

# Role of Sigma 1 Receptor in Retinal Degeneration of the *Ins2<sup>Akita/+</sup>* Murine Model of Diabetic Retinopathy

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**PURPOSE.** Sigma receptor 1 (Sigma1R), a nonopioid putative molecular chaperone, has neuroprotective properties in retina. This study sought to determine whether delaying administration of (+)-pentazocine, a high-affinity Sigma1R ligand after onset of diabetes in *Ins2<sup>Akita/+</sup>* diabetic mice would afford retinal neuroprotection and to determine consequences on retinal phenotype in *Ins2<sup>Akita/+</sup>* diabetic mice in the absence of Sigma1R.

**METHODS.** *Ins2<sup>Akita/+</sup>* diabetic and WT mice received intraperitoneal injections of (+)-pentazocine beginning 4 or 8 weeks after onset of diabetes; eyes were harvested at 25 weeks. Retinal histologic sections were analyzed to determine thicknesses of retinal layers, number of ganglion cells, and evidence of gliosis (increased glial fibrillary acidic protein levels). *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice were generated and subjected to in vivo assessment of retinal architecture (optical coherence tomography [OCT]) and retinal vasculature using fluorescein angiography (FA) at 12 and 16 weeks compared with age-matched *Ins2<sup>Akita/+</sup>* mice. Eyes were then harvested for retinal morphometric assessment and gliosis assessment.

**RESULTS.** Wild-type mice had  $13 \pm 0.06$  cells/100  $\mu\text{m}$  retinal length; cell bodies in *Ins2<sup>Akita/+</sup>* mice injected 4 and 8 weeks after onset of diabetes with (+)-pentazocine retained significantly more ganglion cells compared with *Ins2<sup>Akita/+</sup>* mice ( $9 \pm 0.04$ ) and demonstrated significant attenuation of gliosis. *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mouse retinas, analyzed to determine whether the *Ins2<sup>Akita/+</sup>* phenotype was accelerated when lacking Sigma1R, revealed increased nerve fiber layer thickness (OCT), evidence of vitreal opacities, and vessel beading (FA) compared with *Ins2<sup>Akita/+</sup>* mice. Morphometric analysis revealed significantly fewer ganglion cells in *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice compared with *Ins2<sup>Akita/+</sup>* mice.

**CONCLUSIONS.** Sigma1R may be a novel retinal stress modulator, and targeting it even after disease onset may afford retinal neuroprotection.

**Keywords:** retina, sigma 1 receptor ( $\sigma$ 1R), ganglion cells, GFAP, (+)-pentazocine, OCT, fluorescein angiography, FA, mouse

Diabetic retinopathy is a leading cause of sight-threatening disabilities worldwide.<sup>1</sup> Evidence compiled from clinical and basic research investigations supports the view that diabetic retinopathy constitutes a change in the retinal neurovascular unit, which specifically refers to neurons, glia, and vasculature.<sup>2</sup> The vascular pathology in diabetic retinopathy involves occlusion of retinal vessels and leakiness that can result in macular edema, frequently associated with severe vision loss.<sup>3</sup> Retinal neurons and glial cells are also altered in diabetic retinopathy as microvascular lesions develop, especially loss of neurons in the ganglion cell layer (GCL) and the inner nuclear layer (INL).<sup>4</sup> A mouse model that has proven useful for studies of the retinal neuronal phenotype associated with diabetes is the *Ins2<sup>Akita/+</sup>* mouse.<sup>5</sup> This mouse has a point mutation in the *Ins2* gene causing a conformational change in the protein, which accumulates in the  $\beta$  cells of the pancreas, inducing cell death.<sup>6</sup> The homozygous mice are rarely viable, but the heterozygous mice develop hyperglycemia, hypoinsulinemia, polydipsia, and polyuria at approximately 4 weeks. Subclinical changes occur in the retina of *Ins2<sup>Akita/+</sup>*, consistent with the prodromal phase of diabetic retinopathy. Most notably,

there is at least 20% reduction in the number of cell bodies in the retinal GCL of these diabetic mice as they age.<sup>5</sup> Although this model has increased retinal vascular permeability and a modest increase in acellular capillaries after approximately 8 months, the classical vascular pathology as assessed by scanning laser ophthalmoscopy (SLO), fluorescein angiography (FA), and optical coherence tomography (OCT) is not prominent in this mouse model.<sup>7</sup>

Several years ago, our laboratory used the *Ins2<sup>Akita/+</sup>* mouse to investigate whether targeting a unique protein, sigma 1 receptor (Sigma1R), would attenuate ganglion cell loss.<sup>8</sup> We found that sustained administration of a high-affinity Sigma1R ligand, (+)-pentazocine [(+)-PTZ] conferred significant retinal neuroprotection, reduced evidence of oxidative stress, and preserved retinal architecture. In particular, ganglion cell death was markedly reduced in (+)-PTZ-treated *Ins2<sup>Akita/+</sup>* mice, suggesting that Sigma1R ligands are promising therapeutic agents for intervention in neurodegenerative diseases of the retina, including diabetic retinopathy.

Sigma1R is 223-amino-acid nonopioid protein that shares no homology with any other mammalian protein. It is a unique

TABLE 1. Average Weights and Blood Glucose Levels of Mice Used in the Delayed (+)-PTZ Administration Study

Mouse Strain and Treatment Group	n	Mean Weight ± SEM (g)	Blood Glucose ± SEM (mg/dL)	Age When (+)-PTZ Injection Began (wk)
WT ( <i>Ins2<sup>+/+</sup></i> ) [no (+)-PTZ]	6	25.96 ± 2.65	197.0 ± 46.5	*
WT ( <i>Ins2<sup>+/+</sup></i> ) [0.5 mg kg <sup>-1</sup> (+)-PTZ]	8	25.60 ± 3.10	116.6 ± 55.9	8
<i>Ins2<sup>Akita/+</sup></i> [no (+)-PTZ]	5	22.91 ± 0.66	487.0 ± 26.0	*
<i>Ins2<sup>Akita/+</sup></i> [0.5 mg kg <sup>-1</sup> (+)-PTZ]	5	20.82 ± 5.08	474.5 ± 51.0	8
<i>Ins2<sup>Akita/+</sup></i> [0.5 mg kg <sup>-1</sup> (+)-PTZ]	4	18.02 ± 1.66	376.7 ± 99.3	12

\* These mice received no (+)-PTZ injection.

integral membrane protein.<sup>9</sup> Aside from *N,N*-dimethyltryptamine,<sup>10</sup> no natural ligands for Sigma1R have been identified. Sigma1R is a putative molecular chaperone<sup>11</sup> that regulates Ca<sup>2+</sup> mobilization from endoplasmic reticulum (ER) stores<sup>12</sup> and contributes to the stability of inositol 1,4,5-triphosphate type 3 receptor channels to ensure proper Ca<sup>2+</sup> transport between the ER and the mitochondrial-associated membrane.<sup>13</sup> These cellular survival properties have prompted speculation that ligands for Sigma1R could be promising for treatment of neurodegenerative diseases,<sup>14</sup> including retinal diseases. Sigma1R is present in several ocular tissues including retina,<sup>15–20</sup> cornea, ciliary body, and lens.<sup>15,20</sup> A number of in vitro and in vivo studies demonstrate the powerful neuroprotective effects of Sigma1R ligands, most notably in protection against retinal ganglion cell death.<sup>8,21–24</sup> Ligands for Sigma1R modulate ER stress in retinal ganglion cells<sup>25</sup> and ER stress and osmolar stress in retinal Müller glia cells<sup>26,27</sup>; they also regulate intracellular Ca<sup>2+</sup> calcium levels in retinal ganglion cells.<sup>28</sup> Studies from Tchandre et al.<sup>29</sup> provide evidence that Sigma1R interacts directly with voltage-gated Ca<sup>2+</sup> calcium channels in primary cultures of ganglion cells. Mice lacking Sigma1R (*Sig1R*<sup>-/-</sup> mice) have a late-onset retinal degeneration characterized by loss of ganglion cells.<sup>30</sup> Under stress, such as optic nerve crush<sup>18</sup> or streptozotocin-induced diabetes<sup>31</sup> in *Sig1R*<sup>-/-</sup> mice, there is acceleration of the ganglion cell loss.

The finding that ligands for Sigma1R were neuroprotective in *Ins2<sup>Akita/+</sup>* mice<sup>8</sup> combined with the observation that streptozotocin-induced diabetes accelerated the phenotype in *Sig1R*<sup>-/-</sup> mice prompted the present investigation. First, we asked whether delaying injection of (+)-PTZ to *Ins2<sup>Akita/+</sup>* mice beyond the onset of diabetes would confer neuroprotection. This is relevant clinically because it would be rare that treatment of humans would commence at diabetes onset. Our data suggest that (+)-PTZ has neuroprotective properties even when administered several weeks after the onset of diabetes. Second, we asked whether the retinal phenotypic changes observed in *Ins2<sup>Akita/+</sup>* mice would be accelerated when Sigma1R was absent and whether there would be additional ocular manifestations including vasculopathy. Our data indicate that lack of Sigma1R in *Ins2<sup>Akita/+</sup>* mice worsens the neurodegenerative phenotype and accentuates vasculopathy.

## MATERIALS AND METHODS

### Animals

C57BL/6J *Ins2<sup>Akita/+</sup>* mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA), *Ins2<sup>Akita/+</sup>* female mice were bred to wild-type (WT) male mice in our animal facilities. Genotyping of *Ins2<sup>Akita/+</sup>* mice was performed per the protocol recommended by the Jackson Laboratories. Retinas of male and female mice were examined; however, only males were used in the morphometric retinal analyses because disease progression in females is slower and less uniform.<sup>5</sup> Diabetes onset was

verified by measuring urine glucose using the Diastic Reagent Strips for urinalysis (Bayer, Mishawaka, IN, USA) and blood glucose using a Blood Glucose Monitoring System (Abbott Laboratories, Alameda, CA, USA). Fasting blood glucose levels >240 mg/dL were considered diabetic. Diabetic animals were not maintained on insulin. Age-matched, nondiabetic WT mice were used as controls. All mice received food and water ad libitum and were maintained on a 12-hour light-dark cycle. Body weight and blood glucose were measured at time of death, and data are provided in Tables 1 and 2.

Two studies were conducted. In the first study, we examined the effects of delaying (+)-PTZ injection (after onset of diabetes) on retinal neuronal death in diabetic retinopathy. Details of (+)-PTZ injections are provided below. In the second study, we determined whether the retinal neurodegeneration of *Ins2<sup>Akita/+</sup>* mice would be accelerated if the mice lacked Sigma1R, and we asked whether the retinal phenotype would involve vasculopathy when Sigma1R was absent. C57BL/6-*Ins2<sup>Akita/+</sup>* mice were bred with *Sig1R*<sup>-/-</sup> mice (the generation of which has been previously described<sup>30,32</sup>) to produce *Ins2<sup>Akita/+</sup>/Sig1R<sup>±</sup>* mice, which were crossed with *Sig1R*<sup>-/-</sup> mice to generate *Ins2<sup>Akita/+</sup>/Sig1R*<sup>-/-</sup> mice. Genotyping was performed to confirm gene expression in all mice. In addition, we confirmed that the mice did not carry the rd8 (*Crb1*) mutation, which manifests as disrupted morphology including retinal rosettes.<sup>33</sup> Mice were maintained for either 12 or 16 weeks, after which they were subjected to functional and morphologic analysis. Maintenance of animals adhered to the institutional guidelines for the humane treatment of animals following our Institutional Animal Care and Use Committee (IACUC)-approved protocol and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Administration of (+)-PTZ After Onset of Diabetes

*Ins2<sup>Akita/+</sup>* mice become diabetic at 3–4 weeks.<sup>5,6</sup> Mice were administered (+)-PTZ either 4 or 8 weeks after onset of diabetes (thus, one group of *Ins2<sup>Akita/+</sup>* mice was injected beginning at age 8 weeks and a second was injected beginning at age 12 weeks). Additional groups of mice included *Ins2<sup>Akita/+</sup>* non-injected, WT (*Ins2<sup>+/+</sup>*) noninjected, and WT (*Ins2<sup>+/+</sup>*) injected beginning at 8 weeks (Table 1). (+)-Pentazocine (Cat. No. P127; Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved initially in dimethyl sulfoxide (DMSO) and diluted with 0.01 M PBS, and it was administered via intraperitoneal injection at a dosage 0.5 mg kg<sup>-1</sup> two times per week for 22 weeks. Mice were euthanized at 25 weeks, as was the case in the previous study in which *Ins2<sup>Akita/+</sup>* mice were administered (+)-PTZ at diabetes onset.<sup>8</sup> Retinas were evaluated morphometrically (described below).

### Spectral-Domain OCT Imaging, Fundoscopy, and FA

To evaluate retinal morphology in vivo, spectral-domain (SD)-OCT imaging was performed in *Ins2<sup>Akita/+</sup>/Sig1R*<sup>-/-</sup> mice. Mice

**TABLE 2.** Average Weights and Blood Glucose Levels of Mice Used in the Analysis of *Ins2<sup>Akita/+</sup>*, *Sig1R<sup>-/-</sup>* Mice

Mouse Genotype	n	Mean Weight ± SEM (g)	Blood Glucose ± SEM (mg/dL)	Age at Euthanasia (wk)
WT ( <i>Ins2<sup>+/+</sup></i> , <i>Sig1R<sup>+/+</sup></i> )	5	21.10 ± 1.22	104.4 ± 14.81	12
<i>Ins2<sup>Akita/+</sup></i>	5	19.91 ± 1.66	377.6 ± 137.01	12
<i>Ins2<sup>Akita/+</sup></i> , <i>Sig1R<sup>-/-</sup></i>	3	19.14 ± 1.17	396 ± 91.66	12
WT ( <i>Ins2<sup>+/+</sup></i> , <i>Sig1R<sup>+/+</sup></i> )	5	21.85 ± 1.78	81.2 ± 20.39	16
<i>Ins2<sup>Akita/+</sup></i>	5	21.97 ± 2.04	412 ± 172.97	16
<i>Ins2<sup>Akita/+</sup></i> , <i>Sig1R<sup>-/-</sup></i>	5	20.42 ± 0.70	317 ± 154.29	16

were anesthetized using a rodent anesthesia cocktail (ketamine hydrochloride/xylazine hydrochloride solution [80:12 mm/mL]; Sigma-Aldrich Corp.). Pupils were dilated with 1% tropicamide (Bausch and Lomb, Tampa, FL, USA) followed by application of GenTeal Lubricant Eye Gel, 10 g (Alcon, Ft. Worth, TX, USA). Systane lubricant eye drops (Alcon) were applied to keep the cornea moist. The SD-OCT images were obtained using the Bioptigen Spectral-Domain Ophthalmic Imaging System (SD OIS; Bioptigen Envisu R2200; Bioptigen, Inc., Durham, NC, USA). Imaging included averaged single B-scan and volume intensity scans (VIPs) with images centered on the optic nerve head. Postimaging analysis included auto segmentation report analysis and manual assessment of all retinal layers using InVivoVue™ DIVER 2.4 software (Bioptigen, Inc.).

Fundus imaging was performed in *Ins2<sup>Akita/+</sup>*/*Sig1R<sup>-/-</sup>* mice using the Micron III camera (Phoenix Research Laboratories, Inc., Pleasanton, CA, USA) in mice that had been anesthetized as described for SD-OCT. Pupils were dilated with 1% tropicamide, and the cornea was kept moist. Following fundus imaging, fluorescein angiography was performed following a 20- $\mu$ L 10% fluorescein lite (Apollo Ophthalmic, Newport Beach, CA, USA) intraperitoneal injection, which permitted visualization of retinal vasculature. The procedures for OCT and FA followed our previously reported methods.<sup>34,35</sup>

### Tissue Processing and Morphometric Analysis

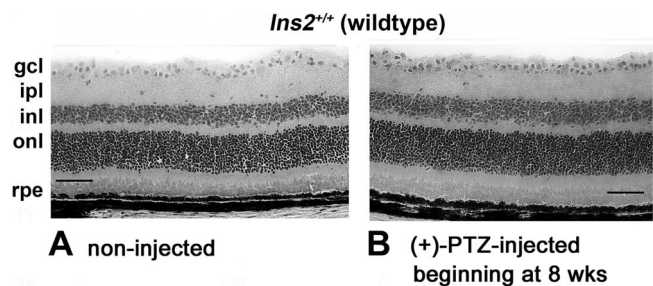
Mice were killed by CO<sub>2</sub> asphyxiation followed by cervical dislocation. Eyes were removed immediately and were either oriented in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN, USA) and were frozen slowly by immersion in liquid nitrogen (without fixation) or were fixed in 4% paraformaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer and embedded in JB-4 embedding compound (EMS, Hatfield, PA, USA) per our method.<sup>30</sup> Sections were stained with hematoxylin and eosin (H&E) and used for systematic morphometric analysis using a Zeiss Axioplan2 fluorescent microscope equipped with HRM camera and the Axiovision 4.6.3 program (Carl Zeiss, Oberkochen, Germany). Measurements included the thickness of the (1) total retina, (2) inner plexiform layer (IPL), (3) INL, (4) outer plexiform layer (OPL), and (5) the photoreceptor inner/outer segments. The number of rows of photoreceptor cell nuclei in the outer nuclear layer (ONL) was counted. The number of cell bodies in the GCL was quantified by counting cells from the temporal ora serrata to the nasal ora serrata and expressing the data as number of cells per 100  $\mu$ m length of retina. Sections analyzed were from the center of the eyeball; thus, the optic nerve was visible. Three measurements were made on each side (temporal and nasal) of the optic nerve at 200- to 300- $\mu$ m intervals, resulting in a total of six measurements obtained per eye. Per mouse, at least two sections were analyzed for the left and right eye. In addition, eyes were examined for evidence of gross pathology.

### Assessment of Gliosis and Cell Death

Glial fibrillary acidic protein (GFAP) is a 54-kDa intermediate filament protein that is a major constituent of retinal astrocytes; under retinal stress conditions, GFAP expression is up-regulated in retinal Müller glial cells in a process termed gliosis.<sup>36</sup> Retinal cryosections were fixed with 4% paraformaldehyde, washed with PBS-Triton X-100, and incubated with Power Block, after which sections were incubated with rabbit anti-GFAP (1:500; Dako, Carpinteria, CA, USA) overnight at 4°C. Sections were washed and incubated Triton X-100, followed by incubation with secondary antibody Alexa Fluor 488-conjugated donkey-anti-rabbit IgG (1:1,000; Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. Sections were washed three times with PBS-Triton X-100 and coverslipped with Fluoroshield with 4',6-diamidino-2-phenylindole (DAPI). Sections were examined by epifluorescence using a Zeiss Axioplan-2 microscope, equipped with the Axiovision program, an HRM camera, and filters to detect FITC and DAPI. Fluorescence intensity was analyzed using Metamorph Image Analysis software (Molecular Devices, Sunnyvale, CA, USA). Additional immunofluorescence experiments were performed to detect dying cells using the ApoptTag Fluorescein In Situ Apoptosis Detection Kit (TUNEL assay; EMD Millipore, Billerica, MA, USA) per our published methods.<sup>30</sup>

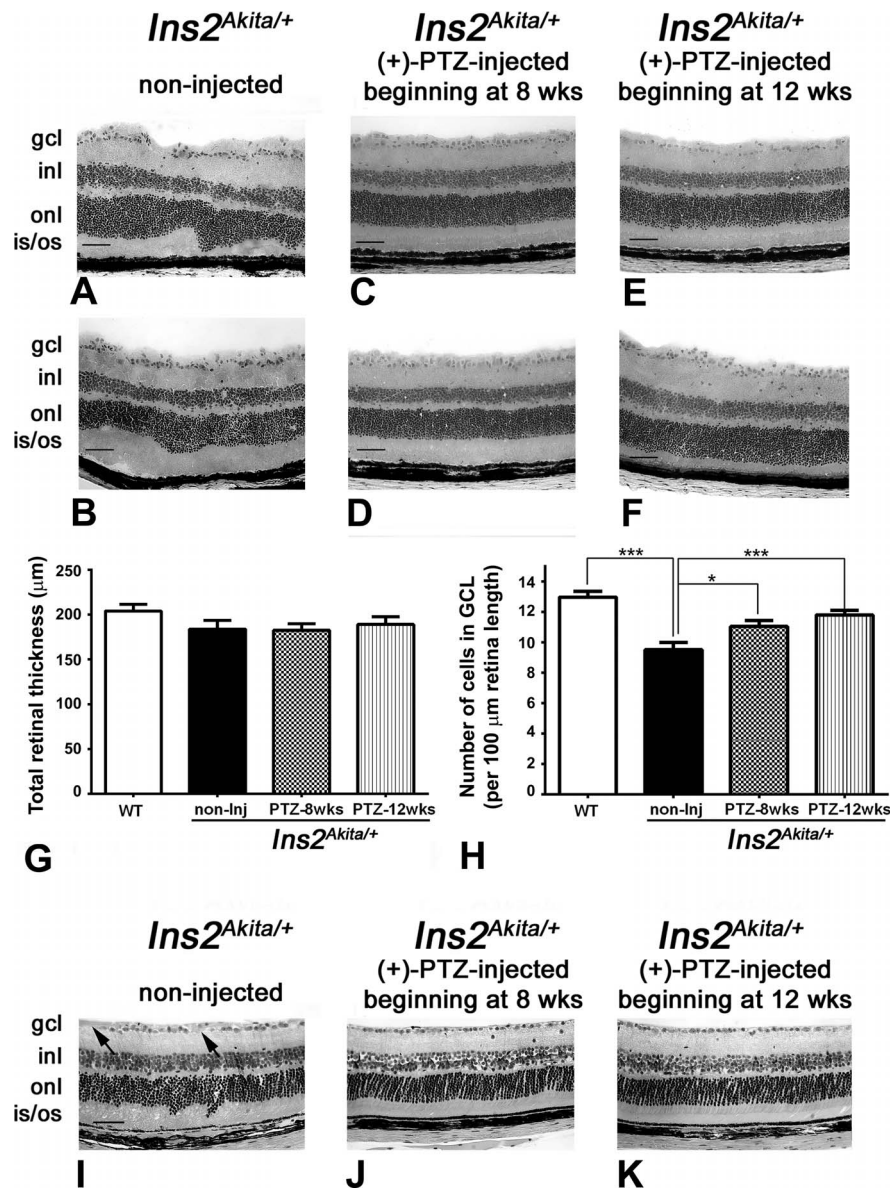
### Statistical Analysis

Analysis of variance was used to determine whether there were significant differences in morphologic measurements in mice administered (+)-PTZ at the two time intervals compared with noninjected animals. For OCT measurements and cell counting in GCL studies, 2-way ANOVA was used to determine whether there were significant differences between WT, *Ins2<sup>Akita/+</sup>*, and *Ins2<sup>Akita/+</sup>*/*Sig1R<sup>-/-</sup>* mouse groups at the two ages studied. Tukey's paired comparison test was the post hoc statistical test. Statistical analysis was conducted using the GraphPad Prism software (version 6; GraphPad Software, Inc., La Jolla, CA, USA).  $P < 0.05$  was considered significant.



**FIGURE 1.** Effects of (+)-PTZ on retinas of WT mice. Representative photomicrographs of H&E-stained retinas of control (C57BL/6) mice (WT) that received (A) no (+)-PTZ injection or (B) 0.5 mg kg<sup>-1</sup> (+)-PTZ injection twice weekly beginning at 8 weeks. Retinal appearance was similar between noninjected and injected WT mice. gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigment epithelial layer.





**FIGURE 2.** Preservation of retinal structure and cells in the GCL in *Ins2<sup>Akita/+</sup>* mice administered (+)-PTZ after diabetes onset. All images are from mice age 25 weeks. (A, B) Representative H&E-stained retinal cryosections of *Ins2<sup>Akita/+</sup>* mice that received no (+)-PTZ for the duration of the study; the INL is slightly disrupted, and GCL density is decreased. Representative H&E-stained retinal cryosections of (C, D) *Ins2<sup>Akita/+</sup>* mice that received 0.5 mg kg<sup>-1</sup> (+)-PTZ beginning 4 weeks after onset of diabetes (at age 8 weeks) or (E, F) *Ins2<sup>Akita/+</sup>* mice that received 0.5 mg kg<sup>-1</sup> (+)-PTZ beginning 8 weeks after onset of diabetes (at age 12 weeks). Retinas of (+)-PTZ injected mice demonstrated marked preservation of retinal structure. Morphometric analysis revealed no significant change in total retinal thickness in any of the eyes evaluated (G), but a significant difference in the number of cell bodies in the GCL per 100 μm length of retina (H). Eyes were processed for JB-4 embedding and stained with H&E to permit visualization of retinal architecture of (D) *Ins2<sup>Akita/+</sup>* mice that received no (+)-PTZ; (J) *Ins2<sup>Akita/+</sup>* mice that received 0.5 mg kg<sup>-1</sup> (+)-PTZ beginning 4 weeks after onset of diabetes (at age 8 weeks); or (K) *Ins2<sup>Akita/+</sup>* mice that received 0.5 mg kg<sup>-1</sup> (+)-PTZ beginning 8 weeks after onset of diabetes (at age 12 weeks). Arrows in I point to areas of cell dropout. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; is/os, inner/outer segments. Magnification bar: 50 μm. Data are means ± SE. Significantly different at \**P* < 0.05 and \*\*\**P* < 0.001.

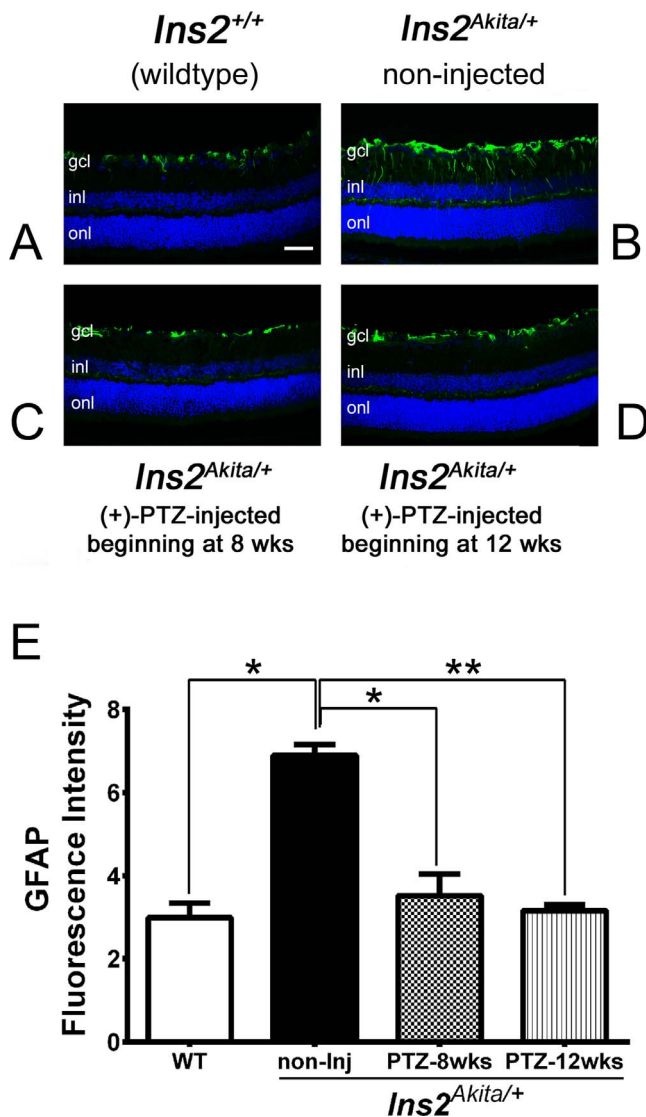
## RESULTS

### Effects of Administration of (+)-PTZ After Diabetes Onset on Neuronal Death in Diabetic Retinopathy

Earlier studies demonstrated that twice weekly administration of (+)-PTZ at the 0.5-mg kg<sup>-1</sup> dosage reduced apoptotic neuron death characteristic of *Ins2<sup>Akita/+</sup>* mice and preserved retinal architecture, when treatment started at diabetes onset.<sup>8</sup> We used this same (+)-PTZ dosage and treatment regimen in the present study, but initiated injections either 4 or 8 weeks after

diabetes onset, which meant that injections began at age 8 or 12 weeks in *Ins2<sup>Akita/+</sup>* mice. Representative photomicrographs of retinas of WT mice receiving either no (+)-PTZ injection or receiving (+)-PTZ twice weekly beginning at age 8 weeks are shown (Fig. 1). There was no deleterious effect on retinal architecture when WT mice were administered the Sigma1R ligand (Fig. 1) and no differences in morphometric analysis of retinal layers (data not shown).

In the case of the *Ins2<sup>Akita/+</sup>* mice that received no (+)-PTZ injections, the retinas showed mild disruption. Figures 2A and 2B show representative photomicrographs of retinas from two



**FIGURE 3.** Immunofluorescent detection of GFAP, a marker of gliosis in *Ins2<sup>Akita/+</sup>* mice administered (+)-PTZ after diabetes onset. Representative photomicrographs of immunofluorescent detection of GFAP in retinal cryosections from (A) *Ins2<sup>+/+</sup>* (WT), (B) *Ins2<sup>Akita/+</sup>* [no (+)-PTZ treatment], (C) *Ins2<sup>Akita/+</sup>* mice that received 0.5 mg kg<sup>-1</sup> (+)-PTZ beginning 4 weeks after onset of diabetes (at age 8 weeks), and (D) *Ins2<sup>Akita/+</sup>* mice that received 0.5 mg kg<sup>-1</sup> (+)-PTZ beginning 8 weeks after onset of diabetes (at age 12 weeks). Immunofluorescence analysis used anti-GFAP followed by incubation with Alexa Fluor 488 (green-labeled secondary antibody), showing marked increase in GFAP in radially oriented fibers of Müller glial cells (radial labeling) in *Ins2<sup>Akita/+</sup>* [no (+)-PTZ treatment], but minimal labeling in Müller cells of the WT or (+)-PTZ-treated mice. Astrocytic labeling with GFAP is typical and is observed in retinas of all mice. (E) Quantification of GFAP fluorescence intensity data obtained from metamorphic analysis (significant difference: \**P* < 0.05, \*\**P* < 0.01).

of the mice in the nontreated group. Other nontreated mice had similar retinal architecture. The alterations in the INL by 25 weeks in the *Ins2<sup>Akita/+</sup>* mice have been reported.<sup>5</sup> The nuclear layers did not have their characteristic uniform stratified appearance, but this was not attributable to presence of the rd8 mutation. Mice that had been injected intraperitoneally beginning at 4 or 8 weeks after onset of diabetes onset with 0.5 mg kg<sup>-1</sup> (+)-PTZ twice weekly showed preservation of

retinal architecture (Figs. 2C–2F). The panels shown are representative of two mice per group at each age studied. The nuclear layers were uniform in appearance. The retinas were subjected to morphometric analysis, and the total retinal thickness did not differ between the WT mice, *Ins2<sup>Akita/+</sup>*-noninjected mice, and *Ins2<sup>Akita/+</sup>*-injected mice beginning at 4 or 8 weeks (Fig. 2G). Nor did the thickness of the individual layers (IPL, INL, OPL, and ONL) differ significantly between groups (data not shown). However, when the numbers of cells in the GCL were quantified, there was a significant difference between the WT mice and the *Ins2<sup>Akita/+</sup>*-noninjected mice (Fig. 2H). Wild-type mice had approximately 13 cells per 100 μm length of retina, whereas the *Ins2<sup>Akita/+</sup>*-noninjected mice had approximately 9 cells for the same retinal length (Fig. 2H). For the mice treated with Sigma1R ligand at either 4 or 8 weeks after onset of diabetes, there were approximately 11 cells per 100 μm retinal length, which was significantly greater than the *Ins2<sup>Akita/+</sup>*-noninjected mice. Although the numbers of cells in the GCL of (+)-PTZ-treated *Ins2<sup>Akita/+</sup>* mice were not equal to that of the WT mice, there was clear preservation of these cells compared with diabetic mice not treated with the Sigma1R ligand. We also noted that there was waveform irregularity of the ONL accompanied by shallow retinal detachment and irregularly elongated photoreceptor outer segments in the retinas of *Ins2<sup>Akita/+</sup>*-noninjected mice. To evaluate whether the waveform appearance and occasional retinal detachment might be a consequence of the cryosectioning method of preparing sections, we examined eyes that had been fixed prior to embedding and processed for JB-4 plastic embedding. In this case, the *Ins2<sup>Akita/+</sup>*-noninjected mice showed minimal detachment, although slight irregularities of the nuclear layers were still detectable (Fig. 2I). There continue to be regions within the GCL devoid of cells (Fig. 2I, arrows). The JB-4-embedded retinas of the (+)-PTZ-treated *Ins2<sup>Akita/+</sup>* mice had a generally uniform appearance (Figs. 2J, 2K) similar to that observed in the cryosections of retinas of these mice (Figs. 2C–2F).

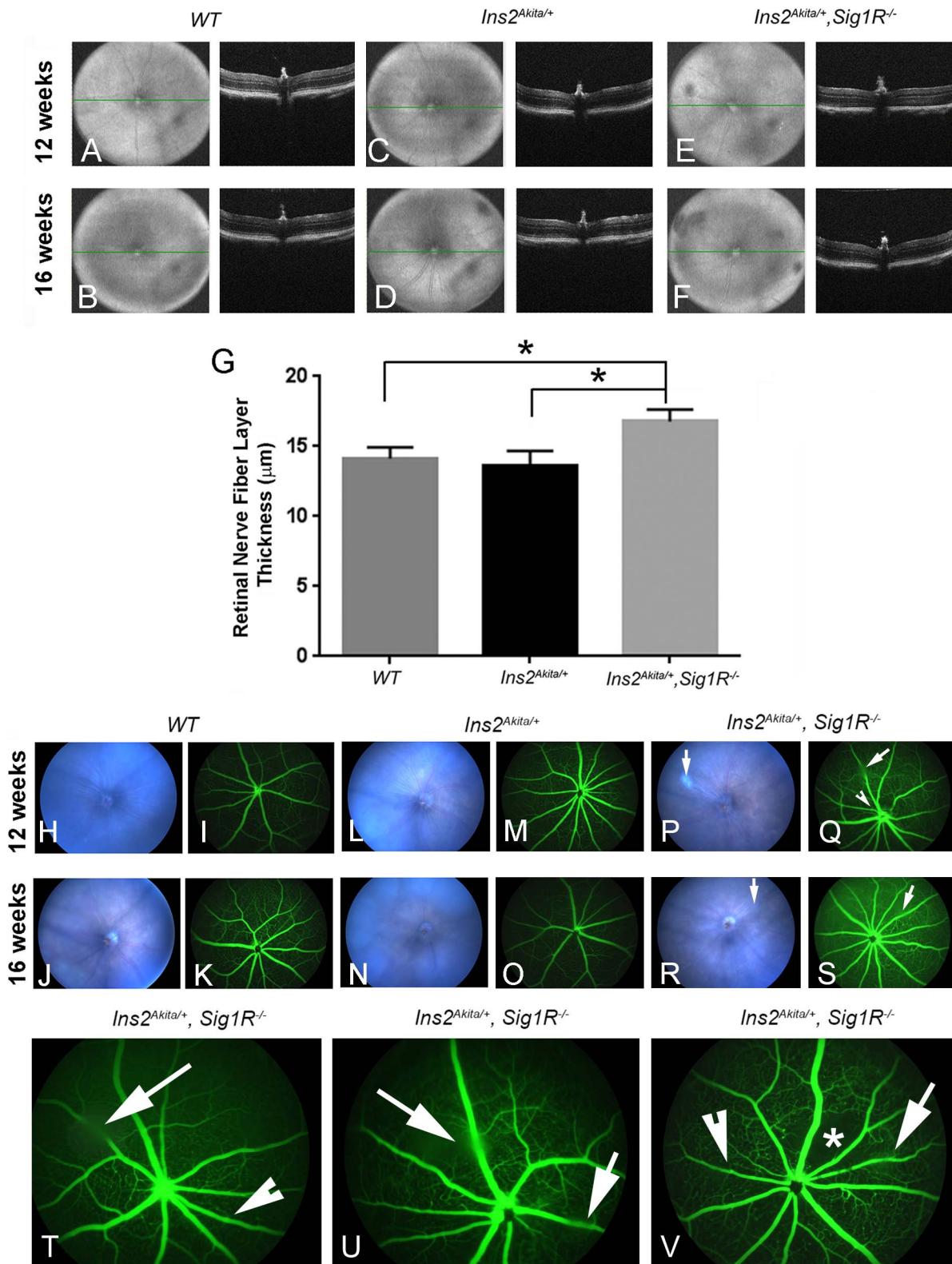
We examined the expression levels of GFAP, which is up-regulated during retinal gliosis.<sup>36</sup> We observed labeling in the WT retina limited to astrocytes, which typically express this protein (Fig. 3A). In the *Ins2<sup>Akita/+</sup>*-noninjected mice, the level of GFAP increased, and the distribution of labeling was detected along the radial fibers of Müller glial cells (Fig. 3B). This is an indication of gliosis characteristic of this diabetic retinopathy model. In the *Ins2<sup>Akita/+</sup>* (+)-PTZ mice, the level of GFAP was very low in the Müller cells. The observation of minimal detection of GFAP (except the normal detection in astrocytes) was true for mice treated beginning at 8 weeks (4 weeks after onset of diabetes; Fig. 3C) and in mice treated beginning at 12 weeks (8 weeks after onset of diabetes; Fig. 3D). The quantification of fluorescence intensity is shown in Figure 3E.

Regarding the monitoring of blood glucose in this study (Table 1), WT mice had blood glucose values below 240 mg/dL and were not hyperglycemic. The (+)-PTZ-treated *Ins2<sup>Akita/+</sup>* mice remained hyperglycemic throughout the study; blood glucose levels were approximately 400 mg/dL (similar to untreated *Ins2<sup>Akita/+</sup>* mice) and were significantly higher than WT mice (116–197 mg/dL), suggesting that hyperglycemia per se may not be sufficient to trigger neuronal loss in diabetes. Treatment with (+)-PTZ does not alter blood glucose levels.

### SD-OCT Imaging, Fundoscopy, and FA in *Ins2<sup>Akita/+</sup>*/*Sig1R<sup>-/-</sup>* Mice

In addition to investigating the effects of delayed administration of (+)-PTZ on the retinal structure in the *Ins2<sup>Akita/+</sup>* diabetic mice described above, we examined the consequences on retinal





**FIGURE 4.** Spectral-domain OCT imaging, funduscopy, and FA in *Ins2<sup>Akita/+</sup>* and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice. Wild-type (*Ins2<sup>+/+</sup>*), *Ins2<sup>Akita/+</sup>*, and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice were anesthetized and subjected to SD-OCT to obtain images of the retina in situ. Representative VIP for WT (*Ins2<sup>+/+</sup>*) mice: age 12 (A) and 16 weeks (B); *Ins2<sup>Akita/+</sup>* mice: age 12 (C) and 16 weeks (D); and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice: age 12 (E) and 16 weeks (F). The panel to the right of each VIP is the B-scans of the same animal. Autosegmentation analysis was performed to determine whether there were significant differences in thickness of retinal layers among the three mouse groups. There was a significant increase in thickness of the nerve fiber layer (G) (\*significantly different at  $P < 0.05$ ). Mice were subjected to funduscopy WT (*Ins2<sup>+/+</sup>*) at 12 (H) and 16 weeks (J); *Ins2<sup>Akita/+</sup>* at 12 (L) and 16 weeks (N); *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* at 12 (P) and 16 weeks (R). Fundus images were normal for WT and *Ins2<sup>Akita/+</sup>*, whereas vitreal opacities (denoted by long arrows) were detected in the *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice (P, R). Mice were subjected to FA to observe vascular filling with a

fluorescent dye. Wild-type (*Ins2<sup>+/+</sup>*) showed no alterations at either 12 (I) or 16 weeks (K). *Ins2<sup>Akita/+</sup>* mice also had normal vascularization patterns at 12 (M) and 16 weeks (O), whereas *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice had beading at 12 (Q) and at 16 weeks (S). The long arrows in Q and S point to areas of vitreal opacity; the indented arrowhead points to a vessel with beading (S). (T) Enlarged image of image shown in S. (U, V) FA images of two additional mice at 16 weeks showing vitreal opacity (long arrow) and occasional beading of vessels (indented arrowhead). The asterisk in V denotes an area of capillary dropout.

phenotype when Sigma1R was absent by generating *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice. We performed SD-OCT in WT (*Ins2<sup>+/+</sup>*), *Ins2<sup>Akita/+</sup>* mice, and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice that were either 12 or 16 weeks of age. Volume image projections and the accompanying B-scans showed generally normal retinal architecture and no evidence of gross disruption in the retinal nuclear or plexiform layers among the three groups (Figs. 4A–4F). Postimage analysis of retinal layers used InVivoVue 2.4 DIVER software (Bioptigen, Inc., Durham, NC, USA) and confirmed that the thickness of most retinal layers was comparable among the three groups of mice, with the exception of the measurement of the nerve fiber layer (NFL). In this case, the NFL was thicker in retinas of *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice compared with WT (*Ins2<sup>+/+</sup>*) or *Ins2<sup>Akita/+</sup>* mice (Fig. 4G). Measurement of other retinal layers did not differ significantly among the three mouse groups (data not shown).

We then examined WT (*Ins2<sup>+/+</sup>*), *Ins2<sup>Akita/+</sup>*, and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice by retinal funduscopy and FA at 12 and 16 weeks of age. The fundus was normal in appearance in WT (Figs. 4H, 4J) and *Ins2<sup>Akita/+</sup>* mice (Figs. 4L, 4N). There was evidence of vitreal opacities in several *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice (Figs. 4P, 4R, long arrows). To visualize retinal vessels, fluorescein dye was injected, and images were acquired 30 seconds after injection and every minute thereafter for 5 minutes. Images were arranged according to capture time, and data were compared among the three mouse groups at ages 12 and 16 weeks. Fluorescein angiography performed in WT mice showed normal vessel filling, no leakage, and uniform capillary network around blood vessels at both ages studied (Figs. 4I, 4K). Similarly, vessel filling in *Ins2<sup>Akita/+</sup>* mice appeared normal, and leakage was not observed at either 12 (Fig. 4M) or 16 weeks (Fig. 4O). In *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice, however, there was evidence of vessel beading at 12 weeks (Fig. 4Q, indented arrowhead) and at 16 weeks (Fig. 4S, indented arrowhead). A larger image of Figure 4S is provided (Fig. 4T). The long arrow in Figure 4T points to a region of vitreal opacity, not an area of leakiness. Higher-magnification FA images for two additional 16-week *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice are shown (Figs. 4U, 4V). In Figure 4V, there appears to be an area of capillary dropout (denoted by an asterisk) in the retina of a 16-week *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mouse. It is noteworthy that all of the 16-week *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice examined showed some evidence of beading and of vitreal opacities. The data suggest that in the absence of Sigma1R subtle retinal vascular abnormalities develop that are not normally observed by FA in the *Ins2<sup>Akita/+</sup>* diabetic mouse retina.<sup>7</sup>

### Retinal Morphometric Analysis in *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* Mice

Eyes from WT (*Ins2<sup>+/+</sup>*), *Ins2<sup>Akita/+</sup>*, and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice were processed in JB-4 embedding compound for light microscopic evaluation and retinal morphometric analysis at 12 and 16 weeks. Although the retinal architecture becomes mildly disrupted by 25 weeks in the *Ins2<sup>Akita/+</sup>* mice as reported previously<sup>5</sup> and Figure 2 of this study, the retinal architecture of *Ins2<sup>Akita/+</sup>* mice at the younger ages (12 and 16 weeks) analyzed in this study (Fig. 5B) was similar to that of WT (*Ins2<sup>+/+</sup>*) mice (Fig. 5A). A notable feature that distinguished the *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice from the WT and *Ins2<sup>Akita/+</sup>* mice was the fragmented appearance and disruption

of the NFL in these mice compared with the WT (*Ins2<sup>+/+</sup>*) and *Ins2<sup>Akita/+</sup>* mice (Fig. 5C). Although the disrupted appearance of the NFL may reflect increased sensitivity to the fixative used in processing the tissue, there appears to be involvement of NFL in the *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice when examined in vivo by SD-OCT imaging (Fig. 4G). The NFL is comprised of the axons of the ganglion cells; thus, the decrease in the numbers of cells observed in the *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice could account for disruption of their axons. The images in Figures 5A–5C are from 16-week mice.

In the mouse retina, the number of cell bodies in the GCL is typically 12–14 per 100  $\mu\text{m}$  length of retina. This was observed in the WT (*Ins2<sup>+/+</sup>*) mouse retina at both 12 and 16 weeks (Figs. 5D, 5E). In *Ins2<sup>Akita/+</sup>* mice, there was minimal difference at 12 and 16 weeks in the number of cells bodies in the GCL compared with WT mice (Figs. 5D, 5E). In *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice, however, there was a significant decrease in the number of cells in the GCL at 12 ( $\sim 11$  cells/100  $\mu\text{m}$  retinal length) and 16 weeks ( $\sim 8$  cells/100  $\mu\text{m}$  retinal length). The data suggest that, in the absence of Sigma1R, the loss of ganglion cells observed at 25 weeks in the *Ins2<sup>Akita/+</sup>* is markedly accelerated. We subjected retinal cryosections from the three mouse groups to immunofluorescence to detect dying cells using the ApoptTag Fluorescein In Situ Apoptosis Detection Kit (TUNEL staining). We did not detect dying cells in GCL of WT (*Ins2<sup>+/+</sup>*) mice (Fig. 5F), indicating minimal cell death in this layer under normal conditions. We observed some TUNEL-positive cells in the GCL of the *Ins2<sup>Akita/+</sup>* retina (Fig. 5G) and many more TUNEL-positive cells in the GCL of *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice (Fig. 5H) at age 16 weeks. The findings confirm the reduced numbers of cells in the GCL determined by morphometric analysis. We also examined the INL for TUNEL-positive cells and observed very few TUNEL-positive cells in retinas of WT (*Ins2<sup>+/+</sup>*) (Fig. 5I) or *Ins2<sup>Akita/+</sup>* mice (Fig. 5J), but a significant increase in TUNEL-positive cells in the INL of retinas of *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice (Fig. 5K).

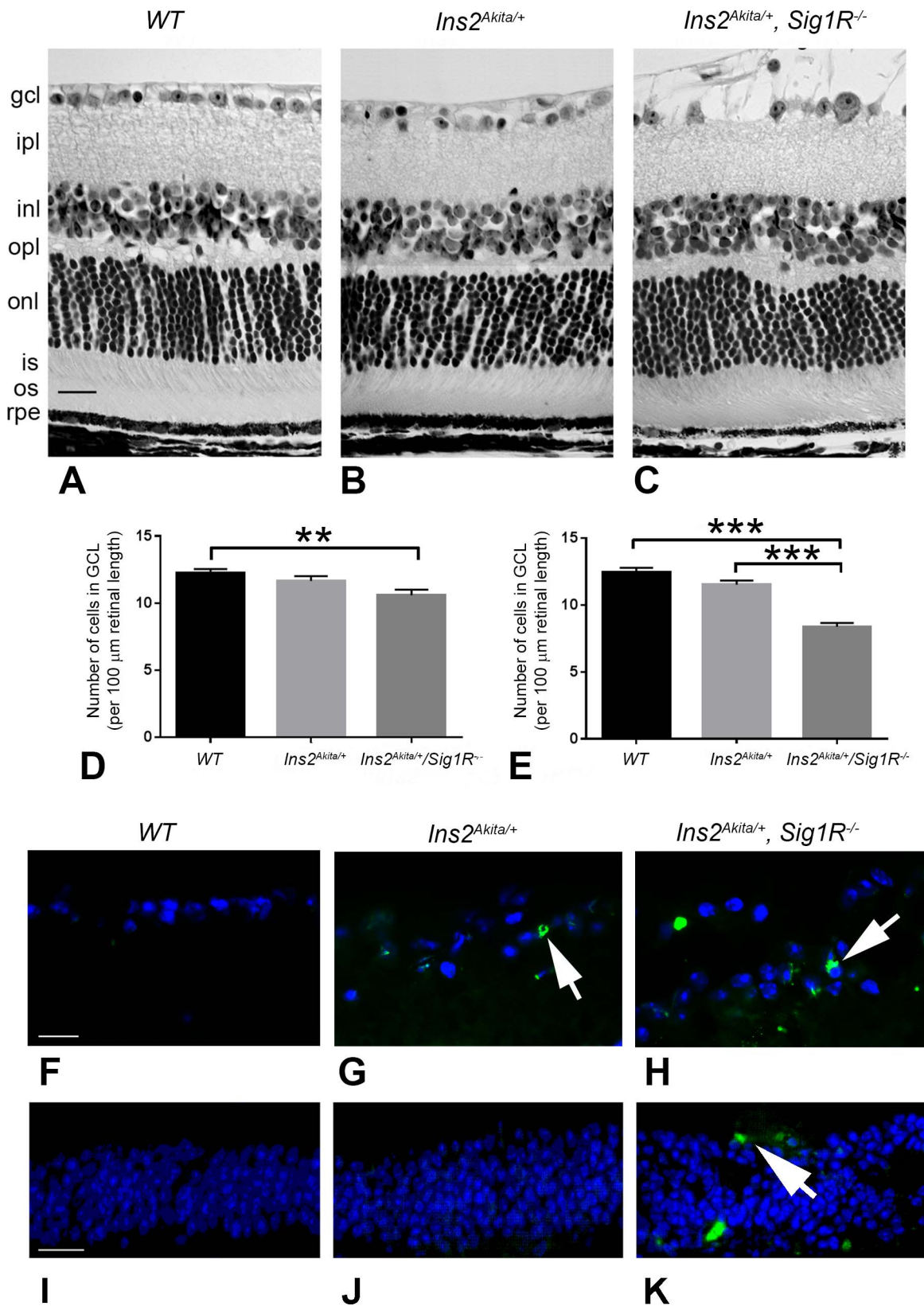
### Assessment of Gliosis in *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* Mice

In the *Ins2<sup>Akita/+</sup>* retina, increased expression of GFAP has been reported in Müller cells at 6 months<sup>7</sup> (and Fig. 3 of this study). In the current study, we investigated the levels of GFAP in retinal cryosections of WT (*Ins2<sup>+/+</sup>*), *Ins2<sup>Akita/+</sup>*, and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice by immunofluorescence at 12 and 16 weeks. Photomicrographs shown in Figure 6 are from 16-week mice. Aside from GFAP labeling typically observed in retinal astrocytes, we detected minimal GFAP in retinas of WT (*Ins2<sup>+/+</sup>*) mice (Fig. 6A). There was a slight increase in GFAP levels in retinas of *Ins2<sup>Akita/+</sup>* mice (Fig. 6B) and a substantial increase in GFAP detected in retinas of *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice (Fig. 6C). The data suggest that, in younger *Ins2<sup>Akita/+</sup>* mice lacking Sigma1R, there is increased retinal gliosis compared with age-matched diabetic mice that have Sigma1R.

### Effects of Sigma1R on Blood Glucose Levels in *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* Mice

The blood glucose levels measured in WT (*Ins2<sup>+/+</sup>*) were within normal limits, whereas levels were hyperglycemic in both *Ins2<sup>Akita/+</sup>* and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice (Table 2). The





**FIGURE 5.** Retinal structure of WT (*Ins2<sup>+/+</sup>*), *Ins2<sup>Akita/+</sup>*, and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice. Eyes of (A) WT (*Ins2<sup>+/+</sup>*), (B) *Ins2<sup>Akita/+</sup>*, and (C) *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice were processed for JB-4 embedding and stained with H&E to permit visualization of retinal architecture. Animals were either 12 or 16 weeks at the time eyes were harvested. Panels shown are from 16-week-old mice. In WT (*Ins2<sup>+/+</sup>*) mice, the retinas are well organized and layers are of normal thickness. The retinas of the *Ins2<sup>Akita/+</sup>* and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice are similar in appearance, with the exception of the nerve fiber layer in the *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice (arrow), which is markedly disrupted. Retinas were subjected to morphometric analysis; there was a significant decrease in the number of cells in the GCL in the *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice at 12 (D) and 16 weeks (E). Retinal cryosections of the same



mice were subjected to immunofluorescent detection of TUNEL-positive cells (green labeling), cell nuclei were blue (DAPI) in (F) WT (*Ins2<sup>+/+</sup>*), (G) *Ins2<sup>Akita/+</sup>*, and (H) *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice. Images were obtained using a high-powered objective focused on the GCL. Additional images of TUNEL staining were obtained using a lower-powered objective focused on the INL for (I) WT (*Ins2<sup>+/+</sup>*), (J) *Ins2<sup>Akita/+</sup>*, and (K) *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice. gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; opl, outer plexiform layer; onl, outer nuclear layer; is, inner segment; os, outer segment; rpe, retinal pigment epithelium. Calibration bar: 50  $\mu$ m. Statistical significance: \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

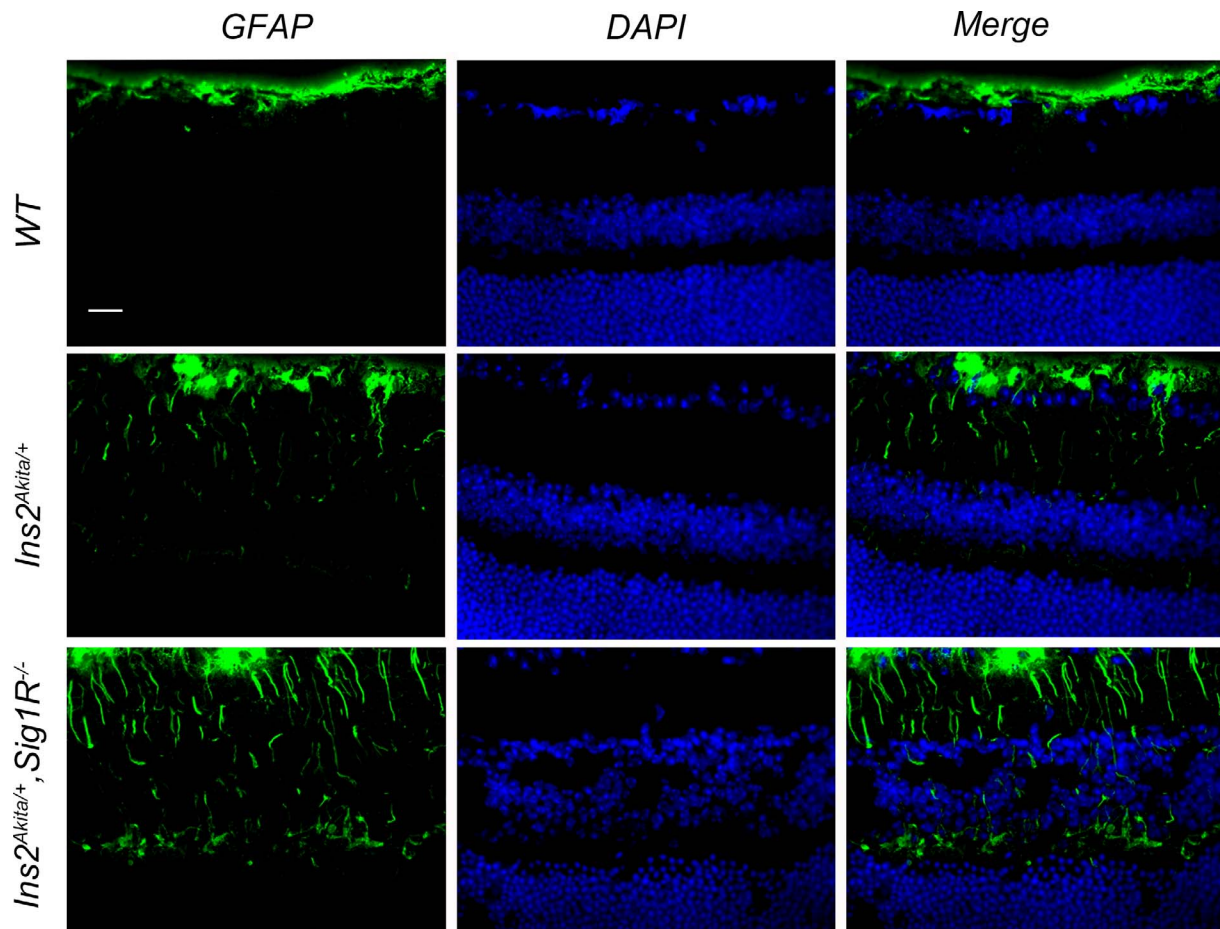
absence of Sigma1R did not alter blood glucose levels in *Ins2<sup>Akita/+</sup>* mice.

## DISCUSSION

This study had two objectives. One was to investigate whether delaying administration of a high-affinity Sigma1R ligand after onset of diabetes could afford neuroprotection in a model of diabetic retinopathy and the second was to evaluate whether absence of Sigma1R would accelerate neuron loss in this model. Diabetic retinopathy is one of the foremost retinopathies afflicting humans.<sup>1</sup> Although diabetic retinopathy has long been recognized as a retinal vascular disease, significant neuronal loss is also a feature of this disease.<sup>37</sup> We chose to use the diabetic *Ins2<sup>Akita/+</sup>* mouse as an endogenous diabetic model with modest progressive loss of cells in the GCL.

In our study examining whether administration of (+)-PTZ after onset of diabetes would be neuroprotective, we investi-

gated four mouse groups. We used WT mice, *Ins2<sup>Akita/+</sup>* mice that received no injection, and two groups of *Ins2<sup>Akita/+</sup>* mice that were injected beginning at 8 or 12 weeks (which was either 4 or 8 weeks after diabetes onset). Earlier studies had shown that by the time *Ins2<sup>Akita/+</sup>* mice were 25 weeks, there was an approximate 20% loss of cells in the GCL.<sup>5,8</sup> We used that same time point to investigate whether (+)-PTZ could attenuate cell loss and found that, in both the 8- and 12-week injection groups, there was attenuation of cell loss. That is, even though (+)-PTZ was not administered at the onset of diabetes, there still appeared to be significant neuroprotection when the retinas were examined at 25 weeks. The retinas of *Ins2<sup>Akita/+</sup>* mice that were in the delayed injection groups retained significantly more cells in the GCL than noninjected *Ins2<sup>Akita/+</sup>* mice. In addition, examination of retinal architecture showed that retinas of (+)-PTZ-injected mice were well organized. Most intervention studies (including our own study with the *Ins2<sup>Akita/+</sup>* mouse several years ago<sup>8</sup>) start therapeutic intervention as early as possible, sometimes prior to onset of disease, to achieve the



**FIGURE 6.** Immunofluorescent detection of GFAP, a marker of gliosis in WT (*Ins2<sup>+/+</sup>*), *Ins2<sup>Akita/+</sup>*, and *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice at 16 weeks. Representative photomicrographs of immunofluorescent detection of GFAP in retinal cryosections from (A) WT (*Ins2<sup>+/+</sup>*), (B) *Ins2<sup>Akita/+</sup>*, and (C) *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice at age 16 weeks. Immunofluorescence analysis used anti-GFAP followed by incubation with Alexa Fluor 488 (green-labeled secondary antibody), showing marked increase in GFAP in radially oriented fibers of Müller glial cells (radial labeling) in *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice but much less GFAP labeling in Müller cells of the WT or *Ins2<sup>Akita/+</sup>* mice at this age. Astrocytic labeling with GFAP is typical and is observed in retinas of all mice. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer.

greatest likelihood of a beneficial outcome. A recent example of this involving a Sigma1R ligand was a report from Shimazawa et al.<sup>38</sup> in which the effects of cutanesine dihydrochloride (SA4503) were investigated in a light-induced mouse model of photoreceptor degeneration. In this case, the mice were pretreated with the Sigma1R ligand (intravitreal injection) 1 hour prior to exposure to intense light. The effects of cutanesine on the photoreceptor cell loss were impressive, but it is not known whether administering the ligand after the exposure to intense light would be protective. Similarly, Sun et al.<sup>39</sup> induced ganglion cell death in rats using episcleral vein cauterization (EVC) and tested whether the Sigma1R ligand pregnenolone sulfate would lower EVC-induced increase in intraocular pressure (IOP). They observed normalization of IOP in this treatment regimen, but again it is not known whether the treatment could lower IOP if administered after the EVC insult. Our findings that administration of (+)-PTZ after onset of diabetes can afford some degree of neuroprotection is important because most human patients would not receive treatment at the onset of disease. Our findings that gliosis was attenuated as shown by decreased GFAP expression in the *Ins2<sup>Akita/+</sup>* mouse retina, even though (+)-PTZ administration was delayed until after diabetes onset, is noteworthy given the recent report that activation of Sigma1R using PRE-084 inhibits osmotic swelling of retinal Müller glial cells and may contribute to the neuroprotective properties of Sigma1R ligands.<sup>26</sup>

(+)-Pentazocine is a potent and highly specific Sigma1R ligand.<sup>40</sup> It binds Sigma1R with a very high affinity and is presumed to be an agonist for the receptor; however, because the actual signaling events associated with Sigma1R activation have not been characterized, it is difficult to ascribe definitively an agonist/antagonist function to this ligand. There is convincing evidence that (+)-PTZ is specific for Sigma1R because in experiments where the gene is absent, (+)-PTZ does not afford protection.<sup>25,28,41,42</sup> The preservation of retinal structure and attenuated neuronal death do not appear to be a direct function of decreased hyperglycemia because (+)-PTZ-injected *Ins2<sup>Akita/+</sup>* mice continue to have elevated blood glucose throughout the study. Retinal neuronal loss associated with diabetes may not involve hyperglycemia directly; rather, it may be due to complications secondary to hyperglycemia such as oxidative stress.<sup>43</sup>

The beneficial effects of (+)-PTZ administered at the onset of diabetes,<sup>8</sup> as well as after onset of diabetes reported herein, suggest that activation of the Sigma1R may be a promising treatment strategy for retinal degenerative diseases. The precise physiologic role of Sigma1R in retina is not known; however, findings from many studies support the notion that Sigma1R is a molecular chaperone and modulates cellular stress. Sigma1R has been shown to modulate oxidative stress via Nrf2 signaling,<sup>42</sup> to activate extracellular signal-regulated kinases1/2,<sup>44,45</sup> to suppress inflammatory responses in microglial cells,<sup>45</sup> to attenuate calcium influx in ganglion cells,<sup>28</sup> to modulate voltage-gated calcium channels,<sup>29</sup> to decrease retinal glial cytokine release and promote cytosolic-to-nuclear NF-κB translocation,<sup>42</sup> and to modulate ER stress.<sup>25,27</sup>

The multiple protective functions of Sigma1R prompted the present study to investigate whether absence of Sigma1R would accelerate the retinal phenotype in *Ins2<sup>Akita/+</sup>* mice. *Sig1R<sup>-/-</sup>* mice have a late-onset retinal degeneration characterized by optic nerve axonal degeneration at approximately 6 months and progressive loss of ganglion cells by 1 year; the retina is normal in appearance in the first few months of life.<sup>30</sup> No retinal vasculopathy has been reported in *Sig1R<sup>-/-</sup>* mice. In the present study, we evaluated retinas of *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice and compared them with *Ins2<sup>Akita/+</sup>* mice at 12 and 16 weeks, time points earlier than the reported ganglion cell loss (25 weeks). We found that ganglion cell loss was minimal in

*Ins2<sup>Akita/+</sup>* mice at 12 and 16 weeks but was significantly worse in *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice, particularly at 16 weeks. The data suggest that in the absence of Sigma1R, retinal neuronal loss is accelerated. The data were confirmed by detection of significantly more TUNEL-positive cells (indicative of apoptosis), in retinas of *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice compared with WT or *Ins2<sup>Akita/+</sup>* mice. In addition, the NFL examined in retinal sections was fragmented and disrupted in *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice. This was not due simply to fixation artifacts because OCT analysis showed that, although the total retinal thickness and most of the individual retinal layers did not differ from WT, there was a significant increase in the NFL thickness in the *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice.

In addition to OCT analysis, imaging of the retinal vessels of *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice showed subtle vascular changes that are not typically observed in *Ins2<sup>Akita/+</sup>* mice.<sup>7,46</sup> Especially at 16 weeks, FA revealed vessel beading and occasional capillary dropout. The findings suggest that despite the minimal vascular involvement of retina in *Ins2<sup>Akita/+</sup>* mice normally,<sup>7,46</sup> lack of Sigma1R increases slightly the incidence of vascular alterations. In addition to neuronal and vascular features, the *Ins2<sup>Akita/+</sup>/Sig1R<sup>-/-</sup>* mice also demonstrate significant increase in GFAP compared with *Ins2<sup>Akita/+</sup>* mice, suggesting that retinal gliosis, which has been reported in the *Ins2<sup>Akita/+</sup>* mice, is more pronounced when Sigma1R is absent. The findings suggest a role for Sigma1R as a modulator of stress; in the absence of Sigma1R, there appears to be heightened sensitivity to stress.

Taken collectively, the data from the present study provide insights into the role of Sigma1R in retinal disease. The findings that (1) administration of a high affinity Sigma1R ligand after onset of diabetes affords a degree of retinal neuroprotection and (2) absence of the receptor in diabetic retinopathy worsens the phenotype suggest that Sigma1R may have an important role in preservation of retinal structure. Ligands for Sigma1R are in clinical trials for treatment of neurodegenerative diseases. It remains to be determined whether other types of retinal degeneration, for example, glaucoma, will respond positively to targeting of Sigma1R. In addition it will be important to explore targeting Sigma1R in treatment of other more severe retinopathies including photoreceptor cell degeneration.

There is an additional aspect of the present study that warrants discussion, which concerns the retinal phenotype of the *Ins2<sup>Akita/+</sup>* diabetic mouse. Several years ago, our group published in *Investigative Ophthalmology and Visual Science* observations about the protective effects of (+)-PTZ<sup>8</sup> in *Ins2<sup>Akita/+</sup>* diabetic mice. In that study, we observed and reported a very severe retinal phenotype in *Ins2<sup>Akita/+</sup>* mice affecting the INL and the GCL. Barber and others<sup>5</sup> had initially reported the ganglion cell loss and decreased thickness of INL in this mouse, although they did not report a pronounced disruption as we had observed. We do not know what factors caused the *Ins2<sup>Akita/+</sup>* mice to have such a disrupted retinal architecture in our colony at that time. For several years thereafter, we did not use the *Ins2<sup>Akita/+</sup>* mice in our studies. When we reestablished our colony more recently, we again purchased the mice from the Jackson Laboratories (the source of the original mice), but the animals derived from establishing the new colony showed a much milder phenotype, which is reported here. The ganglion cell loss, reported initially by Barber and others<sup>5</sup> and by our group,<sup>8</sup> is still a characteristic feature of this mouse model, but the fulminant retinopathy we observed<sup>8</sup> is not present. We cannot account for the apparent phenotypic drift that appears to have occurred between our initial studies and the current work. We do not know whether our original colony harbored the rd8 mutation; however, we do not think this was the case because the characteristic retinal

rosette pattern was not observed. We do not know whether a pathogen in our laboratory animal facilities was responsible. At any rate, the *Ins2<sup>Akita/+</sup>* mice phenotype observed and reported in the present work is very similar to that observed by other investigators,<sup>5</sup> and the severe phenotype is no longer detectable in our animals.

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