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A novel neuroprotective role of a small peptide from flesh fly, 5-S-GAD in the rat retina
in vivo

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Abbreviations:

5-S-GAD, N- β -alanyl-5-S-glutathionyl-3, 4-dihydroxyphenylalanine;

GFAP, glial fibrillary acidic protein;

PKC, protein kinase C;

RGCs, retinal ganglion cells;

TUNEL, terminal transferase dUTP nick end labeling

Abstract

N-β-Alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine(5-S-GAD), an antibacterial substance isolated from flesh fly, has been described as having multipotential biological activities toward various tissues. However, there has been no report testing its action on neural cells. In the present study, we investigate whether 5-S-GAD is neurotoxic or neuroprotective to the rat retina. 5-S-GAD at high doses (more than 200 pmol) induced apoptosis of retinal neurons 7 days after intraocular injection. NMDA (50-100 nmol) induced loss of retinal ganglion cells (RGCs) and thinning of the inner retina 7 days after injection. 5-S-GAD at low doses (2-20 pmol) significantly attenuated the loss of RGCs and the thinning of inner retina induced by NMDA in a dose-dependent manner. To understand the protective mechanism of 5-S-GAD, we investigated the influence of 5-S-GAD on the cell survival molecules, phospho-Akt and Bcl-2. 5-S-GAD (2-20 pmol) rapidly increased phospho-Akt expression 1-7 days and Bcl-2 expression 3-7 days after injection. The cellular localization of this increase was both in bipolar cells and RGCs. This neurosurvival effect of 5-S-GAD was further tested using another toxic model of optic nerve injury. 5-S-GAD significantly blocked the apoptosis of RGCs 7 days after optic nerve crush. These results show that 5-S-GAD (2-20 pmol) protects against the NMDA- and optic nerve crush-induced apoptosis of RGCs. The neuroprotective action of 5-S-GAD in the retina might be mediated by the cell survival phospho-Akt/Bcl-2 system and offers a therapeutic option to rescue RGCs from various types of excitotoxic disease, such as glaucoma.

Running title: Neuroprotection by 5-S-GAD

Key words: retinal ganglion cell, 5-S-GAD, neuroprotection, NMDA, optic nerve injury,

Akt

1. Introduction

N- β -Alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD) was isolated from adult flesh fly as a defense protein produced in response to bacterial injection or body injury (Leem *et al.* 1996). 5-S-GAD was found to inhibit tyrosine phosphorylation of protein tyrosine kinases (Leem *et al.* 1998). Among the protein tyrosine kinases are receptors for growth factors, hormones and viral oncogene products such as v-Src or v-BCR-ABL. This inhibition of tyrosine phosphorylation by 5-S-GAD leads to suppression of tumor cells. 5-S-GAD inhibits cell growth of some human tumor cells *in vitro* (Akiyama *et al.* 2000) and *in vivo* angiogenesis of mouse S180 sarcoma cells (Nishikawa *et al.* 2006). The optimal concentration of 5-S-GAD for the inhibition of cell growth is relatively high, in the order of 10-100 μ M. Although the biological activity of 5-S-GAD has been examined in a variety of cellular systems, its action on nervous system is totally unknown. Therefore, in the present study, we tested how 5-S-GAD affects control retina and retina treated with excessive amounts of the glutamate receptor subtype agonist, (NMDA or optic nerve injury). Excitotoxicity induced by NMDA and delayed cell death by optic nerve injury are thought to be an experimental model for retinal ischemia and glaucoma (Lafuente *et al.* 2001). The toxic effect of 5-S-GAD was examined in the control retina at doses of 2-2000 pmol. The protective effect of 5-S-GAD was examined in the NMDA treated or optic nerve injured retina at doses of 2-20 pmol. 5-S-GAD at high doses (200-2000 pmol) was certainly apoptotic to retinal cells, particularly retinal ganglion cells (RGCs), while 5-S-GAD at low doses (2-20 pmol) was oppositely protective against the NMDA- or optic nerve crush-induced cell damage to RGCs. Such a hormesis-like action of 5-S-GAD is described here for the first time. Furthermore, we sought the signaling cascade(s) operative in the 5-S-GAD-mediated neuroprotection of RGCs, with particular focus on cell survival pathways and glial activation.

2. Results

< ----- Fig. 1

2.1. Induction of apoptosis in the rat retina by 5-S-GAD at high doses

When 5-S-GAD (2-2000 pmol) was injected into rat eye, no terminal transferase dUTP nick end labeling (TUNEL) -positive cells could be seen in the retina until 5 days after injection (data not shown). However, a small number of TUNEL-positive RGCs appeared 7 days after injection of 5-S-GAD (more than 200 pmol, Fig. 1D). 5-S-GAD (2000 pmol) induced apoptosis of almost all retinal neurons, including those in the outer nuclear, inner nuclear and ganglion cell layers (Fig. 1E). No effect could be seen at low doses (2-20 pmol) of 5-S-GAD (Fig. 1B, 2 pmol; C, 20 pmol), like PBS treatment (Fig. 1A).

< ----- Fig. 2

2.2. Prevention of NMDA-induced apoptosis in the retina by 5-S-GAD at low doses

When NMDA (2-100 nmol) was injected into rat eye, concentrations of more than 20 nmol significantly induced apoptosis of retinal cells in the inner nuclear layer and ganglion cell layer neurons within 72h after injection (Fig. 2B, 50 nmol; 2C, 100 nmol). However, to observe the maximum prevention effect of 5-S-GAD, we studied TUNEL assay at 7 days after injection. Interestingly, the apoptosis of retinal neurons induced by 100 nmol NMDA 7 days after injection was significantly reduced by treatment with 2-20 pmol 5-S-GAD (Fig. 2D, 20 pmol). Such a dose of 5-S-GAD alone did not induce any apoptosis of retinal cells (see Fig. 1C), as in the control (Fig. 2A). We qualitatively confirmed that most of apoptotic cells in the ganglion cell layer were RGCs using an immunohistochemical study with anti- β -tubulinIII (Koriyama unpublished data)

< ----- Fig. 3

2.3. 5-S-GAD protects against the NMDA-induced loss of RGCs and thinning of the

inner retina

NMDA (100 nmol) induced RGC apoptosis 7 days after injection (Fig. 2). We next examined actual loss of RGCs in the rat retina with HE staining. RGCs were certainly lost 7 days after NMDA injection (Fig. 3B) as compared with PBS treatment (Fig. 3A). 5-S-GAD (2-20 pmol) prevented the cell loss induced by NMDA (Fig. 3C). Graphical quantification of cell loss induced by NMDA with or without 5-S-GAD is shown in Fig. 3D. Thinning of the inner retina (marked with a vertical line in each figure) 7 days after NMDA treatment was additionally notable in comparison with controls (cf. Fig. 3A and 3B). The thinning of the inner retina by NMDA was also prevented by prior injection of 2-20 pmol of 5-S-GAD (Fig. 3C, E). 5-S-GAD (2-20 pmol) significantly blocked the loss of RGCs and the thinning of the inner retina induced by NMDA. The thickness of the outer nuclear layer was not changed by NMDA treatment (Koriyama, unpublished data).

< ----- Figs 4 & 5

2.4. 5-S-GAD activates cell survival signals in the rat retina

We next examined the cell survival system responsible for the neuroprotective action of 5-S-GAD (2-20 pmol), specifically by analyzing the levels of two representative cell survival molecules, phospho-Akt and Bcl-2, in the rat retina (Homma *et al.* 2007). In control retina, weak immunoreactivity of phospho-Akt could be seen in the bipolar cells and ganglion cells (Fig. 4A). 5-S-GAD rapidly increased phospho-Akt expression in the retina 1 day after injection. This increase in the level of phospho-Akt continued until at least 7 days after injection. The cellular localization of this increase was in both ganglion cells and bipolar cells, as detected with anti-phospho Akt antibody (Fig. 4B). The phospho-Akt activity in bipolar cells was confirmed by double staining for protein kinase C (PKC) (Negishi *et al.* 1988). The immunopositive reactivity of bipolar and ganglion cells induced by 5-S-GAD was unchanged under NMDA treatment (Fig. 4D). No positive signals for phospho-Akt

were seen in the NMDA-treated retina (Fig. 4C). We further examined Bcl-2 expression after 5-S-GAD injection. In control retina, no significant immunoreactivity for Bcl-2 could be seen (Fig. 5A). 5-S-GAD at low doses (2-20 pmol) increased Bcl-2 expression in the rat retina 3 days after injection and this increase in the level of Bcl-2 continued until at least 7 days after injection. The cellular localization of this increase was also observed in both ganglion cells and bipolar cells with anti-Bcl-2 antibody (Fig. 5B). The increase in the level of Bcl-2 in RGCs induced by 5-S-GAD was unchanged by NMDA treatment (Fig. 5D). No positive staining for Bcl-2 was seen in the NMDA-treated retina (Fig. 5C).

< ----- Fig. 6

2.5. Quantitative measurement of phospho-Akt and Bcl-2 levels induced by 5-S-GAD

Since 5-S-GAD rapidly increased the immunoreactivity of phospho-Akt and Bcl-2 in the bipolar and ganglion cells 1-7 and 3-7 days after injection, respectively (Figs 4 & 5), we next measured the levels of these survival molecules by Western blotting analysis. 5-S-GAD alone increased phospho-Akt protein levels 2-fold in the retina 7 days after injection as compared with the control value (Fig. 6A). Conversely, NMDA alone reduced the levels of phospho-Akt by 40% of control in the retina 7 days after injection. The reduced levels of phospho-Akt induced by NMDA treatment was almost recovered by 5-S-GAD (Fig. 6A). 5-S-GAD also increased Bcl-2 levels 1.6 fold in the retina 7 days after injection (Fig. 6B). NMDA alone decreased the levels of Bcl-2 in the retina 7 days after treatment by 40% of control value. The reduced levels of Bcl-2 induced by NMDA treatment was recovered by 5-S-GAD (Fig. 6B).

< ----- Fig. 7

2.6. 5-S-GAD does not affect glial activation induced by NMDA treatment

To investigate the neuroprotective mechanism of 5-S-GAD against NMDA excitotoxicity, we studied the effect of 5-S-GAD on glial activation (Honjo *et al.* 2000).

NMDA induced glial activation, as revealed by positive immunohistochemical staining for glial fibrillary acidic protein (GFAP) in Müller cells 7 days after injection (Fig. 7C), but no glial activation was seen in control retina (Fig. 7A). No significant effect of 5-S-GAD (2-20 pmol) on Müller cells could be seen with or without NMDA treatment (Fig. 7D and Fig. 7B).

< ----- Fig. 8

2.7. 5-S-GAD prevents apoptosis of RGCs after optic nerve crush

Optic nerve crush retrogradely induces cell death of RGCs in the rat retina 6-7 days after injury (Homma *et al.* 2007). As shown in Fig. 8C, many RGCs in the rat retina became TUNEL positive 7 days after optic nerve injury (Fig. 8A). 5-S-GAD (20 pmol) clearly reduced the number of apoptotic RGCs 7 days after injury (Fig. 8D). 5-S-GAD (2-20 pmol) alone did not induce the appearance of any TUNEL-positive cells 7 days after injection (Fig. 8B). Figure 7E shows that 5-S-GAD significantly reduces the number of apoptotic RGCs induced by optic nerve injury in a dose-dependent manner.

3. Discussion

3.1. Hormesis-like action of 5-S-GAD for neuroprotection in the rat retina

5-S-GAD at high doses (200-2000 pmol) clearly induced apoptosis of retinal neurons, including RGCs, 7 days after intraocular injection. The toxic dose of 5-S-GAD delivered to the retina in this study is the same as the dose causing inhibition of cell growth in other cellular systems. The inhibition of tumorigenesis of human melanoma and breast cancers was evoked by 100 μ M 5-S-GAD (Akiyama *et al.* 2000). The inhibition of angiogenesis and monocyte-macrophage lineage cells was also evoked by 100 μ M 5-S-GAD (Nishikawa *et al.* 2006; Hijikata *et al.* 1999). The doses of 5-S-GAD (2-2000 pmol) used in our study are estimated to correspond to a concentration of roughly 0.01-10 μ M 5-S-GAD in the rat ocular fluid. Sublethal doses of 5-S-GAD (2-20 pmol) rather induced neuroprotection of RGCs. The expression levels of the cell survival molecules phospho-Akt and Bcl-2 were enhanced 1-3 or 3-5 days after injection, respectively. The neuroprotective effect of 5-S-GAD was examined under the condition of NMDA excitotoxicity. It is currently accepted that most damaged cells (>70%) by NMDA are RGCs in ganglion cell layer (Siliprandi *et al.*, 1992). Furthermore, displaced amacrine cells in ganglion cell layer can easily be expected because their diameter is extremely smaller (<10 μ m) than that of RGCs. Dying of RGCs and thinning of the inner retina were significantly blocked by 2-20 pmol of 5-S-GAD (Figs 2 and 3). Thus, 5-S-GAD at low doses (2-20 pmol) is neuroprotective toward RGCs, while 5-S-GAD at high doses (200-2000 pmol) stimulates apoptosis in the retina. Such a hormesis-like action of 5-S-GAD is first described in this study. Although the signaling cascade downstream of 5-S-GAD leading to the neuroprotection is unclear, this action could be explained as follows. Recently, Akiyama *et al.* (2007) reported that 5-S-GAD liberated o-semiquinone radical anion for inhibition of cell growth at high concentrations. The apoptosis in the retina induced by 2

nmol 5-S-GAD might be caused by such a cytotoxic radical (H_2O_2). Thus, we propose the following possibilities: (1) low doses (2-20 pmol) of 5-S-GAD liberate a small amount of radical, which in turn activates the cell survival molecule phospho-Akt; (2) 5-S-GAD (2-20 pmol) activates a pathway involving phospho-Akt through the phosphatidylinositol-3 kinase/Akt system in rat RGCs, via either brain-derived neurotrophic factor or insulin-like growth factor-I (Mansoor-Robay *et al.* 1994; Kermer *et al.* 2000; Homma *et al.* 2007); (3) 5-S-GAD inhibits Src protein kinase leading to protection against NMDA excitotoxicity, as shown in hippocampal or striatal neurons after ischemic insult (Hou *et al.* 2007; Crossthwaite *et al.* 2004). Through which possibilities 5-S-GAD is neuroprotective to the RGCs, is uncertain at present. Anyhow, such hormesis like action of 5-S-GAD on the nervous cell system is enough considerable.

3.2. Neuroprotective action of 5-S-GAD on glaucomatous conditions in the rat retina *in vivo*

Glaucoma is an optic nerve neuropathy caused by a complicated etiology including an increase in intraocular pressure, ocular hypertension, optic disc ischemia and oxidative stresses (Weinreb and Levin, 1999; Kuehn *et al.* 2005). Progression of optic nerve degeneration finally results in cell death of RGCs and loss of vision. The main therapeutic target for treating glaucoma is decreasing intraocular pressure (Bakalash *et al.* 2002). An anti-glaucoma drug with neuroprotective or neuroregenerative properties has been awaited for a long time. In this study, 5-S-GAD (2-20 pmol) clearly prevented RGCs from undergoing cell death induced by NMDA and optic nerve injury. These two models of RGCs damage are often used as glaucomatous conditions (Tezel and Wax, 1999). The pathophysiology of NMDA exposure is simulated to be equivalent to ischemia, calcium overload, generation of super oxide or nitric oxide radicals (Lipton *et al.* 1994). It is also well known that optic

nerve injury retrogradely induces apoptosis of RGCs 6-7 days after injury (Berkelaar *et al.* 1994). Interestingly, expression of phospho-Akt was rapidly downregulated in rat RGCs 1 day after optic nerve crush (Homma *et al.* 2007). Since 5-S-GAD first activates phospho-Akt in the retina at 1-3 days (Fig. 4) followed by expression of Bcl-2 3-5 days after injection (Fig. 5), the activation of phospho-Akt plays a key role in the neuroprotection induced by 5-S-GAD. The neuroprotective effect of 5-S-GAD is directly mediated through the phospho-Akt/Bcl-2 systems, but is not mediated by glial activation (Fig. 7). It is well known that phospho-Akt increases synthesis of Bcl-2 protein via phosphorylation of cAMP response element binding protein (Pugazhenti *et al.* 2000). Moreover, another cell survival factor, mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) was not activated by 5-S-GAD (data not shown). Of course, further studies using other types of glaucoma model, such as increasing intraocular pressure (Wang *et al.* 2005), are needed. Taken together, these data strongly indicate that this small peptide from flesh fly might become an epoch-making neuroprotective or neuroregenerative reagent against glaucomatous conditions.

In conclusion, 2-20 pmol (corresponding to 0.01-0.1 μ M in the ocular fluid) of 5-S-GAD clearly protected the RGCs in rat retinas from cell death induced by excitotoxic NMDA or optic nerve injury *in vivo*. The neuroprotective mechanism is thought to involve activation of the cell survival phospho-Akt/Bcl-2 system. Although the exact pathway leading to activation of this cell survival system is not yet clarified, these results strongly indicate that this novel action of 5-S-GAD might become a therapeutic option for rescuing damaged RGCs in glaucoma patients.

4. Experimental procedures

4.1. Animals and surgery

Sprague-Dawley rats (body weights 250-300 g) were used throughout the study. Rats were reared and handled according to NIH guidelines and the ARVO statement on the care and use of laboratory animals. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30-40 mg/kg body weight). Various concentrations of NMDA or 5-S-GAD were injected intravitreally in a 5- μ l solution with a micro-syringe. The optic nerve was crushed 1 mm away from the eyeball with forceps, as described elsewhere (Homma *et al.* 2007). Rats were reared in clear plastic cages and kept under a 12 h/12 h light-dark cycle at 23°C.

4.2. Morphological measurement of surviving RGCs in rats after NMDA and/or 5-S-GAD treatment

Fixation and cryosection of retinal samples have been described elsewhere (Homma *et al.*, 2007). In brief, rat eyes were enucleated and fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 5% sucrose for 2 h at room temperature, followed by incubation in 20% sucrose overnight at 4°C. All eyes were then embedded into OCT compound in vinyl molds, and frozen in liquid nitrogen. Retinal sections in central areas within 300- μ m were cut at 12- μ m thickness, and mounted onto silane-coated slides. To evaluate surviving RGCs after treatment, we performed immunohistochemistry using anti- β -tubulinIII antibody (TUJ1, R & D Systems, Minneapolis, USA) and HE staining. The number of surviving RGCs was performed by counting the cells with more than 10 μ m in diameter to avoid miscounting the displaced amacrine cells under microscopic observation of 25 visual fields per retina (Sabel *et al.* 1995). The thickness of the inner retina (outer

plexiform layer, inner nuclear layer, inner plexiform layer and ganglion cell layer) was calculated from transverse sections.

4.3. TUNEL staining

After fixation and cryosection, retinal tissues were incubated in 0.1% Triton X-100 and 0.1% sodium citrate for 15 min and rinsed in PBS. DNA fragmentation of cells undergoing apoptosis was detected using an *in situ* cell death detection kit (Roche, Mannheim, Germany) containing terminal transferase and fluorescence dUTP. The retinal tissue was incubated in this reaction mixture overnight at 37°C, and rinsed twice in PBS. In each eye, the number of TUNEL-positive cells within 300- μ m of the optic disk was counted. A positive control for the TUNEL staining was performed with DNase I treatment at 37°C for 30 min. A clear positive signal with DNA fragmentation could be observed in all retinal neurons (data not shown).

4.4. Immunohistochemistry

Immunohistochemical staining of cell survival molecules (phospho-Akt and Bcl-2) and retinal cell markers, including PKC for bipolar cells, GFAP for Müller cells, and β -tubulin III for ganglion cells, has been described elsewhere (Koriyama *et al.* 2007). The primary antibodies used were anti-mouse Bcl-2 (1:200), anti-mouse PKC (1:200) were purchased from Santa Cruz. Anti-mouse phospho-Akt (1:500) and anti-mouse GFAP antibody (1:100) were purchased from Sigma-Aldrich, USA. Following washing and blocking, tissue sections were incubated with primary antibodies overnight at 4°C. After washing, retinal sections were incubated with an Alexa Fluor 488 secondary antibody (Molecular Probe, Japan) for visualization at room temperature.

4.5. Western blotting analysis of cell death and survival signals

Retinal extracts were prepared at 7 days after treatment. Retinas were immersed in Tris-HCl buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma), and were then sonicated and centrifuged for 10 min at 1,000 g, as described previously (Koriyama et al., 2003). Following SDS-PAGE electrophoresis in 12.5% gel, proteins were transferred onto a nitrocellulose membrane (Amersham International, U.K.). The membrane was incubated in a blocking buffer (3% bovine serum albumin in PBS) for 1 h at room temperature, and probed with a primary antibody in blocking buffer overnight at 4°C. The membrane was further washed, and probed with a secondary antibody in blocking buffer for 1 h at room temperature, and washed again. The signal (56 kDa of p-Akt and 26 kDa band of Bcl-2 protein) was detected using 3, 3'-diaminobenzidine tetrahydrochloride. The same primary antibodies in immunohistochemical study were used. An appropriate anti-IgG antibody (Santa Cruz Biotechnology, CA) was used as secondary antibody (1:200-1,000). To confirm the amount of protein on acrylamide gel, we performed staining for protein by rapid stain Coomassie brilliant blue kit (Nacalai Tesque, Kyoto). Each protein band was densitometrically analyzed by the NIH imaging software (NIH, Bethesda, MD, USA). All experiments were repeated at least 3 times.

4.6. Statistics

Values of surviving or apoptotic RGCs, retinal thicknesses and density of the band in Western blotting are indicated as means±S.E.M for 3 experiments. Statistical significance was determined as $P < 0.05$ using one way ANOVA followed by the Bonferroni test.

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Figure Legends

Fig. 1. 5-S-GAD induces apoptosis of retinal neurons at high doses.

5-S-GAD (2-20 pmol) did not induce any significant change in TUNEL staining in the rat retina 7 days after intravitreal injection (B, 2 pmol; C, 20 pmol). 5-S-GAD (200 pmol) induced apoptosis of some retinal ganglion cells (RGCs) in rat 7 days after injection (D) unlike the control (A). 5-S-GAD (2 nmol) induced apoptosis of almost all retinal neurons including outer nuclear, inner nuclear and ganglion cell layers 7 days after injection (E). Scale bar=50 μ m.

Fig. 2. NMDA induces apoptosis of RGCs, which is prevented by 5-S-GAD at low doses.

NMDA (50 nmol) induced apoptosis of RGCs 7 days after intravitreal injection, as detected by TUNEL staining (B), unlike the control (A). NMDA (100 nmol) induced apoptosis of almost all RGCs and some inner nuclear neurons 7 days after injection (C). With a combination of 100 nmol of NMDA with 20 pmol of 5-S-GAD, the apoptosis of RGCs and some inner nuclear neurons induced by NMDA was greatly reduced (D). Scale bar=50 μ m.

Fig. 3. 5-S-GAD at low doses protects against NMDA-induced loss of RGCs and thinning of the inner retina, as revealed by HE staining.

NMDA (100 nmol) induced loss of RGCs and thinning of the inner retina (marked with a vertical line) 7 days after intravitreal injection (B) as compared to the control (A). Prior injection of 20 pmol of 5-S-GAD significantly reduced the loss of RGCs and the thinning of the inner retina induced by NMDA (C). Scale bar=50 μ m. Fig. 3D shows a graphical presentation of the loss of RGCs by induced NMDA (100 nmol) and its prevention by 5-S-GAD (2-20 pmol). *P<0.01 compared to the control. +P<0.01 compared to NMDA

(100 nmol) treatment. Fig. 3E shows a graphical presentation of the thinning of the inner retina by NMDA (100 nmol) and its protection by 5-S-GAD (2-20 pmol) 7 days after injection. * $P < 0.01$ compared to the control. + $P < 0.01$ compared to the NMDA (100 nmol) treatment.

Fig. 4. 5-S-GAD induces phospho-Akt expression in the rat retina

5-S-GAD (20 pmol) induced strong immunoreactivity for phospho-Akt in bipolar and ganglion cells 1-7 days after injection (B, 2 days after injection); this was not seen in the control retina, in which weak staining of bipolar and ganglion cells could be seen (A). NMDA (100 nmol) alone did not induce any immunoreactivity for phospho-Akt in the retina 7 days after injection (C). With a combination of NMDA and 5-S-GAD, strong immunoreactivity for phospho-Akt could still be seen in the bipolar and ganglion cells 7 days after injection (D). Scale bar=50 μm .

Fig. 5. 5-S-GAD induces Bcl-2 expression in the rat retina.

5-S-GAD (20 pmol) induced strong immunoreactivity of Bcl-2 in RGCs and weak immunoreactivity in bipolar cells 3-7 days after injection (B, 5 days after injection), which was not seen in the control (A). NMDA (100 nmol) alone did not induce any immunoreactivity of Bcl-2 in the retina 7 days after injection (C). With a combination of NMDA with 5-S-GAD, positive immunoreactivity of Bcl-2 in the RGCs could still be seen 7 days after injection (D). Scale bar=50 μm .

Fig. 6. Quantitative analyses of phospho-Akt and Bcl-2 levels in the retina after 5-S-GAD injection.

The supernatants of retinal samples treated with 5-S-GAD and /or NMDA for 7 days were

subjected to Western blotting analysis. (A) 5-S-GAD alone increased phospho-Akt levels 2-fold in the retina 7 days after injection. NMDA alone decreased phospho-Akt levels by 40% of control value. 5-S-GAD fully recovered the decrease by NMDA treatment. * $P < 0.05$ vs. control. + $P < 0.05$ vs. the NMDA treatment (n=3). (B) 5-S-GAD alone increased Bcl-2 levels 1.6 fold in the retina 7 days after injection. NMDA alone decreased Bcl-2 levels by 40% of control value. 5-S-GAD fully recovered the decrease by NMDA treatment. Electrophoretic bands for phospho-Akt and Bcl-2 were shown in the upper side of each figure.

Fig. 7. NMDA induces glial activation in the rat retina.

NMDA (100 nmol) induced strong immunoreactivity for GFAP in Müller cells 7 days after injection (C) unlike the control (A). With a combination of NMDA and 5-S-GAD (20 pmol), no change in GFAP immunoreactivity in the Müller cells induced by NMDA could be seen in the retina 7 days after injection (D). 5-S-GAD alone did not evoke any GFAP immunoreactivity in the retina 7 days after injection (B). Scale bar=50 μm .

Fig. 8. 5-S-GAD protects RGCs against apoptosis after optic nerve crush.

Optic nerve crush induced specific apoptosis of RGCs 7 days after injury (C), unlike the control (A), as revealed by TUNEL staining. 5-S-GAD (20 pmol) significantly reduced the level of apoptosis of RGCs 7 days after treatment (D). 5-S-GAD alone did not induce apoptosis in the retina (B). Scale bar=50 μm . Fig. 8E shows a graphical presentation of the number of apoptotic RGCs after optic nerve crush and its protection by 5-S-GAD. * $P < 0.01$ compared to the control. + $P < 0.01$ compared to the optic nerve injury.

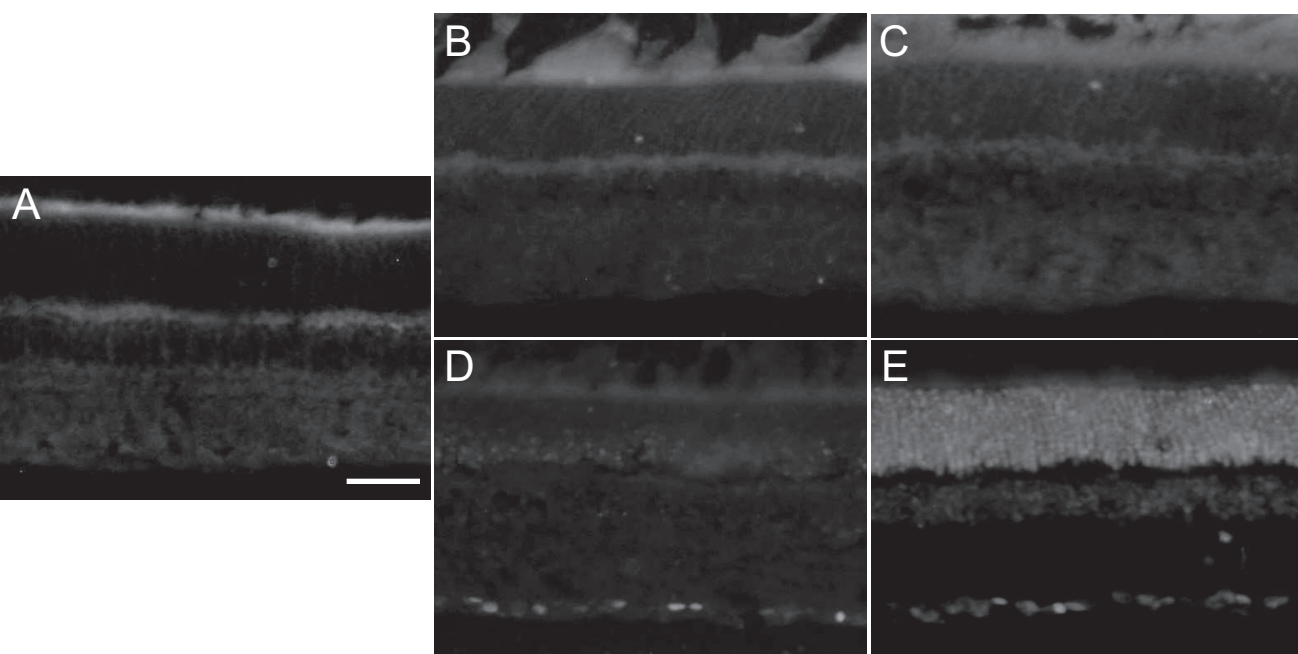


Fig. 1

Figure2

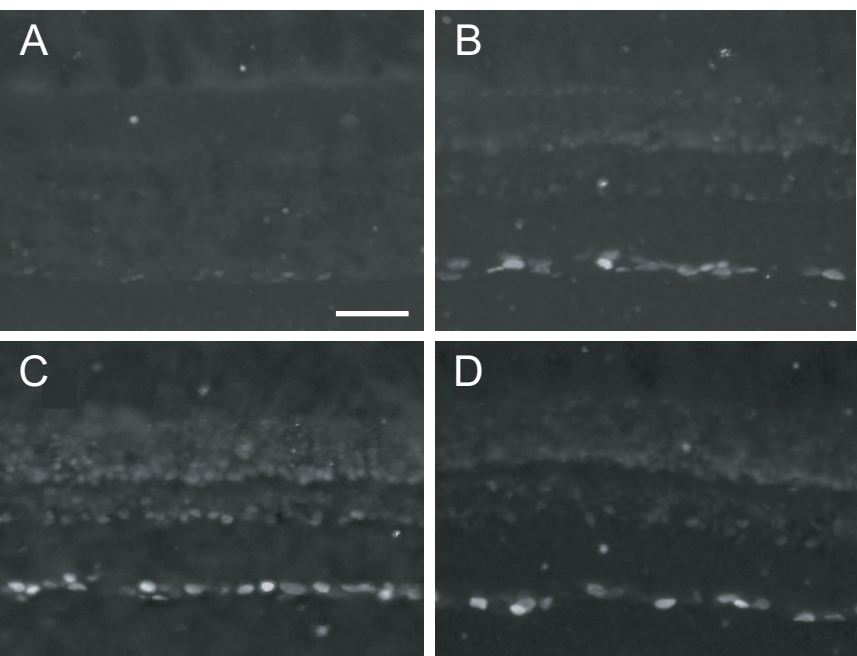


Fig. 2

Figure3

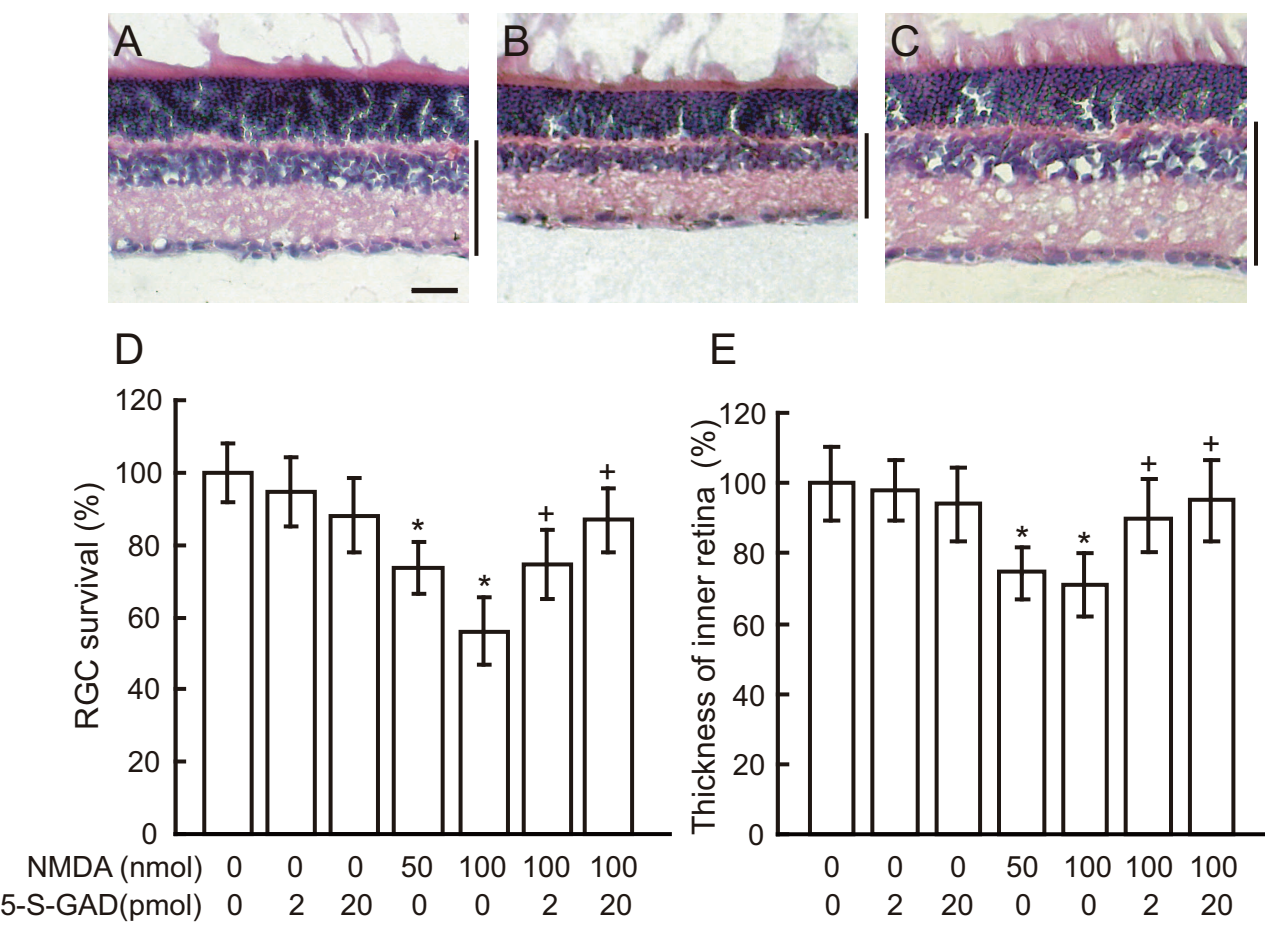


Fig. 3

Figure4

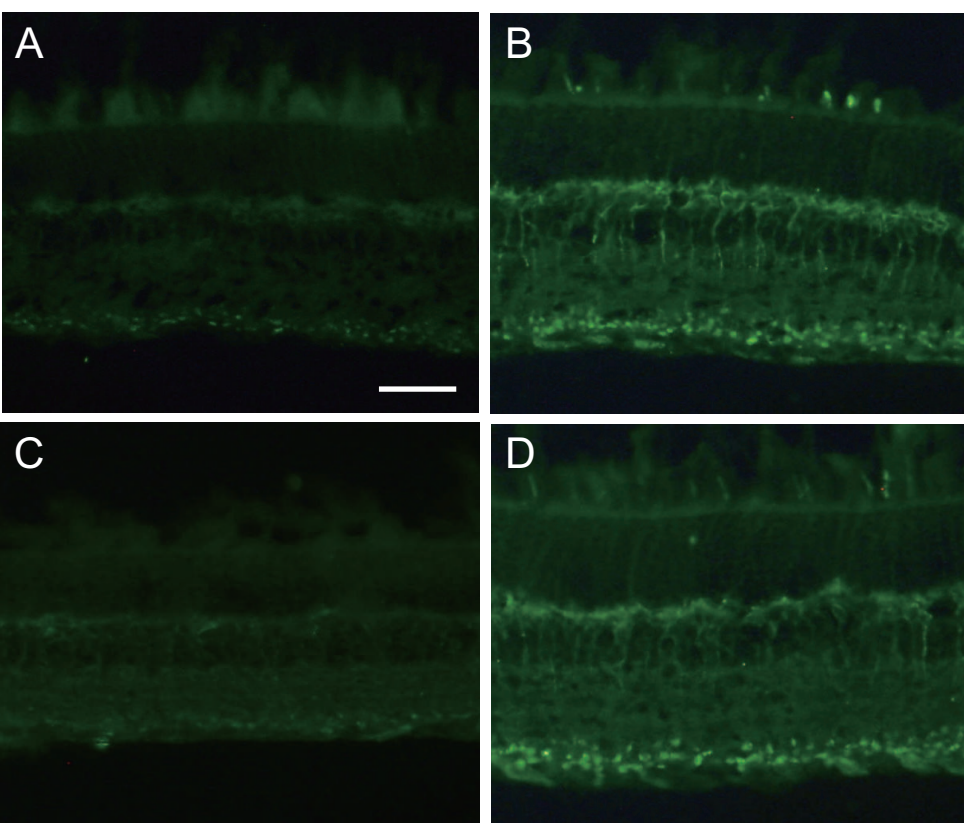


Fig. 4

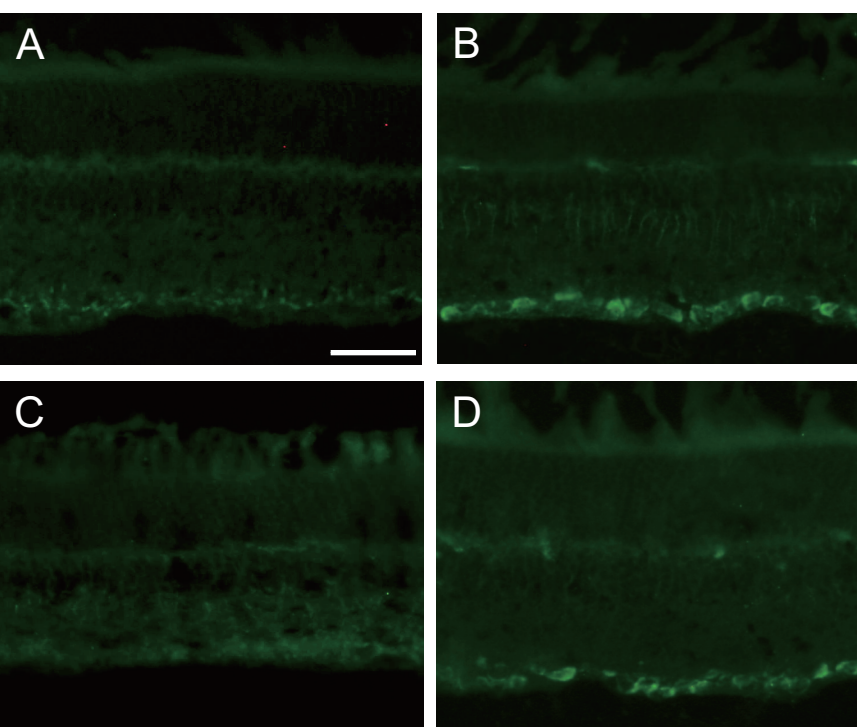


Fig. 5

Figure6

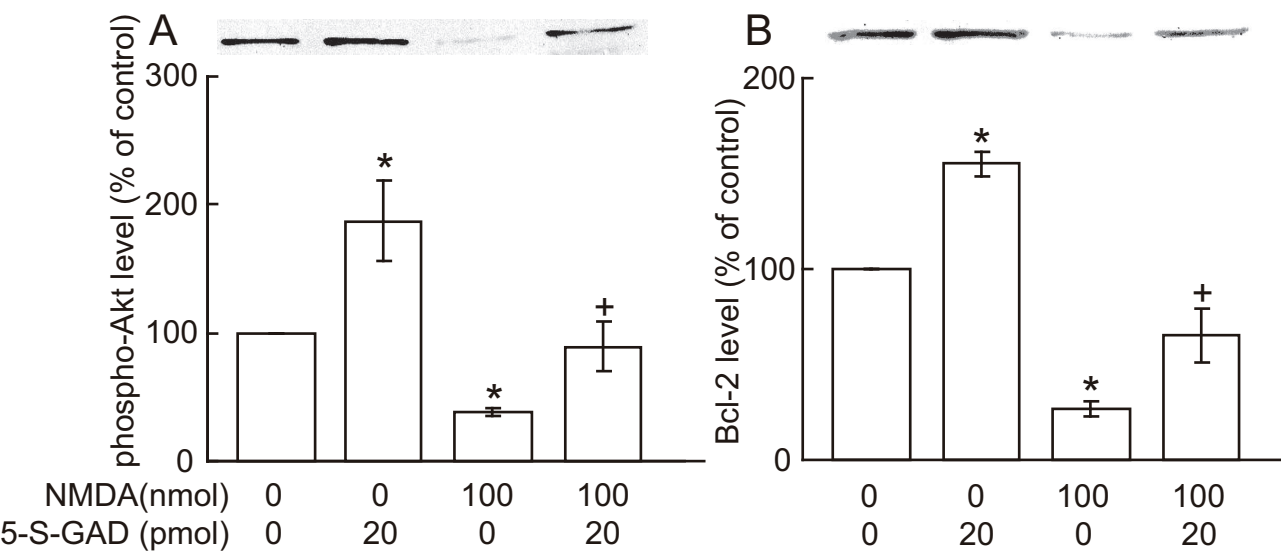


Fig. 6

Figure7

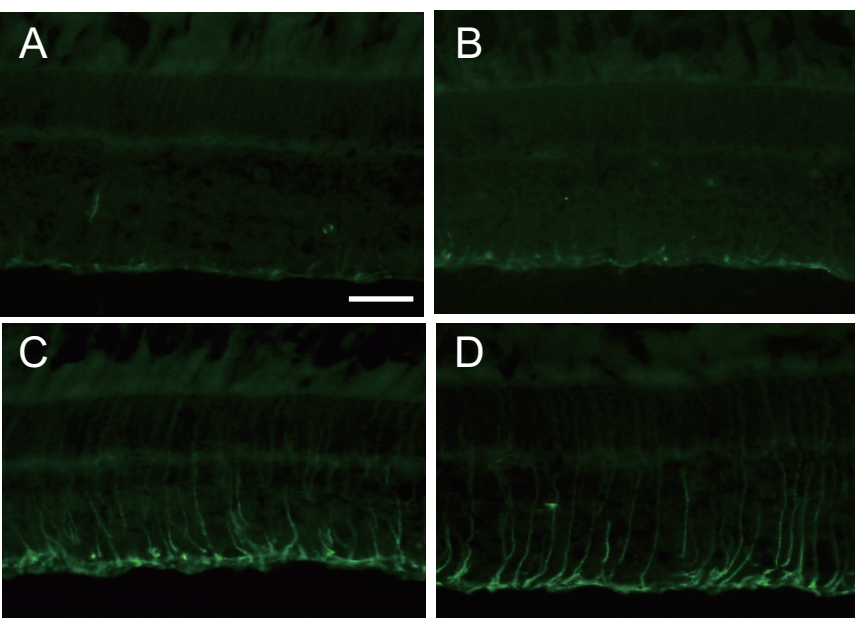


Fig. 7

Figure 8

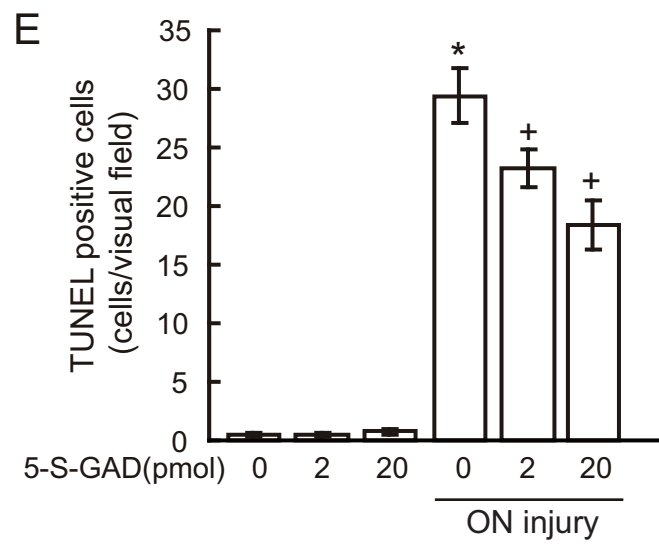
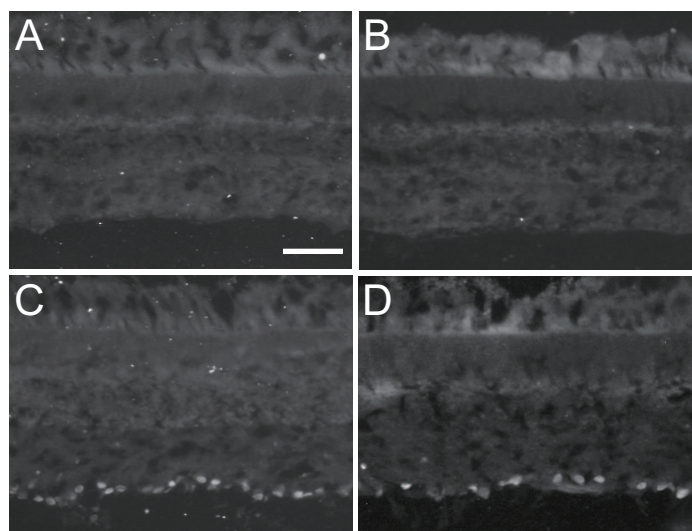


Fig. 8