

Psychological stress induces moderate pathology in the ganglion cell layer in mice

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Purpose: Primary open-angle glaucoma (POAG) is a condition with unclear pathogenesis. Researchers have observed an increased incidence of young Chinese POAG patients who manifest significant psychological stress while their intraocular pressure (IOP) is normal or close to normal; we hypothesize that psychological stress may play a causal role in initiating POAG.

Methods: Twenty-four male C57BL/6 mice were included and divided randomly into two groups. A chronic unpredictable mild stress (CUMS) mouse model was established to evaluate the effect of psychological stress on glaucoma-related retinal pathologies. Body weight and IOP were recorded weekly. At 5 weeks after the CUMS procedure, a behavior test, serum corticosterone level, retinal nerve fiber layer (RNFL) thickness, retinal ganglion cell (RGC) number and neurotrophic factor expression were evaluated and compared between the CUMS group and the control group.

Results: CUMS exposure induced depression-like behaviors, lighter body weight, and increased serum corticosterone levels in mice. RNFL thinning and neural cell loss in the ganglion cell layer (GCL) were observed in CUMS mice without significant IOP elevation. Decreased mRNA expression and protein levels of neurotrophic factors in retinas of CUMS mice were observed, especially brain-derived neurotrophic factor (BDNF).

Conclusions: The CUMS mouse model demonstrated that psychological stress induced glaucoma-like changes in the retinas of CUMS mice. The mechanism by which psychological stress induces retina defects may be due to a reduced expression of retinal neurotrophic factors. Thus, we conclude that psychological stress is causally associated with POAG.

Primary open-angle glaucoma (POAG) is a neurodegenerative optic neuropathy characterized by progressive structural cupping in the optic nerve head, loss of retinal ganglion cells (RGCs) or their axons (nerve fiber layer, RNFL), and corresponding damage to the visual field (VF) [1]. However, the pathogenesis of POAG remains partially unknown. Although elevated intraocular pressure (IOP) has been shown to be the most important risk factor that influences the development and progression of glaucomatous optic neuropathy (GON), a large percentage of clinical POAG cases—especially those with “normal-tension glaucoma”—cannot be

attributed to IOP elevation, suggesting that additional factors may be involved [2].

Over the past decade, a clear trend has been observed in Chinese glaucoma clinics, where an increased number of young patients in their 20s to 40s are diagnosed with POAG. Interestingly, upon repeated examinations, these young POAG patients usually present with normal or close-to-normal IOP, suggesting that elevated IOP may not be a readily accountable etiology for these cases. This calls for further exploration of other possible causative factors. One significant trait that has been repeatedly found in young POAG patients is their markedly overstressed mental status when they visit glaucoma clinics. Extensive studies have been conducted on the correlation between POAG and psychological stress, with anxiety and depression being the two most commonly identified factors. Our recent study also demonstrated an association between psychological stress and a higher prevalence of glaucoma [3]. However, the available evidence can

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only establish a clinical connection between psychological stress and POAG. It is not known whether psychological stress is potentially an initial contributing factor in causing POAG, or in contrast, whether psychological stress is simply a consequence following POAG diagnosis and treatment. To date, no studies have investigated this etiological connection.

To resolve these remaining questions and concerns, we specifically designed a clinical study to assess and quantify psychological stress using the Self-rating Anxiety Scale (SAS) [4] and the Self-rating Depression Scale (SDS) [3] before glaucoma screening tests and diagnosis. Notably, the study only selected subjects aged 20–40 years and patients with early stage POAG, with the overarching goal of establishing a causal connection between psychological stress and POAG at the early disease developmental stage. As expected, we found that psychological stress was significantly higher in the young POAG group compared with the matched controls. Moreover, a nationwide population-based study conducted in South Korea observed a similar phenomenon: Undiagnosed glaucoma subjects were more likely to have anxiety/depression problems than those without glaucoma [5].

Based on the clinical findings, we proposed that psychological stress may play a causative role in at least some cases of POAG. To further investigate whether psychological stress can lead to functional or neural damage in the retina, we established the classic, well-documented mouse model of chronic unpredictable mild stress (CUMS) [6]. The CUMS mouse model is reliable and effective model, and it is currently the most commonly used rodent model of depression. Previous studies have reported that chronic stress might modulate the development of neurodegenerative diseases, such as Alzheimer disease and Parkinson disease [7,8]. However, as a neurodegenerative disease, the CUMS model has not yet been used to explore the causative connection between psychological stress and POAG. If RNFL or neuron cell loss were found in the retinas of the mice induced by CUMS exposure, together with the clinical data, we could reasonably suggest that psychological stress plays a causal role in the development of POAG.

METHODS

CUMS procedure: Twenty-four adult male C57BL/6 mice (8 weeks old) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China), and housed in a standardized animal room (lights on 8:00 AM–8:00 PM, room temperature [RT] at 22 ± 2 °C), with food and water provided ad libitum. Mice were acclimated to the environment for 1 week before a 5-week induction of CUMS, and

then randomly assigned to the control or CUMS group. Mice in the CUMS group were subjected to different mild stressors, which were as follows: food/water deprivation for 12 h, inversion of the light/dark cycle for 24 h, dampened bedding for 12 h, crowded caging for 12 h (putting all mice into one cage), restrained stress for 1 h (putting each mouse into a 50 ml centrifuge tube), empty caging for 12 h (with no bedding), cold swimming for 5 min (at 4 °C), hot water swimming for 5 min (at 45 °C), 45° cage tilting for 12 h, and tail pinch for 1 min. Mice were randomly subjected to one of these stressors at different times every day, and the same stressor was not used consecutively over 2 days; these measures kept animals from predicting the occurrence of next stimulation. Meanwhile, no stressor was induced for the controls except for the necessary routine cage cleaning and the supplying of food and water. Twenty-four hours after the end of CUMS, the animals were subjected to behavioral tests, and they were sacrificed for further testing after 24 h. The protocol was approved by the Animal Experimental Ethics Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University (approval number: 2019–067). In addition, the methods were performed in accordance with the approved guidelines of the Animal Care and Use Committee of Zhongshan Ophthalmic Center and the Association Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Behavioral testing: Depressive-like behaviors in the CUMS models were assessed using the Forced Swim Test (FST) and Sucrose Consumption Test (SCT). In the FST, mice were placed in cylinders (18 cm in height \times 14 cm in diameter) containing 24 ± 1 °C water with a 14 cm depth. Notably, the water was changed after each test. The FST was conducted for 6 min, and immobility time was recorded in the final 4 min. Immobility, reflecting behavioral despair, was defined as the mice floating in the water without struggling but only making necessary movements to keep their heads above the water.

For SCT, mice were trained to adapt to 1% sucrose solution (w/v) as follows: One percent sucrose solution was provided in each cage for 24 h. Water deprivation was then imposed for 12 h. Next, the SCT was conducted, in which each mouse was housed in an individual cage and had free access to 1% sucrose solution for 2 h. The bottles were well sealed, and each bottle contained 50 ml of 1% sucrose solution. Consumption of the 1% sucrose solution was calculated as follows: (weight of 1% sucrose solution before test – weight of 1% sucrose solution after test).

Body weight measurements: The body weight of each mouse was measured at the same time every week using an

electronic scale (ZhuHeng, ZheJiang, China). The measurement was conducted at least 2 h after the latest stressor.

Serum corticosterone measurements: Mice were sacrificed for blood collection at the end of 5 weeks of undergoing the CUMS procedure. The mice were tightly held in one hand, and the whiskers was cut, followed by rapid removal of the right eyes using forceps and collection of blood (about 1 ml) into centrifugal tubes. After 40 min at RT, the tubes were centrifuged (2000 ×g, 5 min, RT) to separate the serum. Serum samples were then stored at −80 °C until they were tested. Serum corticosterone levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Tianyuan, Guangzhou, China) according to the manufacturer's instructions. The optical density (OD) of corticosterone was read at a wavelength of 450 nm, and the concentration of corticosterone was calculated according to the standard curves.

IOP measurements: IOP was measured in CUMS and control mice using the TONOLAB tonometer TV02 (Icare Finland Oy, Helsinki, Finland) under light general anesthesia with 2% isoflurane for about 1 min at the same time every week (3:00 PM–5:00 PM) after CUMS induction. The measurements took place at least 2 h after the last stressor was administered. The average of six consecutive measurements, excluding the highest and lowest values, was recorded six times for each eye.

OCT measurement of mouse retina: To determine RNFL thickness, posterior segment OCT (SPECTRALIS-OCT, Heidelberg, Germany) was used to capture retinal cross-section images upon pupil dilation. After general anesthesia was administered, mice were placed on the rodent alignment stage. Following this, 0.9% sterile saline was applied throughout the procedure to keep the corneal wet. Measurements were performed at 400 μm from the center of the optic nerve head within each quadrant (nasal, temporal, superior, and inferior) by a masked observer. The average of the four readings was considered the final thickness value.

RGC evaluation: Neuronal nuclei (NeuN) were used as a neuronal cell marker to label surviving neurons in retinal flat

mounts. Eyeballs were collected 5 weeks after CUMS exposure and fixed in 4% paraformaldehyde (PFA) for 30 min. Subsequently, eyes were enucleated and fixed in 4% PFA for another 30 min. Finally, eyes were placed in 30% sucrose and incubated overnight. For retinal whole mounts, the retinas were dissected out. The retinas were then blocked with 0.5% Triton X-100/5% bovine serum albumin (BSA) for 2 h at RT, followed by incubation with primary antibody rabbit anti-NeuN (ab177487, 1:500; Abcam, Cambridge, MA) overnight at 4 °C. After washing with 1X PBS, the retinas were incubated with Alexa Fluor 488 conjugated donkey anti-rabbit secondary antibody (A21206, 1:800; Invitrogen, Carlsbad, CA) for 2 h at RT and counterstained with DAPI (Vector Laboratories, Burlingame, CA) for 5 min before mounting. NeuN-positive cells in the central retina (a 40X field away from the optic disc edge) and peripheral retina (a 40X field away from the edge of peripheral retina) were counted and compared between CUMS mice and normal mice. Finally, images were observed and captured using a Zeiss Axiophot fluorescent microscope and Zeiss LSM 780 confocal microscopy system (Carl Zeiss Meditec AG, Jena, Germany).

mRNA expression of neurotrophic factors in the retina: The mRNA level of neurotrophic factors was determined using real-time PCR. Total RNA was extracted from retina tissues using Trizol (TaKaRa, Dalian, China) according to the manufacturer's protocol. Next, first-strand cDNA was produced (Applied Biologic Materials Inc., Richmond, BC, Canada). Real-time PCR was performed in a total volume of 10 μl containing the following: 1 μl of cDNA, 1 μl of the primer pairs (10 μmol/l), 3 μl of double-distilled water, and 5 μl of 2X mix (Roche Life Science, Basel, Switzerland). The PCR amplification protocols were performed according to the manufacturer's instructions. The sequences of the primers used are listed in Table 1.

Protein level of BDNF in the retina: Protein concentrations of the retina samples were assessed using a bicinchoninic acid (BCA) kit according to the manufacturer's protocol (Thermo Scientific, Brookfield, WI). Equal amounts of total protein from each sample were loaded on sodium dodecyl sulfate

TABLE 1. THE SEQUENCE OF THE PRIMERS USED FOR QPCR ANALYSIS.

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>BDNF</i>	AAGGACGCGGACTTGTACAC	CGCTAATACTGTCACACACGC
<i>TrkB</i>	CAGCAACGACGATGACTCTG	CCTTCCCCAAGTTCCTCTT
<i>NGF</i>	ATGGTGGAGTTTGGCCTGT	GTACGCCGATCAAAAACGCA
<i>TrkA</i>	AGGGCCACATCATGGAGAAC	GTGCAGACTCCAAAGAAGC
<i>CNTF</i>	GAGCAATCACCTCTGACCCT	GGTTCTCTTGGAGTCGCTCT
<i>GAPDH</i>	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA

polyacrylamide gel electrophoresis (SDS-PAGE) gradient 4%–12% Bis-Tris gels and transferred to polyvinylidene fluoride (PVDF) membranes (Life Technologies, Carlsbad, CA). The membranes were blocked in 5% milk in Tris-buffer saline (TBS; pH 7.6) and incubated with the primary antibody against BDNF (1:500) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2000) overnight. After washing with 0.15% Tween-20 in TBS, membranes were incubated with secondary antibody (1:5000) diluted in TBS for 2 h. Next, the membranes were washed three times and imaged immediately using the MP ChemiDoc Imaging System (Bio-Rad, Shanghai, China).

Data and statistics: Differences between experimental mice groups (CUMS-induced mice and controls) were tested. Data are shown as mean \pm standard error of the mean (SEM). The Student *t* test or Mann–Whitney U test was performed for continuous variables. Pearson's chi-square test was used for categorical variables. A *p* value of less than 0.05 was considered statistically significant. SPSS software version 21.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

RESULTS

CUMS exposure induced depression-like behaviors, lighter bodyweight, and increased serum corticosterone levels in mice: To evaluate the phenotype of CUMS in each group, behavioral tests were performed at the end of the CUMS procedure; these tests included including FST and SCT. The results showed that the mice subjected to CUMS for 5 weeks displayed depression-like behaviors, including increased immobility time in the FST (Figure 1C, *n* = 9–12, *p* = 0.032) and decreased sucrose consumption (Figure 1D, *n* = 9–12, *p* = 0.038). In addition, body weight was measured on a weekly basis. We observed that mice in both groups gained weight continuously during the CUMS induction period, but the pace of weight gain was slower in the CUMS group than it was in the control group. A significant difference in bodyweight between the two groups was observed in weeks 3, 4, and 5 (Figure 1A, *n* = 9–12). Moreover, the serum corticosterone level was higher in the CUMS mice than in the control group (Figure 1B, *n* = 5–6), indicating that the CUMS mice were more stressful than the control group.

RNFL thinning and neural cell loss in GCL were observed in CUMS mice without significant IOP elevation: In this study, the IOP, RNFL thickness, and neural cell count in the GCL were measured in CUMS-exposed mice. The IOP of the CUMS mice fluctuated slightly during the study, but no significant elevation was detected (Figure 2A, *n* = 9–12). Surprisingly, a significant reduction in RNFL thickness was observed through OCT measurement (Figure 2B, *n* =

9–12, *p* < 0.005). Accordingly, in the retina whole mount, we observed a decrease in ganglion cell layer (GCL) neuron counts in both the central and peripheral retina (Figure 3, *n* = 6–6, *p* = 0.020, *p* = 0.004). RNFL thinning and neural cell loss indicated glaucoma-like damage in the CUMS mice.

There was reduced mRNA expression of neurotrophic factors in the retinas of CUMS mice: Given that RNFL thickness decreased in CUMS mice, we subsequently attempted to elucidate the possible underlying molecular mechanisms for this. Notably, it was previously reported that neurotrophic factors in the brain were reduced after CUMS induction, which was associated with a loss of neuron cells. Therefore, this study conducted quantitative real-time PCR analysis of common retinal neurotrophic factors and their receptors, including BDNF, tyrosine protein kinase receptor B (TrkB), nerve growth factor (NGF), tyrosine protein kinase receptor A (TrkA), and ciliary neurotrophic factor (CNTF), which are necessary for the survival of retinal neurons. Figure 4 shows that the mRNA expressions of *BDNF*, *TrkB*, *NGF*, *TrkA*, and *CNTF* were significantly reduced in the retinas of CUMS mice (*n* = 5–6; *p* = 0.036, 0.034, 0.010, 0.005, and 0.0095, respectively) compared to the control group.

The protein level of BDNF in the CUMS mice was lower than that in the control group: BDNF is the most important neurotrophic factor for retinal neuron cells. Therefore, we further determined the protein level of BDNF in the retina based on the findings of qPCR analyses. The results showed a decreased BDNF protein level in the retina of CUMS mice than in the normal mice (*n* = 3–3), as shown in Figure 5.

DISCUSSION

In this study, a CUMS mouse model was used to investigate the hypothesis that psychological stress may play a causative role in the development of POAG in at least some young patients with normal or close-to-normal IOP. CUMS animal models were constructed to evaluate the causative connection between psychological stress and GON-associated retinal pathologies based on the background of clinical observation. Although the opinion that stress is the cause of visual system diseases was reported in a previous study [9], this is the first time the CUMS mouse model was used to investigate the role of psychological stress in eye diseases. Briefly, the results showed that there were depressive-like behaviors in CUMS-exposed mice, including increased immobility time in the FST and decreased sucrose consumption in the SCT, which was consistent with previous CUMS model studies [10]. The serum corticosterone level was also higher among the CUMS mice, indicating a stressful situation in mice exposed to CUMS.

Glaucoma-like phenotypes were observed in CUMS-exposed mice. Via OCT measurement, we observed that the RNFL thickness of the CUMS mice was thinner than that in normal controls, representing a highly effective indicator in the early diagnosis of POAG [11]. A previous study on major depressive disorder (MDD) patients also demonstrated that GCL and inner plexiform layer (IPL) volumes were significantly smaller in recurrent depression patients than they were in first-episode patients and in all MDD patients compared with healthy controls. In addition, there were significant negative correlations between their volume and disease severity parameters [12]. As expected, our animal results were consistent with previous studies on MDD patients. Meanwhile, whole mount immunofluorescence results showed that there were fewer neural cells in the GCL of CUMS mice compared with normal controls, including the central retina and peripheral retina. RNFL thinning and neuron loss in the GCL were solid markers of POAG. It is worth noting that this is the first time it has been shown that psychological stress can induce a glaucoma-like phenotype in animal model studies.

High IOP was the main risk factor for the development of glaucoma. In the animal model of glaucoma, retinal damage and RGC loss are induced by acute or chronic IOP elevation. However, in this study, the IOP of CUMS mice was not elevated during the experiment. Notably, 2% isoflurane may result in deep anesthesia, leading to an artificial IOP decrease in CUMS mice. However, we observed similar IOPs in both the control and CUMS groups, suggesting that 2% isoflurane may not have affected the analysis. Therefore, it was assumed that RNFL thinning and neuron loss resulted from other factors after CUMS induction.

To the best of our knowledge, no study has examined the expression of neurotrophic factors in the retina in mouse models of depression. The results obtained in this study indicated that the expression of several neurotrophic factors and its receptors in the CUMS retina was reduced at the mRNA level. In addition, the protein level was downregulated. Previous studies have reported that neurotrophic factors, especially BDNF, CNTF, and NGF, are vital for the survival of retinal cells [13-16]. BDNF has also been proven to be the

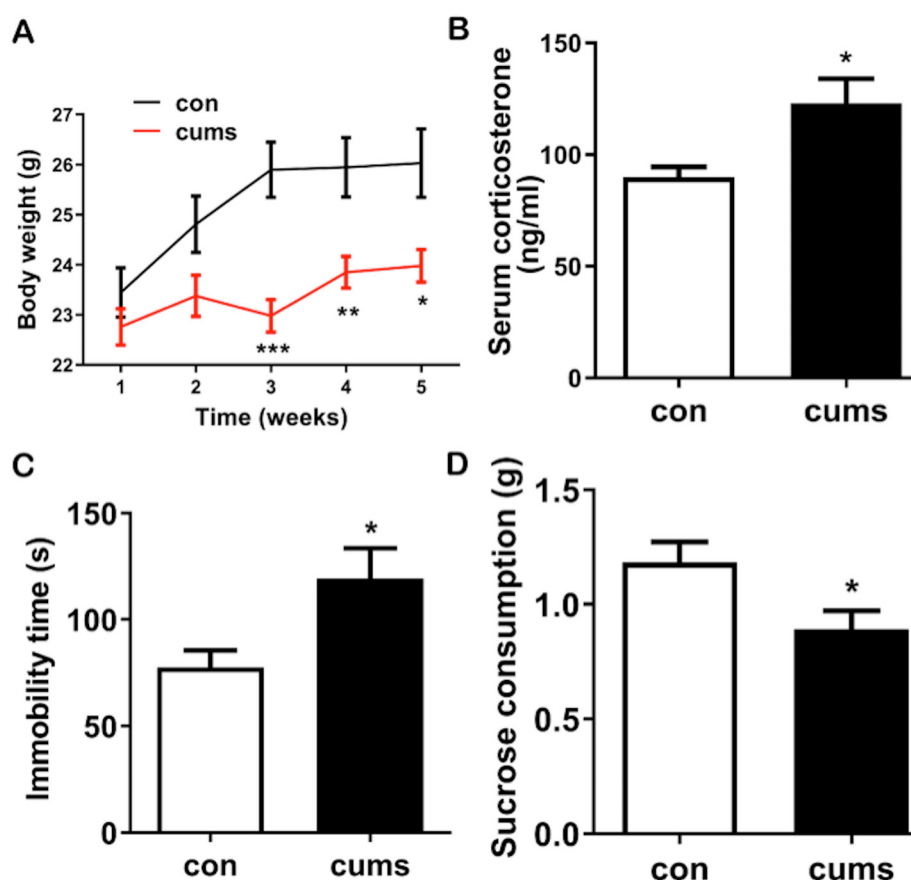


Figure 1. Chronic unpredictable mild stress (CUMS) mice presented depression-like behaviors and reduced weight gain. **A:** Body-weight was measured weekly for both groups. CUMS mice gained weight more slowly than control mice did. The mice exposed to CUMS were significantly lighter than the normal controls as of week 3 ($n = 9-12$). **B:** The blood of the mice was collected and measured at the end of 5 weeks of exposure. The serum corticosterone level was higher in the CUMS mice than in the normal group ($n = 5-5$), which indicated a higher stress level of CUMS mice. **C:** The immobility time was examined in the forced swim test (FST). **D:** Sucrose consumption was determined using the sucrose consumption test (SCT) at the end of 5 weeks of CUMS exposure. Our results showed increased immobility time in the

FST and decreased sucrose consumption in the SCT. Data are presented as mean \pm standard error of the mean (SEM), $n = 9-12$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

best neuroprotectant in the central nervous system and the key neurotrophic factors in glaucoma [17]. Live-cell imaging captured the dynamics of the axonal transport of BDNF in living RGCs [18], and BDNF was consistently reduced in the optic nerve heads of glaucoma patients [19]. Moreover, many studies have reported that antibodies that selectively activate TrkB, the receptor of BDNF, enhance RGC survival in acute and chronic glaucoma models [17]. Therefore, we proposed that psychological stress induces glaucoma-like changes, probably as a result of a lack of neurotrophic factors in CUMS-exposed mice. This is consistent with studies focusing on the relationship between stress and neural cell survival in the brain. Accumulating evidence has demonstrated that stress decreases the expression of BDNF in limbic structures that control mood [20]. One study also proposed that the decreased level of BDNF and other neurotrophic factors could contribute

to the atrophy of certain limbic structures, including the hippocampus and prefrontal cortex, that has been observed in depressed subjects [21]. Conversely, the neurotrophic actions of antidepressants could reverse neuronal atrophy and cell loss, contributing to the therapeutic action of these treatments [22]. Furthermore, RNFL thinning and neuron cell loss in the CUMS mouse can be attributed to reduced retinal neurotrophic factors.

These results collectively suggest, for the first time in an animal model, that psychological stress could be a causative factor in at least some POAG cases. Although psychological stress may just be one of the complex pathogenesis of POAG, we think it plays an important role in initiating and promoting POAG neurodegeneration. Considering that stress is widespread among the younger population in China, this study provides an important perspective to explain the rising

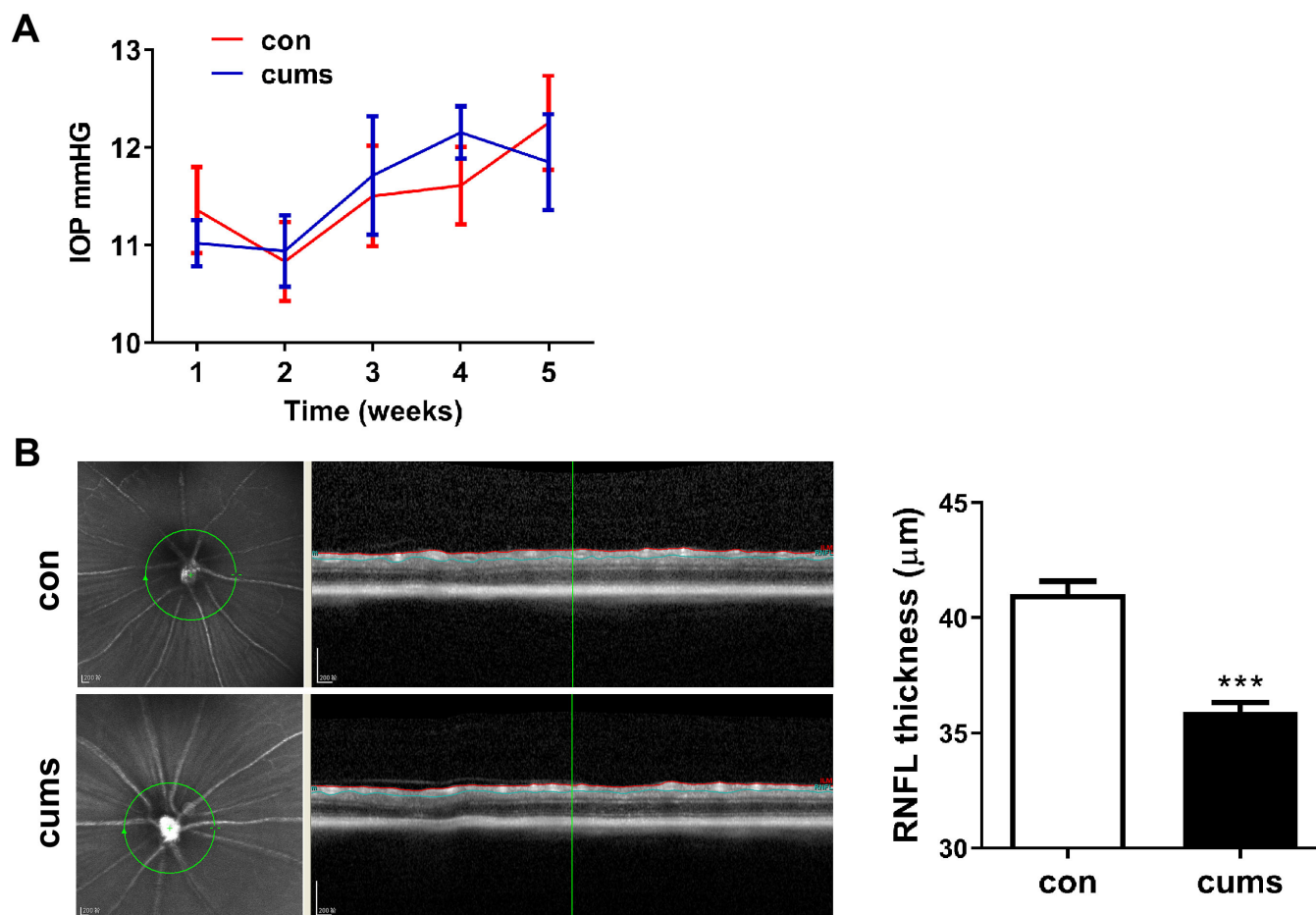


Figure 2. Chronic unpredictable mild stress (CUMS) induced retinal nerve fiber layer (RNFL) thinning without intraocular pressure (IOP) elevation. **A**: IOP was measured weekly. The IOP of both groups of mice fluctuated slightly. No significant elevation of IOP was observed during the 5-week experiment. **B**: The RNFL thickness was determined at the end of 5 weeks of CUMS exposure. We observed a significant reduction in RNFL thickness via optical coherence tomography (OCT) measurement. Data are presented as mean \pm standard error of the mean (SEM), $n = 9-12$. ** $p < 0.01$, *** $p < 0.005$.

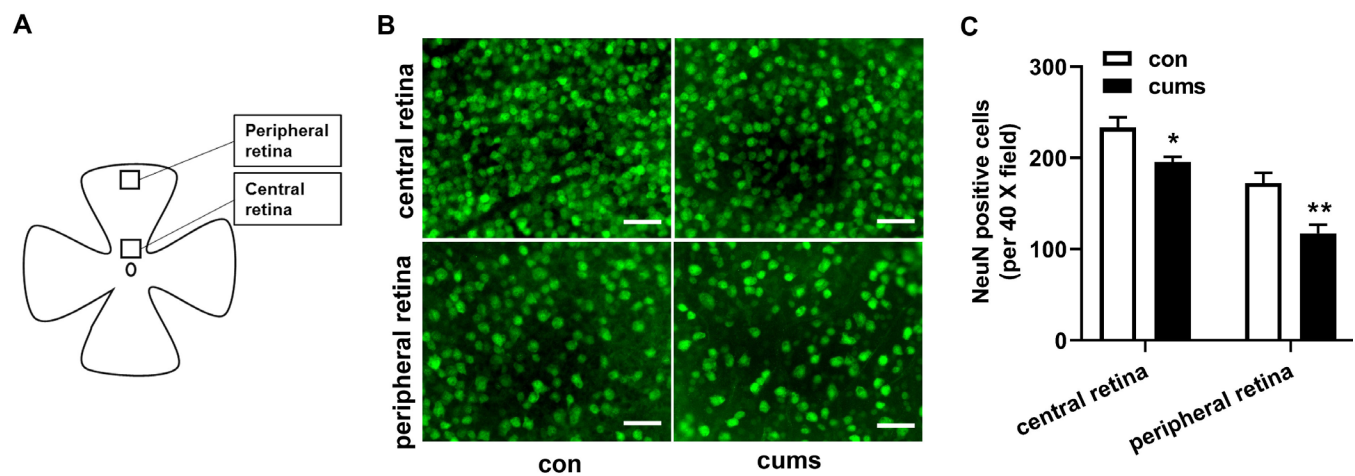


Figure 3. Chronic unpredictable mild stress (CUMS) induced neuronal cell loss in the ganglion cell layer (GCL) of the retina. **A**: The representative area of the selected central and peripheral retina in the whole mount. **B**: The representative neuronal nucleus (NeuN) staining. **C**: Quantification of NeuN-positive cells in the central and peripheral retina in whole mounts at the end of 5 weeks of CUMS exposure. The amount of neural cells in the retina was lower in the CUMS mice than it was in the normal mice in the central and peripheral retina. Data are presented as mean \pm standard error of the mean (SEM), $n = 6-6$. * $p < 0.05$, ** $p < 0.01$. Scale bar = 50 μ m.

incidence of POAG in the young normal/close-to-normal IOP population. Although psychological stress may only explain a small portion of POAG, it provides a new direction for further elucidating this complex disease and for developing new treatment options.

Limitations: This study has some limitations. First, there are no data on changes in the retinal layers other than the

RNFL. Glaucoma-like phenotypes include the thinning of the RNFL, whereas other retinal layers are unaffected. Second, the causal relationship between psychological stress and glaucomatous changes proposed here should be validated using further molecular and pathway studies. Third, this study lacks additional experiments at both earlier and later time points in the course of POAG to prove that the loss of RGC in

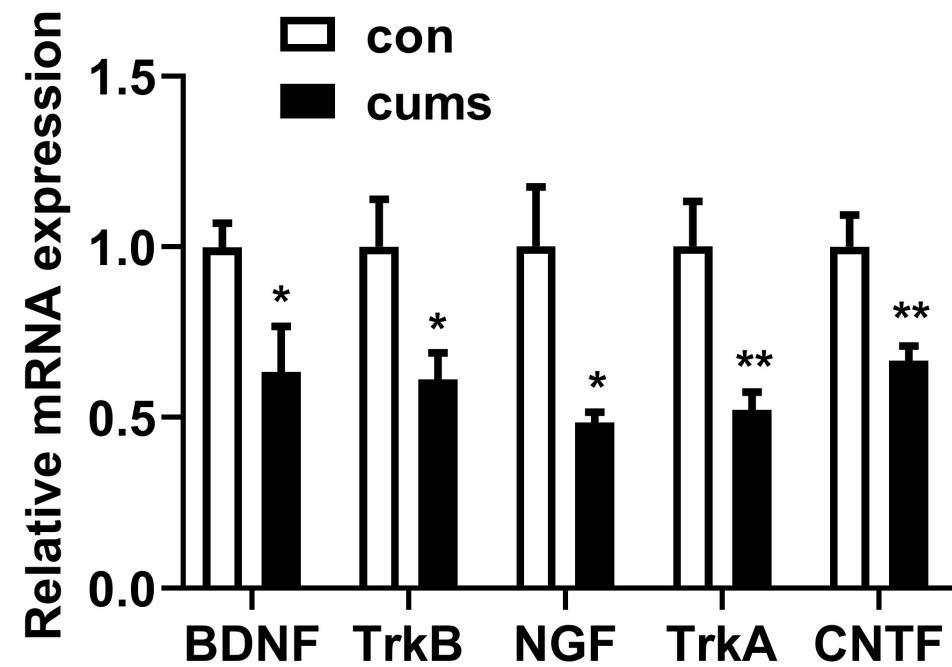


Figure 4. Chronic unpredictable mild stress (CUMS) induced decreased mRNA expression of neurotrophic factors and its receptors in the retina. The mRNA expressions of brain-derived neurotrophic factor (BDNF), tyrosine protein kinase receptor B (TrkB), nerve growth factor (NGF), tyrosine protein kinase receptor A (TrkA), and ciliary neurotrophic factor (CNTF) in the retina decreased at the end of 5 weeks of CUMS exposure compared with normal controls. Data are presented as mean \pm standard error of the mean (SEM), $n = 5-6$. * $p < 0.05$. ** $p < 0.01$.

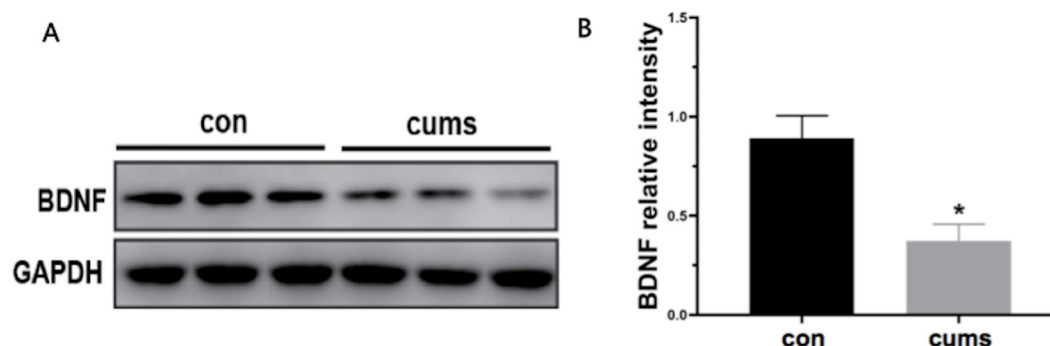


Figure 5. Chronic unpredictable mild stress (CUMS)-induced decreased protein expression of BDNF in the retina. In the western blot analyses, we observed that the protein level of BDNF, the main neurotrophic factor of retina neuron cells, was decreased in the CUMS mice than in the normal group after 5 weeks of exposure (n = 3–3).

the CUMS model is an ongoing process rather than a limited event. Notably, the ongoing loss of RGCs is more similar to the nature of POAG. Finally, in addition to animal experiments and molecular experiments, this study needs clinical data to provide stronger evidence, such as prospective cohort studies.

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