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Elevation of p-NR2A^{S1232} by Cdk5/p35 contributes to retinal ganglion cell apoptosis in a rat experimental glaucoma model

Jie Chen, Yanying Miao, Xiao-Han Wang, Zhongfeng Wang *

Institutes of Brain Science, Institute of Neurobiology and State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai 200032, China

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ABSTRACT

Glaucoma, mainly caused by high intraocular pressure (IOP), is characterized by apoptotic death of retinal ganglion cells (RGCs). We investigated the possible involvement of cyclin-dependent kinase 5 (Cdk5) and its activator p35, which have been implicated in a variety of neurological disorders, in RGC apoptosis in a rat experimental glaucoma model reproduced by blocking episcleral veins. Cholera toxin B subunit (CTB) retrogradely labeled RGCs displayed a dramatic reduction in number both in the central and peripheral retina on day 14 (D14) ($P<0.05$ vs control), D21 ($P<0.01$ vs control) and D28 ($P<0.001$ vs control) after operation. Terminal dUTP nick end labeling (TUNEL)-positive cells were detected on D14 both in the central and peripheral regions, and numerous TUNEL-positive cells were found on D21 and D28 in both the regions (P all <0.001 vs control). As compared with the control eyes, the expression level of Cdk5 was significantly increased on D21 ($P<0.001$), whereas that of p35 displayed a marked increase on D14 ($P<0.01$) and D21 ($P<0.001$). Meanwhile, both NR2A and p-NR2A^{S1232} increased from D14 onwards ($P<0.01$ to 0.001). Co-immunoprecipitation indicated a direct interaction between Cdk5 and p-NR2A^{S1232}. Intraperitoneal injection of the Cdk5 inhibitor roscovitine remarkably inhibited RGC apoptosis ($P<0.001$ vs vehicle group) and increased the number of CTB-labeled RGCs ($P<0.05$ to 0.01 vs vehicle group) in whole flat-mounted retinas, which was accompanied by a significant decrease in expression levels of p35 and p-NR2A^{S1232} (P all <0.01 vs vehicle group). Our results suggest that elevation of p-NR2A^{S1232} by Cdk5/p35 contributes to RGC apoptotic death in experimental glaucoma rats, which could be effectively ameliorated by inhibiting Cdk5/p35.

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Introduction

Glaucoma, the second leading cause of blindness, is a neurodegenerative disease that is characterized by optic nerve degeneration resulting from apoptotic death of retinal ganglion cells (RGCs) (Guo et al., 2005; Hitchings, 2000; Resnikoff et al., 2004). Although elevated intraocular pressure (IOP) is commonly regarded as a hallmark risk factor (Quigley et al., 1995; Weinreb and Khaw, 2004), the pathogenesis of RGC death following intraocular hypertension is still poorly understood. The role of the excitotoxicity induced by glutamate (Glu), a major excitatory neurotransmitter in the retina (Thoreson and Witkovsky, 1999; Yang, 2004), in neurodegeneration in glaucoma models is still a controversial issue (Dreyer et al., 1996; Levkovitch-Verbin et al., 2002; Ullian et al., 2004). Nevertheless, lots of evidence suggest that the excess of extracellular Glu may be a potential risky factor for retinal malfunction in glaucoma (Guo et al., 2006; Levin, 2003; Salt and Cordeiro, 2006). Indeed, expression of Glu transporters is significantly reduced in rat glaucoma models, indicating frustration of the effective buffering of extracellular Glu (Martin et al., 2002; Vorwerk et al., 2000). Moreover, prolonged injection of Glu of low-concentration was shown to induce RGC death in rat (Nucci et al., 2005). Glu-induced apoptotic death of RGCs is known to be primarily mediated by the N-methyl-D-aspartate (NMDA) subtype receptor (Guo et al., 2006; Lipton, 2001; Seki and Lipton, 2008), and the NMDA channel blocker MK-801/memantine indeed prevents RGC death in experimental rat glaucoma models (Calzada et al., 2002; Chaudhary et al., 1998; Guo et al., 2006; Hare et al., 2004; WoldeMussie et al., 2002).

Cyclin-dependent kinase 5 (Cdk5), a proline-directed serine/threonine kinase, has multiple roles in neural development and synaptic plasticity by phosphorylating numerous synaptic substrates (Dhavan and Tsai, 2001; Ko et al., 2001; Lee et al., 2004; Morabito et al., 2004; Tomizawa et al., 2002). Cdk5 is expressed ubiquitously,

Abbreviations: BCA, bicinchoninic acid; Bcl-2, B-cell lymphoma protein 2; Cdk5, cyclin-dependent kinase 5; Co-IP, co-immunoprecipitation; CNS, central nervous system; CTB, cholera toxin B subunit; DMSO, dimethyl sulfoxide; GCL, ganglion cell layer; Glu, glutamate; HRP, horseradish-peroxidase; IOP, intraocular pressure; IP, immunoprecipitate; NMDA, N-methyl-D-aspartate; p-NR2A^{S1232}, NR2A receptor phosphorylation at S1232 site; ONH, optic nerve head; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RGC, retinal ganglion cell; TUNEL, terminal dUTP nick end labeling.

* Corresponding author at: Institutes of Brain Science, Institute of Neurobiology and State Key Laboratory of Medical Neurobiology, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China. Fax: +86 21 5423 7643.
E-mail address: zfwang@fudan.edu.cn (Z. Wang).

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but shows high activity exclusively in the central nervous system (CNS) due to the restricted distribution of its activators (Tang et al., 1995). P35, an essential activator of Cdk5 (Chae et al., 1997; Ko et al., 2001), restricts the expression of active Cdk5 primarily to post-mitotic neurons (Tang et al., 1995; Tsai et al., 1994). The dysregulation of Cdk5/p35 has been implicated in many neurological disorders (Borghi et al., 2002; Bu et al., 2002; Chen and Wang, 2010; Lee et al., 1999; Nguyen et al., 2003; Smith et al., 2003). There is accumulating evidence suggesting that Cdk5 may be a "Jekyll and Hyde" kinase in that it takes different responsibilities under different conditions (Cruz and Tsai, 2004). For instance, activation of Cdk5 induces hippocampal CA1 cell death by directly phosphorylating the NR2A subunit at S1232 site (p-NR2A^{S1232}) in an ischemic rat model (Wang et al., 2003). In contrast, Cdk5 is shown to prevent neuronal apoptosis through ERK-mediated upregulation of B-cell lymphoma protein 2 (Bcl-2) in vitro (Wang et al., 2006) and by directly phosphorylating Bcl-2 at Ser70 site in rat cortical neurons (Cheung et al., 2008), respectively. Furthermore, Cdk5 may exert a pro-survival role by negatively regulating c-Jun N-terminal kinase 3 (Bu et al., 2002) and/or by participating in the neuregulin-dependent activation of PI3K and Akt pathway (Li et al., 2003). In the present study we investigate the possible involvement of Cdk5/p35 in the pathogenesis of RGCs in a rat experimental glaucoma model. Our results demonstrate that activation of Cdk5/p35 causes an elevation of p-NR2A^{S1232} that likely contributes to RGC apoptotic death in this model and this defect is significantly ameliorated by roscovitine, an inhibitor of Cdk5/p35.

Materials and methods

Animals and glaucoma model

All experimental procedures described here were carried out in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals and the guidelines of Fudan University on the ethical use of animals. During this study all efforts were made to minimize the number of animals used and their suffering. Male Sprague–Dawley rats weighing 100–300 g obtained from SLAC Laboratory Animal Co. Ltd (Shanghai, China) were housed on a 12 h light/dark schedule, with standard food and water provided ad libitum. In this study a total of 176 rats were used, among which 44 were for control (24 for whole flat-mounted experiments, 10 for Western blot experiments, 4 for co-immunoprecipitation experiments and 6 for vertical slices) and 132 for reproduction of the glaucoma model (70 for whole flat-mounted experiments, 28 for Western blot experiments and 34 for vertical slices). The detailed animal numbers used in each of the experiments were given in the Results Section.

The rat glaucoma model used in the present study was reproduced following the procedure previously described (Naka et al., 2010; Wu et al., 2010; Yu et al., 2006), with minor modifications. Briefly, rats were anesthetized by intramuscular injection of a mixture of ketamine (25 mg/kg) and xylazine (10 mg/kg), and the right eyes were further anesthetized locally with a topical application of 0.4% oxybuprocaine hydrochloride eyedrop (Benoxil, Santen Pharmaceutical Co. Ltd, Osaka, Japan). After minimum conjunctival incision, three episcleral veins of the right eyes near the superior and temporal rectus muscles were carefully separated, ligated or cauterized under an OPMI VISU 140 microscopy (Carl Zeiss, Jena, Germany). IOPs of both eyes were measured using a handheld digital tonometer (Tonopen XL, Mentor O&O Inc., MA, USA) under general and local anesthesia as described above. The average value of five consecutive measurements with a deviation of less than 5% was accepted. All measurements were performed in the morning to avoid possible circadian difference. The IOPs of both eyes were measured before surgery as a baseline (D0), and then on the next day of the operation (D1), days 3, 7, 11, 14 (D3, D7, D11, D14), and weekly afterwards.

Labeling and quantification of RGCs

Retrograde labeling of RGCs in control and experimental eyes was performed as previously described in detail (Chen et al., 2004; Zhao et al., 2010). Briefly, after rats were deeply anesthetized with 25% urethane (1.25 g/kg), 1% cholera toxin B subunit (CTB) (List Biological Laboratories, Campbell, CA, USA) was injected into the superior colliculus bilaterally (2 μl each site) (6.0 mm posterior and 2.0 mm lateral to the bregma and 4.5–5 mm deep from the cortical surface). After a survival period of 5 days, RGCs could be retrogradely labeled by CTB and detected by the anti-CTB antibody.

CTB immunohistochemistry experiments were performed following the procedure described in detail by Zhang and Diamond (2006). Animals were anesthetized and killed by decapitation. The eyes were removed quickly and fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) for 1 h at 4 °C, and then dehydrated with graded sucrose solutions at 4 °C (1 h each in 15%, 20%, and overnight in 30%). The eyecups were embedded in OCT compound (Tissue Tek, Torrance, CA) and stored at −20 °C for further use. For retinal sections, the tissues were vertically sectioned at 14 μm thickness on a freezing microtome (Leica, Nussloch, Germany). After rinsing with 0.01 M PBS (pH 7.4), the sections were blocked for 1 h in 5% normal donkey serum (v/v) (NDS, Sigma, St. Louis, MO, USA) in PBS plus 0.3% Triton X-100 at room temperature, and then incubated with the primary antibody polyclonal goat-anti-CTB (1:4000 dilution) overnight at 4 °C. Binding sites of the primary antibody were visualized by incubating with Rhodamine Red-X (RRX)-conjugated donkey anti-goat IgG (1:400 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The sections were rinsed and coverslipped with Vectashield (Vector, Burlingame, CA, USA). For whole flat-mounted retina examination, the same procedure was conducted except the incubation time in anti-CTB being 5 days and in RRX-conjugated donkey anti-goat IgG being 1 day. CTB-immunoreactivity and terminal dUTP nick end labeling (TUNEL, see below) signals were visualized with a confocal laser scanning microscope through a 20× objective (Fluroview 1000, Olympus, Monolith, Tokyo, Japan). Counting of CTB-positive RGCs in retinal sections was made using a fluorescence microscope (BX51, Olympus, Monolith, Tokyo, Japan). Final images were assembled using Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA, USA).

Quantification of RGCs was performed on vertical retinal sections following the procedure previously reported (Qiu et al., 2010), with some modifications. For a single preparation, the whole retina was sectioned into 256 slices at 14 μm thickness from the nasal side to the temporal side across the optic disk. The first 8 slices were sequentially mounted on 8 different glass slides. Again, the next 8 slices were mounted in the same way and so on, thereby making the total 32 slices on each of the 8 slides to cover different zones of the whole retina from the nasal side to the temporal side. We randomly selected one slide from the 8 slides to count all CTB-positive RGCs in the 32 slices using the fluorescent microscope through a 20× objective.

Degeneration assay of cells in GCL

To detect cell apoptosis, TUNEL assay (Gavrieli et al., 1992) was performed using the DeadEnd Fluorometric TUNEL System G3250 kit (Promega, Madison, WI, USA) and following the manufacturer's instructions. TUNEL signals were visualized with the confocal laser scanning microscope. Images of DAPI-positive cells were taken simultaneously to confirm the co-localization of the TUNEL signals with the cell nuclei.

In order to get a general profile of the distribution of TUNEL-positive signals and CTB-positive cells in whole flat-mounted retinas, we selected two representative regions, which are named as central and peripheral ones throughout the text as previously described (Neufeld et al., 1999; Sawada and Neufeld, 1999; Yang et al., 2009). The rat retinas used in the present work ranged in diameter from 10.0 to 12.0 mm.

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