Agonist of growth hormone–releasing hormone enhances retinal ganglion cell protection induced by macrophages after optic nerve injury

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Optic neuropathies are leading causes of irreversible visual impairment and blindness, currently affecting more than 100 million people worldwide. Glaucoma is a group of optic neuropathies attributed to progressive degeneration of retinal ganglion cells (RGCs). We have previously demonstrated an increase in survival of RGCs by the activation of macrophages, whereas the inhibition of macrophages was involved in the alleviation on endotoxin-induced inflammation by antagonist of growth hormone–releasing hormone (GHRH). Herein, we hypothesized that GHRH receptor (GHRH-R) signaling could be involved in the survival of RGCs mediated by inflammation. We found the expression of GHRH-R in RGCs of adult rat retina. After optic nerve crush, subcutaneous application of GHRH agonist MR-409 or antagonist MIA-602 promoted the survival of RGCs. Both the GHRH agonist and antagonist increased the phosphorylation of Akt in the retina, but only agonist MR-409 promoted microglia activation in the retina. The antagonist MIA-602 reduced significantly the expression of inflammation-related genes *Il1b*, *Il6*, and *Tnf*. Moreover, agonist MR-409 further enhanced the promotion of RGC survival by lens injury or zymosan-induced macrophage activation, whereas antagonist MIA-602 attenuated the enhancement in RGC survival. Our findings reveal the protective effect of agonistic analogs of GHRH on RGCs in rats after optic nerve injury and its additive effect to macrophage activation, indicating a therapeutic potential of GHRH agonists for the protection of RGCs against optic neuropathies especially in glaucoma.

GHRH | retinal ganglion cells | neuroprotection | macrophage activation | optic nerve injury

Optic neuropathies can occur in common and serious ocular complications, including glaucoma, traumatic injury, inflammation, ischemia, and tumors. They share a common pathology of degeneration of retinal ganglion cells (RGCs) and axonal loss. Optic neuropathies are leading causes of visual impairment and irreversible blindness, affecting more than 100 million people worldwide (1). There is no effective treatment for the complete functional recovery in optic neuropathies. Among them, glaucoma is the top cause of irreversible impaired vision affecting 5% of the world's population across all races and regions (2). The principle of treatment for glaucoma is to minimize progression by lowering the intraocular pressure (3). There is no therapy to date that stops or reverses optic nerve (ON) damage in glaucoma so as to cure the disease (4).

Mammalian retina has low intrinsic regenerative ability, and the presence of myelin-associated inhibitors, scar formation at the injury site, and lack of trophic support further contribute to the failure in regeneration after ON injury (5–7). Research aimed at enhancing the survival of RGCs and axonal regeneration has led to potential therapeutic strategies, including exogenous supplementation of neurotrophic factors (8, 9), inhibition of the apoptotic pathway (10), counteracting the inhibitory signals associated with myelin and glial scar formation (11), deletion of *Pten* and *Socs3* genes (12), as well as stem cell therapy (13). Clinical applications of these findings are under development.

Mild ocular inflammation induced by lens injury or zymosan-induced macrophage activation has been shown to promote the survival of RGCs and axonal regeneration (14, 15). Oncomodulin is the major mediator derived from activated macrophages and neutrophils in the inflammation-induced axonal regeneration (16, 17), with the elevation of cyclic adenosine monophosphate (cAMP). However, oncomodulin does not account for all promoting effects on axonal regeneration by lens injury.

Significance

Optic neuropathies, characterized by the degeneration of retinal ganglion cells (RGCs) and their axons, are leading causes of irreversible blindness and visual impairment, for which currently there is no effective treatment or cure. This study demonstrates that subcutaneous application of an agonist or antagonist of growth hormone–releasing hormone (GHRH) reduces the degeneration of RGCs induced by optic nerve injury in rats. GHRH agonist further enhances the neuroprotective effect of macrophage activation on the survival of RGCs. Our findings demonstrate the therapeutic potential of GHRH agonist for the protection of RGCs against optic neuropathies, including glaucoma, the world's most common cause of irreversible blindness.

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There are still undiscovered factors and mechanisms involved in the inflammation-induced neural recovery after ON injury.

Growth hormone (GH)–releasing hormone (GHRH) controls the secretion of GH in the anterior pituitary, and GH regulates the synthesis of insulin-like growth factor-1 (IGF1) in the liver (18). GHRH signaling also exerts extrapituitary functions, including the involvement in tumor apoptosis (19). Our previous study in endotoxin-induced anterior uveitis rat model have shown that GHRH receptor (GHRH-R) is up-regulated in the ciliary body, macrophages, and leukocytes (20). The GHRH antagonist MIA-602 can reduce the infiltration of endotoxin-induced macrophages and leukocytes into aqueous humor by inhibiting inflammatory cytokine secretion, indicating the participation of GHRH signaling in the regulation of inflammatory responses (20). Notably, we observed the expression of GHRH-R in the RGC layer of the retina. While GH should be neuroprotective against excitotoxic-induced cell death in RGCs (21), the physiological role of GHRH-R signaling in RGCs remains elusive. We previously showed that GHRH agonists regulate the PI3K/Akt and MAPK/Erk pathways (22), which are important for RGC survival. Herein we hypothesized that GHRH-R signaling could be involved in the promotion of neural recovery after ON injury induced by inflammation. In this study, we investigated the effects of GHRH agonists and antagonists on RGCs after ON injury, and its involvement in the promotion of neural repair induced by inflammation after lens injury or zymosan-induced macrophage activation.

Results

Expression of GHRH-R and Its Related Signaling Molecules in Retina. Expression of the GHRH-R protein in the RGC layer of rat retinas was shown by immunofluorescence analysis. The GHRH-R was colocalized with βIII-tubulin$^+$ RGCs and Gfap$^+$ astrocytes (Fig. 1 *A* and *B*). Sybr green PCR analysis showed that the *Ghrh*, *Ghrhr*, and cAMP-responsive element binding protein (*Creb1*) genes were also expressed in rat retinas (Fig. 1*C*), whereas the *Gh* gene and splice variant-1 of *Ghrh* were not detectable. At day 7 after ON injury, both MR-409 and MIA-602 significantly decreased the expression of the *Ghrh* gene by 2.77-fold (ΔCt: 14.42 ± 1.07; $P < 0.01$) and 86% (ΔCt: 13.85 ± 0.14; $P < 0.001$), respectively, compared to the vehicle-treated control (ΔCt: 12.95 ± 0.21). MIA-602 also reduced *Ghrhr* expression by 48% (ΔCt: 12.90 ± 0.43; $P < 0.05$) as compared to the vehicle-treated control (ΔCt: 12.34 ± 0.09). In contrast, the expression of *Creb1* did not show statistical differences among different treatment groups after ON injury. These results suggest that GHRH-R is expressed in the RGCs of rat retinas, which responded to treatment with GHRH agonist and antagonist in acute-phase protein production.

Subcutaneous Injection of GHRH Agonist and Antagonist Promote the Survival of RGCs after ON Injury. Two weeks after the ON injury, the number of surviving RGCs decreased as compared to the untreated rats (Fig. 2 *A* and *B*). The average numbers of surviving RGCs in the untreated rats and the vehicle-treated rats after ON injury were 1,507.0 ± 243.1 and 217.7 ± 38.9 cells/mm^2, respectively (Fig. 2*E*). In rats treated with subcutaneous injection of MR-409, the number of surviving RGCs increased significantly by 3.19-fold to 694.9 ± 52.4 cells/mm^2 ($P < 0.001$) (Fig. 2*C*), compared to the vehicle-treated group. Similarly, the number of surviving RGCs was also increased in rats treated with subcutaneous injection of MIA-602 by 2.61-fold to 568.3 ± 69.8 cells/mm^2 ($P < 0.001$) (Fig. 2*D*). To further evaluate the direct effect of GHRH agonist and antagonist on RGC survival, MR-409 or MIA-602 was intravitreally injected into the rats after ON injury. However, we found that the number of RGCs showed no significant differences in rats treated with MR-409 (228.6 ± 23.7 cells/mm^2) or MIA-602 (222.5 ± 38.5 cells/mm^2) after ON injury as compared to the vehicle-treated rats (210.6 ± 28.9 cells/mm^2, $P > 0.05$) (Fig. 2*F*). Additionally, treatment of GHRH agonist or antagonist showed no significant differences in the numbers of RGCs in the retinal explant culture (*SI Appendix*, Fig. S1). Our results indicate that only subcutaneous injection of GHRH agonist MR-409 and antagonist MIA-602 could promote the survival of RGCs after ON injury.

GHRH Agonist and Antagonist Regulate GH, Inflammation, PI3K/Akt, and MAPK/ERK Pathways in Retina after ON Injury. To delineate the underlying mechanisms of the effects of GHRH agonist and antagonist on RGCs after ON injury, we evaluated the regulation of GH concentration, inflammatory responses, and the GHRH-R–related downstream signaling pathways involving Akt, Erk1/2, and Stat3 after ON injury. In ON-injured rats with MR-409 treatment, GH concentration in the vitreous humor increased significantly by 51% on day 1 at 1.29 ± 0.03 pg/μg total protein as compared to the vehicle-treated rats at 0.86 ± 0.15 pg/μg total protein ($P < 0.01$) (Fig. 3*A*), and by 24% on day 3 at 1.52 ± 0.18 pg/μg total protein as compared to the vehicle-treated rats at 1.22 ± 0.16 pg/μg total protein ($P < 0.05$) (Fig. 3*B*). In contrast, the GH concentration was decreased in rats treated with MIA-602 by 49% on day 1 (0.44 ± 0.18 pg/μg total protein, $P < 0.01$) and 56% on day 3 (0.54 ± 0.19 pg/μg total protein, $P < 0.01$). Our results indicate that GHRH agonist MR-409 can regulate the secretion of GH in the retina after ON injury.

For inflammatory responses, expressions of the *Il1b*, *Il6*, and *Tnf* genes in the retinas of MIA-602–treated rats were significantly reduced by 2.05-fold (ΔCt: 15.24 ± 0.56; $P < 0.01$), 2.32-fold (ΔCt: 17.19 ± 0.67; $P < 0.01$), and 3.66-fold (ΔCt: 16.19 ± 0.14; $P < 0.01$), respectively, compared to that of the vehicle-treated controls (*Il1b* ΔCt: 14.20 ± 0.33; *Il6* ΔCt: 15.97 ± 0.37; *Tnf* ΔCt: 14.31 ± 0.78) (Fig. 4 *A*–*C*). In contrast, the expressions of *Icam1* and *Sele* genes were significantly up-regulated in the retinas of rats treated with MIA-602 by 3.02-fold (ΔCt: 10.98 ± 0.46; $P < 0.01$) and 47% (ΔCt: 14.68 ± 0.33; $P < 0.05$), respectively, as compared to the vehicle-treated controls (*Icam1* ΔCt: 12.58 ± 0.63; *Sele* ΔCt: 15.24 ± 0.19) (Fig. 4 *D* and *E*). There was no significant change in the inflammation-related gene expression in the retinas of rats treated with MR-409. To investigate macrophage activation in the protection of RGCs by the GHRH agonist and antagonist, we examined the ED1$^+$ infiltrating macrophages in the wholemount retina. In the vehicle-treated control group, the density of infiltrating macrophages was 61.4 ± 20.4 cells/mm^2 after ON injury (Fig. 4 *F* and *I*). There were no statistically significant differences in the densities of infiltrating macrophages in the treatment groups for both MR-409 (81.6 ± 9.3 cells/mm^2, $P > 0.05$) (Fig. 4*G*) and MIA-602 (66.0 ± 10.9 cells/mm^2, $P > 0.05$) (Fig. 4*H*). To evaluate the involvement of microglia activation in the protection of RGCs by the GHRH agonist and antagonist, we examined the Iba-1$^+$ microglia in the retinal sections. The number of Iba-1$^+$ microglia in the retina of rats treated with MR-409 (16.3 ± 1.8 cells per section) (Fig. 4 *K*, *N*, and *P*) is significantly higher than that in the vehicle-treated group (9.0 ± 1.4 cells per section, $P < 0.001$) (Fig. 4 *J* and *M*). However, the number of microglia in the retina of rats treated MIA-602 (10.8 ± 0.8 cells per section, $P > 0.05$) (Fig. 4 *L* and *O*) did not show significant difference, compared to the vehicle-treated group. Our results reveal the antiinflammatory effect of MIA-602 and the microglia activation effect of MR-409 on the rat retina after ON injury, but neither of them promoted infiltration of macrophages into the retina.

For the GHRH-R–related signaling pathways, immunoblotting analysis showed a significantly higher ratio of phospho-Akt/Akt in the retinas of rats treated with MR-409 (day 1: 0.795 ± 0.124, $P < 0.05$; day 3: 0.611 ± 0.198, $P < 0.05$) compared with the vehicle-treated control (day 1: 0.619 ± 0.014; day 3: 0.213 ± 0.134) on days 1 and 3 after ON injury, respectively (Fig. 5*A*). At day 1 after ON injury, the phospho-Erk1/Erk1 ratio was also significantly higher in the retinas of rats treated with MR-409 (0.678 ± 0.237, $P < 0.05$) than that in the vehicle-treated control (0.383 ± 0.013) (Fig. 5*B*).

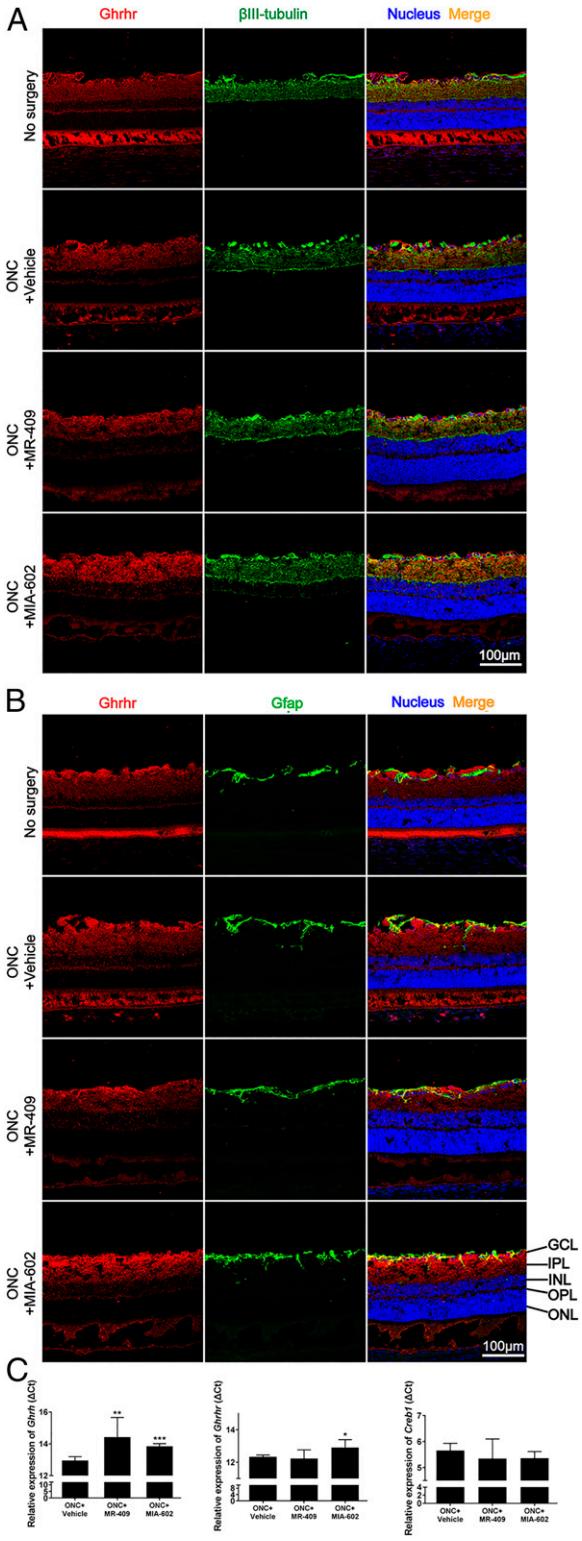

Fig. 1. Expression of GHRH-R and its related signaling molecules in retina. The GHRH-R and related signaling molecules were expressed in adult rat retina. Immunofluorescence analysis of GHRH-R (red) in rat retina and its colocalization with (*A*) the RGC marker (βIII tubulin; green) and (*B*) the astrocyte marker (Gfap; green). (Scale bar, 100 μm.) (*C*) Sybr green PCR analysis of GHRH (*Ghrh*), GHRH-R (*Ghrhr*) and the *Creb1* genes in rat retina. At day 7 after ON injury, the *Ghrh* gene expression was significantly decreased in groups treated with MR-409 and MIA-602, whereas the *Ghrhr* expression was significantly reduced in the MIA-602 treatment group, compared to the control group. Data are presented as mean ± SD; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $n = 5$ in each group.

In contrast, on day 3 after ON injury, only the phospho-Akt/Akt (0.676 ± 0.310, $P < 0.05$) ratio was increased in the retinas of rats treated with MIA-602. However, there was no change in the phospho-Erk2/Erk2 and phospho-Stat3/Stat3 ratios in the retinas of rats treated with MR-409 or MIA-602 compared to the control (Fig. 5 *B* and *C*). Our results suggest that GHRH agonist MR-409 and antagonist MIA-602 activate the PI3K/Akt pathway in the retina after ON injury.

GHRH Agonist Enhances the Effect of Zymosan-Induced Protection of RGCs. Since GHRH agonists and antagonists are involved in the regulation of the inflammatory responses (20), we postulate that they also regulate the protective effect mediated by macrophage activation on the survival of RGCs. In our investigation, intravitreal injection of zymosan drastically increased the number of infiltrating macrophages by 53.3-fold to 3,272.4 ± 668.2 cells/mm^2 ($P < 0.001$) (Fig. 6 *B* and *E*) compared to the control group (61.4 ± 20.4 cells/mm^2) (Fig. 6*A*), indicating induction of macrophage infiltration into the retina by zymosan. However, no significant change in the number of infiltrating macrophages was found after combined treatment of zymosan with agonist MR-409 (2,436.7 ± 664.4 cells/mm^2, $P > 0.05$) (Fig. 6*C*) or with antagonist MIA-602 (2,815.0 ± 1,166.1 cells/mm^2, $P > 0.05$) (Fig. 6*D*). Coherently, the expressions of the *Il1b*, *Il6*, *Tnf*, and *Icam1* genes in the retinas of the rats treated with zymosan as well as in combination with agonist MR-409 or antagonist MIA-602 were significantly up-regulated as compared to those of the vehicle-treated controls (Fig. 6 *F*–*J*). However, there was no statistically significant difference in the inflammation-related gene expressions in the groups with combined treatment of zymosan with MR-409 or MIA-602 as compared to the group with zymosan treatment only.

Consistently with our previous study (23), intravitreal injection of zymosan significantly promoted the survival of RGCs after ON injury by 2.84-fold (619.2 ± 78.2 cells/mm^2, $P < 0.001$) (Fig. 6 *L* and *O*), compared to the vehicle control group (217.7 ± 38.9 cells/mm^2) (Fig. 6*K*). Compared to treatment by zymosan only, combined administration of zymosan and MR-409 further increased the number of surviving RGCs by 38% (854.4 ± 91.7 cells/mm^2, $P < 0.001$) (Fig. 6*M*). In contrast, combined treatment of zymosan and MIA-602 significantly reduced the number of surviving RGCs by 24% (500.6 ± 68.0 cells/mm^2, $P < 0.05$) (Fig. 6*N*). Thus, agonist MR-409 enhanced the protective effects of zymosan on the survival of RGCs after ON injury. However, both agonist MR 409 and antagonist MIA 602 did not influence the macrophage infiltration induced by zymosan.

GHRH Agonist Enhances the Effect of Lens Injury-Induced Protection of RGCs. In addition to zymosan, lens injury can also induce intraocular inflammation. We found that lens injury significantly increased the number of infiltrating macrophages in the retina by 16.28-fold (902.0 ± 345.4 cells/mm^2, $P < 0.01$) (Fig. 7 *B* and *E*) compared to the control group (61.4 ± 20.4 cells/mm^2) (Fig. 7*A*). Combined treatment of lens injury with MR-409 (736.2 ± 492.7 cells/mm^2, $P > 0.05$) (Fig. 7*C*) or MIA-602 (728.5 ± 256.2 cells/mm^2, $P > 0.05$) (Fig. 7*D*) did not significantly affect the number of infiltrating macrophages in the retina. Meanwhile, expressions of the *Il1b*, *Il6*, *Icam1*, and *Sele* genes in the retinas of rats with lens injury, as well as treated in combination with agonist MR-409 or with antagonist MIA-602, were significantly up-regulated as compared to that of the vehicle-treated controls (Fig. 7 *F*–*J*). There was no statistically significant difference in the inflammation-related gene expressions in the groups with combined treatment of lens injury with MR-409 or MIA-602 as compared to the group with lens injury only.

In accord with a previous study (24), we found that lens injury significantly increased the survival of RGCs after ON injury by 3.40-fold (740.3 ± 66.3 cells/mm^2, $P < 0.001$) (Fig. 7 *L* and *O*), compared to the vehicle control group (217.7 ± 38.9 cells/mm^2)

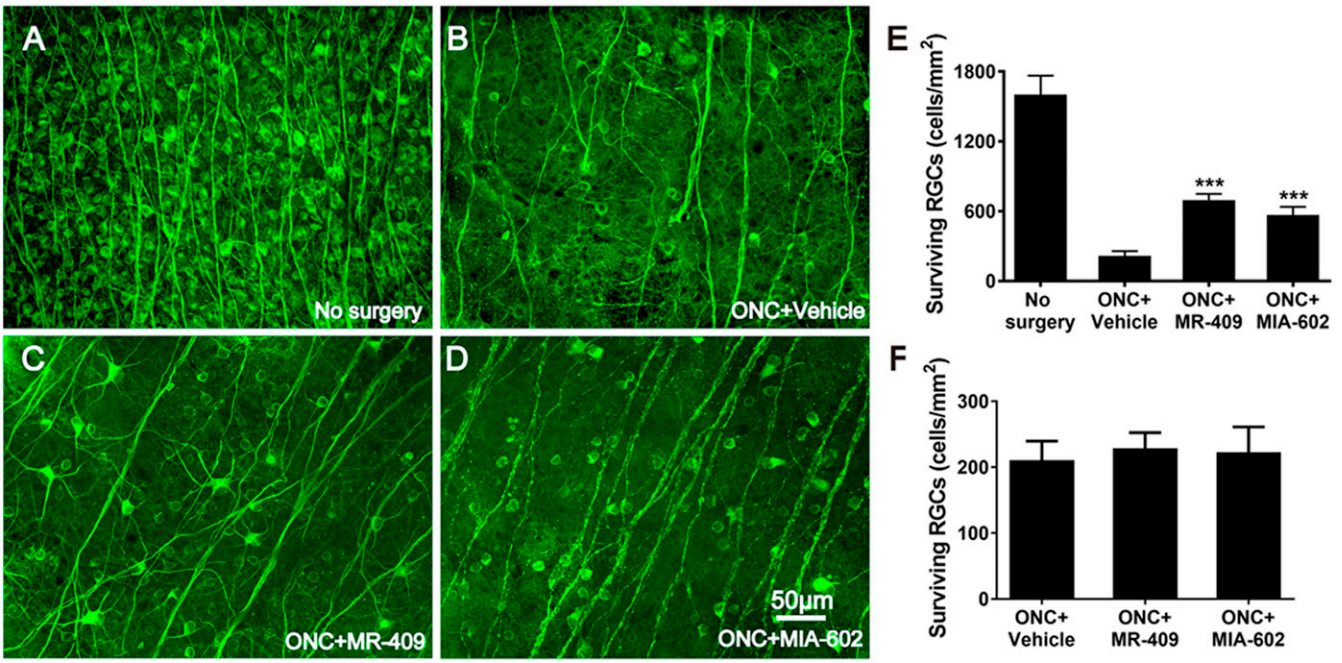

Fig. 2. Subcutaneous injection of GHRH agonist and antagonist promote the survival of RGCs after ON crush (ONC). Surviving RGCs were determined by immunofluorescence analysis in (*A*) untreated rats without ONC and in rats at 2 wk after ONC and subcutaneous injection of (*B*) vehicle, (*C*) MR-409 (GHRH agonist), and (*D*) MIA-602 (GHRH antagonist). (Scale bar, 50 μm.) (*E*) The quantitative analysis of surviving RGCs in different treatment groups. At 2 wk after ON injury, the number of surviving RGCs was significantly increased in MR-409– or MIA-602–treated rats, compared to the vehicle-treated group. (*F*) The quantitative analysis of surviving RGCs at 2 wk after ONC with intravitreal injection of vehicle, MR-409 and MIA-602 in rats. Data are presented as mean ± SD; ***$P < 0.001$. $n = 5$ in each group.

(Fig. 7*K*). Similar to treatment by zymosan, lens injury combined with MR-409 further enhanced the number of surviving RGCs by 24% (921.5 ± 87.4 cells/mm^2, $P < 0.001$) (Fig. 7*M*), compared to lens injury alone. However, lens injury combined with MIA-602 significantly reduced the number of surviving RGCs by 34% (552.4 ± 72.3 cells/mm^2, $P < 0.001$) (Fig. 7*N*). Our results indicate the enhancement of the protective effects of lens injury by agonist MR-409 on the survival of RGCs after ON injury, but macrophage infiltration was not affected.

Discussion

Results of this study show that: 1) both GHRH agonist MR-409 and GHRH antagonist MIA-602 promote the survival of RGCs after ON injury; 2) MR-409 enhances the protective effects of zymosan and lens injury on RGC survival after ON injury; 3) MIA-602 attenuates the promoting effects of zymosan and lens injury on RGC survival after ON injury; 4) both MR-409 and MIA-602 activate the PI3K/Akt pathway in the retina after ON injury; 5) MIA-602 reduces the GH level and the inflammation-related gene expressions in the retina after ON injury; and 6) MR-409 increases the GH level and promotes microglia activation in the retina after ON injury. Collectively, these data suggest the involvement of GHRH-R signaling in the inflammation-induced protection of RGCs in this study on rats (*SI Appendix*, Fig. S2).

GHRH is secreted from the hypothalamus (25) and belongs to a family of neuropeptides that includes vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), secretin, glucagon, glucagon-like peptide (GLP)-1 and GLP-2, and gastric inhibitory peptide. PACAP suppresses the death of RGCs

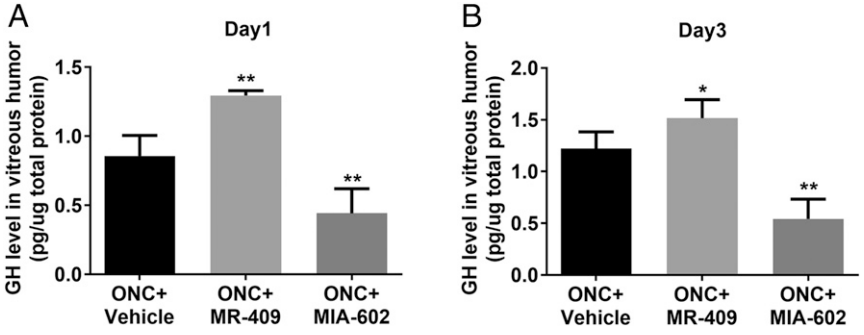

Fig. 3. GH level in the vitreous humor of the ON-injured rats after treatment with GHRH agonist or antagonist. The GH level in vitreous humor of GHRH agonist MR-409–, antagonist MIA-602–, and vehicle-treated rats was determined by ELISA at days (*A*) 1 and (*B*) 3 after ONC. MR-409 significantly increased the GH level, whereas MIA-602 significantly reduced the GH level as compared to the vehicle-treated control rats. Data are presented as mean ± SD; *$P < 0.05$; **$P < 0.01$. $n = 5$ in each group.

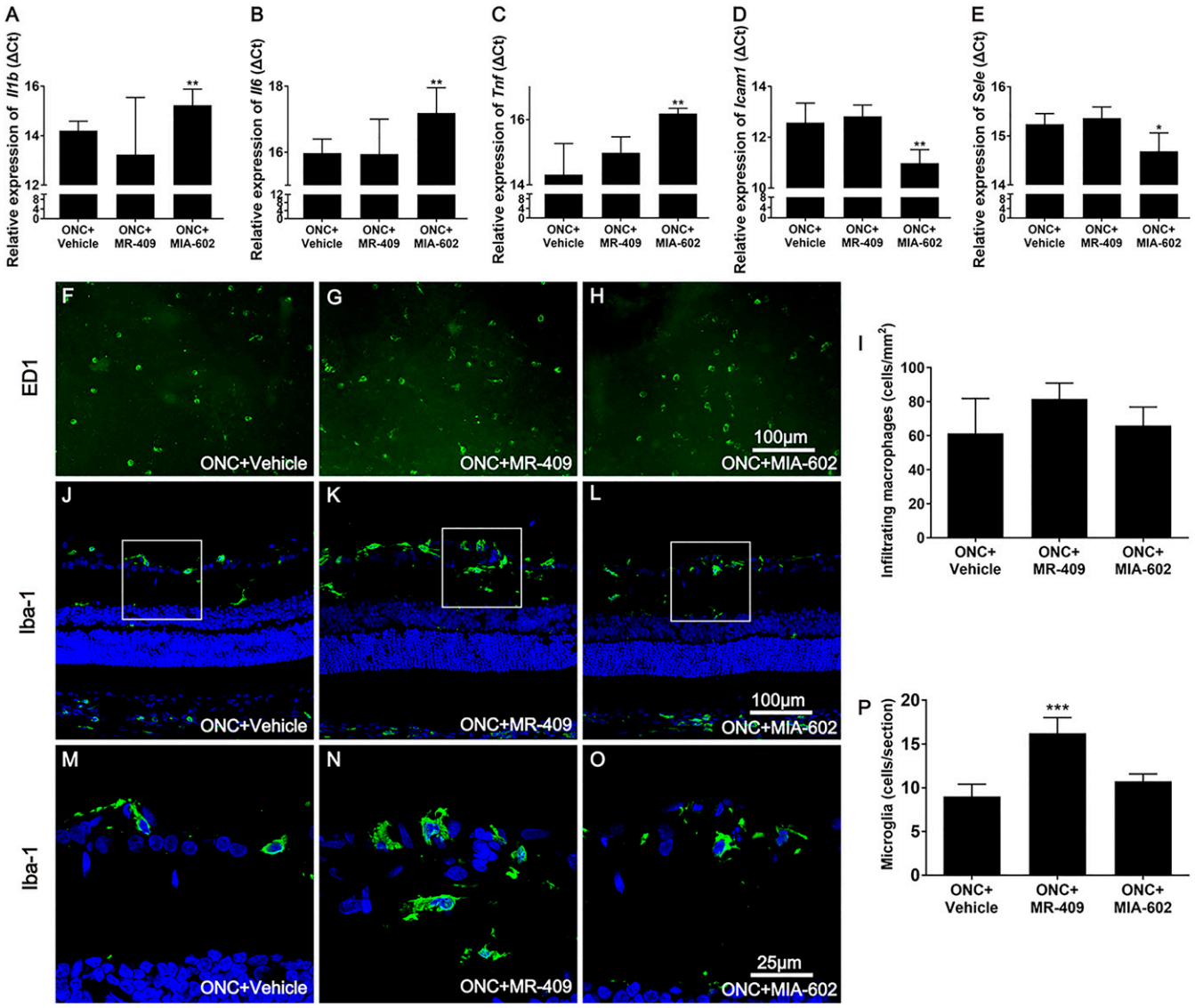

Fig. 4. Inflammation-related gene expression, macrophage infiltration, and microglia activation in the ON-injured retina after treatment with GHRH agonist and antagonist. (*A–E*) Sybr green PCR analysis of inflammation-related genes (*A*) *Il1b*, (*B*) *Il6*, (*C*) *Tnf*, (*D*) *Icam1*, and (*E*) *Sele* in agonist MR-409–, antagonist MIA-602–, and vehicle-treated control rat retinas at day 7 after ONC. (*F–I*) The immunofluorescence analysis of ED1$^+$ infiltrating macrophages into the retinas of the vehicle-treated control, MR-409, and MIA-602 treatment groups after ONC. (Scale bar, 100 μm.) (*J–P*) The immunofluorescence analysis of Iba-1$^+$ microglia in the retinas of the vehicle-treated control, MR-409, and MIA-602 treatment groups after ONC. (Scale bar, 100 μm.) (*M–O*) Magnified images of Iba-1$^+$ microglia. (Scale bar, 25 μm.) Data are presented as mean ± SD; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $n = 5$ in each group.

induced by ON transection in rats (26), while intravitreal injection of VIP protects RGCs from degeneration in the bilateral common carotid artery occlusion-induced ischemic model (27). In addition, implantation of GLP-1 engineered cells promotes the survival of RGCs after ON crush in rats (28). In embryonic chick retina and quail neuroretinal cells, GHRH regulates the production and release of GH from RGCs (29), which in turn enhances the RGC survival (30). In this study, we found that subcutaneous application of GHRH agonist MR-409 increased the GH level in vitreous humor of the rats, whereas antagonist MIA-602 caused its reduction (Fig. 3). Critically, subcutaneous application of both GHRH agonist MR-409 and antagonist MIA-602 promoted the survival of RGCs after ON injury in rats (Fig. 2 *A–D*). However, the RGC protective effect was not observed in the intraocular application of MR-409 or MIA-602 (Fig. 2*E*). In the rat retina, there was colocalization of GHRH-R with RGCs and astrocytes (Fig. 1 *A* and *B*). GHRH-R could mediate the protective effect of GHRH agonists and antagonists on RGC survival after ON injury. Further investigations are required to delineate possible differential mechanisms of GHRH agonist and antagonist for protection of RGCs after ON injury. Whether GHRH agonist and antagonist also regulate RGC axonal regeneration after ON injury could also be investigated.

GHRH-R belongs to the class-II G protein–coupled receptor with seven transmembrane domains (31). Activation of GHRH-R stimulates intracellular accumulation of cAMP and activates protein kinase A (32, 33). cAMP reportedly promotes RGC survival and axonal regeneration through the PI3K/Akt and MAPK/Erk pathways (34, 35), and GHRH agonists regulate the PI3K/Akt and MAPK/Erk pathways (22). In the ON injury model, the protective effect of GHRH agonist on RGCs could be regulated by the cAMP-mediated PI3K/Akt and MAPK/Erk pathways. The results of this study show that both GHRH agonist and antagonist promote the survival of RGCs after ON injury in rats (Fig. 2),

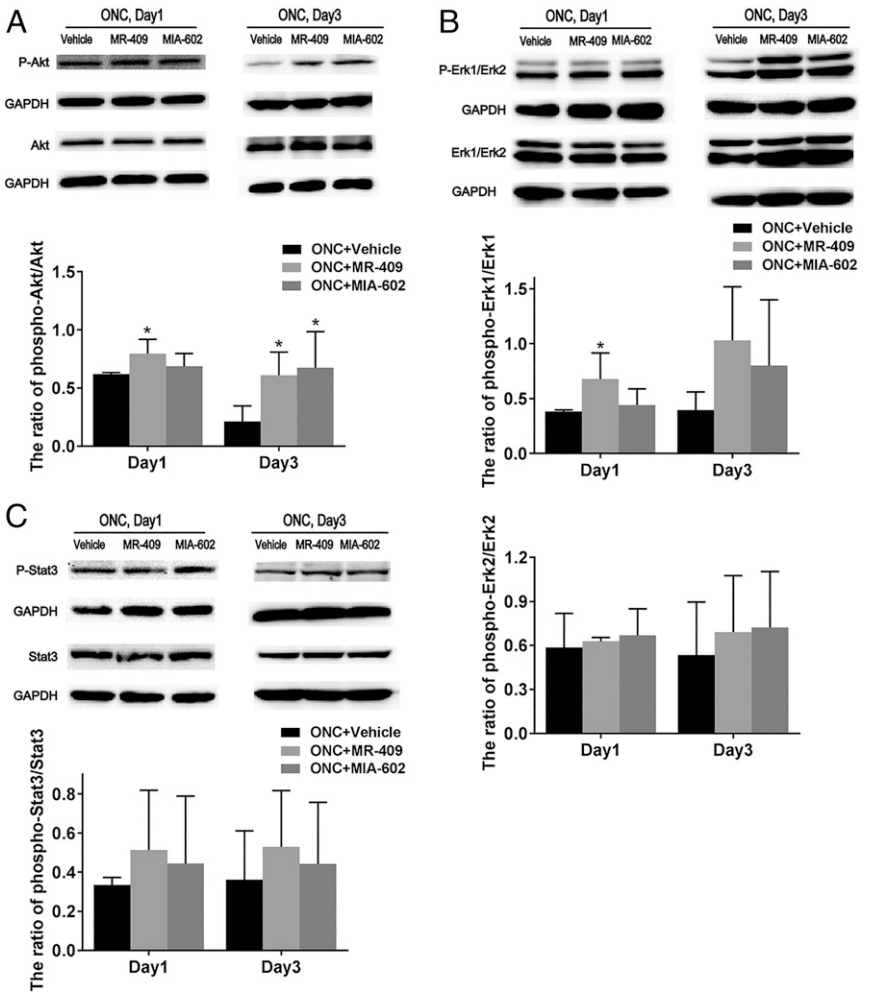

Fig. 5. Activation of Akt, Erk, and Stat3 pathways in the ON-injured retina after treatment with GHRH agonist and antagonist. The immunoblotting analysis of the GHRH-related signaling pathways in the retinas of MR-409– or MIA-602–treated rats after ONC: (*A*) the ratio of phospho-Akt/Akt, (*B*) the ratio of phospho-Erk1/Erk1 and phospho-Erk2/Erk2, and (*C*) the ratio of phospho-Stat3/Stat3. The GHRH agonist activated the Akt and Erk pathways, whereas the GHRH antagonist only activated the Akt pathway. The Stat3 pathway was not affected. Data are presented as mean ± SD; $*P < 0.05$. $n = 5$ in each group.

likely through activation of the Akt and Erk pathways by the agonist and the Akt pathway by the antagonist (Fig. 5 *A* and *B*). Both the agonists and the antagonists are endowed with antioxidative and antiinflammatory actions (20, 36, 37), but only the agonists stimulate the proliferation of various cells and tissues (37). Since the activation of Akt and Erk pathways is associated with the survival of RGCs (38), this could also be related to the promotion of RGC survival by GHRH agonist and antagonist. Furthermore, the promotion of RGC survival by GHRH agonist could also be associated with the microglia activation since only the agonist promotes the microglia activation in the retina after ON injury (Fig. 4*J*). Our observation is consistent with the capability of activated microglia protecting the brain from traumatic injury and other central nervous system diseases (39). How microglial activation by GHRH agonists is related to the protection of RGCs warrants further investigation. Notably, in contrast to our previous study on human gastric cancer (40), GHRH agonist and antagonist did not affect the Stat3 pathway in RGCs after ON injury (Fig. 5*C*).

Mild ocular inflammation promotes RGC survival and axonal regeneration (15, 41). In this study, we confirmed the induction of macrophage infiltration by zymosan and the promotion of RGC survival by lens injury after ON crush (Figs. 6*B* and 7*B*). GHRH agonist MR-409 provided additional protection of RGCs and further enhanced the protective effect on RGCs induced by zymosan and lens injury (Figs. 6*M* and 7*M*). In contrast, the GHRH antagonist MIA-602 attenuated the protective effect of zymosan and lens injury on RGCs (Figs. 6*N* and 7*N*). The GHRH agonist and antagonist showed differential effects in the presence of macrophage infiltration. The GHRH-R signaling pathway, in the presence of macrophage infiltration, could regulate the ocular inflammatory response for RGC protection after ON injury. In accord with this finding, our previous study also demonstrated that antagonist MIA-602 alleviated the endotoxin-induced intraocular inflammation by attenuating macrophage and leukocyte infiltration into anterior chamber and inhibiting inflammatory cytokine secretion (20). GHRH antagonists also inhibited inflammation in mice with autoimmune prostatitis (37). Collectively, these results indicate that the GHRH-R signaling pathway is involved in the inflammation-induced protection of RGCs after ON injury. Further investigations should clarify how the GHRH agonist and antagonist show different regulatory responses in the presence of infiltrating macrophages and how these analogs interact with the infiltrating macrophages after ON injury for protection of RGCs. In addition, whether infiltrating neutrophils would also be regulated by the GHRH agonist and

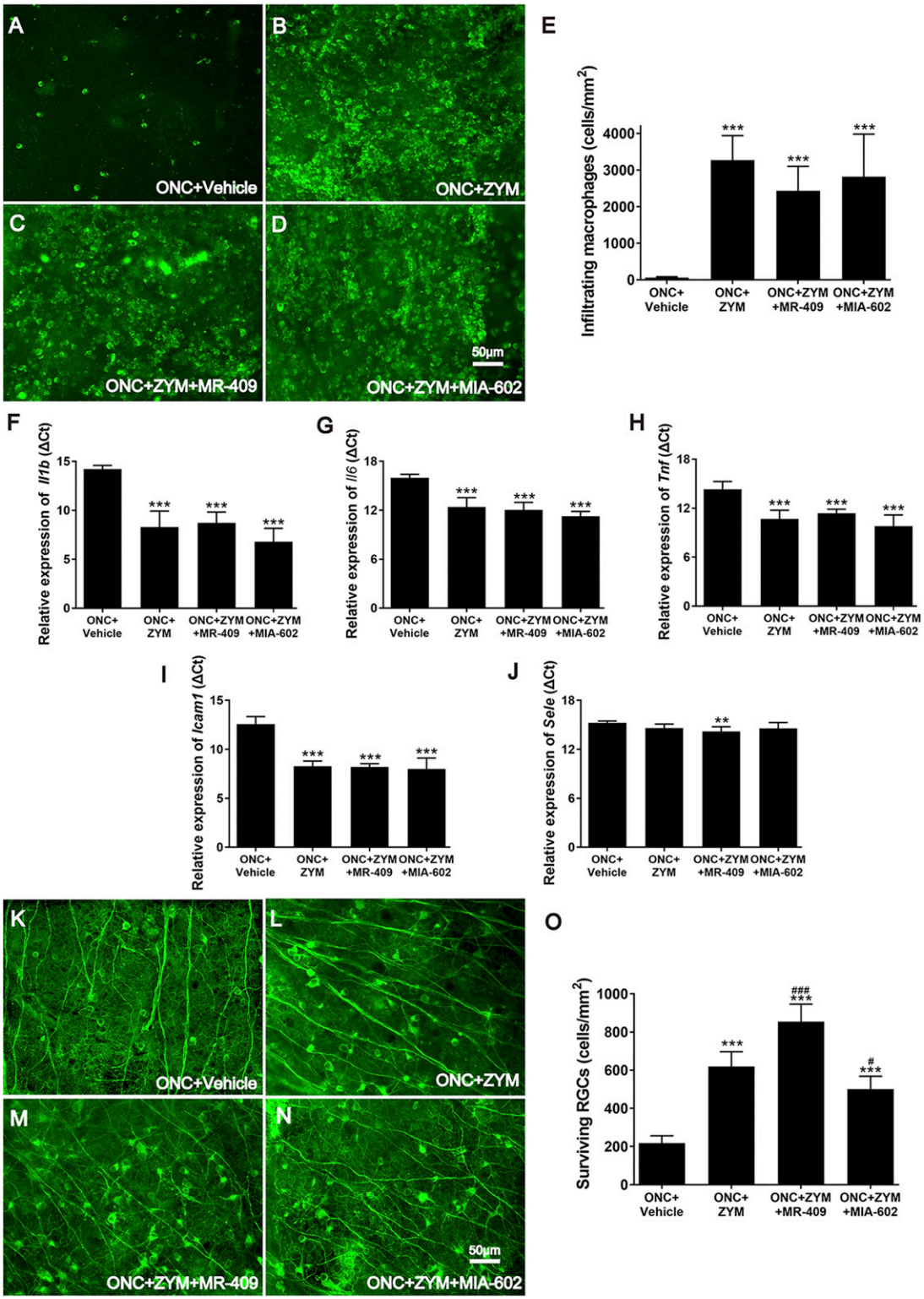

Fig. 6. Enhancements of GHRH agonist on zymosan-induced protection of RGCs. (*A–E*) Macrophage infiltration after ONC, zymosan (ZYM) injection, and the treatment with GHRH agonist or antagonist was evaluated by the immunofluorescence analysis of ED1$^+$ infiltrating macrophages in the retinas of the (*A*) vehicle-treated control (same as Fig. 7*A*), (*B*) zymosan only, (*C*) zymosan and MR-409, and (*D*) zymosan and MIA-602 treatment. (*E*) The ED1$^+$ infiltrating macrophage count in different treatment groups. (*F–J*) Sybr green PCR analysis of inflammation-related genes (*F*) *Il1b*, (*G*) *Il6*, (*H*) *Tnf*, (*I*) *Icam1*, and (*J*) *Sele* in agonist MR-409–, antagonist MIA-602–, and vehicle-treated control rat retinas at day 7 after ONC. (*K–O*) Immunofluorescence analysis of surviving RGC at 2 wk after the ON injury in (*K*) the vehicle-treated control (same as Fig. 7*F*), (*L*) zymosan only, (*M*) zymosan and MR-409, and (*N*) zymosan and MIA-602 treatment. (*O*) The surviving RGC count in different treatment groups. (Scale bars, 50 μm.) Data are presented as mean ± SD; $^{**}P < 0.01$ and $^{***}P < 0.001$ (compared to the control group), $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ (compared to the zymosan only group). $n = 5$ in each group.

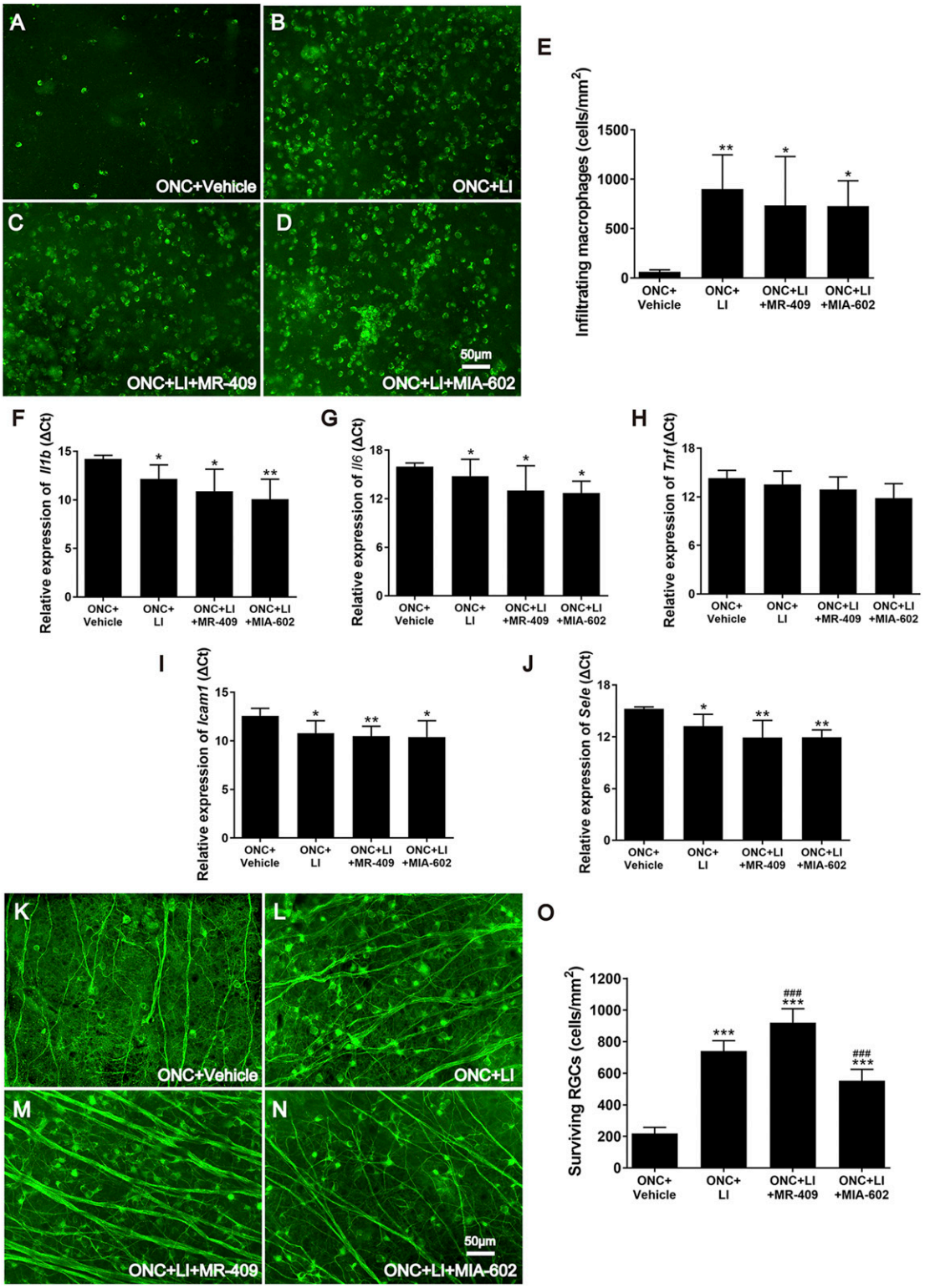

Fig. 7. GHRH agonist enhances the effect of lens injury-induced protection of RGCs. (*A–E*) Macrophage infiltration after ONC, lens injury (LI), and the treatment with GHRH agonist or antagonist was evaluated by the immunofluorescence analysis of ED1$^+$ infiltrating macrophages in the retinas of the (*A*) vehicle-treated control (same as Fig. 6*A*), (*B*) lens injury only, (*C*) lens injury and MR-409, and (*D*) lens injury and MIA-602 treatment. (*E*) The ED1$^+$ infiltrating macrophage count in different treatment groups. (*F–J*) Sybr green PCR analysis of inflammation-related genes (*F*) *Il1b*, (*G*) *Il6*, (*H*) *Tnf*, (*I*) *Icam1*, and (*J*) *Sele* in agonist MR-409–, antagonist MIA-602–, and vehicle-treated control rat retinas at day 7 after ONC. (*K–O*) Immunofluorescence analysis of surviving RGC at 2 wk after the ON injury in (*K*) the vehicle-treated control (same as Fig. 6*F*), (*L*) lens injury only, (*M*) lens injury and MR-409, and (*N*) lens injury and MIA-602 treatment. (*O*) The surviving RGC count in different treatment groups. (Scale bars, 50 μm.) Data are presented as mean ± SD; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (compared to the control group), $^{###}P < 0.001$ (compared to the lens injury only group). $n = 5$ in each group.

antagonist in the inflammation-induced protection of RGCs after ON injury will be investigated in future studies.

In summary, this study reveals that subcutaneous application of GHRH agonist promotes the survival of RGCs after ON injury and enhances the protection of RGCs induced by macrophage activation. The GHRH-R signaling pathway is involved in the inflammation-mediated protection of RGCs in optic neuropathies, and GHRH agonist MR-409 possesses therapeutic potential for the protection of RGCs against optic neuropathies, especially glaucoma, which is the leading cause of irreversible blindness worldwide.

Materials and Methods

Animals. Approval of the Animal Experimentation Ethics Committee of Shantou University Medical College has been obtained. All experiments were conducted according to the guidelines of the Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic and Vision Research. Young adult (8- to 10-wk-old) male Fischer rats were kept under standard conditions with a 12-h light/dark cycle. Surgeries were performed under anesthesia using a mixture (1.5 mL/kg) of ketamine (100 mg/mL) and xylazine (20 mg/mL). Five rats were used in each treatment group.

Peptides and Chemicals. The GHRH agonist MR-409 and antagonist MIA-602 were synthesized in the laboratory of one of us (A.V.S.) at the Miami Veterans Affairs Medical Center. Each peptide was dissolved in DMSO (ACS grade; Sigma-Aldrich) and then diluted (1:500) in 5.5% mannitol. The DMSO concentration never exceeded 0.2%. After ON crush, lens injury, and intravitreal injection of zymosan, 0.2 mL of MR-409 (60 μg per rat) or MIA-602 (20 μg per rat) was injected subcutaneously every 2 d. The control animals were injected subcutaneously with 0.2 mL sterile water with 5.5% mannitol and 0.2% DMSO. For intraocular application, 5 μL of MR-409 (0.15 μg/μL) or MIA-602 (0.05 μg/μL) was injected intravitreally at days 0 and 7 after ON crush.

Immunofluorescence Analysis of GHRH-R. After perfusion of the rats with 4% paraformaldehyde (Sigma-Aldrich), the eyes were enucleated, postfixed overnight at 4 °C, cryoprotected with 10 to 30% sucrose gradient in phosphate buffered saline (PBS), and embedded in a cryoblock. The cryosections of the eyeballs (5-μm thick) were blocked and permeated with 10% normal goat serum (NGS; Biodesign) and 0.2% Triton X-100 (Sigma-Aldrich) for 1 h. They were then colabeled with rabbit anti-GHRH receptor antibody (1:1,000; Abcam) and mouse anti-βIII tubulin (1:1,000; Abcam) or Gfap (1:1,000; Abcam) antibodies for 2 h at room temperature, followed by incubation with the respective secondary antibodies for 1 h at room temperature. The stained sections were mounted (Vector Labs) and imaged under a confocal microscope (Leica TCS SP5 II, Leica).

ON Crush, Intravitreal Injection, and Lens Injury. The ON was crushed under anesthesia through a 1.0- to 1.2-cm incision in the upper conjunctiva of the left eye. The ON was uncovered by separating the extraocular muscles. The dura was opened longitudinally with fine scissors. Using an angled jeweler's forceps (Dumont #5; Roboz), the ON was crushed at 1.5 mm behind the ON head for 5 s. Damage to the ophthalmic artery beneath the ON was avoided. Effective ON injury was confirmed by the presence of a clear gap across the entire ON at the crush site. Zymosan, a yeast cell wall suspension for macrophage activation (Sigma-Aldrich), was sterilized at 90 °C for 10 min prior to intravitreal injection (3 μL at 6.25 mg/mL in saline) behind the corneoscleral limbus of the eyeball using a 10-μL Hamilton syringe (Hamilton) immediately after ON crush (14, 15). Lens damaging was avoided. To induce lens injury, the tip of a needle (30 G) was bent at an angle of 90° and inserted behind the corneoscleral limbus of the eyeball, perpendicular to the sclera, intentionally puncturing the lens surface. Lens injury was verified by direct observation through the cornea and confirmed by the occurrence of opacification within 1 wk.

All the rats in each treatment group were maintained on the treatments with GHRH agonist and antagonist for 14 d after ON crush before further analyses.

RGC and Infiltrating Macrophage Analyses after ON Injury. At 2 wk after ON crush, when RGC death is stable, the rats were killed with an overdose of ketamine and xylazine and perfused with 4% paraformaldehyde. The eyes were enucleated and postfixed overnight at 4 °C. The retinas were dissected and stained to visualize the surviving RGCs and macrophages. Briefly, after fixation, the retina was blocked in phosphate buffered saline (PBS) supplemented with 10% NGS (Biodesign) and 0.2% Triton X-100 for 1 h. The retina was then separated into two parts: one-half for RGC labeling with the anti-βIII tubulin antibody (1:400) and the other half for the infiltrating macrophages with the ED1 antibody (1:400, Serotec) overnight at 4 °C, followed by the incubation of the secondary antibodies conjugated with FITC (1:400; Sigma-Aldrich) or Cy3 (1:400; Jackson Laboratory) overnight at 4 °C. The stained retinas were mounted (Vector Labs) and imaged under a confocal microscope (Leica TCS SP5 II). The numbers of viable RGCs and macrophages were counted in each field (0.443 mm × 0.334 mm) of a grid in the microscope. In each half of retina, 25 fields were counted, and the mean density of fluorescent-labeled RGCs and macrophages in each retina calculated.

Microglia Analysis. Seven days after ON crush, the rats were killed with an overdose of ketamine and xylazine and perfused with 4% paraformaldehyde. The eyes were enucleated, postfixed overnight at 4 °C, cryoprotected with 10 to 30% sucrose gradient in PBS, and embedded in a cryoblock. The eyeball slices (10 μm) were sectioned using a cryostat (Leica), permeabilized and blocked with 10% NGS and 0.2% Triton X-100 in PBS for 1 h, and incubated with the anti–Iba-1 antibody (1:400; Abcam) for 2 h at room temperature, followed by incubation with the secondary antibodies conjugated with FITC (1:400) for 1 h at room temperature. The stained sections were mounted and imaged under a confocal microscope (Leica TCS SP5 II). For each sample, five retinal sections were used, and total 10 fields were imaged for each sample.

RGC Survival in Retinal Explant Culture. Retina explants for RGC survival analysis were dissected from intact eyeballs. After mounting onto nitrocellulose filter paper with the RGC layer upmost, without cutting and sticking onto substrates, the retinal explants were cultured in Neurobasal-A medium (Gibco BRL) with B27 supplement (Gibco BRL) at 37 °C in 5% CO_2 and treated with MR-409 (10 μM in 2 mL) or MIA-602 (10 μM in 2 mL) for 7 d in 24-well plate. The retina explants treated with vehicle were served as the negative control.

GH Concentration Analysis. The concentration of GH protein was determined by ELISA. At 1 and 3 d after ON crush, which are the critical times for RGC survival, the vitreous humor and the retina were collected and soaked in saline for 15 min on ice with gentle mixing. The retina was removed and the supernatant collected after centrifugation. Total protein in the supernatant was determined by BCA Protein Assay (Thermo Fisher Scientific). GH level was determined by a commercially available rat GH ELISA kit (Invitrogen) according to the manufacturer's protocol and calculated as the amount of GH (picograms) divided by the amount of total protein (micrograms).

Expression Analysis of Inflammation and GHRH-R–Related Genes. At 7 d after ON crush, when there was prominent macrophage infiltration, the retinas were collected and stored in the TRIzol reagent (Invitrogen) at −80 °C before use. Total RNA was isolated and treated with RNase-free DNase I (Qiagen) according to the manufacturer's protocol. Total RNA (0.5 μg) was reverse-transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). Gene expressions were examined by Sybr green PCR (Roche) in the LightCycle 480 II real-time PCR apparatus (Roche) with specific primers (*SI Appendix*, Table S1) according to the manufacturer's protocol. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method as compared to the negative control group, and presented by the relative expression (ΔCt).

PI3K/Akt, MAPK/ERK, and JAK/STAT3 Pathway Analysis. The activation of PI3K/Akt, MAPK/ERK, and JAK/STAT3 pathways was examined by immunoblotting. At 1 and 3 d after ON crush, which are the critical times for RGC survival, the retinas were homogenized with a lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 1mM EDTA, 150 mM sodium chloride, 1 mM PMSF, 5 mM sodium pyrophosphate, 0.2 mM sodium molybdate, 0.05 mM sodium fluoride, and 1 mM sodium orthovanadate). The protein was extracted, and total protein concentration determined by the Bio-Rad protein assay (Bio-Rad). Equal amount of the denatured total protein (50 μg) was separated on 10% SDS/PAGE gel (Bio-Rad). The separated proteins were then transferred onto the Hybond-C Super membrane (Amersham Pharmacia) and blocked in Tris-buffered saline supplemented with 0.1% Tween-20 (ICN Biochemicals; TBST) and 5% skimmed milk. The membranes were incubated with anti-Akt (1:1,000; Cell Signaling), antiphospho-Akt (1:1,000; Cell Signaling), anti-Erk1/2 (1:1,000; Cell Signaling), antiphospho-Erk1/2 (1:1,000; Promega), anti-Stat3 (1:1,000; Cell Signaling), or antiphospho-Stat3 (1:1,000; Cell Signaling) primary antibodies in TBST supplemented with 5% bovine serum albumin (BSA) overnight at 4 °C, followed by incubation with horseradish peroxidase (HRP)–conjugated antibodies (Babco; 1:20,000) for 1 h at room temperature before the enhanced chemiluminescence (ECL) signal detection (Amersham Pharmacia) by the ChemiDoc XRS$^+$ system (Bio-Rad). The relative expression levels of Akt, phospho-Akt, Erk1/2, phospho-Erk1/2, Stat3 and phospho-Stat3 were normalized with the expression of Gapdh (1:1,000; Sigma-Aldrich).

Statistical Analysis. Means of data from five rats ± SD were obtained. One-way ANOVA with post hoc Tukey test (for multiple testing correction) was used to compare the means among different treatment groups. All statistical analyses were performed using commercially available software (IBM SPSS Statistics 21; SPSS Inc). Significance was defined as $P < 0.05$.

Data Availability. All study data are included in the article and *SI Appendix*.

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