Supplemental methods

Western blot analysis

At 5 days after I/R, mice were euthanized by cervical dislocation; their eyeballs were rapidly removed, and the retinas were carefully separated from the eyeballs and quickly frozen in dry ice. Following methods were carried out as main manuscripts.

Immunohistochemistry

The eyes were enucleated, fixed in 4% paraformaldehyde 24 h at 4°C, immersed in 25% sucrose for 48 h at 4°C, and embedded in optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Transverse cryostat sections of 10 μm thickness were cut and placed on slides (MAS COAT; Matsunami glass Ind., Ltd., Osaka, Japan). The retinal sections were blocked in non-immune serum from species of secondary antibody and incubated with primary antibody for overnight 4°C. Then, they were washed with PBS and incubated secondary antibody for 1 h. Finally, they were counterstained by Hoechst 33342, and mounted in fluoromount (Diagnostic BioSystems, Pleasanton, CA, USA). For immunostaining, the following primary antibodies were used: TLR4 mouse monoclonal antibody (1:100; IMGENEX), Brn-3a goat polyclonal antibody (1:500; Santa Cruz Biotechnology), glial fibrillary acidic
protein (GFAP; 1:100; SHIMA Laboratories Co., Ltd., Tokyo, Japan), and they were incubated overnight at 4°C. Alexa Flour 488 F (ab’)2 fragment of goat anti-mouse IgG (H+L) antibody (1:1000), Alexa Flour 546 F (ab’)2 fragment of donkey anti-rabbit IgG (H+L) antibody (1:1000), and Alexa Flour 546 F (ab’)2 fragment of rabbit anti-goat IgG (H+L) antibody (1:1000) were used as a secondary antibody, and incubated for 1 h at room temperature. The sections were observed under a confocal microscope (FLUOVIEW FV10i; Olympus Tokyo, Japan).
Supplemental Figure Legends

Supplemental figure 1. Localization of TLR4 after I/R

(A) The expression of TLR4 was upregulated around the Brn-3a positive retinal ganglion cells after I/R. (B) TLR4 did not merge with GFAP after I/R. Scale bar represents 20 μm.

Supplemental figure 2. Downstream TLR4 signaling pathway 5 days after I/R

(A, C, E) Representative band images for (A) NF-κB, (C) p-Syk, and (E) Syk in retinas of non-treated WT mice (WT control), I/R-treated WT mice (WT I/R), non-treated TLR4 KO mice (TLR4 KO control), and I/R-treated TLR4 KO mice (TLR4 KO I/R).

(B, D, E) Quantitative analysis of the band densities for (B) NF-κB, (D) p-Syk, and (E) Syk. Data are shown as mean ± S.E.M. (n = 3 or 6). *P < 0.05 versus I/R-treated WT mice (WT I/R) (t-test). #P < 0.05 versus non-treated WT mice (WT control).

Supplemental figure 3. Piseanannol inhibited p-Syk activation

(A) Representative band images for p-Syk in retinas treated with vehicle, piceatannol, I/R plus vehicle, or I/R plus piceatannol. (B) Quantitative analysis of the band densities for p-Syk. Data are shown as mean ± S.E.M. (n = 5 or 6). *P < 0.05, versus
I/R plus vehicle-treated mice (I/R) (t-test). *P < 0.05 versus vehicle-treated (control) mice (t-test).
Supplemental figure 3

A

B

Density p-Syk / β-actin (Fold increase)

Control  I/R  Control  I/R

Piceatannol

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