoms and molecular biological features of PD [1–6].

Fabomotizole (5-ethoxy 2-[2-(morpholino) ethylthio]-benzimidazole dihydrochloride) is an original anxiolytic developed at the Zakusov Institute of Pharmacology (the Institution of the Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies) that acts on a number of important receptor systems of the brain [7–9]. In the rotenone-induced Parkinsonism model in rats, fabomotizole reduced the severity of postural instability and prevented death of rats. Immediately after the completion of the rotenone administration and its combined administration with fabomotizole, changes in the relative content of brain proteins associated with neurodegeneration were detected [10]. Changes in behavioral reactions and the proteomic profile of brain proteins in the delayed period of time after the cessation of the toxin and neuroprotector administration have not been studied yet.

The aim of this study was to investigate the effect of fabomotizole on the manifestations of PD in rats, induced by the administration of rotenone (behavioral reactions and quantitative changes in the proteomic profile of the brain), 5 days after the last administration of the neurotoxin.

MATERIALS AND METHODS

Reagents

The following reagents have been used in this study: Tris (hydroxymethyl) aminomethane, ammonium bicarbonate, dithiothreitol, guanidine hydrochloride, urea, sodium chloride, Triton X-100, 4-vinylpyridine, Coomassie Brilliant Blue G-250, rotenone (Merck, USA); formic acid, sodium

hydroxide (Acros Organics, USA); acetonitrile (Fisher Chemical, UK); isopropanol, trifluoroacetic acid (Fluka, USA); tris-(2-carboxyethyl)-phosphine (Pierce, USA); modified trypsin (mass spectrometry grade, Promega, USA); fabomotizole (Afobazole, tablets, 10 mg, Otisifarm Pro, Russia).

Other reagents of the highest purity available were from local suppliers.

Experimental Animals

The study was performed using outbred albino rats obtained from the Stolbovaya branch of the Federal State Budgetary Scientific Institution "Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency" (Moscow Region). The animals were kept under standard vivarium conditions with free access to food and water under a 12-h light regime in accordance with GOST 33215 and 33216.

Modeling of Experimental Parkinsonism in Rats

Modeling of PD using systemic administration of rotenone was carried out according to [11]. The animals were randomly divided into four groups:

1) the Control group (10 rats); animals were treated with daily intraperitoneal (i.p.) injections (for 7 days) with saline, 0.2 ml per 100 g of body weight;

2) the Rotenone group (12 rats); the animals were treated with i.p. injections of rotenone 2.75 mg/kg (7 days);

3) the Rotenone + Fabomotizole group (9 rats); the animals were treated with daily i.p. injections of 2.75 mg/kg rotenone (i.p.) and 10 mg/kg fabomotizole (7 days);

4) the Fabomotizole group (10 rats); the animals were treated with daily i.p. injections of 10 mg/kg fabomotizole (7 days).

Rotenone was administered in a solution in the neutral triglyceride miglyol (Miglyol 840). The solution was prepared as described previously [4]. After behavioral tests on day 12 of the experiment (5 days after the last administration of rotenone), the animals were decapitated under light ether anesthesia, brain homogenate lysates were obtained, and samples were prepared for mass spectrometric analysis.

Behavioral Tests

Oligokinesia was assessed in the Open Field and Rotating Rod tests on day 12 of the experiment, 5 days after the last administration of the neurotoxin and neuroprotector, as described earlier [4].

Statistical data processing was performed using the Statistica v. 10.0 program. The results in the tables are presented as mean ± standard error of the mean (Mean ± SEM). Differences between groups were considered significant at $p<0.05$.

Preparation of Samples for Mass Spectrometric Analysis

Brain tissue (cerebral hemispheres) was homogenized using a Heidolph SilentCrusher homogenizer (Heidolph, Germany, 50,000 rpm) in 0.05 M potassium phosphate buffer (pH 7.4) to a final concentration of 30 mg/ml. To assess the relative quantitative changes in the brain protein content in animals of different experimental groups, the same amount of total protein was used in sample preparation; it was controlled using the Bradford method [12]. After incubation in the presence of 3% Triton X-100 (4°C, 1 h), the lysates were diluted 3-fold with the same buffer and centrifuged for 30 min at 16,000 *g* to obtain a cleared supernatant. Protein extraction, alkylation, and trypsinolysis have been described in detail previously [5, 13].

Mass Spectrometric Analysis

Mass spectrometric analysis was performed using the equipment of the "Human Proteome" Core Facility at the Institute of Biomedical Chemistry (IBMC): the Ultimate 3000 RSLCnano high-efficiency liquid peptide separation system (Thermo Scientific, USA) in the nanoflow mode of the Q-Exactive HFX mass spectrometric detector (Thermo Scientific) as described previously [4]. Bioinformatics data processing was performed according to [4]. Each of the proteins presented in the tables was identified in at least three independent experiments.

RESULTS AND DISCUSSION

A course of rotenone administration to rats led to the development of severe parkinsonian syndrome, accompanied by motor disorders characteristic of the advanced stage of PD [4, 13]. The correcting effect of fabomotizole, administered intraperitoneally simultaneously with rotenone, reduced the manifestations of postural instability and oligokinesia to the level characteristic of animals in the control group [11]. Motor impairments in animals treated with rotenone administration persisted at least five days after the end of the administration of this neurotoxin (Table 1).

Rotenone-treated animals demonstrated a decrease in the parameters of motor, orientation-exploratory activity, and impaired movement coordination, compared to the control group, also observed 5 days after the last administration of this neurotoxin (Table 1). In the group of animals treated with fabomotizole simultaneously with rotenone, a decrease in oligokinesia parameters persisted 5 days after the last administration of rotenone (Table 1). The exception was vertical motor activity, which no longer differed from the control level. In rats treated with fabomotizole simultaneously with rotenone, a statistically significant increase

Table 1. The effect of fabomotizole on motor activity and motor coordination of rats with a PD model induced by rotenone administration for 7 days followed by a 5-day interval after the last injections of the neurotoxin and neuroprotector

Groups and the number of animals in each group	Motor activity, units		Duration of retention of animals on a rotating rod, s
	Horizontal activity (number of movements)	Vertical activity (number of stands)	
Control, n=10	14.67±2.42	9.67±2.22	177.75±2.25
Rotenone, n=12	6.92±0.95*	5.67±0.54	87.17±12.43**
Rotenone + Fabomotizole, n=9	8.78±1.45	8.00±1.26	128.88±15.50*#
Fabomotizole, n=10	14.67±3.87	5.89±1.02	—

The results in the tables are presented as mean ± standard error of the mean (Mean ± SEM). *,** – $p<0.01$, $p<0.001$, as compared with control, respectively; # – $p<0.05$, as compared with group Rotenone.

in motor activity, assessed by the duration of time the animals could hold on to a horizontal rod, was noted five days after the last administration. In rats treated with fabomotizole only, the main indicators of motor activity insignificantly differed from those in control animals (Table 1).

Previously, a comparative proteomic identification of brain proteins in control rats and rats with rotenone-induced PD, performed immediately after the end of the rotenone course, showed a change in the level of 86 proteins belonging to different functional groups [10]. These included 65 upregulated and 21 downregulated proteins. A significant increase in the relative content was found in mitochondrial proteins (components of the cytochrome *c* oxidase complex (Uniprot database numbers S5RZM8, P10888, P11951, P12075, P20788) and voltage-dependent ion channels (P81155, Q9Z2L0, A0A0G2JSR0)). This is consistent with literature data on the key role of mitochondrial dysfunction in the pathogenesis of PD [10]. Quantitative changes were also detected for proteins associated with neurodegeneration: alpha-synuclein (P37377), DJ-1 protein (O88767), glyceraldehyde-3-phosphate dehydrogenase (P04797), TRIM2 (D3ZQG6), and prohibitin-2 (Q5XIH7) [10]. In the case of combined administration of rotenone and fabomotizole, a less pronounced (compared to the administration of rotenone alone) increase in the relative content of a number of components of voltage-dependent anion-selective channel proteins (Vdac) 1, 2, and 3 (Q9Z2L0, P81155, A0A0G2JSR0) was noted.

Five days after the last administration of rotenone to rats, changes in the relative content of 17 brain proteins were detected. Interestingly, only one protein (excitatory amino acid transporter 2, P31596) was previously identified in the group of proteins with altered relative content immediately after the end of the rotenone administration. In the case of the acute effect of rotenone, the relative content of the amino acid transporter increased significantly [10], while in the case of the delayed effect, it demonstrated a 2-fold decrease (Table 2). For the group of animals treated with both rotenone and fabomotizole, changes in the content at the end of the course and after five days were detected only for the V-type proton ATPase subunit a (Q8R2H0), as well as for excitatory amino acid transporter 2 (P31596). The relative content of subunit a of this ATPase increased by 22.6 and 2.3 times, respectively, and transporter 2 by 2 times in both cases ([10] and Table 3). In the group of rats treated with fabomotizole, only one common protein showed altered relative content at both time points of the experiment. This was the membrane glycoprotein M6-a (Q812E9); its content increased by 3 and 2 times, respectively) ([10] and Table 4).

Five days after the end of rotenone administration, 8 brain proteins were upregulated (by more than 2 times), and 9 proteins were slightly downregulated versus control (Table 2). In rats treated with rotenone and fabomotizole changes in relative content were found for 31 brain proteins: 23 proteins were upregulated (by 2–3 times) and eight proteins were downregulated (by 2–3 times) (Table 3). Fabomotizole administration caused a delayed increase in relative content (less than 3.5 times) in 10 proteins, and a decrease (2–4.5 times) in 5 brain proteins (Table 4). It should be noted that the sets of proteins demonstrating altered content in response to rotenone administration alone and in combination with the neuroprotector fabomotizole had virtually no common elements (Fig. 1, Venn diagram). In animals treated only with fabomotizole, almost half of the proteins with an altered relative content (versus control) basically coincided with the brain proteins demonstrating altered relative content in response to the combined administration of rotenone and fabomotizole to rats. This suggests that the changes in protein levels in these cases are obviously determined by the effect of fabomotizole (Fig. 1).

Table 2. The delayed change in the relative content of brain proteins of rotenone treated rats

#	Uniprot accession number	Uniprot gene name	Uniprot protein name	Function	Localization	–LOG(P-value)	Fold change	Association with neurodegeneration
1	P11240	*Cox5a*	Cytochrome *c* oxidase subunit 5A, mitochondrial	1	MCh	4.46	3.29	[14]
2	D3ZD09	*LOC120103152*	Cytochrome *c* oxidase subunit 6B1-like	1	MCh	1.81	2.92	[14]
3	P63045	*Vamp2*	Vesicle-associated membrane protein 2	2	Ve, PM	5.23	2.50	[15, 16]
4	P62775	*Mtpn*	Myotrophin	3	C, N	2.37	2.48	—
5	Q63028	*Add1*	Alpha-adducin	2	C, PM	1.11	2.16	—
6	Q6AYS2	*Sfxn1*	Sidoreflexin	2	MCh	2.15	2.16	[17]
7	Q63654	*Ubc*	Polyubiquitin	6	C, N	3.38	2.08	[18]
8	Q7M079	*UniProtKB unreviewed (TrEMBL)*	Calcium-binding protein 4	3	C	1.85	2.07	[19, 20]
9	G3V846	*Slc1a3*	Amino acid transporter	2	M, PM	1.88	0.48	[21]
10	P04636	*Mdh2*	Malate dehydrogenase, mitochondrial	1	MCh	3.28	0.48	[22]
11	P10111	*Ppia*	Peptidyl-prolyl cis-trans isomerase A	6	C, N, S	5.33	0.48	[23]
12	P07632	*Sod1*	Superoxide dismutase [Cu-Zn]	4	C, N	3.74	0.47	[24, 25]
13	P18418	*Calr*	Calreticulin	3	C, ER, Ve, S	1.46	0.45	[26]
14	Q5BJ93	*Eno1*	Phosphopyruvate hydratase	1	C	5.38	0.44	[27]
15	P00507	*Got2*	Aspartate aminotransferase, mitochondrial	6	MCh, PM	3.89	0.41	[28, 29]
16	Q5BJT9	*Ckmt1*	Creatine kinase U-type, mitochondrial	1	MCh	3.42	0.36	[30]
17	P27139	*Ca2*	Carbonic anhydrase 2	3	C, PM	4.25	0.26	[31]

Here and in other tables changes in the relative content are given in comparison with control. Numbers in the column "Function" designate the following functional groups of proteins: *1*. Proteins/enzymes involved in energy generation and carbohydrate metabolism. *2*. Proteins involved in cytoskeleton formation and exocytosis. *3*. Proteins involved in signal transduction and regulation of enzyme activity. *4*. Antioxidant and protective proteins/enzymes. *5*. Protein regulators of gene expression, cell division and differentiation. *6*. Enzymes involved in metabolism of proteins, amino acids and other nitrogenous compounds.

Localization: C – cytoplasm, N – nucleus, M – membranes, PM – plasma membrane, ER – endoplasmic reticulum, G – Golgi complex, MCh – mitochondria, Ve – vesicles, S – secretory protein, Extr – extracellular space.

Thus, 5 days after the completion of the course of administration of the neurotoxin rotenone and the neuroprotector fabomotizole, administered both together and separately, the relative content of a number of proteins in rats changed. The vast majority of these proteins did not coincide with the proteins demonstrating an altered relative content immediately after the completion of the course of administration of the drugs. However, almost all proteins with altered relative content are known to be associated with neurodegeneration, including PD (references to Tables 2–4).

Such dynamics obviously suggests a wave-like change in pathogenetically important brain proteins involved in the mechanisms of neurodegeneration and neuroprotection.

ACKNOWLEDGMENTS

Mass spectrometry analysis of proteins was carried out using the equipment of the "Human Proteome" Core Facility at IBMC.

Table 3. The delayed change in the relative content of brain proteins of rats treated with rotenone and fabomotizole

#	Uniprot accession number	Uniprot gene name	Uniprot protein name	Function	Localization	–LOG(P-value)	Fold change	Association with neurodegeneration
1	F1LMW7	*Marcks*	Myristoylated alanine-rich C-kinase substrate	3	C, M	2.85	2.97	[32, 33]
2	P11505	*Atp2b1*	Plasma membrane calcium-transporting ATPase 1	2	M	3.35	2.69	[34]
3	Q64715	*Map2*	Microtubule-associated protein	2	C	2.10	2.59	[35]
4	Q505J6	*Slc25a18*	Mitochondrial glutamate carrier 2	2	MCh	2.10	2.53	[36]
5	P97546	*Nptn*	Neuroplastin	3	M	3.32	2.44	[37]
6	P0DP31	*Calm3*	Calmodulin-3	3	C	1.32	2.37	[38]
7	D4A565	*Ndufb5*	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial	1	MCh	1.12	2.37	[39]
8	A0A0G2JT78	*Rab6b*	RAB6B, member RAS oncogene family	2	G	1.38	2.36	[40, 41]
9	Q2I6B2	*Atp6v0a1*	V-type proton ATPase subunit a	1	M, Ve, S	1.94	2.32	[42]
10	P07936	*Gap43*	Neuromodulin	5	C, M	2.07	2.28	[43]
11	P32736	*Opcml*	Opioid-binding protein/ cell adhesion molecule	3	M	4.63	2.27	[44]
12	Q6AYS2	*Sfxn1*	Sidoreflexin	2	MCh	2.43	2.19	[17]
13	Q8VHS9	*Cacna2d1*	L-type calcium channel alpha2/delta subunit	2	M	1.43	2.19	[45]
14	Q02563	*Sv2a*	Synaptic vesicle glycoprotein 2A	3	Ve	2.03	2.13	[46]
15	Q5M7T6	*Atp6v0d1*	V-type proton ATPase subunit d	1	M	4.73	2.13	[42]
16	P32851	*Stx1a*	Syntaxin-1A	2	M, Ve	3.63	2.11	[47]
17	P97710	*Sirpa*	Tyrosine-protein phosphatase non-receptor type substrate 1	3	M	2.58	2.11	[48]
18	Q6PW35	*Nrcam*	Neuronal cell adhesion molecule long isoform Nc7	3	M	1.68	2.08	[49]
19	D4A435	*Icam5*	Intercellular adhesion molecule 5	3	M	1.91	2.05	[50]
20	P31596	*Slc1a2*	Excitatory amino acid transporter 2	2	M	2.38	2.05	[51]
21	Q05175	*Basp1*	Brain acid soluble protein 1	5	PM	1.98	2.05	[52, 53]
22	P01830	*Thy1*	Thy-1 membrane glycoprotein	4	M	3.01	2.01	—
23	Q8R2H0	*Atp6v1g2*	V-type proton ATPase subunit G	1	M	1.54	2.01	[42]
24	Q08163	*Cap1*	Adenylyl cyclase-associated protein 1	5	M	1.35	0.50	[54, 55]
25	A0A8I5Y2B8	*ENSRNOG00000062500*	Thioredoxin-dependent peroxiredoxin	4	C	2.08	0.46	[56, 57]
26	Q9JI66	*Slc4a4*	Electrogenic sodium bicarbonate cotransporter 1	2	M	2.83	0.44	—
27	Q63910	*Hba-a3*	Globin c1	2	Extr	5.32	0.39	[58]
28	P47819	*Gfap*	Glial fibrillary acidic protein	2	C	5.18	0.37	[59]
29	M0RC65	*Cfl2*	Cofilin 2	2	C	1.21	0.37	[60]
30	Q6P7S0	*UniProtKB unreviewed (TrEMBL)*	Pyruvate kinase	1	C, N	1.60	0.33	[61]
31	A0A1K0H3R5	*Hbb*	Globin a4	2	Extr	4.68	0.32	[58]

Table 4. The delayed change in the relative content of brain proteins of rats treated with fabomotizole

#	Uniprot accession number	Uniprot gene name	Uniprot protein name	Function	Localization	–LOG(P-value)	Fold change	Association with neurodegeneration
1	P0DP31	*Calm3*	Calmodulin-3	3	C	2.44	3.46	[38]
2	Q63654	*Ubc*	Polyubiquitin	6	C, N	4.09	3.32	[18]
3	B2RYS0	*Cox7a2*	Cytochrome *c* oxidase subunit 7A2, mitochondrial	1	M, MCh	1.95	2.48	[14]
4	F1LMW7	*Marcks*	Myristoylated alanine-rich C-kinase substrate	3	C, M	1.49	2.47	[32, 33]
5	Q64542	*Atp2b4*	Plasma membrane calcium-transporting ATPase 4	2	M	4.42	2.31	[34]
6	B5DFC1	*Vps35*	Vps35 protein	2	M	2.02	2.18	[62, 63]
7	P61589	*Rhoa*	Transforming protein RhoA	2	C, M	2.11	2.13	[64]
8	Q812E9	*Gpm6a*	Neuronal membrane glycoprotein M6-a	5	M	1.36	2.07	[65]
9	Q8VHS9	*Cacna2d1*	L-type calcium channel alpha2/delta subunit	2	M	2.00	2.06	[45]
10	P97710	*Sirpa*	Tyrosine-protein phosphatase non-receptor type substrate 1	3	M	1.17	2.05	[48]
11	A0A8I5Y2B8	*ENSRNOG00000062500*	Thioredoxin-dependent peroxiredoxin	4	C	1.34	0.44	[56, 57]
12	Q08163	*Cap1*	Adenylyl cyclase-associated protein 1	5	M	1.73	0.43	[54, 55]
13	M0R757	*Eef1a1l1*	Elongation factor 1-alpha	6	C	1.72	0.40	[66]
14	Q6P7S0	*UniProtKB unreviewed (TrEMBL)*	Pyruvate kinase	1	C, N	1.43	0.36	[61]
15	Q6AYZ1	*Tuba1c*	Tubulin alpha-1C chain	2	C	1.58	0.22	[67, 68]

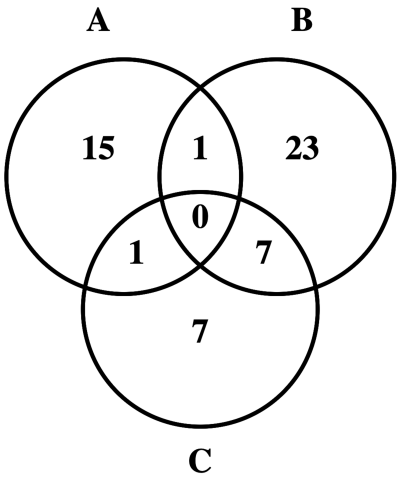

Figure 1. A Venn diagram showing the amount of rat brain proteins with altered relative content (versus control) 5 days after the administration of the neurotoxin rotenone, the neuroprotector fabomotizole and their combination to animals. A – rotenone, B – rotenone + fabomotizole, C – fabomotizole.

FUNDING

The work was performed within the framework of the Program for Basic Research in the Russian Federation for a long-term period (2021–2030) (No. 122030100170-5).

COMPLIANCE WITH ETHICAL STANDARDS

The experiments were carried out in compliance with generally accepted norms of humane treatment of laboratory animals, in accordance with the provisions presented in the "Guidelines for Working with Laboratory (Experimental) Animals in Preclinical (Non-Clinical) Studies" (Appendix to the Recommendation of the Board of the Eurasian Economic Commission dated November 14, 2023, No. 33).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Received: 11. 05. 2025.
Revised: 28. 05. 2025.
Accepted: 29. 05. 2025.

ОТСРОЧЕННОЕ ДЕЙСТВИЕ НЕЙРОПРОТЕКТОРА ФАБОМОТИЗОЛА НА ПРОТЕОМ МОЗГА КРЫС В РОТЕНОНОВОЙ МОДЕЛИ ПАРКИНСОНИЗМА

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Фабомотизол — оригинальный анксиолитик, разработанный в НИИ фармакологии имени В.В. Закусова и действующий на ряд важных рецепторных систем мозга. В модели болезни Паркинсона, вызванной у крыс курсовым введением пестицида ротенона, фабомотизол ослаблял нарушения поведенческих реакций животных, влиял на профиль и относительное содержание белков мозга. Через 5 дней после завершения курсового введения ротенона позитивное влияние фабомотизола на поведенческие реакции крыс сохранялось. По данным протеомного исследования, профиль белков мозга и изменения их относительного содержания существенно отличались от результатов, полученных сразу после завершения курсового введения ротенона, а также ротенона и фабомотизола. Изменения относительного содержания почти всех белков, обнаруженные сразу после завершения курсового введения ротенона или ротенона и фабомотизола, через пять дней уже не определялись. В то же время было обнаружено изменение относительного содержания других белков, ассоциированных с нейродегенерацией при болезнях Паркинсона и Альцгеймера. Такая динамика свидетельствует о волнообразном изменении содержания патогенетически важных белков мозга, вовлечённых в механизмы нейродегенерации и нейропротекции.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: фабомотизол; нейропротектор; белки мозга; болезнь Паркинсона; ротенон; нейродегенерация; протеомное профилирование

Финансирование. Работа выполнена в рамках Программы фундаментальных научных исследований в Российской Федерации на долгосрочный период (2021–2030 гг.) (№ 122030100170-5).

Поступила в редакцию: 11.05.2025; после доработки: 28.05.2025; принята к печати: 29.05.2025.