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ORIGINAL RESEARCH
ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ

Study of the neuroprotective effect of conditioned medium from human iPSCs-derived glial progenitor cells in a model of glutamate excitotoxicity

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Abstract. *Relevance*. In modern biomedicine, therapeutic strategies for neurological disorders are increasingly focused on the development of multitarget therapeutic approaches. Among the promising avenues, preparations based on conditioned media stand out. In the present work, for the first time, a genome-wide transcriptomic analysis of differential gene expression was performed in primary cortical neuron cultures incubated with conditioned medium from human glial progenitor cells under conditions of induced glutamate excitotoxicity. This study allows us to infer potential molecular mechanisms underlying the observed protective effect. *Materials and methods*. Conditioned medium was collected from human glial progenitor cell cultures and subjected to purification and concentration by tangential ultrafiltration. Excitotoxic glutamate exposure was modeled in primary cortical neurons from newborn (P0) rats by adding sodium glutamate to a final concentration of 100 μM. Cell viability was assessed using the MTT assay. Transcriptomic libraries were sequenced on the NextSeq 1000 platform (Illumina, USA); read quality control was performed with the FastQC tool. The R package edgeR was used for differential gene expression analysis (FDR < 0.05). *Results and discussion*. The conditioned medium was found to exert a dose-dependent neuroprotective effect: at a preparation concentration of 45 μg/mL, cell viability recovered to the level of the intact control. According to transcriptomic profiling data, the addition of conditioned medium against a background of glutamate stress was accompanied by upregulation of 173 genes and downregulation of 479 genes. The identified genes were grouped into functional categories with statistical significance. Among the most represented biological processes associated with activated genes were regeneration, cell–cell adhesion, response to oxidative stress, inhibition of apoptosis, and stimulation of the TGF-beta signaling pathway. Downregulated

genes were predominantly linked to calcium transport, glutamatergic signal transmission, and regulation of neurite outgrowth and branching. The results obtained may indicate that components of the conditioned medium trigger antioxidant defense and repair cascades in neurons while simultaneously limiting excessive activation of glutamate receptors and calcium ion influx. *Conclusion.* The conditioned medium exhibits neuroprotective properties under conditions of glutamate-induced excitotoxicity. Presumably, this effect is based on the simultaneous induction of genes promoting survival and suppression of genes mediating glutamatergic neurotransmission. The totality of the data obtained confirms the promise of further development of therapeutic agents based on conditioned media from human glial progenitor cells for the treatment of neurological disorders.

Keywords: conditioned medium, glutamate excitotoxicity, transcriptome analysis, glial progenitor cells, iPSCs

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Conflict of interest statement. The authors declare no conflicts of interest.

Ethics approval. Animal experiments are conducted in accordance with these principles and regulations, the Foundation's preferred low standards (ESF) and the Declaration on Humane Animal Nutrition, as well as in accordance with the Order of the Ministry of Health and Social Development of Russia dated August 23, 2010, No. 708n "On approval of laboratory practice". Animal care, breeding and experimental procedures are carried out in accordance with the requirements of the Ethics Committee of the A.P. Avtsyn Research Institute of Human Morphology of the B.V. Petrovsky Russian Scientific Center of Surgery. Protocol No. 38 (14) dated May 31, 2022. Informed consent was obtained from all patients participating in the treatment when taking skin biopsies from donors. The protocol was approved by this Institutional Institutional Review Board of the Medical Genetics Research Center (Protocol No. 2019-2/3 dated October 13, 2020).

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Consent for publication — Voluntary written consent was obtained from the patient for the investigation and publication of relevant medical information according to WMA Declaration of Helsinki — Ethical Principles for Medical Research Involving Human Subjects, 2013.

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Introduction

Glutamate is a key excitatory neurotransmitter of the central and peripheral nervous system, involved in neurotransmission and synaptic plasticity, which determines its fundamental role in the physiology of nervous tissue and cognitive functions. The signaling function of glutamate is realized through binding to ionotropic and metabotropic receptors, which trigger cascades of intracellular reactions controlling axonal growth, dendritic branching and dendritic spine formation, mechanisms of neuroplasticity, as well as the mitochondrial pathway of apoptosis [1]. Maintaining the homeostatic concentration of glutamate in the synaptic cleft is a necessary condition for stable

neuronal functioning. An increase in the amount of glutamate in the extracellular space (excitotoxicity) results in overstimulation of the receptors of this neurotransmitter, leading to the opening of voltage-gated calcium channels and an avalanche-like influx of Ca^{2+} into the cell [2]. An excess of free Ca^{2+} disrupts the homeostasis of this ion and initially overloads the cytoplasmic buffer system, followed by the mitochondrial system, which functions via the calcium uniporter [3]. The consequence is depolarization of the inner mitochondrial membrane, leading to an increase in the number of molecules associated with oxidative stress, such as reactive oxygen species, free radicals, or hydrogen peroxide [4]. This disrupts the function of the electron transport chain, ATP production, and triggers endoplasmic reticulum (ER) stress, contributing to the accumulation of misfolded proteins within the cell and destabilizing the redox balance [5]. Furthermore, mitochondrial dysfunction leads to a decrease in NAD^+ levels, which is necessary for the proper function of the DNA repair system [6]. All of these impairments lead to the activation of intracellular cascades of caspase proteins responsible for programmed neuronal cell death. Cell death via apoptosis leads to extensive damage to nervous tissue, often culminating in necrosis, and is also accompanied by cognitive dysfunctions. Dysregulation of the glutamatergic system exerts a profound negative impact on neuronal physiology and acts as a key pathogenetic factor in a range of neurological disorders, such as dementia, stroke, traumatic brain injury, and amyotrophic lateral sclerosis [3, 7]. Given the multifactorial nature of the excitotoxic action of glutamate, the current strategy for pharmacotherapy of neurodegenerative diseases is increasingly shifting towards the use of drugs with a multimodal mechanism of action, allowing for the simultaneous modulation of various components of the pathological process.

Conditioned media (CM) are currently considered as novel multicomponent agents that target several pathological pathways simultaneously. Using a model of glutamate excitotoxicity, the neuroprotective properties of CM derived from mesenchymal stem cells, neural stem cells, oligodendrocytes, and astrocytes have already been demonstrated [8–10]. Owing to their diverse composition, which includes extracellular vesicles, microRNAs, proteins, peptides, and metabolites, CM exhibit multitargeted effects aimed at stabilizing oxidative stress, inhibiting apoptosis, and reducing ER stress. Previously, our laboratory demonstrated the therapeutic properties of conditioned media from glial derivatives of human induced pluripotent stem cells (GPCs) in a stroke model and a neuritogenesis model [11–14].

The aim of this study: evaluation of the neuroprotective effect of CM derived from human glial progenitor cells and analysis of the main biological pathways and signaling cascades contributing to neuroprotection in a glutamate excitotoxicity model using transcriptomic analysis.

Materials and methods

Preparation of conditioned media from glial progenitor cells (CM-GPC)

The cellular material for obtaining the conditioned media from glial progenitor cells preparation (CM-GPC) consisted of glial progenitor cells previously derived from induced pluripotent stem cells of a healthy donor according to the protocol described in [15]. GPCs were cultured in a media of the following composition: 1% N2, 1 mM L-glutamine, 50 U/mL penicillin/streptomycin (PanEco, Russia), 1% fetal bovine serum (Gibco, USA), supplemented with growth factors: 10 ng/mL FGF-2, 20 ng/mL EGF, and 20 ng/mL CNTF (Peprotech, USA). Upon reaching 80–90% confluence, the cells were washed three times with Hanks' solution (PanEco, Russia) to remove residual serum and transferred to a media devoid of serum, N2, and growth factors for 16 hours. After incubation, the conditioned media was collected and then centrifuged for 30 minutes at 10,000 × g to remove apoptotic bodies and debris. Subsequently, the supernatant was collected and subjected to sequential vacuum filtration through membrane filters with pore diameters of 0.45 μm and 0.22 μm (Millipore, Germany), after which it was concentrated by tangential ultrafiltration using a Labscale TFF system (Millipore,

Germany) with a Pellicon XL 3 kDa cartridge to a final protein concentration of 250 μg/mL. The finished CM preparations were stored at –80 °C. Protein concentration was measured using a ready-to-use Quick Start™ Bradford Protein Assay kit (Biorad, USA).

Establishment of a Neuroglial Culture and Induction of Glutamate Excitotoxicity In Vitro

Modeling of glutamate excitotoxicity *in vitro* was performed on primary neuroglial cultures of the cerebral cortex isolated from newborn rat pups (P0) [15]. Cells were cultured in Neurobasal medium (Gibco, USA) supplemented with 1 mM L-glutamine, 50 U/mL penicillin-streptomycin (PanEco, Russia), and B27 supplement (Gibco, USA). On day 10 after plating, CM-GPC preparations were added to the cultures at final concentrations of 5, 15, and 45 μg/mL. An equivalent volume of serum-free DMEM/F12 medium was used as a negative control to exclude any neuroprotective effect of the vehicle medium itself. On the following day (11 DIV), glutamate excitotoxicity was induced according to a previously described protocol [15]. The experimental protocol involved the use of three types of solutions:

(1) a normalized buffer (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES) for washing the cultures;

(2) a glutamate solution (100 μM glutamate, 10 mM glycine, 2 mM $CaCl_2$ in normalized buffer);

(3) a calcium-free solution (normalized buffer with 2 mM $MgCl_2$ and without $CaCl_2$ addition).

The modeling procedure was as follows: after removing the growth medium and washing with solution (1), solution (1) was left in the control wells, while the experimental wells received solution (2), followed by incubation for 1 hour. After incubation, the cells were washed twice with solution (3) and the previously collected native culture medium was returned to the wells.

Assessment of Cell Viability by a Biochemical Method (MTT Assay)

Assessment of cellular metabolic activity was performed using the MTT assay. For this purpose, the reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Merck, Germany) was preliminarily dissolved in dimethyl sulfoxide (DMSO, ThermoFisher, USA) and added to the culture medium to a final concentration of 0.1 mg/mL. The samples were incubated for 1 hour in a CO_2 incubator at 37 °C. Following incubation, the supernatant was removed, and the formed formazan crystals were dissolved in DMSO. The absorbance of the resulting solution was measured using a ClarioStar plate spectrophotometer (BMG Labtech, Germany) at 520 nm and 690 nm. The final optical density values were expressed as a percentage relative to the control (intact cultures were taken as 100%).

Statistical analysis

Each experiment was repeated at least four times using cultures derived from unrelated cells. Data processing was performed using GraphPad Prism 10 software (GraphPad Software, USA). Comparisons between groups were conducted using one-way analysis of variance (one-way ANOVA) followed by Dunnett's post hoc test for multiple comparisons correction. The Shapiro–Wilk test was used to assess the normality of distribution. Final values are expressed as mean ± standard deviation (Mean ± SD).

Transcriptomic Analysis

Isolation and Purification of Total mRNA

To identify intracellular signaling pathways modulated by the CM-GPC preparation under conditions of glutamate excitotoxicity, total RNA sequencing was performed. CM-GPC was added to the cultures 24 hours prior to damage induction, and glutamate stress was induced the following day according to the procedure described above. Four hours after completion of the glutamate incubation, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The obtained samples were stored and transported at –80 °C.

Residual genomic DNA was removed from the RNA preparations using the Turbo DNA-Free Kit (Thermo Fisher Scientific, USA) in a reaction volume of 50 μL. Subsequent nucleic acid purification was performed using Agencourt RNAClean XP reagent (Beckman Coulter, USA), strictly following the

manufacturer's instructions. RNA concentration was measured fluorometrically using the Quant-iT RiboGreen RNA Assay Kit (Thermo Fisher Scientific, USA). The integrity and quality of the isolated RNA were assessed on an Agilent bioanalyzer using Agilent RNA 6000 Pico Chips (Agilent Technologies, USA).

Preparation of Transcriptomic Libraries and RNA Sequencing

Procedures for library preparation and bioinformatic analysis were performed according to approaches previously validated in our studies [16]. Starting aliquots of 250 ng of total RNA were used for transcriptomic library synthesis. Libraries were prepared using a poly(A)-mRNA enrichment module on magnetic particles (NEBNext) and the KAPA RNA Hyper Kit (Roche, Switzerland) according to the manufacturer's instructions. Intermediate RNA purification was performed using RNA Clean XP, and final purification of the completed libraries was carried out using Agencourt AMPure XP magnetic particles (Beckman Coulter, USA). Fragment size distribution and quality parameters of the libraries were determined using the Agilent High Sensitivity DNA Kit (Agilent Technologies); quantification was performed fluorometrically using the Quant-iT DNA Assay Kit, High Sensitivity (Thermo Fisher Scientific). The resulting libraries were pooled in equimolar ratios to a final pool concentration of 750 pM. Sequencing was performed on a NextSeq 1000 platform (Illumina) using a NextSeq 1000/2000 P2 Reagents Kit (200 cycles) v3 with the addition of 2% PhiX control.

Bioinformatic Processing of Sequencing Results

Initial read quality assessment was performed using the FastQC utility [17]. Low-quality regions and technical adapter sequences were removed using Trimmomatic [18]; additional read processing was conducted using the Cutadapt utility with the parameter — nextseq-trim 20. Quantification of gene expression levels was performed using Salmon (mapping-based mode) [19]. The complete transcript set for the R. norvegicus genome assembly rn6 from the ENSEMBL database version 106 was used as the reference transcriptome. During reference transcriptome indexing, the genomic nucleotide sequence was included as a decoy sequence to prevent erroneous read mapping. Aggregation of expression values from the individual transcript level to the gene level was performed using the R package tximport [20]. Statistical analysis of differential gene expression between compared groups was performed using the R package edgeR with the glmLRT test [21]. The threshold for statistical significance was set at FDR < 0.05.

Results and discussion

Assessment of the Neuroprotective Effect of CM-GPC

Twenty-four hours after exposure to 100 μM glutamate on primary rat cortical neuron cultures, a significant decrease in the number of viable cells was recorded. Their viability relative to the intact control (taken as 100%) decreased to 61.7 ± 12% (Figure 1). In this case, pre-incubation of cultured neurons for 24 hours with CM-GPC at concentrations of 5, 15, and 45 μg/mL led to a significant increase in the number of viable neurons to 86.2 ± 15% ($p = 0.0005$) for 15 μg/mL and to control values upon addition of 45 μg/mL ($p < 0.0001$). Furthermore, upon addition of DMEM/F12 medium alone as a negative control (since glial progenitor cells were incubated in DMEM/F12 during conditioned medium preparation), no difference was observed compared to the group incubated with glutamate only (Figure 1).

Transcriptomic analysis of neuroglial cultures in a model of glutamate excitotoxicity

Analysis of differential gene expression upon addition of conditioned medium to the cultures against the background of glutamate exposure

The addition of conditioned medium led to a statistically significant change in the expression of 652 genes: of these, 173 exhibited upregulated transcription in the group treated with conditioned medium from glial progenitor cells (CM-GPC), whereas 479 genes, conversely, showed downregulated expression ($p < 0.05$, FC > 1.5; FC < –1.5).

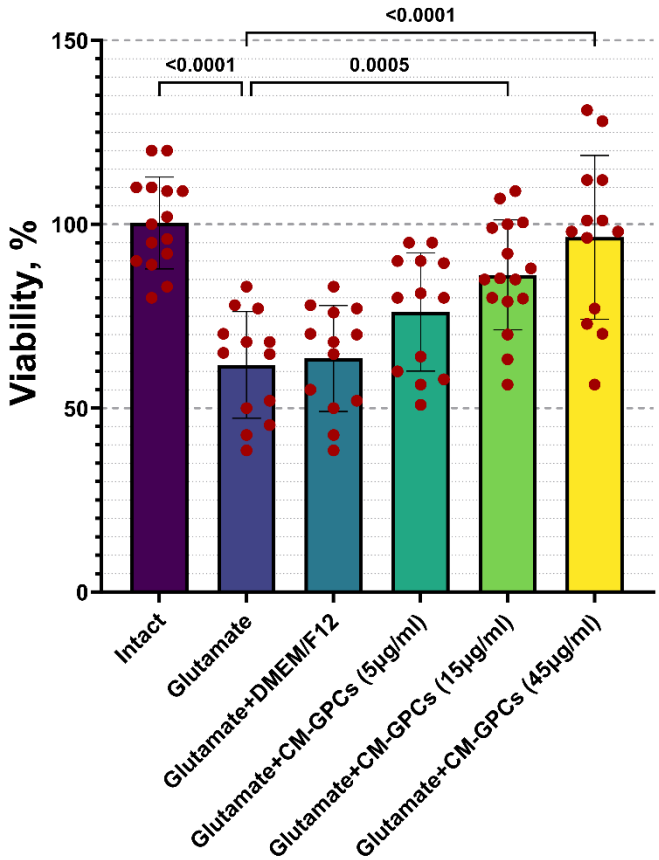


Figure 1. Determination of the viability of cortical neurons using the MTT-test

Note: Intact — intact group, Glutamate — addition of glutamate (100 μM), Glutamate+DMEM/F12 — addition of DMEM/F12 24 hours before glutamate, Glutamate+CM-GPCs (5, 15, 45 μg/ml) — addition of CM-GPCs (5, 15, 45 μg/ml) 24 hours before glutamate. Data were analyzed using one-way ANOVA with Dunnett's post-hoc test. Data are presented as means and standard deviations.

Functional annotation of differentially expressed genes using the Gene Ontology and KEGG databases revealed their significant enrichment in categories related to regeneration, wound healing, negative regulation of apoptosis, response to oxidative stress, and response to growth factors. Specifically, the upregulated genes were grouped into the following functional clusters: positive regulation of cell adhesion (24 genes, including *Fbln2* and *Fstl3*, which encode proteins involved in brain extracellular matrix remodeling during reparative processes; *Lgals1* and *Fn1* — the genes encoding galectin-1 and fibronectin, which are essential for the growth of damaged axons; *Efemp2*, whose product ensures the structural organization of the extracellular matrix [22–25]); wound healing (16 genes, including *Nog* and *Igf1*, which control neuronal differentiation and survival; *Csrp1* — a regulator of the actin cytoskeleton; *Timp1* — a metalloproteinase inhibitor that activates anti-apoptotic genes of the Bcl-2 family; *Ccn1*, which stabilizes synapses during brain development and under neurotoxic conditions [26–30]); regeneration (12 genes: *Spp1*, required for mitochondrial function and activated upon neuronal injury; *Ptpru*, whose phosphatase dephosphorylates β-catenin, thereby affecting intercellular adhesion and stabilizing focal contacts; *Bace2* — an α-secretase that reduces β-amyloid production and its associated toxicity [31, 32]); gliogenesis (11 genes: *Olig1* — a transcription factor of oligodendrocytes that promotes astrocyte survival and axonal growth; *Metrn*, which acts as a neurotrophic factor, stimulating

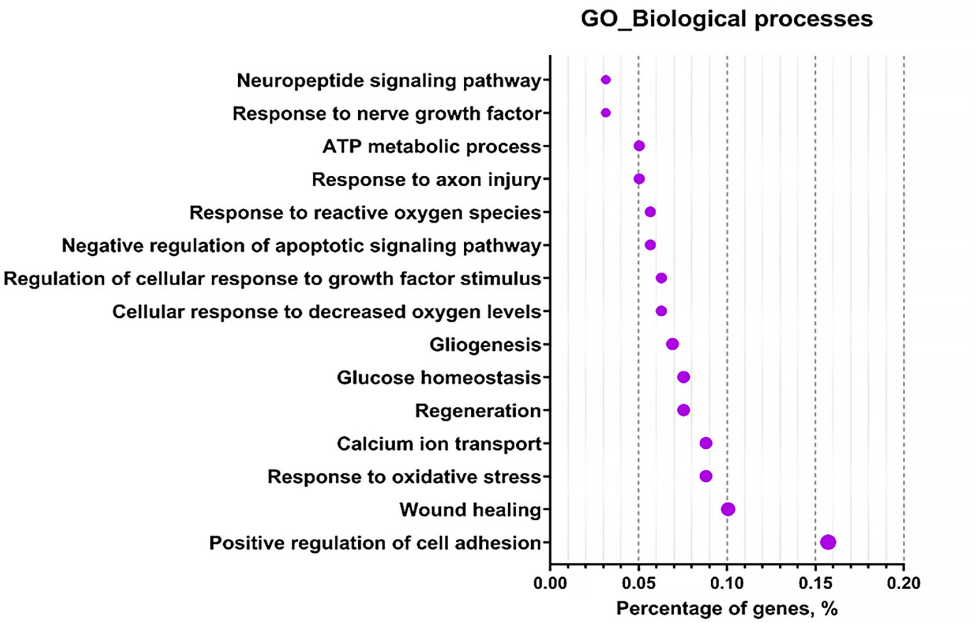
the proliferation of neural precursors and neuroblast migration; *Apcdd1* — a potent inhibitor of the Wnt/β-catenin and BMP signaling pathways, which play a key role in neuronal development and differentiation [33–35]); and response to axon injury (8 genes: *Spp1*, the same osteopontin whose expression is upregulated in motoneurons upon axonal damage and stimulates regeneration; *Tspo* — the gene encoding the 18 kDa mitochondrial translocator protein, which is involved in neurosteroidogenesis [36, 37]). The increased expression of these gene groups indicates that CM-GPC initiates programs aimed at maintaining intercellular adhesion and restoring damaged processes, which is critically important for preserving the integrity of neuronal networks and the overall viability of nerve cells.

The presence of the CM-GPC preparation also contributed to an increase in the expression of genes associated with the response to oxidative stress, the regulation of cellular respiration, and apoptosis. These genes were reliably classified into the following categories: Response to oxidative stress (14 genes: *Gpx3* — the gene encoding glutathione peroxidase 3, one of the key antioxidants; *G6pd* — the gene for glucose-6-phosphate dehydrogenase, which plays a crucial role in neurons by ensuring NADPH production; *Txn1* — the gene for thioredoxin 1, another antioxidant in nerve cells; *Atox1* (Antioxidant 1) encodes a chaperone protein that plays a critical role in copper transport in neurons to maintain homeostasis and protect against oxidative damage [38–41]); Calcium ion transport (14 genes: *Rgs4* — its protein interacts with the G-protein of metabotropic receptors, reducing calcium influx; Bestrophin-1 (*Best1*) is a calcium-activated anion channel that controls the transmission of calcium signals from glutamate [42, 43]); Glucose homeostasis (12 genes: *Serpinf1* — the gene for pigment epithelium-derived factor (PEDF), which maintains glucose homeostasis in neurons; *VGF* — a gene induced by neurotrophins that regulates energy balance and glucose metabolism [44, 45]); Cellular response to decreased oxygen levels (10 genes, including *Higd1a* (Hypoxia-inducible gene domain family-1a), a mitochondrial protein that plays a crucial role in ensuring neuronal survival under hypoxic stress [46]); Response to reactive oxygen species (10 genes, including the aforementioned peroxidase and thioredoxin genes); Negative regulation of apoptotic processes (9 genes: *Hspb1* (heat shock protein beta-1) — a critical chaperone gene that acts as a neuroprotectant by maintaining proper protein folding; *Ier3*, a gene encoding a protein activated by ER stress, which plays a role in protecting cells from apoptosis; *Uchl1* — a gene encoding a protein that contributes to the recovery process after neuronal injury [47, 48]); and ATP metabolic processes (8 genes). The increased expression of these gene groups ndicates that the presence of CM-GPC promotes the activation of protective mechanisms in response to oxidative stress and cell death against the background of glutamate toxicity. These mechanisms are associated with the stabilization of cellular respiration and ATP production, as well as the activation of antioxidant production, which facilitates the elimination of reactive oxygen species. Furthermore, genes were identified that reduce the influx of free calcium ions into the cytoplasm, thereby inhibiting the intracellular stress that leads to apoptosis.

Genes with increased expression related to the response to growth factors were also identified. Among the categories, the following can be highlighted: Regulation of cellular response to growth factor stimuli (10 genes: *Fstl3* (follistatin-like 3) — a secreted glycoprotein that functions as a regulator of TGF-β family signaling pathways; *Dok5* — a gene required for the NT-3 signaling pathway, which helps block TrkC-induced neuronal apoptosis; *Tgfb1i1* — the gene encoding the focal adhesion protein Hic-5, associated with the structural, neuroprotective, and regenerative role of the TGF-β1 signaling pathway; *Fgfbp3* (fibroblast growth factor-binding protein 3) — a secreted protein responsible for neurodevelopment, particularly for the formation and regeneration of motor neurons, through the modulation of FGF signals [49–52]). The activated genes also included 6 genes associated with the response to nerve growth factor, among them *Coro1A*, whose product is required for proper axon navigation and branching during development [53], and 6 genes involved in neuropeptide signaling. The increased expression of these gene groups demonstrates that the

addition of CM-GPC activated processes associated with the effects of various growth factors. This suggests that CM-GPC contains growth factors that activate intracellular signaling cascades within nerve cells, promoting survival and regeneration (Figure 2*a*).

For a more detailed investigation of the intracellular signaling pathways activated by the preparation, the upregulated genes were mapped onto canonical signaling cascades using the KEGG database. The analysis revealed significant enrichment in the categories "Phagosome" (10 genes), "Cell adhesion molecules (CAMs)" (7 genes), and "TGF-β signaling pathway" (6 genes). These results are consistent with the classification data based on biological processes, as the identified cascades are functionally associated with intercellular adhesion and growth factor signal transduction (Figure 2*b*).



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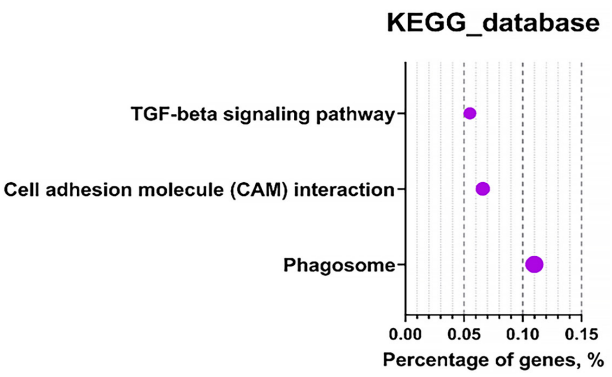

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Figure 2. Results of classification of genes whose expression was increased in the group with the addition of CM-GPC

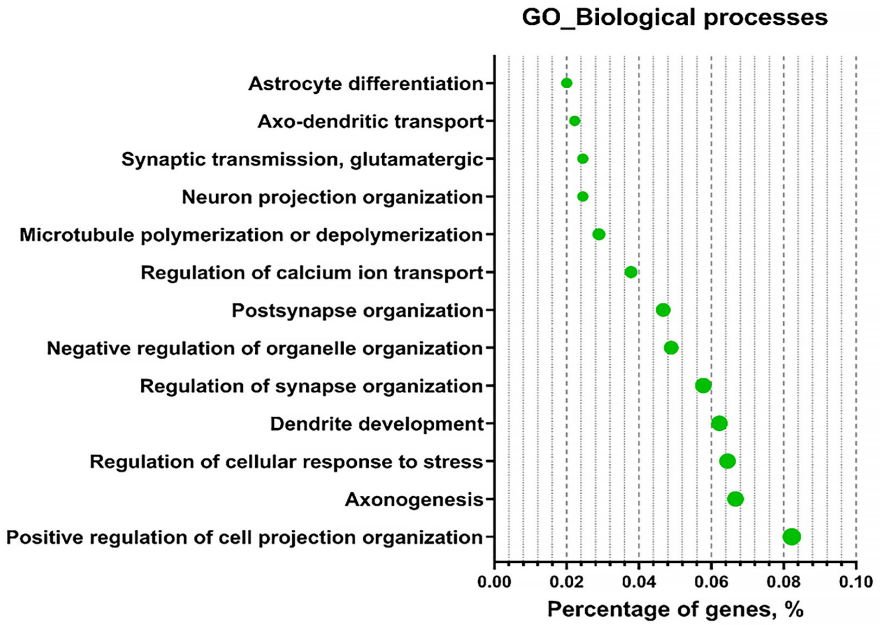
Note: ***a*** — classification with the Gene Onthology database; ***b*** — classification with the KEGG database. The number of genes is indicated on the X-axis, and the selected signaling pathways are shown on the Y-axis.

Conversely, the genes whose expression was found to be decreased in the group treated with CM-GPC were functionally grouped into categories associated with the processes of neuronal activation during synaptic transmission. Among these, the following categories stood out: positive regulation of cellular projection organization (37 genes, including *Clip1*, encoding the CLIP-170 protein, a multifunctional regulator of microtubule dynamics that tracks their plus ends; *Zeb2*, which is actively expressed in the peripheral nervous system and controls process branching; *Sirt1*, which stimulates neurite elongation and branching; *Enpp2*, also responsible for neurite branching; *Shtn1*, involved in the generation of asymmetric intracellular signals that determine neuronal polarization and neurite outgrowth [54–58]); axonogenesis (30 genes: *Tnr*, whose product modulates interactions with extracellular matrix components, which can lead either to stable adhesion and differentiation or to repulsion and inhibition of neurite growth; *Ephb1* — a regulator of directed axon migration; *Sema5a* — a bifunctional axon guidance signal whose activity depends on proteoglycan sulfation; *Plxnb3* (plexin-B3), a receptor for SEMA5A involved in axon growth direction and cell migration; *Nfasc* (neurofascin) — an ankyrin-binding cell adhesion protein implicated in neurite outgrowth, synaptogenesis, myelination, and neuro-glial interaction; *Robo1*, a receptor for SLIT1/SLIT2 that mediates responses to guidance cues during axonal navigation [59–64]); regulation of cellular response to stress (29 genes, including *Tmem33* — an ER stress-inducible molecule that modulates the unfolded protein response cascade and associated apoptosis; *Nfe2l1* — another gene activated under ER stress conditions; *Rock1* and *Rock2*, associated with the execution of the apoptotic program [65–67]); dendrite development (28 genes: *Mef2c* and *Mef2a*, which regulate the formation and elimination of dendritic spines, especially under conditions of excitotoxicity; *Ppp1r9a* (neurabin-1), which interacts with F-actin and is involved in neurite formation [68, 69]); regulation of synapse organization (26 genes: *Septin11*, which plays a role in neuronal cytoarchitecture, including dendritic branching and spine formation, as well as in GABAergic synaptic transmission; *Afdn* — a component of the adhesion system that presumably acts together with the cadherin-catenin complex in the formation of intercellular contacts [70, 71]); calcium ion transport (18 genes: *Cacna1c, Cacna1d, Cacna2d1, Ryr2*, encoding key ion channels and transporters that control Ca^{2+} entry into the neuronal cytoplasm [72]); postsynapse organization (21 genes: *Homer1*, a scaffolding protein of the postsynaptic density; *Tanc2*, a gene encoding a dendritic spine protein involved in the formation of stable postsynaptic contacts [73, 74]); negative regulation of organelle organization (22 genes); microtubule polymerization/depolymerization (17 genes: *Mapt, Map2, Map1a, Kif21a*, whose products regulate microtubule homeostasis and dynamic reorganization [75]); neuronal projection organization (13 genes); axo-dendritic transport (11 genes, predominantly encoding motor proteins of the kinesin family that mediate organelle transport along processes: *Kif5c, Kif3a, Kif21b, Kif2a, Kif21a, Kif5b*); as well as processes related to the glial component, in particular astrocyte differentiation (10 genes: *Plp1* — the major myelin proteolipid protein of the CNS; *Qki* — a regulator of myelination [76, 77]) (Figure 3*a*).

Analysis of signaling cascades using KEGG confirmed the suppression of a few key pathways: cAMP signaling pathway (15 genes), MAPK signaling pathway (15 genes), axon guidance (14 genes), regulation of actin cytoskeleton (14 genes), glutamatergic synapse (9 genes), dopaminergic synapse (9 genes), and long-term potentiation (6 genes) (Figure 3*b*).

The regenerative potential of the brain is extremely limited, as the complete restoration of lost nervous tissue remains unattainable to date. This circumstance significantly complicates the treatment of neurological diseases, including ischemic stroke, traumatic brain injury, traumatic spinal cord injuries, and progressive neurodegenerative pathologies [78, 79]. Existing therapeutic approaches for these conditions are primarily aimed at alleviating symptoms and improving the quality of life of patients but do not ensure the recovery of lost functions [80–82]. In this regard, there is an urgent need for the development of fundamentally new therapeutic strategies for

diseases of the central nervous system. Cell therapy is regarded as one of the most promising directions, largely driven by progress in the field of induced pluripotent stem cells (iPSCs) and the development of protocols for their directed differentiation into various cell types, including neuronal and glial lineages. Numerous studies have confirmed the efficacy of cell therapy with various routes of stem cell administration [83–85]. However, studies have shown that the therapeutic effect is largely attributable to the paracrine activity of the transplanted cells rather than their replacement of vacated cellular niches [86]. Thus, the use of conditioned medium may serve as a promising alternative to the direct implantation of stem cells. It contains growth factors, neurotrophins, and exosomes with therapeutic potential [87, 88].



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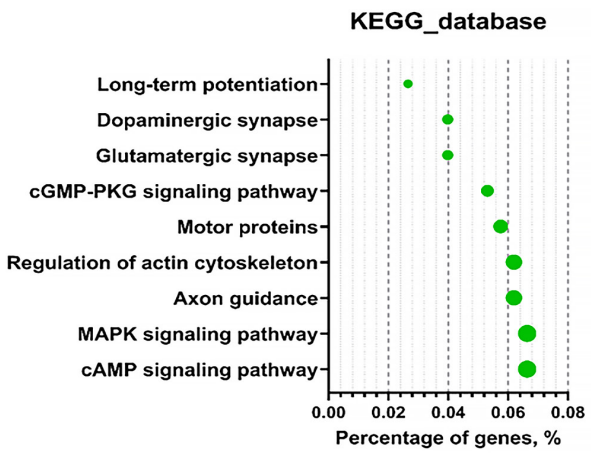

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Figure 3. Results of classification of genes whose expression was decreased in the group with the addition of CM-GPC

Note: a — classification with the Gene Onthology database; *b* — classification with the KEGG database The number of genes is indicated on the X-axis, and the selected signaling pathways are shown on the Y-axis.

To date, the therapeutic effects of conditioned medium from many cell lines have been studied; however, the role of the glial cell conditioned medium in the restoration of brain function in neurological diseases remains insufficiently explored. One of the key characteristics of glia is their ability to exert neuroprotective effects through paracrine mechanisms mediated by the secretion of extracellular vesicles, cytokines, and other signaling molecules [89]. Through these factors, glial cells regulate CNS activity by performing metabolic, structural, and homeostatic functions, including the control of synaptic transmission, maintenance of neuronal homeostasis, and regulation of blood-brain barrier permeability [90]. The activation of these mechanisms contributes to the maintenance of normal neuronal activity and ensures the protection of nervous tissue from damaging influences. Consistent with previous studies on PC12 cells, neuroprotective properties of conditioned medium from glial progenitor cells against the toxic action of glutamate have also been demonstrated in rat cortical neurons. The decrease in the level of cell death observed in the presence of CM is consistent with the transcriptomic data, which revealed increased expression of genes associated with the negative regulation of apoptosis, including genes encoding chaperone proteins. Furthermore, it was previously shown that the presence of CM reduces oxidative stress in PC12 cells exposed to glutamate *in vitro* and promotes the increased expression of a number of genes responsible for the activation of the antioxidant defense system, among them *Hmox1* [91]. The results obtained in the present study are consistent with the transcriptomic data showing that the presence of CM under conditions of glutamate excitotoxicity is accompanied by increased expression of genes classified according to Gene Ontology into the categories “Response to oxidative stress” and “Response to reactive oxygen species”. Among the activated genes were those encoding glutathione peroxidases, thioredoxins, and a number of antioxidant proteins. Moreover, these results align with evidence on the protective functions of glial cells and their ability to stimulate the synthesis of antioxidants, leading to the protection of neurons from oxidative stress [92, 93]. Presumably, such a neuroprotective effect may be associated with the activation of TGF-beta growth factor signaling cascades in nerve cells, as the addition of CM increases the expression of the *Tgfb1* gene. According to studies, this growth factor is secreted by astrocytes and, by interacting with its receptors on the plasma membrane of neurons, activates the c-Jun/AP-1 signaling cascade, which leads to enhanced neuronal survival by activating the antioxidant defense system [94].

Furthermore, according to the transcriptomic data, the addition of CM reduces the expression of genes classified by the Gene Ontology database into the categories “Regulation of calcium ion transport into the cell”, “Regulation of synapse organization”, and “Postsynaptic organization”, which included genes encoding calcium channel proteins within the postsynaptic membrane. A number of genes with decreased expression upon CM addition were assigned by the KEGG database to the groups “Glutamatergic synapse” and “Long-term potentiation”, which included genes of glutamate receptors. These results are consistent with previously obtained data on the effect of CM proteins on calcium transport into neurons during glutamate exposure. Using real-time calcium imaging, it was demonstrated that proteins of the conditioned medium from glial progenitor cells reduce the efficiency of calcium ion entry into the neuronal cytoplasm upon glutamate exposure [95]. Such an effect may be associated with the decreased expression of genes encoding calcium transporters and glutamate receptors and a corresponding reduction in these channels on the postsynaptic membrane of cells, which also serves as a protective mechanism against glutamate hyperstimulation and the pathological accumulation of free calcium in the cell cytoplasm, consistent with the findings of other researchers. It has been shown that astrocytes protect neurons from excessive calcium influx into the cytosol and subsequent excitotoxicity by maintaining ionic homeostasis, especially after glutamate excitotoxicity, through the regulation of the ionic environment of neurons and the synthesis of neurotrophic factors that promote neuronal survival [94–97].

The investigation of the mechanisms underlying the neuroprotective action of conditioned media derived from various cellular sources remains one of the key challenges in modern cell biology and neurobiology. The data accumulated to date indicate that conditioned media possess a number of advantages over other therapeutic agents, which is attributable to their multi-target mode of action and their ability to modulate a broad spectrum of intracellular signaling cascades. A detailed elucidation of the mechanisms by which CM influences nerve cells provide a foundation for the development of innovative and effective therapeutic agents intended for the treatment of neurological diseases.

Conclusion

The obtained data indicate that human CM-GPC exerts a neuroprotective effect mediated through the activation of mechanisms associated with counteracting oxidative stress, the suppression of calcium ion entry into the cytosol, and the stimulation of growth factor signaling pathways, in particular transforming growth factor beta (TGF-β). The use of this therapeutic agent allows for the partial compensation of the consequences of pathological glutamate excitotoxicity, which opens up prospects for improving the efficacy of therapy for neurodegenerative diseases.

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Исследование нейропротективного действия кондиционированной среды от глиальных производных, полученных из индуцированных плюрипотентных стволовых клеток человека, на модели глутаматной эксайтотоксичности

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Аннотация. Актуальность. В современной биомедицине стратегии лечения неврологических расстройств все чаще фокусируются на разработке мультитаргетных терапевтических подходов. Среди перспективных направлений выделяются препараты на основе кондиционированных сред. В данной работе впервые осуществлен полногеномный транскриптомный анализ дифференциальной экспрессии генов клеток первичной культуры кортикальных нейронов, проинкубированных с кондиционированной средой от глиальных клеток-предшественников человека на фоне индукции глутаматной эксайтотоксичности. Данное исследование позволяет предположить потенциальные молекулярные механизмы наблюдаемого защитного действия. *Материалы и методы*. Кондиционированную среду собирали от культуры глиальных предшественников человека и подвергали очистке и концентрированию методом тангенциальной ультрафильтрации. Эксайтотоксическое воздействие глутамата моделировали на первичных кортикальных нейронах новорожденных (P0) крыс путем внесения натриевой соли глутамата в конечной концентрации 100 мкМ. Жизнеспособность клеток оценивали

с помощью МТТ-теста. Транскриптомные библиотеки секвенировали на платформе NextSeq 1000 (Illumina, США), контроль качества прочтений производили с помощью утилиты FastQC. Для оценки дифференциальной экспрессии генов был использован R-пакет edgeR (значение FDR менее 0,05). *Результаты и обсуждение*. Исследование показало, что добавление 45 мкг/мл кондиционированной среды восстанавливает уровень выживаемости клеток до контрольных значений, то есть препарат обладает дозозависимым нейропротекорным действием. По результатам тракскриптомного профилирования добавление среды на фоне глутаматного стресса приводило к повышению экспрессии 173 генов и подавлению 479 генов. Дифференциально экспрессированные гены статистически достоверно кластеризовались по функциональным группам. К числу наиболее представленных биологических процессов, связанных с активированными генами, относились регенерация, межклеточная адгезия, ответ на окислительный стресс, ингибирование апоптоза и активация TGF-β-сигнального каскада. Гены, экспрессия которых снижалась, преимущественно участвовали в транспорте ионов кальция, глитаматергической нейропередаче и регуляции роста и ветвления нейритов. Полученные данные позволяют предположить, что компоненты кондиционированной среды запускают в нейронах программы антиоксидантной защиты и восстановления, одновременно ограничивая чрезмерную активацию глутаматных рецепторов и приток ионов кальция. *Выводы.* Исследуемый препарат кондиционированной среды обладает нейропротекторными свойствами в условиях глутамат-индуцированной эксайтотоксичности. Предположительно, в основе данного эффекта лежит одновременная индукция экспрессии генов, способствующих выживанию, и подавление генов, обеспечивающих глутаматергическую нейротрансмиссию. Совокупность полученных данных подтверждает перспективность дальнейшей разработки лекарственных средств на базе кондиционированных сред глиальных клеток-предшественников человека для терапии неврологических расстройств.

Ключевые слова: кондиционированная среда, глутаматная эксайтотоксичность, транскриптомный анализ, глиальные клетки-предшественники, иПСК

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