Pin1 promotes regulated necrosis induced by glutamate in rat retinal neurons via CAST/calpain2 pathway

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The purpose of the current study was to investigate whether peptidyl-prolyl cis/trans isomerase NIMA-interacting 1 (Pin1) can interact with calpastatin (CAST) and regulate CAST/calpain2, under excessive glutamate conditions, and subsequently regulate necrosis in rat retinal neurons. Glutamate triggered CAST/calpain2-mediated necrosis regulation in primary cultured retinal neurons, as demonstrated by propidium iodide-staining and lactate dehydrogenase assay. Co-IP results and a computer simulation suggested that Pin1 could bind to CAST. Western blot, real-time quantitative polymerase chain reaction, immunofluorescence, and phosphorylation analysis results demonstrated that CAST was regulated by Pin1, as proven by the application of juglone (i.e., a Pin1 specific inhibitor). The retinal ganglion cell 5 cell line, combined with siRNA approach and flow cytometry, was then used to verify the regulatory pathway of Pin1 in CAST/calpain2-modulated neuronal necrosis that was induced by glutamate. Finally, *in vivo* studies further confirmed the role of Pin1 in CAST/calpain2-modulated necrosis following glutamate excitation, in the rat retinal ganglion cell and inner nuclear layers. In addition, a flash electroretinogram study provided evidence for the recovery of impaired visual function, which was induced by glutamate, with juglone treatment. Our work aims to investigate the involvement of the Pin1-CAST/calpain2 pathway in glutamate-mediated excitotoxicity.

Figure 1. Effect of glutamate on Pin1-CAST/calpain2 pathway and the interaction of Pin1 with CAST. **(A)** Protein surface electrostatic potential energy were calculated using APBS (figures were produced by using PyMOL). **(B)** IP assay of CAST and Pin1. **(C)** *Pin1* and *CAST* mRNA levels in retinal neurons following glutamate treatment. Data are expressed as means $\pm$ SD ($n = 4$, each). $*p < 0.05$ vs. CTL group. **(D)** Pin1, CAST, calpain2, PTEN, and tAIF expression in

retinal neurons following glutamate treatment. **(E)** P-CAST and CAST levels in retinal neurons following glutamate treatment. **(F)** The statistical analysis of p-CAST/CAST and p-CAST/ β -Actin in retinal neurons following glutamate treatment. Data are expressed as means $\pm$ SD ($n = 4$, each). $*p < 0.05$ vs. CTL group. **(G)** The statistical analysis of Pin1, CAST, calpain2, PTEN, and tAIF expression in retinal neurons following glutamate treatment. Data are expressed as means $\pm$ SD ($n = 5$, each). $*p < 0.05$ vs. CTL.

Figure 1

Figure 2. Pin1 regulates CAST/calpain2 pathway. **(A)** IP assay of CAST and Pin1 after glutamate and pretreated with juglone. **(B)** *Pin1* and *CAST* mRNA levels in retinal neurons following glutamate and pretreated with juglone before glutamate treatment. Data are expressed as means $\pm$ SD ($n = 4$, each). $*p < 0.05$ vs. CTL group, $\#p < 0.05$ vs.

Glu 4 h group. **(C)** Pin1, CAST, calpain2, PTEN, and tAIF expression in retinal neurons following glutamate and pretreated with juglone before glutamate treatment. **(D)** P-CAST and CAST expression in retinal neurons following glutamate and pretreated with juglone before glutamate treatment. **(E)** The statistical analysis of p-CAST/CAST and p-CAST/ β -Actin in retinal neurons following glutamate and pretreated with juglone before glutamate treatment. Data are expressed as means $\pm$ SD ($n = 4$, each). $*p < 0.05$ vs. CTL group, $\#p < 0.05$ vs. Glu 6 h group. **(F)** The statistical analysis of Pin1, CAST, calpain2, PTEN, and tAIF expression in retinal neurons following glutamate treatment. Data are expressed as means $\pm$ SD ($n = 5$, each). $*p < 0.05$ vs. CTL group, $\#p < 0.05$ vs. Glu 6 h.

Figure 2

Figure 3. Necrosis was determined by propidium iodide staining and LDH release, while visual function was detected using flash electroretinogram (fERG) in retina after

glutamate treatment *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 $\pm$ SD ($n = 4$, each). $*p < 0.05$ vs. CTL group, $\#p < 0.05$ vs. Glu group. **(N)** The percentage of necrosis cells were determined by LDH release, Data are expressed as means $\pm$ 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 $\pm$ 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 μ m in all panels.

Figure 3