Annals of Anatomy 221 (2019) 57–67

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.com/locate/aanat

RESEARCH ARTICLE

# Calpain2 but not calpain1 mediated by calpastatin following glutamate-induced regulated necrosis in rat retinal neurons

Shuchao Wang[a,1], Yanxia Huang[a,1], Yuhan Yan[b], Hongkang Zhou[a], Mi Wang[a], Lvshuang Liao[a], Zhen Wang[a], Dan Chen[a], Dan Ji[c], Xiaobo Xia[c], Fengxia Liu[d], Jufang Huang[a,*], Kun Xiong[a,*]

[a] Department of Anatomy and Neurobiology, School of Basic Medical Sciences, Central South University, Changsha 410013, China
[b] Xiangya School of Medicine, Central South University, Changsha 410008, China
[c] Department of Ophthalmology, Xiangya Hospital, Central South University, Changsha 410008, China
[d] Department of Human Anatomy, School of Basic Medical Science, Xinjiang Medical University, Urumqi 830001, China

ARTICLE INFO

*Article history:*
Received 23 April 2018
Received in revised form 15 August 2018
Accepted 16 August 2018

*Keywords:*
Glutamate
Regulated necrosis
Calpain1
Calpain2
Calpastatin

ABSTRACT

The purpose of this study is to investigate whether calpastatin (CAST) plays an important role in the regulated necrosis (RN) in rat retinal neurons under an excessive glutamate condition and furthermore to investigate whether this process is regulated by calapin1 and calpain2. In the present study, glutamate triggered CAST inhibition, calpain2 activation and retinal neuronal RN after injury. The application of CAST active peptide could provide protective effects against activated calpain2 mediated RN. However, the calpain1 activity was not changed in these processes. Finally, *in vivo* studies further confirmed the role of the CAST-calpain2 pathway in cellular RN in the rat retinal ganglion cell layer and inner nuclear layer after glutamate excitation. In addition, flash electroretinogram results provided evidence that the impaired visual function induced by glutamate could recover after CAST peptide treatment. This research indicated that excessive glutamate may lead to CAST inhibition and activated calpain2, but not calpain1 activation, resulting in RN.



## 1. Introduction

Glutamate, one of the twenty essential amino acids, has extensive numbers of physiological functions, such as regulating neurogenesis, signaling and neuronal survival (Martinez et al., 2014; Mattson, 2008; Yan et al., 2016). However, under some neurodegenerative conditions such as Alzheimer's disease (AD), Huntington's disease (HD), glaucoma and diabetic retinopathy, glutamate may exist in high concentrations (Haas et al., 2017; Miao et al., 2012; Yan et al., 2016). The inefficient clearance of excessive glutamate from the synaptic cleft initially activates different types of glutamate receptors, including *N*-methyl-D-aspartic acid receptor (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR) and kainic acid receptors (KAR) (Lopez-Colome et al., 2016; Quillinan et al., 2016), leading to massive calcium accumulation. Overloading calcium can trigger a cascade of downstream neurotoxic events (Suarez-Pozos et al., 2017), such as proteases activation, energy deficiency, and oxidative stress, resulting in neuronal death known as necrosis, apoptosis and regulated necrosis (RN) (Del Rosario et al., 2015; Leon et al., 2009; Xu et al., 2007). RN is a form of programmed cell death that mainly includes necroptosis, ferroptosis, parthanatos, and so on, which exhibit morphological features similar to those of necrosis (Cheng et al., 2018; Ding et al., 2015; Galluzzi et al., 2014; Kong et al., 2017; Pasparakis and Vandenabeele, 2015; Wang et al., 2018b). However, so far, the molecular pathways underlying excessive glutamate modulated neuronal RN are still not clearly defined.

The calpastatin (CAST)-calpains system has drawn wide attention because of the discovery of relationships between changes in its genes and human diseases (Suzuki et al., 2004). Calpains, which exist ubiquitously in organisms, are a family of calcium dependent cysteine proteases that are vital for varieties of calcium-related cellular processes, such as survival, proliferation, differentiation, apoptosis and signal transduction (Agudo et al., 2009; Curcio et al., 2016; Martensson et al., 2017; Senter et al., 1991). There are two major calpain isoforms in the nervous system – calpain1 (μ-calpain) and calpain2 (m-calpain) –, which differ in their

* Corresponding authors at: Department of Anatomy and Neurobiology, Morphological Sciences Building, Central South University, 172 Tongzi Po Road, Changsha, Hunan 410013, China.
*E-mail addresses:* huangjufang@csu.edu.cn (J. Huang), xiongkun2001@163.com (K. Xiong).
[1] These authors made equal contributions to this manuscript.

https://doi.org/10.1016/j.aanat.2018.08.005
0940-9602/

activation of micromolar and millimolar levels of calcium requirements, respectively (Hoque et al., 2016; Liu et al., 2005; Wang et al., 2016a). From previous research, activation of calpain1/2 may lead to various neuronal degenerative diseases such as AD, PD, epilepsy, glaucoma and diabetic retinopathy (Ahn et al., 2016; Kaur et al., 2011; Liu et al., 2005; Pandey et al., 2016; Wang et al., 2016b). Our previous results also demonstrated that glutamate accumulation could lead to calpain2-mediated RN in primary retinal neurons (Wang et al., 2018a). However, some recent studies showed that calpain1 activation could promote cell survival under some conditions, such as cerebellar ataxia, epileptic activity and ischemia/reperfusion injury (Seinfeld et al., 2016; Wang et al., 2016b; Wang et al., 2016c). In fact, some of the studies indicating the distribution of calpain1 in the retina have not addressed the role of calpain1 in neuronal RN. Therefore, in this study, we first aimed to investigate whether activity of calpain1 was changed in rat retinal neurons after glutamate injury. We are also interested in the distinction and relationship between calpain1 and calpain2 in neuronal RN. In addition, levels of calpain1/2 are physiologically strictly regulated by CAST, a specific endogenous inhibitor of calpains (Averna et al., 2001; Volbracht et al., 2005). Although some reports have indicated that calpain1 and calpain2 play opposite roles in cell death, there are still no studies that have investigated whether the different regulatory role of CAST on calpain1 and calpain2 exist in retinal neuronal RN after glutamate injury. Our previous study has demonstrated that Pin1 may interact with CAST/calpain2 complex and play a regulatory role in complex-mediated retinal RN (Wang et al., 2018a). However, we did not investigate whether CAST directly participate into retinal RN induced by glutamate. Thus, the second aim of this study is to further address the direct role of CAST in retinal RN and the specific distinct roles of CAST in calpain1 or/and calpain2 mediated retinal RN following glutamate excitotoxicity.

To further demonstrate the potential differential roles of CAST in calpain1 or/and calpain2 activation, we first verify the effects of CAST on RN in retinal neurons after glutamate injury *in vitro*. Further, we investigated the effect of active CAST peptide in activation of calpain1 or/and calpain2 under the excessive glutamate condition *in vitro*. Finally, *in vivo* studies were performed to verify the role of CAST-calpain1/2 pathways in RN in retinal GCL and INL after glutamate excitatory transmission. We expect that the results will provide a better mechanism for understanding neuronal RN in glutamate excitatory in the future.

## 2. Methods and materials

### 2.1. Primary retinal neuron cultures and in vitro model preparation

All experimental procedures were approved by the Ethics Committee of the 3rd Xiangya Hospital of Central South University in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. 1-day-old Sprague-Dawley (SD) rats were used for five studies (Li et al., 2016; Morgan-Warren et al., 2016; Wang et al., 2017). Briefly, the retinae were first removed aseptically and placed in Dulbecco's modified Eagle's medium (DMEM, GE Healthcare, Logan, Utah USA) containing 0.02% papain and digested at 37 °C for 10 min. Then, the retinal tissue was triturated 30 times and filtered with a 70-mm nylon cell strainer. Last, the cells were plated at a density of $6 \times 10^5$ cells/mL. Four hours after plating, the DMEM medium was replaced with neurobasal medium (Thermo Scientific, Waltham, USA) supplemented with B27 (Thermo Scientific). On the seventh day, the neuronal cultures were treated with 50 μM glutamate (Sinopharm, Beijing, China) for 2 h and then allowed to recover for 0, 2, 4, 6, 12, or 24 h as our previous research described (Aihara et al., 2014; Kim et al., 2016; Wang et al., 2018a).

### 2.2. Animal model of GT and tissue preparation

The SD rat glutamate models were prepared following our previous protocol (Wang et al., 2018a). Briefly, rats were anesthetized and a single dose of 5 μL of 100 mM glutamate in 0.01 M PBS was injected into the vitreous cavity using a 32-gauge Hamilton needle and syringe (Sisk and Kuwabara, 1985; Wang et al., 2018a). A sham operation was performed as a control. Animals were allowed to survive for 6 h. Each group was composed of 5 animals. At each sacrifice time point, animals were anesthetized and perfused transcardially with 0.9% sodium chloride, followed by 4% paraformaldehyde (PF). After perfusion, eyes were enucleated, the anterior segments were removed and the posterior eyecups were post-fixed in 4% PF overnight at 4 °C. Then, the eyes were placed sequentially in 15% and 30% sucrose at 4 °C. Next, the eyecups were embedded in Tissue-Tek optimal cutting temperature medium and frozen in liquid nitrogen. Afterwards, cryosections were cut into 6 mm thickness using a microtome (Thermo Scientific).

### 2.3. Drug application

CAST active peptide and Negative peptide were obtained from MilliporeSigma (Darmstadt, Germany). All drugs were dissolved in sterile phosphate-buffered saline (PBS, GE Healthcare) as stock solutions. CAST peptide and Negative peptide were used at a concentration of 50 nM. Drugs were administered directly to primary retinal neuronal cultures or intravitreal injection of rats 1 h before GT.

### 2.4. Lactate dehydrogenase (LDH) release

For *in vitro* experiments, a LDH cytotoxicity assay kit (Beyotime, Shanghai, China) was used to measure the LDH released from necrotic cells into the extracellular space/supernatant upon the rupture of the plasma membrane (Shang et al., 2017; Xiong et al., 2016) after different treatments. Cell-free culture supernatants were collected from 96-well microtiter plates and incubated with the appropriate reagent mixture according to the manufacturer's instructions at room temperature (RT) for 30 min. For *in vivo* experiments, a LDH cytotoxicity assay kit was purchased from Jiancheng institutes (Nanjing, China) and assays were performed according to the instructions (Wang et al., 2018a). Briefly, the retinae were homogenized by sonication in 0.86% NaCl, and incubated with the appropriate reagent mixture at 37 °C for 30 min. The intensity of red color formed in the assay, measured at a wavelength of 490 or 450 nm, was proportional to both LDH activity and the percentage of necrotic cells. The percentage of necrotic cell death was calculated by the color intensity of treated cells minus control cells/LDH releasing reagent treated cells minus control cells from four independent experiments.

### 2.5. Propidium iodide (PI) staining

PI staining was performed to determine necrotic neurons (Li et al., 2016; Pietkiewicz et al., 2015; Rosenbaum et al., 2010). Cell cultures were washed twice with ice-cold PBS and incubated with PI at RT for 20 min in the dark. For *in vivo* experiments, 5 μL PI were intravitreally injected 30 min prior to sacrifice (Huang et al., 2013). Rats were euthanized at indicated time points. Eyes were frozen in nitrogen vapor and cryostat sections were cut. The coverslips or retinal sections were washed three times and covered with an anti-fading mounting medium with DAPI (Vector Laboratories, Burlingame, USA). Images were captured by a fluorescence

microscope (Olympus, Tokyo, Japan). The percentages of cells were analyzed by Image J software (NIH, Baltimore, Maryland USA). The number of PI staining cells were assessed by calculating the number of PI-positive cells in every intact captured images.

### 2.6. Immunofluorescence staining

Cell cultures were fixed for 20 min with 4% PF and the rat retinal sections were recovered at RT. The samples were blocked for 1 h in blocking buffer that consisted of PBS containing 5% normal bovine serum and 0.3% Triton X-100 and then incubated with combinations of the primary antibodies against the following targets: CAST (1:50, Santa Cruz, Dallas, USA), calpain1 (1:100, Abcam, Cambridge, UK), calpain2 (1:100, Abcam), PTEN (1:50, Boster, Wuhan, China), overnight at 4 °C. The samples were shifted to RT for 30 min and then washed three times with PBS and incubated with Alexa-conjugated secondary antibodies (1:200, Jackson Immuno Research, West Grove, Pennsylvania USA) for 2 h. After washing three times in PBS, the samples were covered with Vectashield mounting medium (Vector Laboratories, Burlingame, California USA) containing DAPI. Images were taken under the fluorescence microscope.

### 2.7. Western blot

Cell cultures and rat retinal tissues were lysed in RIPA containing 1% protease inhibitors (CWBIO, Beijing, China). The extracts were centrifuged at 12,000 × *g* for 20 min at 4 °C. The protein concentration was determined by BCA assay and 10 μg of each protein sample was loaded per lane. Proteins were separated by 4–6% or 10% SDS-PAGE gel and transferred to nitrocellulose membranes (GE Healthcare). The membranes were blocked with 5% nonfat milk with 0.1% Tween 20 for 1 h or 3 h at RT and then incubated overnight at 4 °C with primary antibodies: CAST (1:200), calpain1 (1:1,000), calpain2 (1:500), PTEN (1:500), HSP90, (1:1,000, Proteintech, Wuhan, China) GAPDH (1:5,000, Beyotime), β-Actin (1:5,000, Beyotime). The membranes were then shifted to RT for 30 min. After washing three times, the membranes were incubated with HRP-conjugated secondary antibodies (1:2000, Beyotime) for 2 h at RT. The immunoreactive bands were visualized by chemiluminescence reagent (CWBIO). Integrated density values of specific proteins were quantified using Image J software and normalized to the values of GAPDH or β-Actin.

### 2.8. Calpain activity

Pan-calpain activity: pan-calpain activity was measured by a calpain activity assay kit (Abcam) according the manufacturer's protocol. Briefly, cell cultures were harvested and homogenized in ice-cold extraction buffer. Then, tissues were centrifuged at 12,000 × *g* for 5 min at 4 °C and the supernatant was incubated with reaction buffer for 1 h at 37 °C in the dark. Last, pan-calpain activity was shown by fluorescence and was detected at 365 nm excitation and 460 nm emission measured using a Bio-rad microplate reader (Hercules, USA). Changes in pan-calpain activity are normalized to the saline control and expressed as fold change of relative fluorescent units (RFU) (Wolhuter et al., 2018).

Calpain2 activity: calpain2 activity was assessed by measuring the fold change of PTEN expression (Hanouna et al., 2017; Wang et al., 2016c). The activity of calpain2 in the control group was set to m (= 1). The increased activity of calpain2 (= n) was the absolute value of quantitative difference between the fold change of PTEN in control group and treated group. The total activity of calpain2 is m + n.

Calpain1 activity: there is no commercial kit or specific substrates to detect calpain1 activity. As calpain1 and calpain2 are the main forms of the calpain family (Wang et al., 2016c), the calpain1 activity is approximately the quantitative difference between the activity of pan-calpain and calpain2 (Wang et al., 2016c). The activity of calpain1 in the control group is set to p (= 1). The increased activity of calpain1 (= q) is the quantitative difference between increased activity of pan-calpain and calpain2. The total activity of calpain1 was p + q.

### 2.9. Flash electroretinogram (fERG)

Rats were adapted in the dark for 6 h after drug application. All of the following procedures were conducted under dim red illumination. Recordings were performed by the RM6240 system (Chengdu Instrument Factory, Chengdu, China). The recording electrode was inserted into the anterior chamber. The reference and ground electrodes were placed on the subcutaneous layer of the forehead and tail base respectively. Bandpass was filtered at 10 Hz. Flash luminance ranged 1.6 cd/s/$m^2$. Each eye was exposed to flashes three times and the interval was 5 min. When one eye was recorded, the contralateral eye was covered. All procedures were repeated at least 4 times. The amplitude of the b wave was calculated from the bottom of a wave to the peak of b wave.

### 2.10. Statistical analysis

Figure panels were assembled by using Photoshop (Adobe Systems Incorporated, San Jose, USA). The measurement data were presented as the mean ± SD. One-way analysis of variance and independent sample *t*-tests were used to analyze the data using GraphPad Prism 5 software (GraphPad Software Inc, San Diego, USA). A value of $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Glutamate induces necrosis in primary retinal neurons

Primary retinal neurons were treated with 50 μM glutamate for 0, 2, 4, 6, 12, or 24 h. PI staining was used to label the necrotic neurons. In our observation time points, the number of increased PI-positive cells was observed at 2 h and peaked at 6 h after GT (Fig. 1A, B). A LDH assay was also conducted to evaluate the percentage of necrotic neurons. Compared with 0 h, the percentage of necrotic neurons was increased in a time-dependent manner at all observation time points (Fig. 1C). These results indicated that glutamate treatment (GT) could induce necrosis in primary retinal neurons.

### 3.2. CAST, calpain1 and calpain2 activities following GT

We first examined whether the expression of CAST, calpain1 and calpain2 in cultured primary retinal neurons could be changed after GT. Double immunofluorescence results showed that CAST, calapin1 and calapin2 are all predominantly located in cytoplasm and nucleus, and slightly in dendrites (Fig. 2A–C). Western blot results showed that the expression of CAST was remarkably decreased after 2, 4, or 6 h of GT and then tended to be at normal levels at 12 and 24 h (Fig. 2D, E). In parallel to the decreased CAST expression, the calpain2 expression was increased compared to the control group and exhibited a peak at 6 h after GT (Fig. 2D, G). However, following GT, the expression of calpain1 did not change remarkably at all time points (Fig. 2D, F). These results suggest that CAST expression was inhibited and calpain2 expression was enhanced after GT, while the expression of calpain1 was not changed after GT.

We next analyzed the activity of calpain1 and calpain2. PTEN is the specific substrate of proteolysis of calpain2 and used to be

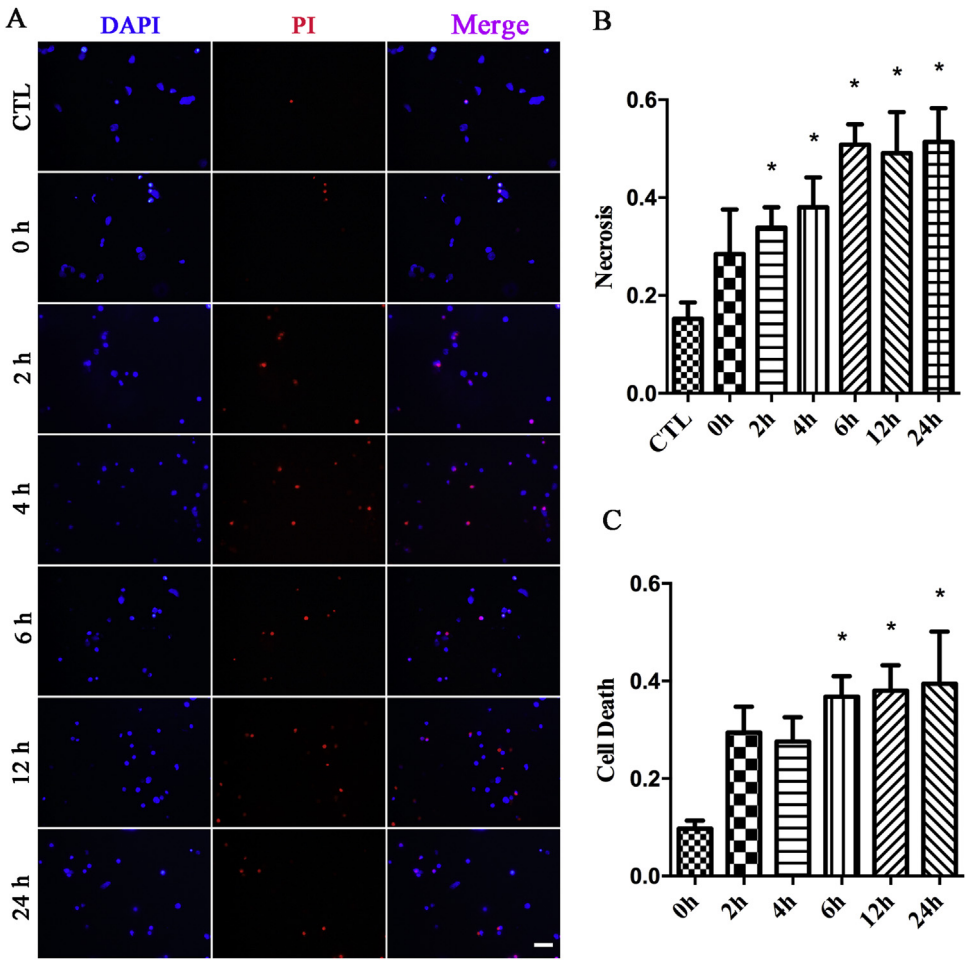


**Fig. 1.** Necrotic retinal neurons determined by PI staining and LDH release after GT. (A) Glutamate treated retinal neurons were stained with PI (red). Nuclei were counterstained with DAPI (blue). (B) Quantitative analysis of PI stained neurons after GT. (C) The percentage of necrotic neurons were determined by LDH release. $^{*}p < 0.05$ *vs* respective CTL or 0 h group. Data are expressed as means ± SD (n = 5). *Scale Bar* = 20 μm in all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

considered the specific marker of calpain2 activation (Briz et al., 2013; Liu et al., 2016). The decreased PTEN levels indicated that calpain2 is activated (Fig. 2D, H). However, as described in Section 2, there is no specific calpain1 substrate, therefore we first detected the pan-calpain activation by a commercial kit, which showed that the pan-calpain activation was also enhanced after GT. As calpain1 and calpain2 are the main forms of calpain, thus we calculated the calpain1 activity by the quantitative difference of pan-calpain and calapin2 (see the materials). Although the calapin1 activity was increased slightly and accurately, the difference was not significant. Together, this results indicate that calpain1 was activated, but the calpain2 activity was not changed after GT.

Some researchers have reported that heat shock protein 90 (HSP90) interacts with calpain1 and plays a regulatory role in calpain1 activation, in conditions of increased calcium (Averna et al., 2015; Martines et al., 2017). Thus, we further investigate whether HSP90 was changed in our study. From the western blot results (see Supplement figure), it is clear that the expression of HSP90 was not changed after GT.

### 3.3. The role of CAST in activities of calpain1 and calpain2

To investigate the underlying different roles of CAST in regulating activities of calpain1 or/and calpain2, we cultured retinal neurons with active CAST peptide before GT. Correlated with previous reports, our results first showed that CAST was efficiently blocked the calpain2 activity, which was shown by decreased calpain2 expression and increased PTEN level (Fig. 3A–E). Next we investigated the effect of CAST on calpain1. However, the expression and activation of calpain1 were not significant compared to the GT group (Fig. 3A–C). This results suggest that the calpain1 activity might not be affected by CAST in this glutamate injury model. Altogether, these results suggest that CAST may lead to differential activation of calpain1 and calpain2 in retinal neurons after GT.

### 3.4. The role of CAST in retinal neuronal RN

To evaluate the effect of CAST on retinal neuronal RN, the neurons were cultured with active CAST peptide. PI staining and LDH assay were performed to determine the necrosis. Fig. 4 shows that RN in CAST treatment group was reduced compared with the GT group, indicated by decreased LDH and PI counts. Meanwhile, the CAST Negative peptide did not change the percentage of RN in the DMSO group, as compared to GT group (Fig. 4). Collectedly, these results demonstrate that decreased CAST activity plays an important role in retinal neuronal RN after GT and that application of active CAST peptide has a protective effect against glutamate-induced RN.

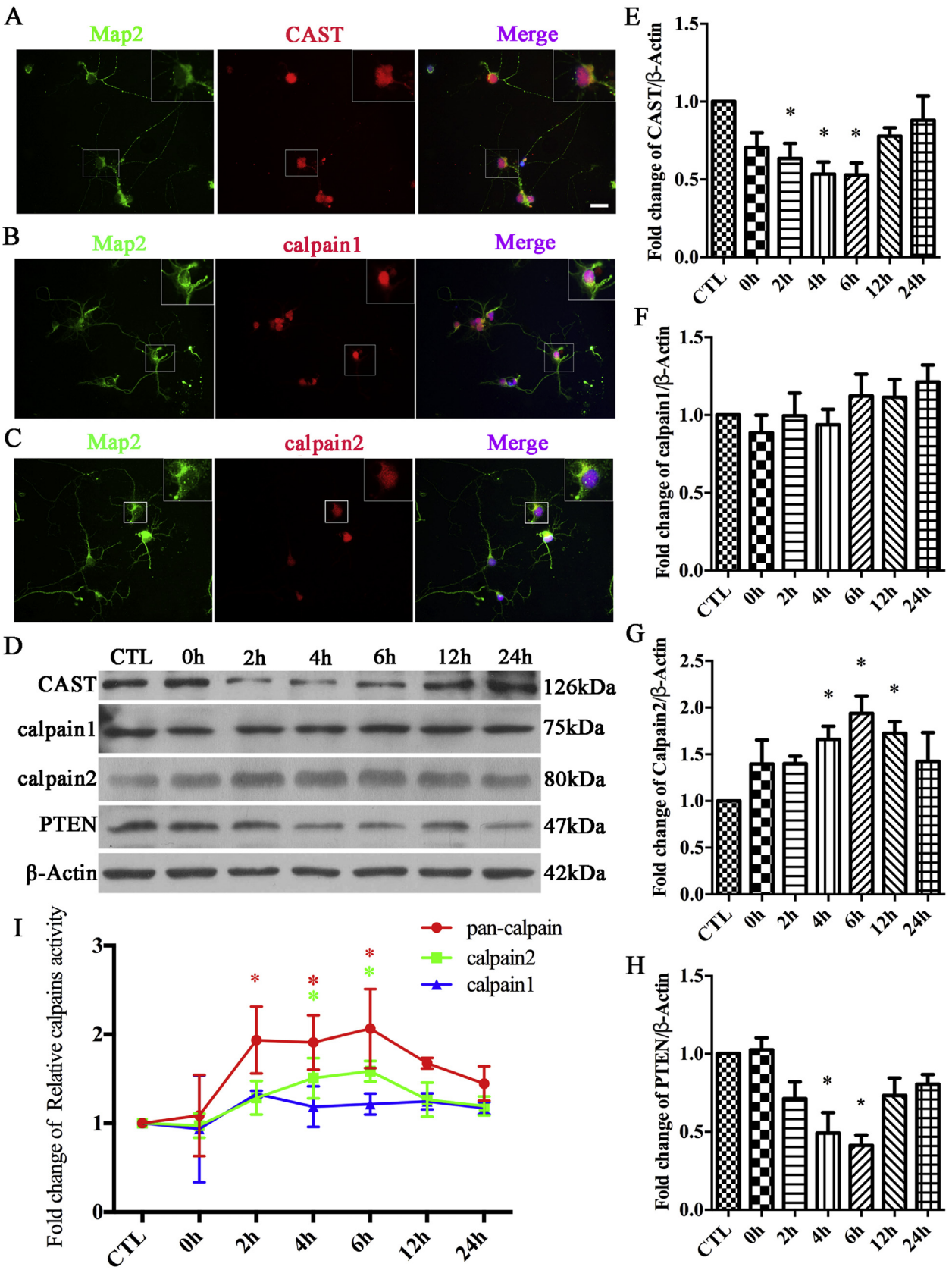


**Fig 2.** Activities of CAST, calpain1, calpain2 and PTEN in retinal neurons following GT. (A–C) Double-immunofluorescence staining of MAP2 with CAST, calpain1 and calpain2, *Scale Bar* = 20 μm. The small frame is the enlargement of the indicated area, *Scale Bar* = 10 μm. (D) Western blot of CAST, calpain1, calpain2 and PTEN expression in retinal neurons following GT. (E–H) The statistical analysis of CAST, calpain1, calpain2 and PTEN expression by western blot in retinal neurons following GT. $^{*}p < 0.05$ *vs* CTL group. (I) The statistical analysis of pan-calpain2, calpain1 and calpain2 activities after GT for 6 h. Data are expressed as means ± SD (n = 5). $^{*}p < 0.05$ *vs* CTL group.

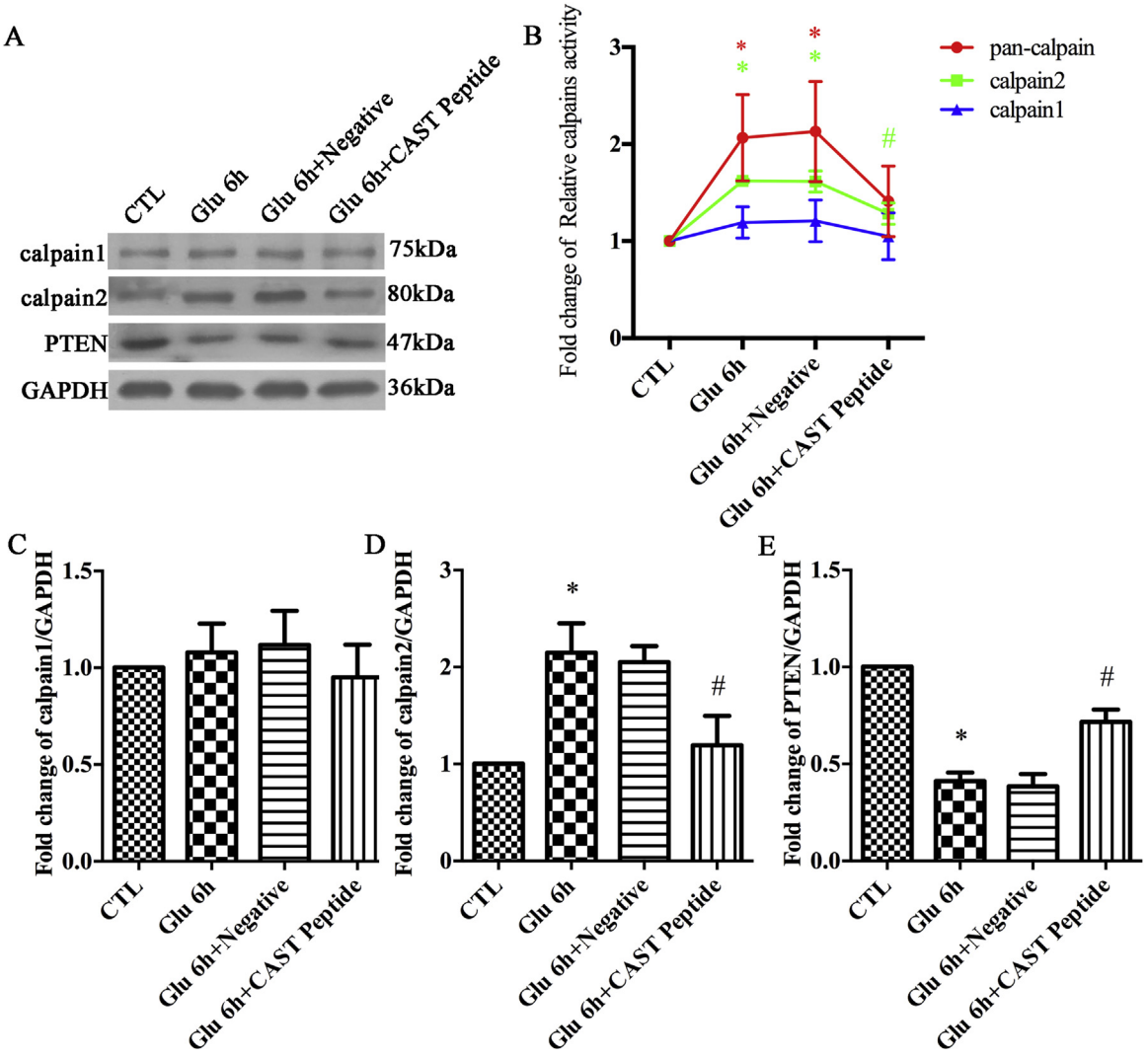


**Fig. 3.** Activities of calpain1, calpain2 and PTEN in retinal neurons following GT and pretreated with CAST peptide before GT for 6 h. (A) Western blot of calpain1, calpain2 and PTEN expression in retinal neurons following GT. (B) The statistical analysis of pan-calpain2, calpain1 and calpain2 activities pretreated with CAST peptide before GT. $^{*}p < 0.05$ *vs* CTL group. (C, E, F) The statistical analysis of calpain1, calpain2 and PTEN expression by western blot pretreated with CAST peptide before GT. Data are expressed as means ± SD (n = 5). $^{*}p < 0.05$ *vs* CTL group, $^{\#}p < 0.05$ *vs* Glu 6 h group.

### 3.5. *CAST modulates RN in retinal GCL and INL* in vivo

Fig. 5 shows that PI stained necrotic cells in the ganglion cell layer (GCL) and the inner nuclear layer (INL) were increased in the glutamate group (Fig. 5E, F), compared with controls (Fig. 5A, B) or sham groups (Fig. 5C, D). Next, we investigated the effect of active CAST peptide in RN. PI staining results showed significant reduction of necrotic cells in retinal GCL and INL following CAST peptide application (Fig. 5G, H). Quantitative analysis of PI positive cells in GCL and INL is shown in Fig. 5M. The LDH assay *in vivo* also demonstrated that increased necrotic cells were observed in the glutamate group compared with controls or sham groups. And the increased LDH release was decreased in the CAST peptide pretreatment group (Fig. 5N). Altogether, these results indicated that CAST could provide a protective role in retinal cells in GCL and INL against glutamate injury. Last, fERG was performed to evaluate the visual function (Liao et al., 2016). FERG results showed that the amplitudes of b wave in the glutamate group were significantly decreased compared to controls or sham groups (Fig. 5I–K). Meanwhile, the amplitudes of b wave were enhanced in CAST peptide treated groups (Fig. 5L). Quantitative analysis of the b wave amplitudes is shown in Fig. 5O. This results demonstrated that CAST has protective effects on retinal function after GT.

## 4. Discussion

In this study, we investigated the role of the CAST-calpain2 pathway in rat retinal neuronal RN induced by glutamate. Cultured retinal neurons were exposed to glutamate, which was able to trigger inhibition of CAST, calpain2 activation and neuronal RN. After application of active CAST peptide, both calpain2 activity and neuronal RN were decreased. Additionally, our study indicated that CAST may have no significant effect on calpain1 activity in such a glutamate injury model. Finally, by using an animal glutamate treatment model, we demonstrated that CAST has a neuroprotective role against glutamate induced retinal RN. These results suggest that CAST and calpain2 could be potential targets to protect neurons from RN in some neurodegenerative diseases such as glaucoma, diabetic retinopathy, and even central nervous system diseases.

Excessive glutamate accumulation could activate glutamate receptors and subsequently lead to calcium overloading, eventually resulting in programmed cell death (Lalkovicova and Danielisova,

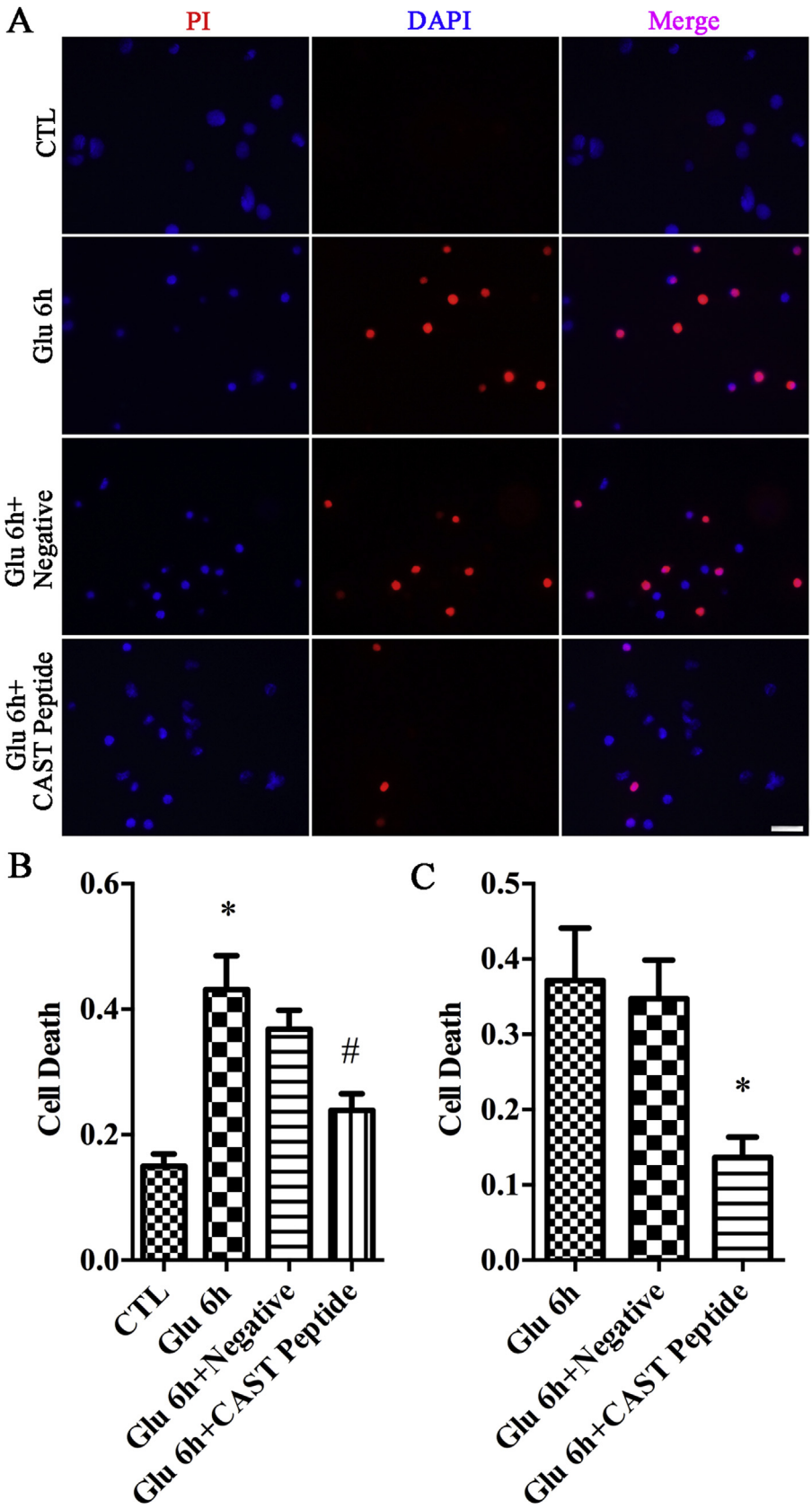


**Fig 4.** Necrosis determined by PI staining and LDH release in retinal neurons pretreated with CAST peptide before GT for 6 h. (A) Retinal necrotic neurons were stained with PI (red). Nuclei were counterstained with DAPI (blue). (B) Quantitative analysis of PI stained retinal neurons. $^{*}p < 0.05$ *vs* CTL, $^{\#}p < 0.05$ *vs* Glu 6 h group (C) the percentage of necrotic neurons were determined by LDH release. Data are expressed as means ± SD (n = 5). $^{*}p < 0.05$ vs Glu 6 h group. *Scale Bar* = 20 μm in all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

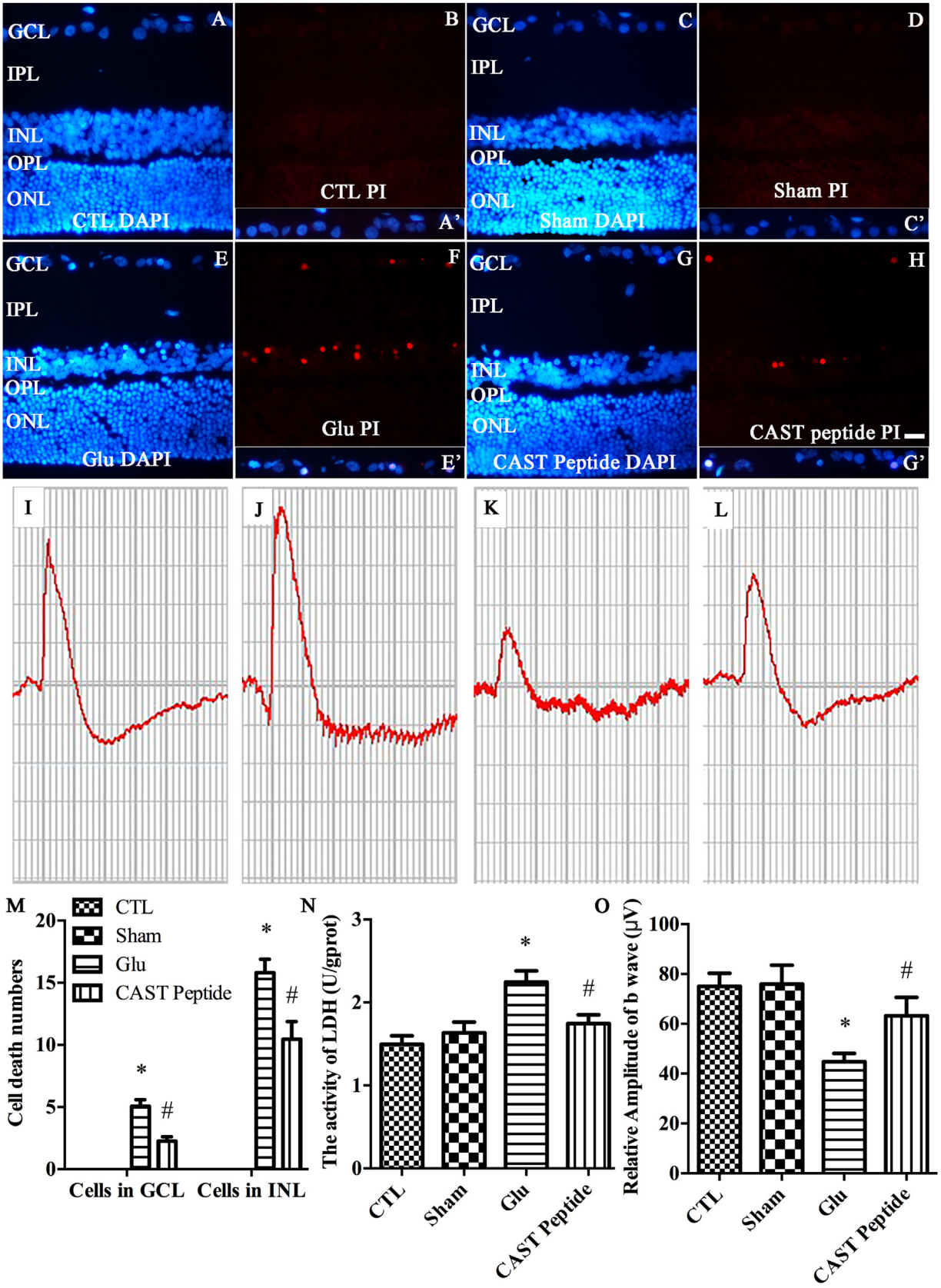


**Fig. 5.** Necrosis determined by PI and LDH, visual function detected by fERG *in vivo*. (A, C, E, G) Nuclei were stained with DAPI (blue). (B, D, F, H) Retinal necrotic neurons were stained with PI (red). (A', C', E', G') The framed areas show merged nuclei and PI staining in the GCL as indicated. (I-L) Representative fERG results. (M) Quantitative analysis of PI-stained cells in GCL and INL in retina, Data are expressed as means ± SD (n = 4, each). *$p < 0.05$ *vs* CTL group, #$p < 0.05$ *vs* Glu group. (N) The percentage of necrotic cells was determined by LDH release, Data are expressed as means ± SD (n = 4, each). *$p < 0.05$ *vs* CTL group, #$p < 0.05$ *vs* Glu group. (O) Quantitative analysis of the b wave amplitudes. Data are expressed as means ± SD (n = 4, each). *$p < 0.05$ *vs* CTL group, #$p < 0.05$ *vs* Glu group. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer. *Scale Bar* = 20 μm in all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

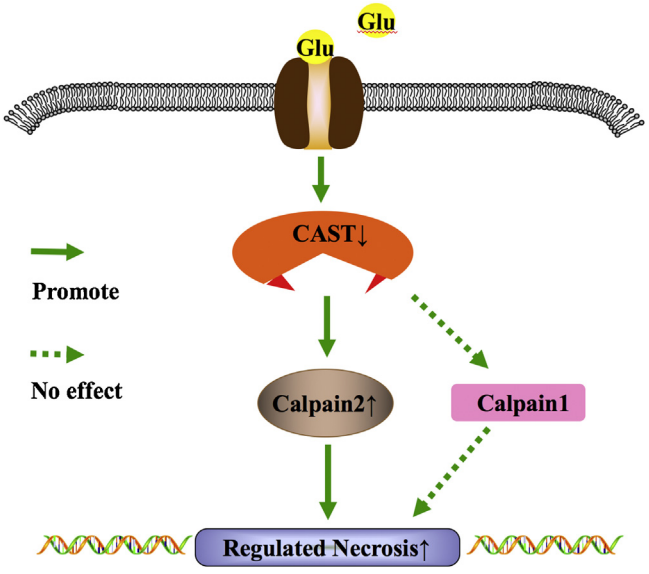


**Fig 6.** Possible mechanisms underlying the effect of glutamate in RN in retinal neurons. The role of CAST-calpain2 pathway induced by excessive glutamate in the RN in retinal neurons.

2016; Ozaki et al., 2016). In addition to apoptosis, RN, another form of programed cell death, could occur in glutamate injury (Pasparakis and Vandenabeele, 2015). The RN was further divided into several types, such as receptor interacting protein 3 (RIP3) dependent necroptosis (Degterev et al., 2008; Jiang et al., 2014; Liao et al., 2017; Wang et al., 2018c), erastin induced ferroptosis, and mitochondrial permeability transition (MPT) dependent RN (Gao et al., 2015; Kong et al., 2017). Although these RN types do not share completely the same signaling pathways, they exhibit some degree of crosstalk (Galluzzi et al., 2014; Zhao et al., 2015).

Calpain is considered as one of the controllers in neural injury induced by calcium overloading (Chen et al., 2016; Cheng et al., 2018; Sanges et al., 2006). In the traditional view, calpain could be over-expressed in neurodegeneration (Curcio et al., 2015; Shang et al., 2014). However, recent studies reported that calpain2 is neurodegenerative but calpain1 is neuroprotective under excessive neurotoxic conditions (Wang et al., 2016c; Wang et al., 2014). In our research, we found that calpain2 activity was enhanced, accompanied by increased retinal neuronal RN, after GT. Besides, there was a decrease in the expression of CAST, an endogenous inhibitor of calpain. Like most studies demonstrating that CAST is the inhibitor of calpain, the decreased CAST activity can explain the calpain2 activation in glutamate treated neurons. In our previous research, we found that Pin1 may modulate glutamate-induced retinal RN through CAST/calpain2 pathway (Wang et al., 2018a). While we did not investigate whether CAST could modulate retinal RN directly. Therefore, in this research, we further uncovered the role of CAST in RN induced by glutamate. CAST active peptide was directly applied to neuronal cultures and able to inhibit calpain2 activity and necrotic neurons. This was consistent with the ability of CAST peptide to interfere with calpain activation mediated cell death as previously reported (Comitato et al., 2014). Last, the visual function was also recovered, as shown by an increased amplitude of b wave, after CAST peptide treatment. Collectively, these results provided strong evidences for CAST as an inhibitor for calpain2 mediated retinal neuronal RN after GT. Lots of evidences have reported that calpain2 activation is involved in series of neurodegenerative diseases. Like in AD process, calpain2 activation could modulate cytoskeletal turnover, remodeling and kinases activity, such as CDK5, which play important role in β-amyloid plague generation and tau protein abnormalities (Kling et al., 2017; Stevenson et al., 2017). It also has been reported that calpain2 activation participate in Wolfram Syndrome, a genetic neurodegeneration, which is due to the loos of Syndrome 2 gene, a negative regulator of calpain2 (Lu et al., 2014). Calpain2 upregulation also has been found in patients with diabetic retinopathy (DR). Increased calpain2 induces pro-death proteins such as Bcl-2 family and caspase-12, eventually leading to the DR genesis (Ahn et al., 2016). Thus, our study about abnormal CAST-calpain2 activities might provide a beneficial target to inhibit excessive glutamate related neuronal damages. In fact, some calpain inhibitors have been tested in clinical experiment (Ono et al., 2016; Wang et al., 2016c). However, none of them are further used in clinical therapy. Major limitations are lack of specificity, cellular penetration and sufficient bioavailable (Kling et al., 2017). As our results indicate that application of selective calpain2 inhibitors may be specific neuroprotective strategies.

However, our data also indicated that calpain1 activity was not significantly changed in the retinal neuronal neurons after GT. Consistent with our results, some other researchers reported that, in rat models employing ventral root transection, calpain2 but not calpain1 was activated in motor-neurons, thus initiating neuropathic pain (Chen et al., 2018; Zang et al., 2015). In particular, some reports indicated that calpain2 but not calpain1 activation was involved in neurofibrillary tangles, tau protein abnormalities and cholinergic impairments in AD patients (Grynspan et al., 1997; Yin et al., 2016). Similar studies also found that calpain2, not calpain1, was activated in the tissues of gastric and colorectal cancer patients (Ivanova et al., 2014; Liu et al., 2017). These different changes of calpain1 and calpain2 activity may be likely due to the different responses and roles of calpain1 and calpain2 to the glutamate excitotoxicity. As previous studies proposed by Braudry and Wang et al. considered that the opposite roles of calpain1 and calpain2 were due to their different regulatory signaling pathways (Wang et al., 2016c; Wang et al., 2014). Activation of synaptic NMDAR-coupled calpain1 is neuroprotective while activation of extrasynaptic NMDAR-coupled calpain2 is neurodegenerative (Wang et al., 2013; Wang et al., 2016c). According to these reports it is conceivable that the defense mechanism of calpain1 may be inhibited by glutamate injury. Further studies with the assistance of genetic tools to manipulate the specific calpain types would be necessary to clarify the distinct roles of calpain1 and calpain2 in neuronal RN after glutamate.

Although the different changes of calpain1 and calpain2 were found in some previous research (Chen et al., 2018; Zang et al., 2015), the distinct regulatory mechanism of CAST on the changes of calpain1 and calpain2 is still unclear. In our study, we found that CAST application might be unaffected the calpain1 activity significantly in this *in vitro* model. Some researchers have reported that HSP90 interacts with calpain1 by competing with CAST and plays a regulatory role in calpain1 activation, in conditions of increased calcium (Averna et al., 2015; Martines et al., 2017). In this context, the CAST is not present in the HSP90-calpain1 complex and could not regulate the calpain1 activity (Grieve et al., 2016; Martines et al., 2017). As a result, the calpain1 cannot be resistant to glutamate-mediated injuries. In the present study, we speculated that HSP90 is involved in the process of calpain1 activity. Thus we also investigate the expression change of HSP90. However, the expression of HSP90 remains unaffected after GT (see Supplement figure). The unchanged HSP90 expression could protect calpain1 from activation and explain the stable calpain1 activity. Although the precise mechanism of glutamate-mediated effect of HSP90 has not been fully investigated, we showed that the HSP90-calpain1 molecular pathway may be unchanged in primary retinal neuronal cultures under the excessive glutamate context. Thus, it can be speculated that the underlying neuroprotective role of calpain1 failed to work in this retinal neuronal cultures. Further investigation is needed to clearly define the regulatory mechanism of HSP90 in calpain1-mediated RN.

In conclusion, we demonstrated that excessive glutamate may lead to CAST inhibition, resulting in activated calpain2, but not calpain1, and mediation of retinal neuronal RN (Fig. 6). This is a complementary mechanism for neurodegeneration in the glutamate induced injury and expands our understanding of the CAST-calpain2 neurodegenerative mechanism. Due to the prominent role of glutamate in a series of neurologic diseases (Gu et al., 2014), therapies that target blocking glutamate induced cell necrosis by the CAST-calpain2 pathway could be novel therapeutic agents for prevention of glutamate excitotoxicity.

## Funding

This study was funded by the National Key Research and Development Program of China (No. 2016YFC1201800), the National Natural Science Foundation of China (Nos. 81571939, 81671225, 81772134, 81871089 and 81860781), the Key Research and Development Program of Hunan Province (No. 2018SK2090, 2018SK2091), Wu Jie-Ping Medical Foundation of the Minister of Health of China (No. 320.6750.14118), Teacher Research Foundation of Central South University (No. 2014JSJJ026), and the Project of Graduate Innovative Plan of Central South University (2018zzts228).

## Acknowledgments

We would like to thank Dr. Jim Vaught, Editor-in-Chief of *Biopreservation and Biobanking*, Bethesda, Maryland, USA, who edited the manuscript for us. Shuchao Wang would like to thank Yuhan Yan for her past companion and encouragement.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aanat.2018.08.005.

## References

Agudo, M., Perez-Marin, M.C., Sobrado-Calvo, P., Lonngren, U., Salinas-Navarro, M., Canovas, I., Nadal-Nicolas, F.M., Miralles-Imperial, J., Hallbook, F., Vidal-Sanz, M., 2009. Immediate upregulation of proteins belonging to different branches of the apoptotic cascade in the retina after optic nerve transection and optic nerve crush. Invest. Ophthalmol.Vis. Sci. 50, 424–431.

Ahn, Y.J., Kim, M.S., Chung, S.K., 2016. Calpain and caspase-12 expression in lens epithelial cells of diabetic cataracts. Am. J. Ophthalmol. 167, 31–37.

Aihara, M., Chen, Y.N., Uchida, S., Nakayama, M., Araie, M., 2014. Hyperbaric pressure and increased susceptibility to glutamate toxicity in retinal ganglion cells in vitro. Mol. Vis. 20, 606–615.

Averna, M., de Tullio, R., Passalacqua, M., Salamino, F., Pontremoli, S., Melloni, E., 2001. Changes in intracellular calpastatin localization are mediated by reversible phosphorylation. Biochem. J. 354, 25–30.

Averna, M., De Tullio, R., Pedrazzi, M., Bavestrello, M., Pellegrini, M., Salamino, F., Pontremoli, S., Melloni, E., 2015. Interaction between calpain-1 and HSP90: new insights into the regulation of localization and activity of the protease. PLoS One 10, e0116738.

Briz, V., Hsu, Y.T., Li, Y., Lee, E., Bi, X., Baudry, M., 2013. Calpain-2-mediated PTEN degradation contributes to BDNF-induced stimulation of dendritic protein synthesis. J. Neurosci. 33, 4317–4328.

Chen, S., Yan, J., Deng, H.X., Long, L.L., Hu, Y.J., Wang, M., Shang, L., Chen, D., Huang, J.F., Xiong, K., 2016. Inhibition of calpain on oxygen glucose deprivation-induced RGC-5 necroptosis. J. Huazhong Univ. Sci. Technol. Med. Sci. 36, 639–645.

Chen, S.X., Liao, G.J., Yao, P.W., Wang, S.K., Li, Y.Y., Zeng, W.A., Liu, X.G., Zang, Y., 2018. Calpain-2 regulates TNF-alpha expression associated with neuropathic pain following motor nerve injury. Neuroscience 376, 142–151.

Cheng, S.Y., Wang, S.C., Lei, M., Wang, Z., Xiong, K., 2018. Regulatory role of calpain in neuronal death. Neural Regen. Res. 13, 556–562.

Comitato, A., Sanges, D., Rossi, A., Humphries, M.M., Marigo, V., 2014. Activation of Bax in three models of retinitis pigmentosa. Invest. Ophthalmol. Vis. Sci. 55, 3555–3562.

Curcio, M., Salazar, I.L., Inacio, A.R., Duarte, E.P., Canzoniero, L.M., Duarte, C.B., 2015. Brain ischemia downregulates the neuroprotective GDNF-Ret signaling by a calpain-dependent mechanism in cultured hippocampal neurons. Cell Death Dis. 6, e1645.

Curcio, M., Salazar, I.L., Mele, M., Canzoniero, L.M., Duarte, C.B., 2016. Erratum to "Calpains and neuronal damage in the ischemic brain: the swiss knife in synaptic injury" [Progress in Neurobiology 143 (2016) 1–35]. Prog. Neurobiol. 147, 20.

Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I.L., Korkina, O., Teng, X., Abbott, D., Cuny, G.D., Yuan, C., Wagner, G., Hedrick, S.M., Gerber, S.A., Lugovskoy, A., Yuan, J., 2008. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat. Chem. Biol. 4, 313–321.

Del Rosario, J.S., Feldmann, K.G., Ahmed, T., Amjad, U., Ko, B., An, J., Mahmud, T., Salama, M., Mei, S., Asemota, D., Mano, I., 2015. Death associated protein kinase (DAPK)—mediated neurodegenerative mechanisms in nematode excitotoxicity. BMC Neurosci. 16, 25.

Ding, W., Shang, L., Huang, J.F., Li, N., Chen, D., Xue, L.X., Xiong, K., 2015. Receptor interacting protein 3-induced RGC-5 cell necroptosis following oxygen glucose deprivation. BMC Neurosci. 16, 49.

Galluzzi, L., Kepp, O., Krautwald, S., Kroemer, G., Linkermann, A., 2014. Molecular mechanisms of regulated necrosis. Semin. Cell Dev. Biol. 35, 24–32.

Gao, M., Monian, P., Quadri, N., Ramasamy, R., Jiang, X., 2015. Glutaminolysis and transferrin regulate ferroptosis. Mol. Cell 59, 298–308.

Grieve, S., Gao, Y., Hall, C., Hu, J., Greer, P.A., 2016. Calpain genetic disruption and HSP90 inhibition combine to attenuate mammary tumorigenesis. Mol. Cell Biol. 36, 2078–2088.

Grynspan, F., Griffin, W.R., Cataldo, A., Katayama, S., Nixon, R.A., 1997. Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease. Brain Res. 763, 145–158.

Gu, L., Xu, H., Wang, F., Xu, G., Sinha, D., Wang, J., Xu, J.Y., Tian, H., Gao, F., Li, W., Lu, L., Zhang, J., Xu, G.T., 2014. Erythropoietin exerts a neuroprotective function against glutamate neurotoxicity in experimental diabetic retina. Invest. Ophthalmol. Vis. Sci. 55, 8208–8222.

Haas, L.T., Salazar, S.V., Smith, L.M., Zhao, H.R., Cox, T.O., Herber, C.S., Degnan, A.P., Balakrishnan, A., Macor, J.E., Albright, C.F., Strittmatter, S.M., 2017. Silent allosteric modulation of mGluR5 maintains glutamate signaling while rescuing Alzheimer's mouse phenotypes. Cell Rep. 20, 76–88.

Hanouna, G., Mesnard, L., Vandermeersch, S., Perez, J., Placier, S., Haymann, J.P., Campagne, F., Moroch, J., Bataille, A., Baud, L., Letavernier, E., 2017. Specific calpain inhibition protects kidney against inflammaging. Sci. Rep. (UK) 7.

Hoque, A., Hossain, M.I., Ameen, S.S., Ang, C.S., Williamson, N., Ng, D.C., Chueh, A.C., Roulston, C., Cheng, H.C., 2016. A beacon of hope in stroke therapy-blockade of pathologically activated cellular events in excitotoxic neuronal death as potential neuroprotective strategies. Pharmacol. Ther. 160, 159–179.

Huang, J.F., Shang, L., Zhang, M.Q., Wang, H., Chen, D., Tong, J.B., Huang, H., Yan, X.X., Zeng, L.P., Xiong, K., 2013. Differential neuronal expression of receptor interacting protein 3 in rat retina: involvement in ischemic stress response. BMC Neurosci. 14, 16.

Ivanova, E.V., Kondakova, I.V., Spirina, L.V., Afanas'ev, S.G., Avgustinovich, A.V., Cheremisina, O.V., 2014. Chymotrypsin-like activity of proteasomes and total calpain activity in gastric and colorectal cancer. Bull. Exp. Biol. Med. 157, 781–784.

Jiang, S.H., Shang, L., Xue, L.X., Ding, W., Chen, S., Ma, R.F., Huang, J.F., Xiong, K., 2014. The effect and underlying mechanism of Timosaponin B-II on RGC-5 necroptosis induced by hydrogen peroxide. BMC Complement Altern. Med. 14, 459.

Kaur, J., Mencl, S., Sahaboglu, A., Farinelli, P., van Veen, T., Zrenner, E., Ekstrom, P., Paquet-Durand, F., Arango-Gonzalez, B., 2011. Calpain and PARP activation during photoreceptor cell death in P23H and S334ter rhodopsin mutant rats. PloS One 6, e22181.

Kim, B.J., Silverman, S.M., Liu, Y., Wordinger, R.J., Pang, I.H., Clark, A.F., 2016. In vitro and in vivo neuroprotective effects of cJun N-terminal kinase inhibitors on retinal ganglion cells. Mol. Neurodegener. 11, 30.

Kling, A., Jantos, K., Mack, H., Hornberger, W., Drescher, K., Nimmrich, V., Relo, A., Wicke, K., Hutchins, C.W., Lao, Y., Marsh, K., Moeller, A., 2017. Discovery of novel and highly selective inhibitors of calpain for the treatment of Alzheimer's disease: 2-(3-Phenyl-1H-pyrazol-1-yl)-nicotinamides. J. Med. Chem. 60, 7123–7138.

Kong, D., Zhu, J., Liu, Q., Jiang, Y., Xu, L., Luo, N., Zhao, Z., Zhai, Q., Zhang, H., Zhu, M., Liu, X., 2017. Mesenchymal stem cells protect neurons against hypoxic-ischemic injury via inhibiting parthanatos, necroptosis, and apoptosis, but not autophagy. Cell. Mol. Neurobiol. 37, 303–313.

Lalkovicova, M., Danielisova, V., 2016. Neuroprotection and antioxidants. Neural Regen. Res. 11, 865–874.

Leon, R., Wu, H., Jin, Y., Wei, J., Buddhala, C., Prentice, H., Wu, J.Y., 2009. Protective function of taurine in glutamate-induced apoptosis in cultured neurons. J. Neurosci. Res. 87, 1185–1194.

Li, N., Shang, L., Wang, S.C., Liao, L.S., Chen, D., Huang, J.F., Xiong, K., 2016. The toxic effect of ALLN on primary rat retinal neurons. Neurotox. Res. 30, 392–406.

Liao, L., Shang, L., Li, N., Wang, S., Wang, M., Huang, Y., Chen, D., Huang, J., Xiong, K., 2017. Mixed lineage kinase domain-like protein induces RGC-5 necroptosis following elevated hydrostatic pressure. Acta Biochim. Biophys. Sin. (Shanghai) 49, 879–889.

Liao, R., Yan, F., Zeng, Z., Farhan, M., Little, P., Quirion, R., Srivastava, L.K., Zheng, W., 2016. Amiodarone-induced retinal neuronal cell apoptosis attenuated by IGF-1 via counter regulation of the PI3k/Akt/FoxO3a pathway. Mol. Neurobiol. 54 (9), 1–13.

Liu, B., Zhou, Y., Lu, D., Liu, Y., Zhang, S.Q., Xu, Y., Li, W., Gu, X., 2017. Comparison of the protein expression of calpain-1, calpain-2, calpastatin and calmodulin between gastric cancer and normal gastric mucosa. Oncol. Lett. 14, 3705–3710.

Liu, F., Grundke-Iqbal, I., Iqbal, K., Oda, Y., Tomizawa, K., Gong, C.X., 2005. Truncation and activation of calcineurin A by calpain I in Alzheimer disease brain. J. Biol. Chem. 280, 37755–37762.
Liu, Y., Wang, Y., Zhu, G., Sun, J., Bi, X., Baudry, M., 2016. A calpain-2 selective inhibitor enhances learning & memory by prolonging ERK activation. Neuropharmacology 105, 471–477.
Lopez-Colome, A.M., Lopez, E., Mendez-Flores, O.G., Ortega, A., 2016. Glutamate receptor stimulation up-regulates glutamate uptake in human Muller glia cells. Neurochem. Res. 41, 1797–1805.
Lu, S., Kanekura, K., Hara, T., Mahadevan, J., Spears, L.D., Oslowski, C.M., Martinez, R., Yamazaki-Inoue, M., Toyoda, M., Neilson, A., Blanner, P., Brown, C.M., Semenkovich, C.F., Marshall, B.A., Hershey, T., Umezawa, A., Greer, P.A., Urano, F., 2014. A calcium-dependent protease as a potential therapeutic target for Wolfram syndrome. Proc. Natl. Acad. Sci. U. S. A. 111, E5292–E5301.
Martensson, L.B., Blom, C.L., Dahlin, L.B., 2017. Ca2+ involvement in activation of extracellular-signal-regulated-kinase 1/2 and m-calpain after axotomy of the sciatic nerve. Neural Regen. Res. 12, 623–628.
Martines, A., Stifanese, R., Faelli, E.L., Perasso, L., Melloni, I., Ruggeri, P., Averna, M., 2017. Calpain-1 resident in lipid raft/caveolin-1 membrane microdomains plays a protective role in endothelial cells. Biochimie 133, 20–27.
Martinez, D., Garcia, L., Aguilera, J., Ortega, A., 2014. An acute glutamate exposure induces long-term down regulation of GLAST/EAAT1 uptake activity in cultured Bergmann glia cells. Neurochem Res. 39, 142–149.
Mattson, M.P., 2008. Glutamate and neurotrophic factors in neuronal plasticity and disease. Ann. N. Y. Acad. Sci. 1144, 97–112.
Miao, Y., Dong, L.D., Chen, J., Hu, X.C., Yang, X.L., Wang, Z., 2012. Involvement of calpain/p35-p25/Cdk5/NMDAR signaling pathway in glutamate-induced neurotoxicity in cultured rat retinal neurons. PloS One 7, e42318.
Morgan-Warren, P.J., O'Neill, J., de Cogan, F., Spivak, I., Ashush, H., Kalinski, H., Ahmed, Z., Berry, M., Feinstein, E., Scott, R.A., Logan, A., 2016. siRNA-mediated knockdown of the mTOR inhibitor RTP801 promotes retinal ganglion cell survival and axon elongation by direct and indirect mechanisms. Invest. Ophthalmol. Vis. Sci. 57, 429–443.
Ono, Y., Saido, T.C., Sorimachi, H., 2016. Calpain research for drug discovery: challenges and potential. Nat. Rev. Drug Discov. 15, 854–876.
Ozaki, T., Yamashita, T., Tomita, H., Sugano, E., Ishiguro, S., 2016. The protection of rat retinal ganglion cells from ischemia/reperfusion injury by the inhibitory peptide of mitochondrial mu-calpain. Biochem. Biophys. Res. Commun. 478, 1700–1705.
Pandey, A.K., Shukla, S.C., Bhattacharya, P., Patnaik, R., 2016. A possible therapeutic potential of quercetin through inhibition of mu-calpain in hypoxia induced neuronal injury: a molecular dynamics simulation study. Neural Regen. Res. 11, 1247–1253.
Pasparakis, M., Vandenabeele, P., 2015. Necroptosis and its role in inflammation. Nature 517, 311–320.
Pietkiewicz, S., Schmidt, J.H., Lavrik, I.N., 2015. Quantification of apoptosis and necroptosis at the single cell level by a combination of imaging flow cytometry with classical Annexin V/propidium iodide staining. J. Immunol. Methods 423, 99–103.
Quillinan, N., Herson, P.S., Traystman, R.J., 2016. Neuropathophysiology of brain injury. Anesthesiol. Clin. 34, 453–464.
Rosenbaum, D.M., Degterev, A., David, J., Rosenbaum, P.S., Roth, S., Grotta, J.C., Cuny, G.D., Yuan, J., Savitz, S.I., 2010. Necroptosis, a novel form of caspase-independent cell death, contributes to neuronal damage in a retinal ischemia-reperfusion injury model. J. Neurosci. Res. 88, 1569–1576.
Sanges, D., Comitato, A., Tammaro, R., Marigo, V., 2006. Apoptosis in retinal degeneration involves cross-talk between apoptosis-inducing factor (AIF) and caspase-12 and is blocked by calpain inhibitors. Proceedings of the National Academy of Sciences of the United States of America 103, 17366–17371.
Seinfeld, J., Baudry, N., Xu, X., Bi, X., Baudry, M., 2016. Differential activation of Calpain-1 and Calpain-2 following kainate-induced seizure activity in rats and mice. eNeuro 3.
Senter, P.D., Wallace, P.M., Svensson, H.P., Kerr, D.E., Hellstrom, I., Hellstrom, K.E., 1991. Activation of prodrugs by antibody-enzyme conjugates. Adv. Exp. Med. Biol. 303, 97–105.
Shang, L., Ding, W., Li, N., Liao, L., Chen, D., Huang, J., Xiong, K., 2017. The effects and regulatory mechanism of RIP3 on RGC-5 necroptosis following elevated hydrostatic pressure. Acta Biochim. Biophys. Sin. (Shanghai) 49, 128–137.
Shang, L., Huang, J.F., Ding, W., Chen, S., Xue, L.X., Ma, R.F., Xiong, K., 2014. Calpain: a molecule to induce AIF-mediated necroptosis in RGC-5 following elevated hydrostatic pressure. BMC Neurosci. 15, 63.
Sisk, D.R., Kuwabara, T., 1985. Histologic changes in the inner retina of albino rats following intravitreal injection of monosodium L-glutamate. Graefes Arch. Clin. Exp. Ophthalmol. 223, 250–258.
Stevenson, A., Lopez, D., Khoo, P., Kalaria, R.N., Mukaetova-Ladinska, E.B., 2017. Exploring erythrocytes as blood biomarkers for Alzheimer's disease. J. Alzheimers Dis. 60, 845–857.
Suarez-Pozos, E., Chi-Castaneda, D., Ortega, A., 2017. Glial glutamate transporters as signaling molecules. Adv. Neurobiol. 16, 185–198.
Suzuki, K., Hata, S., Kawabata, Y., Sorimachi, H., 2004. Structure, activation, and biology of calpain. Diabetes 53 (Suppl. 1), S12–S18.
Volbracht, C., Chua, B.T., Ng, C.P., Bahr, B.A., Hong, W., Li, P., 2005. The critical role of calpain versus caspase activation in excitotoxic injury induced by nitric oxide. J. Neurochem. 93, 1280–1292.
Wang, C., Shi, D., Song, X., Chen, Y., Wang, L., Zhang, X., 2016a. Calpain inhibitor attenuates ER stress-induced apoptosis in injured spinal cord after bone mesenchymal stem cells transplantation. Neurochem. Int. 97, 15–25.
Wang, S., Hu, T., Wang, Z., Li, N., Zhou, L., Liao, L., Wang, M., Liao, L., Wang, H., Zeng, L., Fan, C., Zhou, H., Xiong, K., Huang, J., Chen, D., 2017. Macroglia-derived thrombospondin 2 regulates alterations of presynaptic proteins of retinal neurons following elevated hydrostatic pressure. PLoS One 12, e0185388.
Wang, S.C., Liao, L.S., Wang, M., Zhou, H.K., Huang, Y.X., Wang, Z., Chen, D., Ji, D., Xia, X.B., Wang, Y., Liu, F.X., Huang, J.F., Xiong, K., 2018a. Pin1 Promotes regulated necrosis induced by glutamate in rat retinal neurons via CAST/calpain2 pathway. Front. Cell. Neurosci. 11.
Wang, Y., Briz, V., Chishti, A., Bi, X., Baudry, M., 2013. Distinct roles for mu-calpain and m-calpain in synaptic NMDAR-mediated neuroprotection and extrasynaptic NMDAR-mediated neurodegeneration. J. Neurosci. 33, 18880–18892.
Wang, Y., Hersheson, J., Lopez, D., Hammer, M., Liu, Y., Lee, K.H., Pinto, V., Seinfeld, J., Wiethoff, S., Sun, J., Amouri, R., Hentati, F., Baudry, N., Tran, J., Singleton, A.B., Coutelier, M., Brice, A., Stevanin, G., Durr, A., Bi, X., Houlden, H., Baudry, M., 2016b. Defects in the CAPN1 gene result in alterations in cerebellar development and cerebellar ataxia in mice and humans. Cell Rep. 16, 79–91.
Wang, Y., Lopez, D., Davey, P.G., Cameron, D.J., Nguyen, K., Tran, J., Marquez, E., Liu, Y., Bi, X., Baudry, M., 2016c. Calpain-1 and calpain-2 play opposite roles in retinal ganglion cell degeneration induced by retinal ischemia/reperfusion injury. Neurobiol. Dis. 93, 121–128.
Wang, Y., Zhu, G., Briz, V., Hsu, Y.T., Bi, X., Baudry, M., 2014. A molecular brake controls the magnitude of long-term potentiation. Nat. Commun. 5, 3051.
Wang, Z., Guo, L.M., Wang, Y., Zhou, H.K., Wang, S.C., Chen, D., Huang, J.F., Xiong, K., 2018b. Inhibition of HSP90alpha protects cultured neurons from oxygen-glucose deprivation induced necroptosis by decreasing RIP3 expression. J. Cell. Physiol. 233, 4864–4884.
Wang, Z., Guo, L.M., Zhou, H.K., Qu, H.K., Wang, S.C., Liu, F.X., Chen, D., Huang, J.F., Xiong, K., 2018c. Using drugs to target necroptosis: dual roles in disease therapy. Histol. Histopathol., 11968.
Wolhuter, K., Whitwell, H.J., Switzer, C.H., Burgoyne, J.R., Timms, J.F., Eaton, P., 2018. Evidence against stable protein *S*-nitrosylation as a widespread mechanism of post-translational regulation. Mol. Cell 69, 438–450, e435.
Xiong, K., Liao, H., Long, L., Ding, Y., Huang, J., Yan, J., 2016. Necroptosis contributes to methamphetamine-induced cytotoxicity in rat cortical neurons. Toxicol. In Vitro 35, 163–168.
Xu, X., Chua, C.C., Kong, J., Kostrzewa, R.M., Kumaraguru, U., Hamdy, R.C., Chua, B.H., 2007. Necrostatin-1 protects against glutamate-induced glutathione depletion and caspase-independent cell death in HT-22 cells. J. Neurochem. 103, 2004–2014.
Yan, M., Zhu, W., Zheng, X., Li, Y., Tang, L., Lu, B., Chen, W., Qiu, P., Leng, T., Lin, S., Yan, G., Yin, W., 2016. Effect of glutamate on lysosomal membrane permeabilization in primary cultured cortical neurons. Mol. Med. Rep. 13, 2499–2505.
Yin, Y., Wang, Y., Gao, D., Ye, J., Wang, X., Fang, L., Wu, D., Pi, G., Lu, C., Zhou, X.W., Yang, Y., Wang, J.Z., 2016. Accumulation of human full-length tau induces degradation of nicotinic acetylcholine receptor alpha4 via activating calpain-2. Sci. Rep. 6, 27283.
Zang, Y., Chen, S.X., Liao, G.J., Zhu, H.Q., Wei, X.H., Cui, Y., Na, X.D., Pang, R.P., Xin, W.J., Zhou, L.J., Liu, X.G., 2015. Calpain-2 contributes to neuropathic pain following motor nerve injury via up-regulating interleukin-6 in DRG neurons. Brain Behav. Immun. 44, 37–47.
Zhao, H., Ning, J., Lemaire, A., Koumpa, F.S., Sun, J.J., Fung, A., Gu, J., Yi, B., Lu, K., Ma, D., 2015. Necroptosis and parthanatos are involved in remote lung injury after receiving ischemic renal allografts in rats. Kidney Int. 87, 738–748.