• Basic Research •

Exploration of the glutamate-mediated retinal excitotoxic damage: a rat model of retinal neurodegeneration

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Abstract

• AIM: To explore the more suitable concentration of glutamate or N-methyl-D-aspartic acid (NMDA) for intravitreal injection to establish a rat model of retinal neurodegeneration.

• METHODS: We injected different doses of glutamate (20 or 50 nmol) or NMDA (40 nmol) into the vitreous chambers of rats, then measured the concentration of glutamate and retinal thickness, quantified apoptotic cells and determined the degree of tau hyperphosphorylation at different time points. *T*-test was used for comparison of two groups. One-way ANOVA and Turkey's multiple comparisons test were used for comparisons of different groups, and *P* values below 0.05 were considered statistically significant.

• RESULTS: The glutamate level in the rats treated with 50 nmol of glutamate was twice that of the control group and persisted two weeks. Seven days after intravitreal injection of 50 nmol of glutamate, three parameters [inner retinal thickness (IRT), retinal thickness (RT) and ganglion cell layer (GCL) cell number] were reduced significantly. Furthermore, numerous TUNEL-positive cells were observed in the GCL one day after intravitreal injection of 50 nmol of glutamate, the expression of the apoptosis-related factor cleaved casepase-3 was markedly increased compared with the expression levels in the other treatment groups, and the expression levels of tau s396 and tau s404 were significantly increased compared with those in the control group.

• CONCLUSION: This study demonstrates that the intravitreal injection of 50 nmol of glutamate can establish the more effective retinal neurodegeneration animal model relative to other treatment groups.

• KEYWORDS: retinal neurodegeneration; glutamate; excitotoxicity; animal model; glaucoma

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INTRODUCTION

Primary glaucoma has become the second leading cause of blindness worldwide. Elevated intraocular pressure (IOP) is the only factor that can be controlled in clinical practice, and many basic studies of glaucoma have been based on an animal model of elevated IOP. However, vision and the visual field gradually deteriorate even in the presence of stable IOP in some patients[1], indicating that damage to the optic nerve is progressive. Currently, the exact pathogenesis of glaucoma is in-completely understood. A pathophysiological hallmark of glaucoma is gradual retinal neurodegeneration, particularly the degeneration and death of retinal ganglion cells (RGCs)[2], which are central nervous system (CNS) neurons that have their soma in the inner retina and their axons gathered in the optic nerve. RGCs convey visual information from the retina to the brain. Similar to other CNS neurons, RGCs are difficult to regenerate if they die. To some extent, glaucoma can be viewed as a neurodegenerative disease, similar to other degenerative CNS diseases such as Alzheimer's and Parkinson's disease, and is ultimately caused by deficits in neuronal function[3-6]. The degeneration of retinal neurons, including RGCs degeneration in glaucoma, occurs as a result of apoptotic mechanisms triggered by multiple factors. In addition to elevated IOP, other factors play important roles in glaucoma pathogenesis, including elevated glutamate concentrations, ischemia, oxidative stress, excessive production of nitric oxide, and reductions in neurotrophic factors[2].

Excitatory amino acids (EAAs) are considered to have an important role in the pathophysiology of CNS degeneration[7-8]. Glutamate is the most common EAA in the CNS, and N-methyl-

D-aspartic acid (NMDA) is an analogue of glutamate. Many studies have shown that intravitreal injection of glutamate or NMDA can lead to rapid RGC degeneration and apoptosis[9-11]. Multiple studies have established glutamate-induced RGC-degeneration animal models of glaucoma[10]. However, the dose and duration of treatment varies greatly, and no comparisons of NMDA and glutamate are available; most studies have not tested the actual concentration of glutamate in vitreous fluid. Researchers have shown that glutamate concentrations in excess of 25 μmol/L cause excitotoxic damage to neurons[12]. According to the literatures[9,12-15], the effective glutamate concentration, the actual vitreous volume of rat and time for test were taken into consideration, we selected 50 nmol and 20 nmol glutamate as well as 40 nmol NMDA for injection into the vitreous chambers of rats to induce retinal excitotoxic damage. Then, we detected the concentration of glutamate, measured retinal thickness (RT), counted apoptotic neurons of retina and assessed the degree of tau hyperphosphorylation at different time points[16]. The purpose of this study was to explore the more suitable concentration of glutamate or NMDA for injection into the vitreous chamber to establish an excitotoxic damage related rat retinal neurodegeneration model.

MATERIALS AND METHODS

Animals and Anesthesia Adult male Sprague-Dawley rats weighing 180-200 g were purchased from the Laboratory Animal Center of Daping Hospital of Army Medical University. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals. The rats were housed in cages with free access to food and water and were maintained on a 12h light/dark cycle in a temperature-controlled room (22℃). Light intensity within the cages ranged from 9 to 24 lx. The animal experiments also followed our institutional guidelines. All surgical procedures were carried out under general anesthesia induced by intraperitoneal (i.p.) injection of 10% chloral hydrate (350 mg/kg). The animals were sacrificed with an i.p. injection of an overdose of 10% chloral hydrate at various time points after intravitreal injection.

Methods

Intravitreal injection The rats were anesthetized by i.p. injection of 10% chloral hydrate (350 mg/kg). Before injection, pupillary dilatation was maintained by administering tropicamide phenylephrine eye drops 3 times for a total of 90 μL (Santen Pharmaceutical Co., Ltd., Japan), and topical anesthesia was achieved by administering 0.5% oxybuprocaine hydrochloride eye drops 3 times for a total of 90 μL (Santen Pharmaceutical Co., Ltd., Japan). Levofloxacin eye drops (Santen Pharmaceutical Co., Ltd., Japan) were administered 3 times for a total of 90 μL to clean conjunctival sacs.

Three microlitres of different drugs [40 nmol NMDA: 20 mmol/L (Sigma-Aldrich); 50 nmol glutamate (Solarbio): 25 mmol/L; 20 nmol glutamate: 25 mmol/L; when injecting less than 3 μL, 0.1 mol/L sterile phosphate-buffered saline (PBS; HyClone) was added to bring the volume to 3 μL] was injected into the right eye with a 30-gauge (G) needle (Hamilton). The needle punctured the center of the vitreous chamber approximately 1.5 mm from the limbus in the superior temporal quadrant. The tip of the needle was pointed in the direction of the optic nerve to avoid injuring the lens. The tip was visible through the dilated pupil, and the drug was slowly injected. The needle was left in the eye for an additional 20s to allow the eye to adjust to the increase in volume and then pulled out. The control group received the same volume of 0.1 mol/L of sterile PBS (HyClone) as this was used as the solvent for NMDA and glutamate.

The left eyes remained untreated. After injection, the eyes were treated 3 times with a total of 90 μL of levofloxacin eye drops (Santen Pharmaceutical Co., Ltd., Japan). While recovering from anesthesia, the animals were placed in their cages.

At least six animals were used for each experimental condition, and eyes were enucleated and processed for further analysis.

Determination of the glutamate concentration in vitreous humor samples by high-performance liquid chromatography Vitreous humor samples were obtained from each eye of all animals immediately after euthanasia. A 30-G micro syringe was used to obtain each sample from the central region of the vitreous chamber. Care was taken to avoid contaminating the vitreous humor sample with blood or any other intraocular tissue. Samples were centrifuged at 13 000 rpm for 10min at 4℃. The supernatant was then transferred to new centrifuge tubes. Subsequently, 2 μL of $HClO_4$ and 1 μL of K_2CO_3 (ratio: 10:2:1) were added to the vitreous humor samples (10 μL), followed by centrifugation twice at 13 000 rpm for 10min at 4℃. The glutamate concentrations in the vitreous humor samples were measured *via* high-performance liquid chromatography (HPLC; Waters e2695 Separations Module, WATERS Co., USA). The samples were run through a C18 column and analyzed by a 157 spectrofluorometric detector with the excitation wavelength set at 330 nm and the emission wavelength fixed at 450 nm[17].

Histology and Analysis

Paraffin sectioning Eyes were immediately enucleated after euthanasia *via* anesthetic overdose followed by cervical dislocation. The cornea, lens and vitreous humor were removed after incubation with Davidson's fluid (2% of a 37%-40% solution of formaldehyde, 35% ethanol, 10% glacial acetic acid and 53% distilled water) for approximately 3h. The remainder of each eye cup was incubated in Davidson's fluid overnight. The tissue samples were dehydrated and embedded in paraffin wax. Microtomy was performed in the sagittal plane (parallel

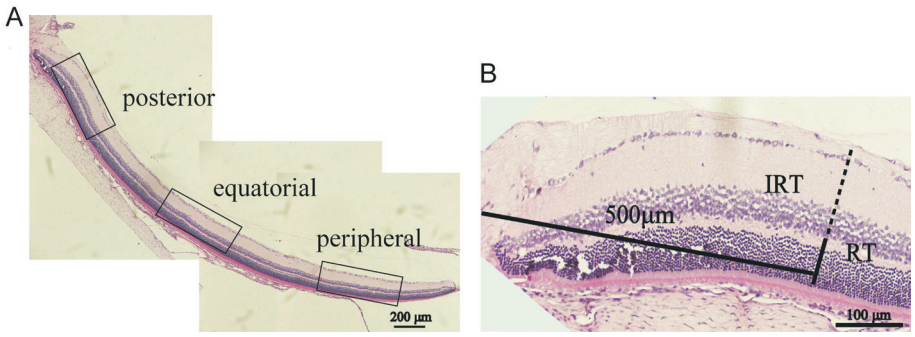

Figure 1 Method of measurement A: The cell counting unit was defined as the total cell number of one posterior, one equatorial and one peripheral picture of a section; B: Approximately 500 μm from the center of optic disc of posterior images were used for analysis of IRT and RT.

to the visual axis) from the temporal to nasal sides of the eye. Sections through the optic nerve, at a thickness of 5 μm, were mounted on slides for hematoxylin and eosin (H&E), TUNEL and immunofluorescence assays.

H&E: cell counting and morphometry Sections were gradient dewaxed, washed with PBS 3 times and stained with H&E. To count the number of neurons and for morphometric analysis of RT, thirty-six images of six sections, with an interval of 30 μm per retina, were examined under 200× magnification, and six images were taken of each section from one ora serrata through the optic nerve to another ora serrata (two peripheral, two equatorial and two posterior images) using a microscope (Leica DM 1000). Images were processed with Image-Pro Plus 6.0 (Media Cybernetics, USA).

For cell counts, only cells with a generally round shape and a discernable nucleus and cytoplasm were selected. No attempt was made to distinguish the types of RGCs or displaced amacrine cells. Morphologically distinguishable glial cells and vascular endothelial cells were excluded from the cell count. The cell counting unit was defined as the total cell number of one posterior; one equatorial and one peripheral picture of a section (Figure 1A). The sum of twelve units from six sections was averaged to obtain the value of one eye. Six eyes were used for each time point in all groups.

Inner retinal thickness (IRT) was defined from the inner boundary of the ganglion cell layer (GCL) to the outer boundary of the inner nuclear layer (INL). Overall RT was defined from the inner boundary of the GCL to the outer boundary of the outer nuclear layer (ONL). For standardization, a site approximately 500 μm from the center of the optic disc in the posterior images was used for the analysis of RT (Figure 1B). Measurements of six posterior pictures from three sections were averaged to obtain the value of one eye. Six eyes were used for each time point in all groups.

Immunofluorescence Sections were then prepared for immunofluorescence according to standard techniques. Briefly, specimens were sequentially dewaxed, washed with PBS 3 times and subjected to antigen retrieval in heated sodium citrate. Specimens were then treated with 0.1% TritonX-100 (Solarbio) and blocked in PBS containing 10% goat serum to reduce nonspecific binding. Then, sectioned specimens were treated with primary antibodies diluted with antibody dilution solution (Solarbio; rabbit anti-tau phospho s396, ab109390, Abcam; dilution 1:300 and rabbit anti-tau phospho s404, ab131338, Abcam; dilution: 1:300) overnight at 4℃. Sections were washed with PBS 3 times and incubated with a secondary antibody (ab150077, Abcam; Alexa Fluor 488-conjugated goat anti-rabbit; dilution: 1:300) for two hours at room temperature. The sections were then washed in PBS, and Hoechst 33342 (C0030, Solarbio; dilution: 1:2000) was used for nuclear staining for three minutes. After staining, the sections were washed with PBS and mounted on slides with florescent mounting medium. Negative controls for all antibody stains were performed using secondary antibodies only. Images were obtained with identical parameters with an SP-8 confocal microscope (Leica, Germany) at 200× magnification and were processed with Leica LAS AF Lite software (Leica, Germany) and Image-Pro Plus 6.0 (Media Cybernetics, USA). For further evaluations, six images per retina (two peripheral, two equatorial, and two posterior images) and six eyes for each time point were examined.

TUNEL assay Apoptotic cell death was detected in retinal sections following the protocol provided with the TUNEL kit (Roche, Switzerland). Briefly, the deparaffinized sections were washed three times in double distilled water and PBS for 5min each. Specimens were incubated with proteinase K (20 mg/L) diluted with 10 mmol/L Tris-HCl for 15min on ice for permeabilization. For double immunofluorescent staining of Brn-3a and TUNEL, samples were treated with mouse anti-Brn-3a antibody (MAB1585, Millipore; dilution: 1:50) and goat anti-mouse IgG H&L (Cy3; ab97035, Abcam; dilution: 1:300). Subsequently, the TUNEL detection solution was added to the sections, and the samples were incubated for 40min at 37℃ under dark and humid conditions. The sections were washed with PBS and incubated with Hoechst 33342 (C0030, Solarbio; dilution: 1:2000) for nuclear staining. After washing in PBS, the sections were mounted, and images were taken using an SP-8 confocal microscope (Leica, Germany)

at 200× magnification and were processed with Leica LAS AF Lite software (Leica, Germany). Image-Pro Plus 6.0 (Media Cybernetics, USA) was used to quantify the total number of Brn-3a positive cells, TUNEL-positive cells and the total number of nuclei in the GCL. The apoptotic index was calculated as the number of TUNEL-positive cells/total number of cells in the GCL within one image (×100%). Three sections, with an interval of 30 μm per retina, were examined under 200× magnification, and six images were taken of each section from one ora serrata through the optic nerve to another ora serrata (two peripheral, two equatorial and two posterior images) with a microscope (Leica DM 1000). Six eyes were used for each time point of all groups.

Western blotting For Western blotting, samples were boiled at 100℃ for 5min in loading buffer. The proteins were electrophoresed in 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore Ltd.). The membranes were then blocked with 5% bovine serum albumin (BSA; Solarbio, Beijing, China) for 1h and probed with primary antibodies diluted with antibody dilution solution (Beyotime, Shanghai, China) rabbit anti-tau phospho s396 (ab109390, Abcam; dilution 1:10 000), rabbit anti-tau phospho s404 (ab131338, Abcam; dilution: 1:1000), rabbit anti-tau (ab32057, Abcam; dilution: 1:5000), rabbit anti-caspase-3 (#9662, Cell Signaling; dilution: 1:1000), and mouse anti-beta actin (ab6276, Abcam; dilution: 1:5000) at 4℃ overnight. Secondary antibodies diluted with antibody dilution solution (Beyotime, Shanghai, China) goat anti-mouse IgG H&L (HRP; ab6789, Abcam; dilution: 1:5000) or goat anti-rabbit IgG H&L (HRP; ab6721, Abcam; dilution: 1:5000) were applied for 1h. Images were taken with a gel imaging system (Aplegen, Gel Company, Inc, CA, USA). The protein bands were recorded and analyzed with LabWorks (version 4.6.0.65, UVP Company). The protein signal intensity was normalized to β-actin. We performed three repeats per analysis.

Statistical Analysis Statistical analysis was performed with GraphPad Prism 6.01 (GraphPad Software, USA). The data are presented as the mean±standard deviation (SD) with a minimum of n=3. The t-test was used for comparison of two groups. One-way ANOVA and Turkey's multiple comparisons test were used for comparisons of different groups, and P values below 0.05 were considered statistically significant.

RESULTS

Five eyes that suffered hypotension because of drug leakage, cataract and infection were excluded.

Glutamate Concentrations of Vitreous Humor Injection of 50 nmol or 20 nmol of glutamate or 40 nmol of NMDA all caused significantly increased glutamate concentrations in the vitreous humor samples (P<0.05) compared with those in the PBS group. Injection of 50 nmol of glutamate resulted in a twofold greater level of glutamate than the normal level, which lasted for approximately 2wk (approximately 1wk following the other two treatments). The concentrations then gradually decreased to normal levels (Figure 2).

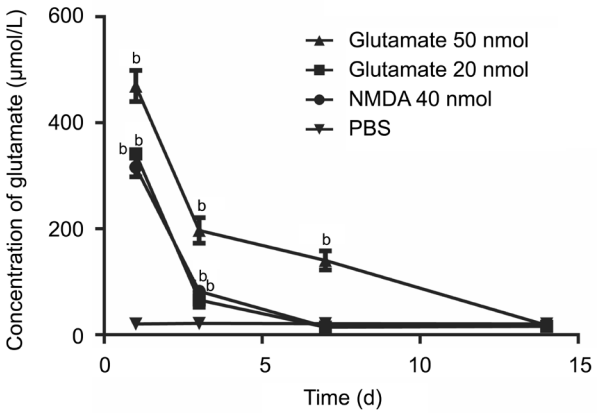

Figure 2 A rat model of glutamate analyses The glutamate concentration reached the peak the day after injection, then decreased gradually. There was good agreement between the injected values and the values measured by HPLC. [a]P<0.05; [b]P<0.01.

Changes in Retinal Morphology in the Glutamate-mediated Retinal Excitotoxic Damage Model Decrease in cell number, disorganized cell arrangements and the appearance of vacuoles in the GCL were observed in the treated eyes (Figure 3A).

Except for the PBS group, the RT, IRT and GCL cell number all decreased 7d after intravitreal injection of 50 nmol or 20 nmol of glutamate or 40 nmol of NMDA, and these changes were time-dependent (Figure 3B, 3D and 3F).

Significant reductions in RT (μm) were noted 7d after intravitreal injections of 40 nmol of NMDA (210.833±5.037, P<0.01), 20 nmol of glutamate (210.833±6.080, P<0.01) and 50 nmol of glutamate (204.667±6.861, P<0.01) compared with that in the PBS group (226.833±3.430; Figure 3C). A decrease in IRT (μm) was apparent 7d after intravitreal injections of 40 nmol of NMDA (135±5.657, P<0.01), 20 nmol of glutamate (133.5±4.506, P<0.01), and 50 nmol of glutamate (120.167±5.648, P<0.01) compared with those in the PBS group (149±5.060; Figure 3E). There were statistically significant reductions in IRT (Figure 3E) and GCL cell number (Figure 3G) in the eyes treated with 50 nmol of glutamate compared with those in the other two treated groups (P<0.01). However, no statistically significant differences in RT were observed among the three treated groups (Figure 3C), although the RT with injection of 50 nmol of glutamate decreased more than those in the other two treated groups. Moreover, no statistically significant differences between the effects of 40 nmol of NMDA and 20 nmol of glutamate were observed in these three parameters (P>0.05).

Apoptosis in the Retina of the Glutamate-mediated Retinal Excitotoxic Damage Model Only a few scattered TUNEL-

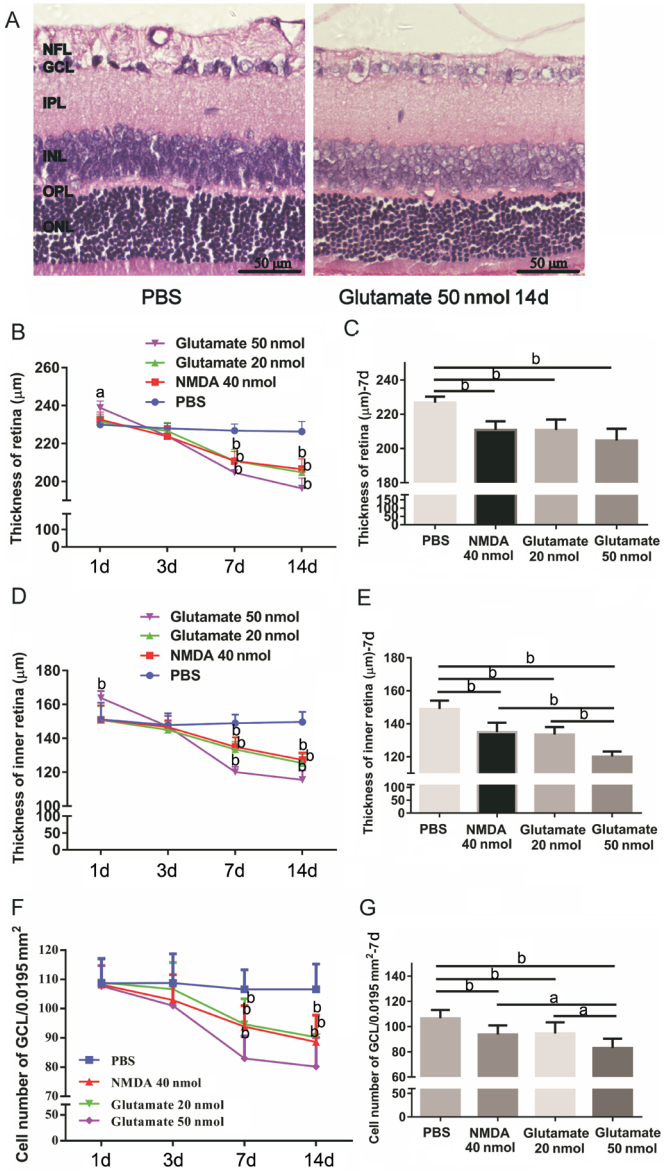

Figure 3 Time-dependent loss of inner retinal elements postinjection A: H&E stain of sagittal semithin section through the retina of rat eye 2wk after intravitreal injection of PBS and glutamate 50 nmol; B, D, F: Change of RT, IRT and cell number of GCL; C, E, G: RT, IRT and cell number of GCL 7d after intravitreal injection. $^{a}P<0.05$; $^{b}P<0.01$.

positive cells were detected in the sections of eyes that received intravitreal injections of PBS (Figure 4A). Numerous TUNEL-positive cells observed in the GCL were Brn-3a positive, with only a few TUNEL-positive cells in the inner plexiform layer (IPL) and INL 7d after intravitreal injections of 50 nmol of glutamate (Figure 4B). The expression of the apoptosis-related factor cleaved caspase-3 was detected 7d after intravitreal injection of 50 nmol of glutamate (Figure 4C). In addition, these changes were time-dependent, and the apoptosis index peaked 7d after intravitreal injection of 50 nmol of glutamate and 3d for 20 nmol of glutamate or 40 nmol of NMDA, then decreased gradually (Figure 4D). There were no statistically significant differences between the effects of 40 nmol of NMDA and 20 nmol of glutamate at any time point ($P>0.05$; Figure 4D). Moreover, when compared with 20 nmol of glutamate, there was a significant increase in the apoptosis index of 50 nmol of glutamate 7d after intravitreal injection ($P<0.01$; Figure 4E). Very few TUNEL-positive cells were detected in the ONL, which may suggest that the excitotoxicity of glutamate has little effect on the outer retina in this glutamate-mediated retinal excitotoxic damage animal model.

Tau Hyperphosphorylation in the Glutamate-mediated Retinal Excitotoxic Damage Model Normally, few hyperphosphorylated tau proteins are expressed in retinas. However, 14d after intravitreal injection of 50 nmol of glutamate, the expression of p-tau s396 and s404 increased significantly compared with that in the PBS group (Figure 5A-5D). The immunofluorescence assays revealed significant differences between the glutamate injection group and the PBS injection group in terms of the mean retinal expression densities of p-tau s396 (1.883±0.007712 *vs* 1.767±0.01809, $P<0.01$; Figure 5E) and p-tau s404 (1.874±0.005286 *vs* 1.752±0.02443, $P<0.01$; Figure 5F). The Western blot data were consistent with the results of the immunofluorescence assay, demonstrating significant differences between the glutamate group and the PBS group in the retinal expression of p-tau s396 (2.112±0.086 *vs* 1.048±0.257, $P<0.05$; Figure 6B) and p-tau s404 (1.354±0.141 *vs* 0.953±0.027, $P<0.05$; Figure 6C).

DISCUSSION

Although most studies have demonstrated that intravitreal EAA concentrations increase in many ocular diseases, such as diabetic retinopathy, glaucoma, retinal detachment, retinal artery occlusion and central retinal vein occlusion[18-21], a few studies have shown that there were no significant changes in vitreous glutamate concentration between glaucoma eyes and control eyes[20,22-23]. However, these studies were limited by a small number of study subjects[22], the use of some of the subjects included in several other protocols[23], and abnormalities in the control group[22]. It is possible that the epiretinal membrane or macular hole by itself could have elevated vitreous glutamate levels, thus lessening the difference between the study and control groups. Moreover, inappropriate handling of the samples before analysis and vitreous sample storage may have interfered with the results. Even if not statistically significant, the elevation of glutamate levels in vitreous glutamate may be physiologically sufficient to damage the retinal neurons, resulting in retinal neurodegeneration. Therefore, the possibility of glutamate-related excitotoxic effects on RGCs as a pathogenic factor of glaucoma should not be dismissed.

Glutamate levels are elevated not only in patients with visual field loss but also in those with glaucomatous optic nerve damage without field loss[24], suggesting that this aminoacidopathy may be present in the earlier stages of glaucoma and the elevated

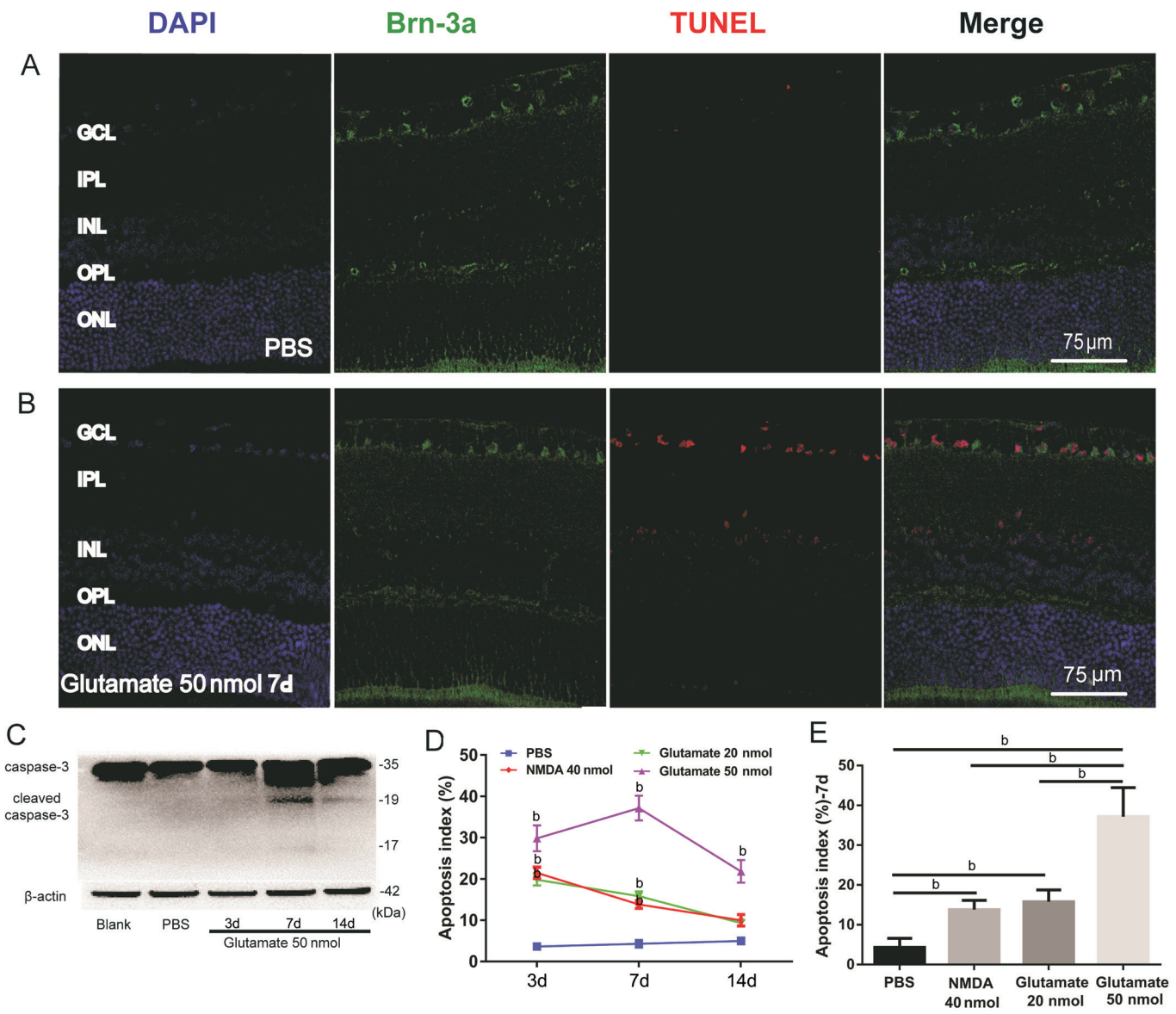

Figure 4 TUNEL of retinae at various time points after intravitreal injection A, B: TUNEL of reinae from PBS group (B) and 50 nmol glutamate group (C); C: Relative protein level of cleaved caspase-3 (n=3); D-E: The apoptosis index of each group (n=6). Apoptosis index= (TUNEL positive cells/total cells of GCL) ×100%. [a]P<0.05; [b]P<0.01.

IOP may not be a necessary factor for glaucoma. Therefore, this glutamate-mediated retinal excitotoxic damage model as a normotensive glaucoma model may better represent the early stages of glaucoma. However, the dose and duration of glutamate or NMDA injected into the vitreous chamber varies greatly[9,13-14,25]. To explore the more suitable concentration of glutamate or NMDA to establish a rat retinal excitotoxic damage model, we injected different doses of those compounds into the vitreous chambers of rats and observed several indices in this study. According to the literature (Table 1)[9,12-15,25], the effective glutamate concentration, the true vitreous volume of the rat and time of test should be taken into consideration, and we selected 50 nmol and 20 nmol of glutamate as well as 40 nmol of NMDA to explore the more suitable concentration of glutamate or NMDA for establishing a rat retinal excitotoxic damage model.

A recent study by Kuehn *et al*[9], that 20 nmol of NMDA had no effect on the percentage of RGCs and that injection of more than 40 nmol of NMDA reduced the percentage of RGCs in the retina. Some investigators have reported that a single injection of as little as 20 nmol of NMDA can be highly toxic to the RGC layer in the eyes of rats[13]. However, most of these studies did not measure the final concentrations of glutamate in vitreous samples. We observed that injections of 20 or 50 nmol of glutamate or 40 nmol of NMDA caused significantly increased glutamate concentrations in vitreous humor samples (P<0.05) compared with levels in the untreated group. However, 40 nmol of NMDA has effects that were approximately equivalent to those of 20 nmol of glutamate; almost all tested indices were similar, including the concentration of glutamate in vitreous samples, GCL cell number and IRT after intravitreal injection at each time

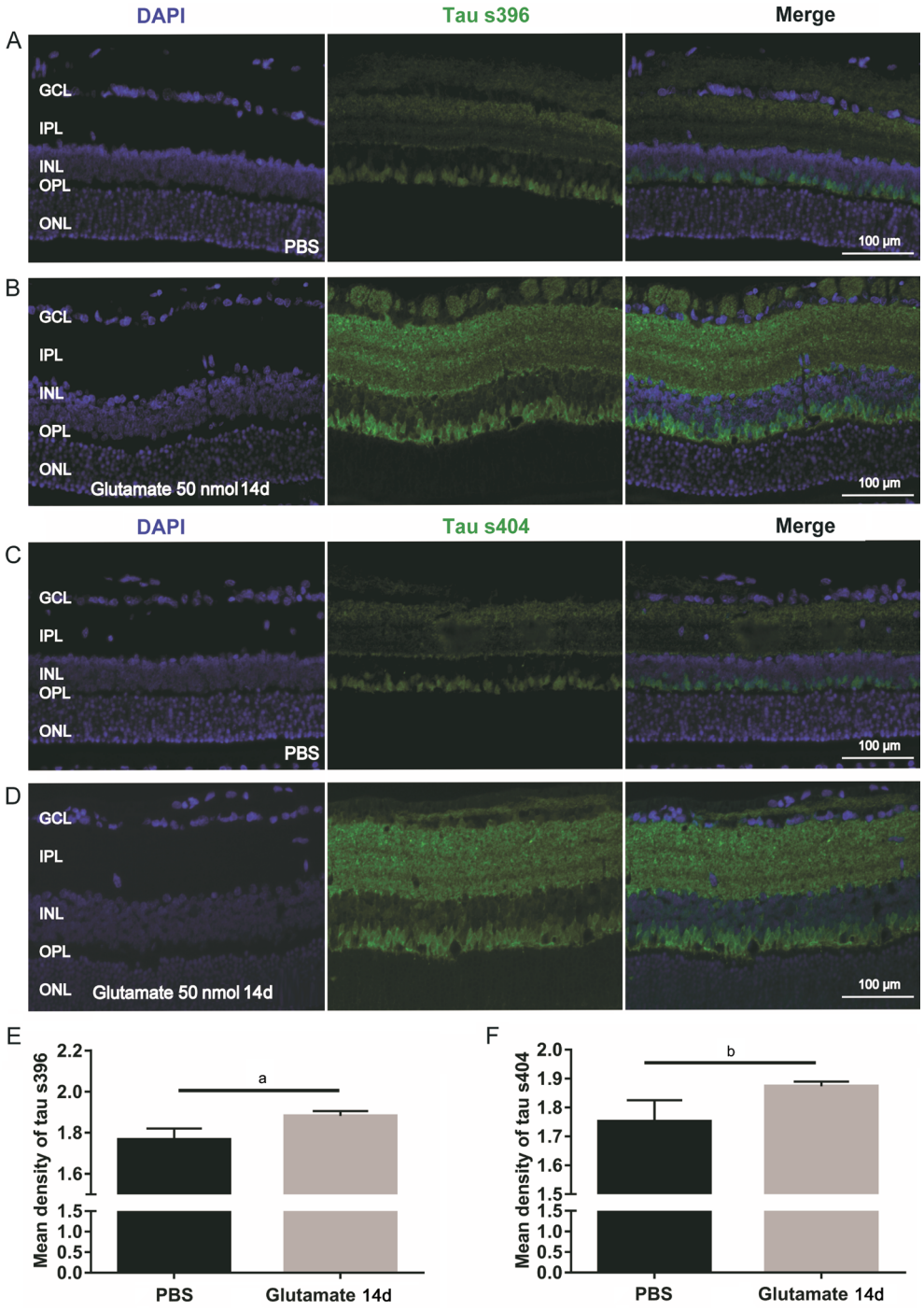

Figure 5 Upregulation of phosphorylated tau s396 and s404 14d after intravitreal injection of glutamate 50 nmol (Immunofluorescence) A, C: Phosphorylated tau s396 (A) and s404 (C) of PBS groups; B, D: Phosphorylated tau s396 (B) and s404 (D) 14d after intravitreal injection of 50 nmol glutamate; E-F: Mean density of phosphorylated tau s396 and tau s404. $^{a}P<0.05$; $^{b}P<0.01$.

point. More obvious changes in these parameters occurred in the group that received intravitreal injections of 50 nmol of glutamate, which resulted in twofold greater levels of glutamate than normal levels for approximately 2wk, while the elevated levels in the other two groups only lasted approximately 1wk. The levels then gradually decreased to normal. The predominant form of excitotoxicity of retinal neuron is mediated by overstimulation of the NMDA subtype of glutamate receptor, which, in turn, leads to excessive levels of intracellular calcium[26]. Non-NMDA receptors also may play a role in glutamate excitotoxicity[12,21]. Intravitreal injection of glutamate may be better than NMDA with respect to the induction of excitotoxicity.

Many of the changes in retinal morphology after glutamate injection were similar to the pathological changes associated with glaucoma: a dramatic decrease in the thickness of the nerve fiber layer (NFL), a decrease in the GCL cell number, and a large number of vacuole-containing cells in the GCL. In addition, the number of TUNEL-positive cells in the GCL increased after injection, and 7d later, the number gradually

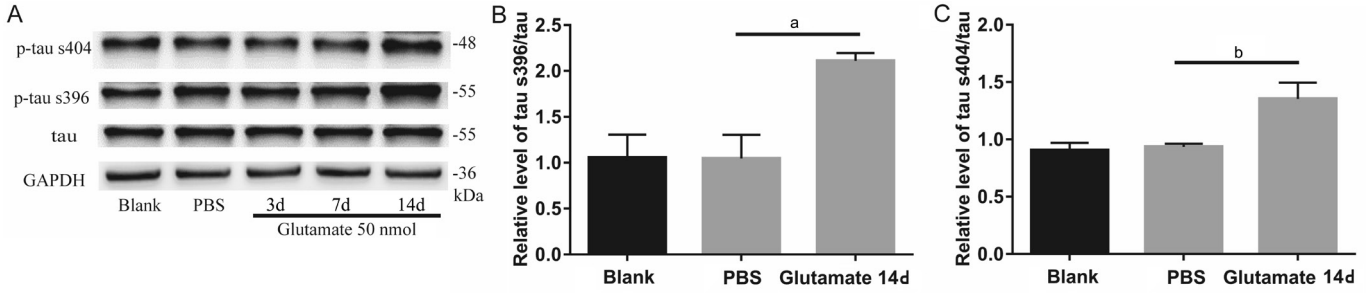

Figure 6 Upregulation of phosphorylated tau s396 and s404 14d after intravitreal injection of glutamate 50 nmol (Western blotting) Relative protein level of phosphorylated tau s396 (n=3; B) and phosphorylated tau s404 (n=3; C). $^aP<0.05$; $^bP<0.01$.

Table 1 Literatures on excitatory toxicity of glutamate

Literature	Subject	Methods	Time of observation	Results
Kuehn *et al*, 2017[9]	rat	Intravitreal injection 20, 40 and 80 nmol NMDA or PBS	days 3 and 14	NMDA 20 nmol/L had no effect on the percentage of Brn-3a+ cells.
Aihara *et al*, 2014[25]	rat RGCs	5 or 25 μmol/L of glutamate; hyperbaric pressure	72h	The survival rate of RGCs in the presence of 25 μmol/L of glutamate was significantly reduced; Hyperbaric pressure increased RGC susceptibility to glutamate toxicity.
Otori *et al*, 1998[12]	rat RGCs	5-500 μmol/L of glutamate	3d	Low doses of glutamate can activate AMPA-KA receptors in RGCs, which causes decreases in cell survival.
Vorwerk *et al*, 1996[14]	rat	Every 5d intravitreal injection of 2.5 nmol (1 μL) glutamate	1h, 1, 3, 5, 10d, 1, 2, 3mo	No retinal pathology was observed 10d after intravitreal injection; About 42% of the RGCs were killed 3mo after intravitreal injection.
Siliprandi *et al*, 1992[13]	rat	5 μL different concentrations of NMDA (2-200 nmol per injection)	8d	Administration of NMDA resulted in a dose dependent loss of cells in the GCL and a reduction in thickness of the IPL.
Dureau *et al*, 2001[15]	rat	Intravitreal injections of 1, 3, 5, or 10 μL China ink	immediately	A 3 or 5 μL volume appears to have the best reproducibility with minimum loss of solution.

NMDA: N-methyl-D-aspartic acid; AMPA-KA: Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-kainic acid; IPL: Inner plexform layer.

decreased. Furthermore, the expression of the apoptosis-related factor cleaved caspase-3 was detected 7d after intravitreal injection of 50 nmol of glutamate. These data are consistent with the concept that three weeks after intravitreal injections, apoptotic processes in the retina are largely complete, and cellular deposits have been removed[11]. Seitz and Tamm[11] reported that a few hours after injections of 30 nmol of NMDA into the vitreous chambers of mice, the first apoptotic cells could be observed in the retina; 24h later, at least half of the cells in the GCL were TUNEL-positive. Given that the volume of the rat vitreous chamber is much larger than that of mice, the differences in the final concentrations of glutamate may cause different results.

Tau proteins are essential for microtubule assembly and microtubule stabilization. We suggest that tau proteins are closely related to retinal neurons degeneration, which is supported by the increased levels of tau proteins in the vitreous bodies of patients with glaucoma and diabetic retinopathy, the susceptibility of retinal neurons to tau-mediated neurodegeneration[27], and the fact that glaucoma exhibits signature pathological features of tauopathies, including tau accumulation, altered tau phosphorylation and tau protein missorting[28]. Phosphorylation is a critical posttranslational modification of tau proteins during development and in pathological conditions. Phosphorylation on residues s396 and s404 has been described in the brains of patients with tauopathies, and both residues are known to impair microtubule binding and facilitate tau aggregation[28]. Normally, few hyperphosphorylated tau proteins are expressed in the retina. In our experiment, after intravitreal injection of 50 nmol of glutamate, the expression of p-tau s396 and s404 in the retina increased significantly compared with that in the control group, which was accompanied by a sustained high concentration of glutamate in the vitreous humor for at least 2wk. These data suggest that the glutamate-induced retinal excitotoxic damage animal model is a good tool for investigating the pathogenesis of glaucoma.

In summary, our study demonstrates that intravitreal injections of glutamate or NMDA can cause glaucomatous retinal neurodegeneration. Moreover, treatment with 50 nmol of glutamate *via* rat intravitreal injection establishes a more effective retinal excitotoxic damage animal model.

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