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Original Article

Proteomic changes and apoptosis involved in the human meningothelial cells respond to glutamate stress

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Abstract: Meningothelial cells (MECs) play a crucial role in maintaining the integrity of the optic nerve-cerebral spinal fluid (CSF) barrier in the retrobulbar part of optic nerve, but the detailed functions of MECs are far from being understood. Our present study investigated the excitatory effect induced by glutamate on the cellular function of MECs. We determined cell viability, cell apoptosis, caspase9 activity, and proteomic changes after MECs were exposed to glutamate. We found that MECs viability was inhibited following exposure to glutamate at different concentration at different time point. Increased apoptosis as well as elevated caspase9 level were found in the glutamate-treated cells compared with controls. Proteomic analyses showed that 7 proteins were identified from 26 differentially expressed proteins through TOF/MS. Among those, transketolase (TK), collapsin response mediator proteins (CRMPs), GAIP-interacting protein (GIPC1), and lamin (LMNA) were presented to be down-regulated. Cathepsin B (CatB), chloride intracellular channel 1 (CLIC1), and actin regulatory protein (CAPG) showed a specific expression in non-glutamate-exposed cells. These differential proteins potentially contribute to the cellular functions such as proliferation and apoptosis. The work presented herein demonstrates that MECs are vulnerable to glutamate stress by changing their physiological function, which probably influences the integrity of optic nerve-CSF barrier.

Keywords: Glutamate, meningothelial cells, proliferation, casepase9, proteomic changes

Introduction

Optic nerve diseases cause visual impairment; however, the pathophysiology of these disorders is not yet fully understood. Functions of subarachnoid space and cerebral fluid (CSF) dynamics were recently demonstrated to be essential in the pathogenesis of optic nerve disorders [1-3].

Optic nerve, a white matter tract of the central nerve system extending into the orbit, is enclosed with dural, arachnoid as well as pial sheaths. Subarachnoid space surrounding the retrolaminar optic nerve behind the eye is filled with CSF. Meningothelial cells (MECs) present as a major cellular component of three meninges of the optic nerve and build an optic nerve-CSF barrier that prevents the free flow of CSF from the subarachnoid space. MECs are supposed to be one of key elements involved in pathophysiological mechanisms of the subarachnoid space and considered to have a capacity of removing active molecules or waste products from the subarachnoid space by ingesting large amounts of particulate matter to modulate CSF composition [4]. MECs are highly active to pathologic conditions [1, 2]. Impairment of MECs might result in the failure of cellular function and induce the accumulation of waste products in the subarachnoid space of optic nerve, which probably contributes to optic nerve diseases such as progressive anterior, posterior ischemic optic neuropathy and optic nerve sheath compartment syndrome [5-7].

Glutamate, a major component of CSF and a crucial excitatory neurotransmitter in the central nervous system (CNS), exerts a series of pathophysiological processes [8]. Excessive release of glutamate can lead to excitotoxicity

in neurons, which may result in cellular calcium overload, mitochondrial membrane depolarization, caspase activation, nitrogen free radicals production, and even cell death [9, 10]. Many neurodegenerative conditions including Alzheimer's disease (AD), Parkinson's disease, stroke and traumatic brain injury have been reported to be associated with glutamate excitotoxicity [11, 12].

MECs, as the major cellular populations covering the orbital optic nerve sheath, directly contact the CSF and are therefore more susceptible to be affected by the alteration of the CSF components. The aim of our present work was to investigate the changes of physiological function of MECs subjected to glutamate-induced excitotoxicity and to identify proteins which were significantly altered in the stress.

Materials and methods

Cell culture

Meningothelial cell line (Ben Men cell line) was grown in 75-cm^2 flasks (Sarstedt, USA) and cultured in Dulbecco modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), penicillin/streptomycin (100 U/ml, 100 μg/ml; Sigma, Germany) until confluence. Cells were washed with phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) and trypsinized, centrifuged, and resuspended. Cell suspension was mounted and added to the 96-well plates (Falcon, USA) with cell concentration at 1×10^4 cells/well.

Cell viability assay

MECs were seeded in a 96-well plate (1×10^4 cells/well). Glutamate (Sigma-Aldrich, USA) was added to the culture medium at concentration raging from 200 μM to 1000 μM. Cell viability was determined by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-terazolium, inner salt] (MTS) Cell Proliferation Assay Kit (BestBio, China) after cells were exposed to glutamate for 24 h and 48 h respectively. Cell viablily was calculated by $(A_{treatment}\text{-}A_{blank})/(A_{control}\text{-}A_{blank}) \times 100\%$, where A represents the absorbance recorded at 490 nm.

Assessment of apoptosis

Cells were seeded in 96-well plates at a density of 1×10^4 cells/well. The cells incubated with medium alone were used as a control group; the experimental group was exposed to 600 μM glutamate. After the treatment for 24 h and 48 h respectively, cells were washed with cold PBS. The supernatant was removed and the cells were stained with 5 μl of Annxin-V-FITC and 10 μL of propidium iodide (PI) for 10 min at room temperature in the dark according to the manufacturer's instructions (KeyGEN BioTECH, China). The cells were observed under an invert fluorescence microscope (Olymbus, Japan), and semi-quantitative immunostaining analysis was evaluated.

Determination of the activity of caspase9

Cells were seeded in discs with 10 cm diameter (1×10^6 cells/disc). After MECs were subjected to glutamate (600 μM) for 24 h and 48 h respectively, the measurement of caspase9 activity was performed according to the manufacturer's instructions (Uscnlife Science & Technology Company, USA). The activity of caspase9 was quantified spectrophotometrically at 405 nm using an ELISA reader (Nanjing, China).

Protemic analysis

Reagents: Tris hydrochloride (Tris/HCl), urea, thiourea, dithiothreitol (DTT), 3[(3-Cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS), ethylenedinitrilotetraacetic acid (EDTA), glycerol, sodium dodecyl sulfate (SDS), iodoacetamide, bromophenol blue dye, silver nitrate solution, $K_3Fe(CN)_6$, trypsin, NH_4HCO_3, and α-cyano-4-hydroxycinnannic acid (CHCA) were obtained from Sigma-Alrich (USA). Acetonitrile anhydrous (ACN) was obtained from Fisher (USA). Trifluoroacetic acid (TFA) was obtained from Merck (Germany).

Extraction and quantification of proteins

Cells were seeded in discs with 10 cm diameter (1×10^6 cells/disc) and cultured in the presence of glutamate (600 μM). After incubation for 48 h, cells were collected, centrifuged and washed with PBS three times at room temperature.

Cell suspensions were thawed and centrifuged at 12000 rpm for 30 min at 4°C. The supernatant was collected for quantification of proteins in suspension using the Bradford assay. Cell pellets were resuspended in a 10-fold volume of lysis buffer solution containing 40 mM Tris/

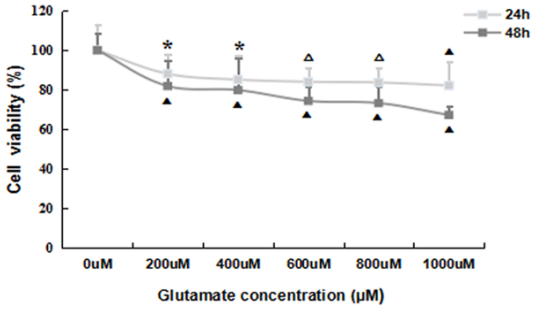

Figure 1. Effect of glutamate on the cell viability of meningothelial cells (MECs). Cell viability decreased compared with controls after MECs were exposed to glutamate at different concentration for 24 h and 48 h respectively. Results are expressed as mean ± SD (n=6). *P < 0.05; ΔP < 0.01; ▲P < 0.001.

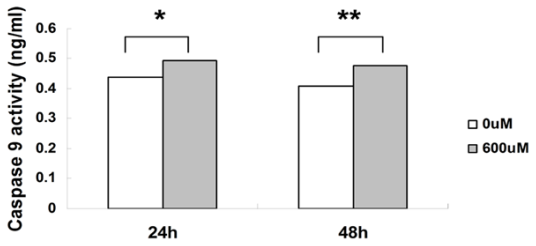

Figure 2. Influence of glutamate on the caspase9 activity of meningothelial cells (MECs). The caspase9 activity of MECs increased after cells were exposed to glutamate (600 μM) compared with control cells for 24 h and 48 h respectively. Data are presented as mean ± SD (n = 6) *P < 0.05; **P < 0.01.

HCl, 7 M urea, 2 M thiourea, 2 M CHAPS, 20 mM DTT, 1 mM EDTA, and 10 μl protease inhibitor cocktail. The mixtures were sonicated for 3 s following immersion for 10 s in an ice-water bath until the suspensions were limpid, and then 50 μl nuclease stock solution was added to each sample suspension. The suspensions were centrifuged at 4°C for 10 min at 12000 rpm. The supernatant fractions were extracted from the homogenized samples and used for 2-DE detection according to the instructions of non-interference protein assay kit (Millipore Sigma, USA).

Two-dimensional gel electrophoresis (2DE) separation

One-dimensional isoelectric focusing (IEF) was carried out with an Amersham Biosciences Ettan IPGphor IEF system (GE Healthcare, USA). Samples (100 μg) were loaded using 13-cm pH-3-10 IPG strips. IEF was performed at 30 V for 12 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 8 h, and 500 V for 4 h. After IEF, the IPG strips were equilibrated with equilibration buffer containing 50 mmol/l Tris-HCl, 6 mol/l urea, 30% glycerol, 2% SDS, and 1% DTT for 15 min at room temperature, followed by 2.5% iodoacetamide instead of 1% DTT in equilibration buffer for another 15 min and subsequently applied to SDS-polyacrylamide gel electrophoresis (PAGE) gels. The second-dimensional SDS-PAGE was performed until the bromophenol blue dye front reached 0.5 cm from the bottom of the gel. The gels were run in triplicate for each sample and stained with silver nitrate solution. For image analysis, gels were scanned at a resolution of 300 dpi, and Image Master $_{TM}$ 2D Platinum (Version 7.0) (GE Healthcare, USA) was used to detect altered protein expression levels.

Protein identification by MALDI-TOF MS

Protein spots were excised from gels, destained, and washed until the gels became clear. Gel plugs were destained using 30 mmol/l $K_3Fe(CN)_6$ and 100 mmol/l NaS_2O_3 mixed in a 1:1 ratio, and then incubated in the dark for 30 min followed by 3×15 min H_2O washes. Spots were dried by lyophilization and digested for 20 h in 10 ng/ml trypsin/0.1 mol/l NH_4HCO_3. Peptides were extracted three times with 50% ACN - 0.1% TFA and dried in a vacuum. The peptide mixture was dissolved in 0.1% TFA and desalted using a C18 ZipTip (Millipore, Bedford, MA). The eluted peptides in 0.1% TFA/50% ACN mixed with an equal volume of 0.1% TFA/30% ACN saturated with CHCA solution were applied onto the target and air-dried. The peptide mixtures from samples were analyzed by 4800 MALDI-TOF MS Proteomics Analyzer (Applied Biosystems Inc, Foster City, CA) with TOF/TOF Optics Applied Biosystems. Tandem mass spectrometry (MS/MS) was used to search protein identity from NCBI human database using Mascot search engine.

Statistical analysis

ANOVA was used for analysis of MECs viability, caspase9 activity, and apoptotic cells. Independent *t*-test was performed for a comparison between two groups as needed. Statistical analyses were conducted with SPSS 18.0 statistical analysis software (SPSS Inc., Chicago,

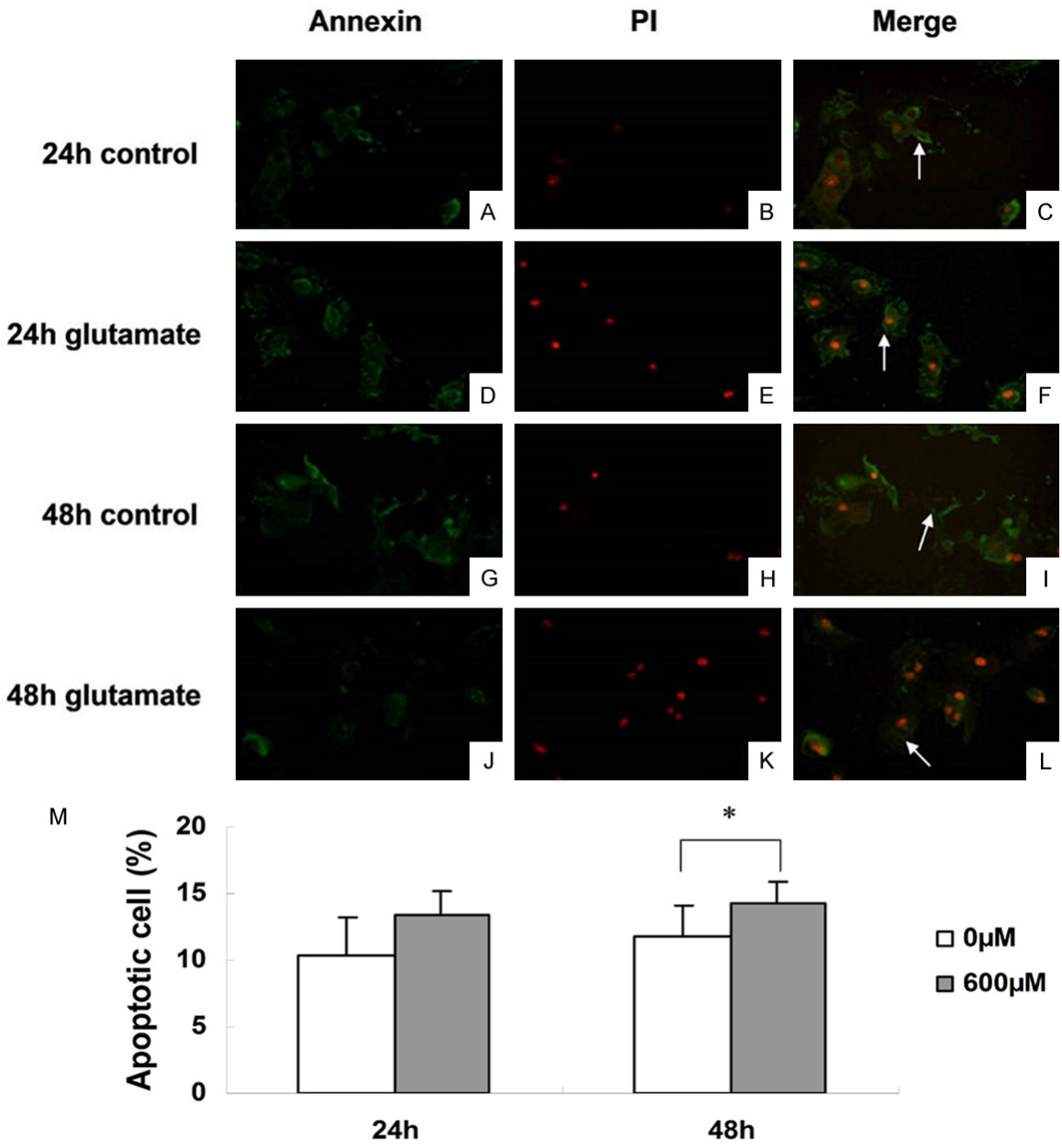

Figure 3. Glutamate-induced cell apoptosis in meningothelial cells (MECs). Cells were treated with 600 μM glutamate for 24 h and 48 h respectively and then subjected to annexin-V and propidium iodide (PI) double- staining for cell apoptosis detection. Each white arrow indicates apoptotic cells. (24 h control, see A-C; 48 h control see G-I; 24 h glutamate-treated cells, see D-F; 48 h glutamate-treated cells, see J-L). (M) is the percentages of apoptosis for the glutamate-exposed and unexposed cells. Data are presented as mean ± SD. *P < 0.05.

IL), with data presented as mean ± SD. Statistical significance was defined as $P < 0.05$.

Results

Glutamate suppressed MECs viability

To investigate the effects of glutamate on the cell viability of MECs, cells exposed to various concentrations of glutamate from 200 to 1000 μM for 24 h and 48 h were detected by MTS. The subsequent assessment of the cell viability indicated that cell growth was evidently inhibited by glutamate in a dose- and time-dependent manner. There was a significant difference among different groups after cells were treated with glutamate; the obvious inhibition effect

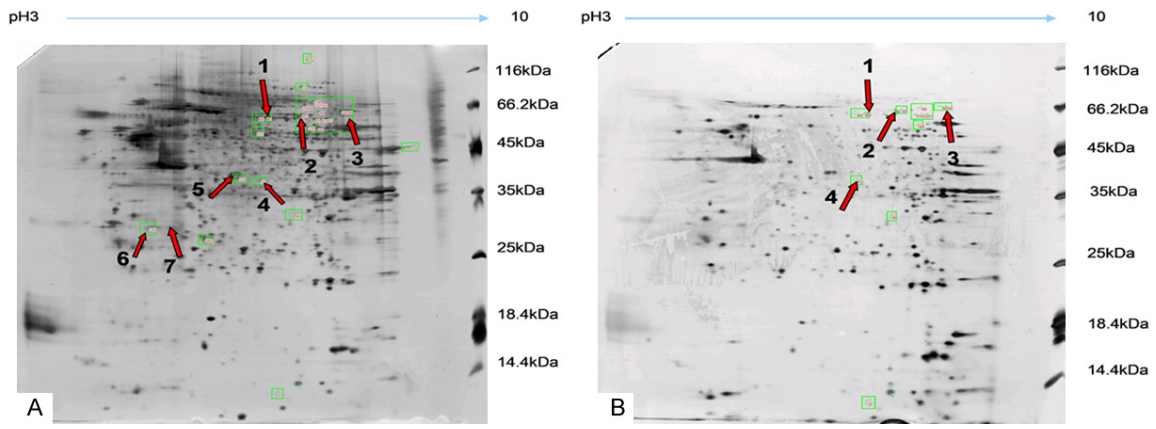

Figure 4. Two-dimensional gel electrophoresis maps of meningothelial cells (MECs). Arrow point spots represent differentially expressed proteins in control cells (A) and glutamate-treated cells (B) in the 2-DE profile. Spot numbers refer to the proteins identified and listed in **Table 1**.

exhibited after cells were incubated with 600 μM glutamate for 24 h ($P < 0.01$). Moreover, with the increase both of exposure time and treatment concentration, glutamate markedly reduced the cell viability when compared to controls (**Figure 1**).

Glutamate enhanced the caspase9 activity of MECs

In order to assess the effect of glutamate on MECs, cells were treated with glutamate at a concentration of 600 μM for 24 h and 48 h respectively. In comparison with unexposed cells, increased caspase9 activity was presented in glutamate-treated cells at 24 h time point ($P < 0.01$). Incubation with glutamate for 48 h elevated the level of caspase9 as compared to control cells ($P < 0.05$) (**Figure 2**).

Glutamate induced the apoptosis of MECs

Annexin-V-PI double staining assay was performed to assess a possible involvement of apoptosis in glutamate-triggered stress on MECs. As shown in **Figure 3**, annexin V generates green fluorescence representing early apoptosis, and cells stained with both PI and annexin V-FITC demonstrate late stage apoptosis and early necrosis. Compared with controls, apoptosis increased after cells were exposed to 600 μM glutamate for 24 h, but there was no significant difference between the two groups ($P > 0.05$). Under glutamate-induced stress for 48 h, the percentage of apoptotic cells was significantly increased relative to those in controls ($P < 0.05$) (**Figure 3M**).

Alterations in MECs protein in response to glutamate stress

Spots which changed consistently and significantly with more than two-fold difference were selected for analysis. Compared with the untreated cells (**Figure 4A**), MECs with glutamate administration (**Figure 4B**) exhibited a significant reduction in the number of protein spots in the 2-DE profiles. 7 proteins were identified from 26 differentially expressed proteins through TOF/MS analysis (**Table 1**). Among these identified proteins, transketolase (TK), collapsin response mediator proteins (CRMPs), GAIP-interacting protein (GIPC1), and lamin (LMNA) were found to be down-regulated. Cathepsin B (CatB), actin regulatory protein (CAPG), and chloride intracellular channel 1 (CLIC1) showed a specific expression in cells with the absence of glutamate.

Discussion

The retrobulbar side of the optic nerve is a compartment occupied with CSF within the subarachnoid space. Compartmentation of the subarachnoid space of the optic nerve might result in an impairment of the integrity of the optic nerve-CSF barrier, which contributes to the development of optic nerve sheath compartment syndrome and might be related to optic neuropathy and optic nerve neurodegenerative diseases [13]. MECs, the predominant cell types lining the inner surface of the dura mater, both sides of arachnoid layer, and the outer surface of pia mater surrounding the optic nerve, directly contact with CSF and might

Table 1. Differentially expressed proteins in control and glutamate-treated cells

Spot No	NCBI No	Protein	Mw	Protein coverage (%)	Isoelectric point	Score	Function
(1) Proteins down-regulated with glutamate							
1	gi|4503377	CRMP-2 (DPYL2)	62711	10	5.95	64	Neuronal growth and migration, apoptosis, proliferation, and differentiation
2	gi|27436946	LMNA	74380	7	6.57	59	DNA replication, DNA repair, chromatin organization, proliferation, mitogenesis, and differentiation
3	gi|388891	TK	68519	14	7.58	108	Cell proliferation and cell cycle
4	gi|545809004	GIPC1	36141	3	5.90	34	Trafficking of transmembrane proteins, cell-cycle, apoptosis, cell adhesion, and motility
(2) Proteins specifically expressed in control with absence of glutamate							
5	gi|63252913	CAPG	38760	8	5.82	55	Cell motility, proliferation, and apoptosis
6	gi|14251209	CLIC1	27248	26	5.09	218	Ion channel protein and cell cycle control
7	gi|4503139	CatB	38766	5	5.88	64	Cell proliferation, apoptosis, invasion, and metastasis

Note: CRMPs, collapsin response mediator proteins; LMNA, lamin; TK, transketolase; GIPC1, GAIP-interacting protein; CAPG, actin regulatory protein; CLIC1, chloride intracellular channel 1; CatB, cathepsin B.

therefore perform an essential role in keeping the optic nerve-CSF barrier intact and maintaining the balance of the substance in the CSF.

In the current study, assessment of the cell viability in the proliferation of MECs under the glutamate stress indicates that glutamate suppressed MECs growth. Upregulation of caspase9 activity in glutamate-exposed cells implies that apoptosis might be involved in the pathogenesis of the glutamate-stress on MECs. Caspase9, an indicator of intrinsic pathway of apoptosis, is regarded as the early symbol via transmitting the cellular apoptosis signaling. Additionally, our data regarding the Annexin-V-PI double staining showed that exposure to glutamate was capable of inducing apoptosis, which was consistent with the elevated level of caspase9 in glutamate-treated cells, suggesting the role of apoptosis might be involved in the pathological event elicited by glutamate excitotoxicity.

In our study, proteomic changes were detected in MECs after glutamate exposure. The identification of protein spots discovered by mass spectrometry techniques allows a more detailed analysis of molecular mechanisms involved in the glutamate-induced stress on the proteomic changes of MECs. 7 proteins were identified from 26 differentially expressed proteins through TOF/MS analysis. Among those, down-regulated proteins including TK, CRMPs, GIPC1, and LMNA were recently demonstrated to be involved in proliferation, apoptosis and cell structure modulation [14-17]. TK is abnormally and characteristically degraded during extraction of AD fibroblasts and proposed to be closely related with the onset of AD [18]. TK knockdown has been reported to result in cell-cycle delay, thereby inhibiting cell proliferation [14]. CRMPs are important brain-specific proteins linked with nervous system development and neurodegenerative disorders [19]. Other investigations have implicated CRMP-2 in a range of cellular functions, such as apoptosis, proliferation, cell migration, differentiation, modifying microtubule assembly, and neuronal survival [15, 19-22]. GIPC1 has emerged as a structural protein by inducing multiple biological responses such as protein trafficking, endocytosis, and receptor clustering [23]. GIPC1 silencing promotes G2 cell-cycle arrest, apoptosis, and alternations in cell adhesion and motility [16]. Nuclear LMNA family provides a structural scaffolding for the cell nucleus and interacts with both nuclear membrane proteins and chromatin [24], and it also plays central roles in DNA replication, formation of the mitotic spindle, gene transcription, cell proliferation, and cell differentiation [17, 25, 26]. Silencing of LMNA expression slows cell proliferation [17]. The down-regulation of TK, CRMP-2, GIPC1 and LMNA in our study gives a clue that glutamate might be involved in the degradation of these proteins, leading to cell growth inhibition and apoptosis during the oxidative stress. Glutamate might therefore restraint the protective role of MECs in the optic nerve-CSF barrier.

In this study, the other 3 identified proteins showed a specific expression in non-glutamate-exposed cells include CatB, CAPG and CLIC1, which are demonstrated to be related with cell

structure and cellular process regulation functions, such as proliferation and apoptosis [27-29]. CatB has been demonstrated a role for proliferation, invasion, and metastasis of tumor development [27]. Down-regulation of CatB has been found to induce apoptosis in glioma cells [28]. CAPG is an actin-regulatory protein and plays important roles in cell motility through interaction with actin monomers and filaments. Moreover, depletion of CAPG has been associated with decreased cell motility, cellular proliferation, and apoptosis [29]. CLIC1, regarded as a real chloride channel in plasma and nuclear membrane, is postulated to play a role in ion homeostasis modulation, cell cycle mediation, transepithelial transport, and regulation of electrical excitability [30, 31]. In our study, glutamate-induced absence expression of CatB, CAPG, and CLIC1 may be part responsible for the MECs dysfunction via interference of cell structure, deregulation of cell cycle, and inhibition of cell motility, which resulted in an inhibitory effect of glutamate on the proliferation of MECs.

Altogether, these in vitro observations provide an evidence that glutamate poses an impact on MECs through inhibiting cell viability, triggering apoptosis, and down-regulating several key proteins. These altered proteins are most likely involved in cell-mediated biological functions such as modulating cell proliferation, apoptosis, regulating cell structure, cell cycle, and cell motility. The down-regulated proteins probably contribute to the suppression of MECs growth and cellular function, which probably influence the intact of optic nerve-CSF barrier. These results suggest a role of MECs in the pathogenic mechanisms of optic nerve diseases.

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Disclosure of conflict of interest

None.

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