# Topical Administration of Somatostatin Prevents Retinal Neurodegeneration in Experimental Diabetes

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Retinal neurodegeneration is an early event in the pathogenesis of diabetic retinopathy (DR). Somatostatin (SST) is an endogenous neuroprotective peptide that is downregulated in the diabetic eye. The aim of the study was to test the usefulness of topical administration of SST in preventing retinal neurodegeneration. For this purpose, rats with streptozotocin-induced diabetes mellitus (STZ-DM) were treated with either SST eye drops or vehicle for 15 days. Nondiabetic rats treated with vehicle served as a control group. Functional abnormalities were assessed by electroretinography (ERG), and neurodegeneration was assessed by measuring glial activation and the apoptotic rate. In addition, proapoptotic (FasL, Bid, and activation of caspase-8 and caspase-3) and survival signaling pathways (BclxL) were examined. Intraretinal concentrations of glutamate and its main transporter glutamate/aspartate transporter (GLAST) were also determined. Treatment with SST eye drops prevented ERG abnormalities, glial activation, apoptosis, and the misbalance between proapoptotic and survival signaling detected in STZ-DM rats. In addition, SST eye drops inhibited glutamate accumulation in the retina and GLAST downregulation induced by diabetes mellitus. We conclude that topical administration of SST has a potent effect in preventing retinal neurodegeneration induced by diabetes mellitus. In addition, our findings open up a new preventive pharmacological strategy targeted to early stages of DR. ***Diabetes*** **62:2569–2578, 2013**

Diabetic retinopathy (DR) has been classically considered to be a microcirculatory disease of the retina. However, there is growing evidence to suggest that retinal neurodegeneration is an early event in the pathogenesis of DR that participates in the microcirculatory abnormalities that occur in DR (1–8). We have contributed to this field by demonstrating that the main hallmarks of retinal neurodegeneration (apoptosis and glial activation) are already present in the retinas of diabetic donors without any microcirculatory abnormalities in ophthalmoscopic examinations performed during the year before death (9–11).

Glutamate is the major retinal excitatory neurotransmitter for the photoreceptor-bipolar-ganglion cell circuit. However, elevated glutamate levels in the retina (which result in excessive stimulation) are implicated in the so-called "excitotoxicity," which leads to neurodegeneration (12). The excitotoxicity of glutamate is the result of overactivation of ionotropic glutamate receptors, which have been found to be overexpressed in rats with streptozotocin-induced diabetes mellitus (STZ-DM) (13,14). Apart from glutamate, oxidative stress (15), advanced glycation end product receptor upregulation (16,17), and renin-angiotensin system activation (18–20) play an essential role in retinal neurodegeneration induced by diabetes mellitus. Finally, recent evidence indicates that diabetes mellitus–induced downregulation of neuroprotective factors synthesized by the retina is also involved in the neurodegenerative process of the diabetic eye (21). On these bases, it is reasonable to hypothesize that therapeutic strategies based on neuroprotection will be effective in preventing or arresting DR development.

Somatostatin (SST) is one of the most important neuroprotective factors synthesized by the retina, retinal pigment epithelium (RPE) being its main source in the human eye. The human retina produces significant amounts of SST, as deduced by the strikingly high levels reported within the vitreous fluid (22,23). Besides SST, its receptors (SSTRs) are also expressed in the retina, with SSTR1 and SSTR2 being the most widely expressed (24). The production of both SST and its receptors simultaneously suggests a relevant autocrine action in the human retina. SST acts as a neuromodulator in the retina through multiple pathways, including intracellular $Ca^{2+}$ signaling, nitric oxide function, and glutamate release from the photoreceptors. Apart from neuroprotection, SST has potent antiangiogenic properties and regulates various ion/water transport systems (24). Therefore, SST seems to be essential in preventing both proliferative DR (PDR) and diabetic macular edema (DME).

In the early stages of DR, there is a downregulation of SST that is associated with retinal neurodegeneration (9). In fact, it has recently been reported that intravitreal administration of SST and SST analogs protects the retina from AMPA-induced neurotoxicity (25). In addition, the lower expression of SST in RPE and neuroretina is associated with a dramatic decrease of intavitreal SST levels in both DME (26) and PDR (22,23). As a result, the physiological role of SST in preventing both fluid accumulation within the retina and neovascularization could be reduced, and consequently, the development of DME and PDR would be favored. For all these reasons, SST replacement treatment can be considered a new target not only for preventing the neurodegenerative process but also for more advanced stages of DR such as DME and PDR.

When the early stages of DR are the therapeutic target, it would be inconceivable to recommend in the clinical practice an aggressive treatment such as intravitreous injections. On the other hand, the use of eye drops has not

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TABLE 1
Experimental design of the dose-efficacy study

| Group | STZ dose (mg/kg) | Treatment | Dose formulation (mg/mL) |
|---|---|---|---|
| A | 0 | Vehicle | — |
| B | 60 | Vehicle | — |
| C | 60 | SST | 0.5 |
| D | 60 | SST | 2 |
| E | 60 | SST | 10 |

$n$ = 8 in all groups.

been considered an appropriate route for the administration of drugs aimed at preventing or arresting DR because of the general assumption that they do not reach the posterior chamber of the eye. However, there is emerging evidence showing the capacity of a lot of drugs to reach the retina in pharmacologically effective concentrations, at least in animal models (27).

On this basis, the aim of the current study was to determine whether topical administration of SST prevents the retinal neurodegenerative process that occurs in STZ-induced diabetic rats. In addition, the effects of SST eye drops on apoptotic/survival signaling pathways and retinal glutamate levels have been examined. Finally, the possibility that SST acts through preventing glutamate accumulation by upregulating glutamate/aspartate transporter (GLAST) has also been explored.

## RESEARCH DESIGN AND METHODS

A total of 24 male Sprague-Dawley rats were obtained from Charles River Laboratories (Senneville, Quebec, Canada). A minimum acclimatization period of 14 days preceded treatment. STZ (60 mg/kg) was administered to 8-week-old ($n$ = 16) rats by intravenous injection via tail vein on day −2 prior to starting SST treatment. The remaining eight rats were injected with vehicle and served as a nondiabetic control group. Animals with blood glucose levels >250 mg/dL the day after STZ administration were considered diabetic. On day −1, all animals were weighed and randomly assigned to treatment groups (vehicle or SST) using a computer-based randomization procedure. Randomization was performed by stratification using body weight as the parameter.

SST or vehicle eye drops were administered directly onto the superior corneal surface of each eye using a syringe. One drop (20 μL) of SST (1%; 10 mg/mL) or vehicle (20 μL of 0.9% sodium chloride) was administered once daily for 14 days. On day 15, the animals' eyes were instilled with a drop of SST or vehicle ~1 h prior to necropsy. Therefore, the elapsed time between the onset of diabetes mellitus until necropsy was 16 days.

A dose-response experiment assessing the hallmarks of retinal neurodegeneration (apoptosis and glial activation) was performed using SST eye drops at 10, 2, and 0.5 mg/mL (Table 1).

The animals used in this study were treated in accordance with the Association for Research in Vision and Ophthalmology Statement.

**Pharmacokinetic analyses.** A pharmacokinetic analysis after a single topical dose of radiolabeled $^{125}$I-somatostatin was performed. For this purpose, seven rats were anesthetized and placed in a supine position. After they were placed in the experimental area, 10 μL (2.5 μCi) $^{125}$I-somatostatin was applied in each eye with a micropipette. The animals were killed at 15, 30, and 60 min and 4, 6, 12 and 24 h after the application of the labeled SST solution. After the animals were killed, the eyes were obtained and gently washed in a volume of 20 mL physiologic saline. A small incision was performed in the rear portion of the eye ball, and the eye content was extracted. The vitreous humor, retina, and choroid-RPE were collected. The sclera was placed in different tubes for independent measurements. All the samples were weighted and counted in the γ counter in order to obtain the percentage of injected dose for each gram of tissue. Samples of blood were also obtained.

In addition, absorption of carboxyfluorecein-labeled SST (CF-SST) eye drops through the ocular structures was also evaluated in 8-week-old Sprague-Dawley rats. For this purpose, three rats were treated with vehicle eye drops in both eyes. Three rats were treated with the fluorescent label (CF) eye drops. The eye drops of CF-SST (10 mg/mL; one drop) were administered to the right eye, and the vehicle was administered to the left eye of three rats. One animal of each group was killed at 10, 30, and 60 min after the application of the corresponding eye drop. The eyes were enucleated, fixed in 4% paraformaldehyde, submerged in optimum cutting temperature Tissue-teck, frozen in liquid nitrogen, and stored at −80°C until microtome sectioned. The whole eyes were cryosectioned to 50-μm sections, mounted with antifade-mounting medium with DAPI, and visualized in fluorescent and confocal microscopes (Olympus). All images were taken with the same settings.

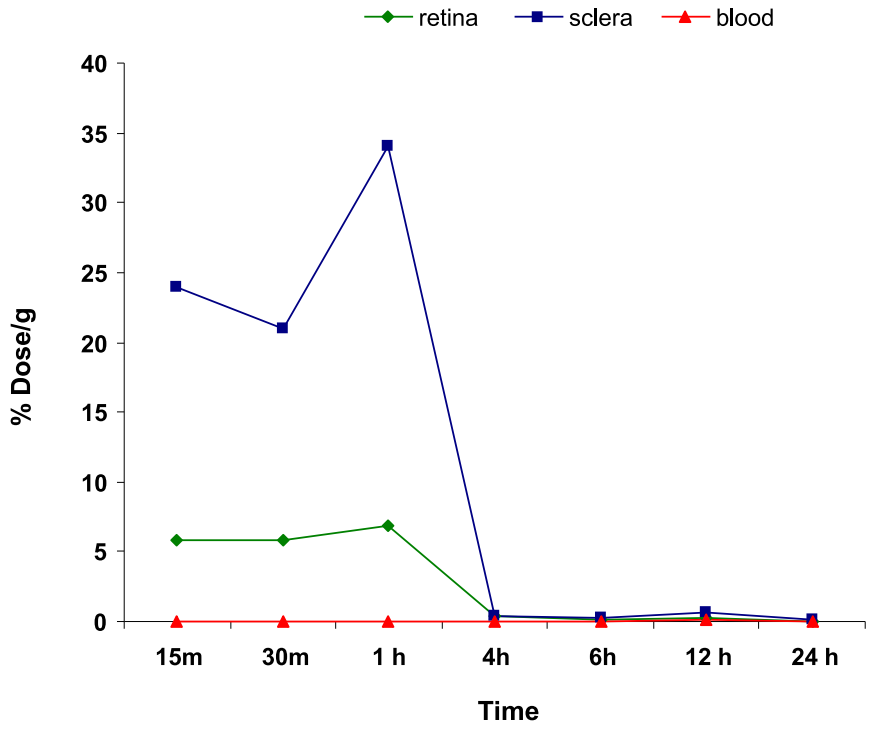



**FIG. 1. Ocular absorption of $^{125}$I-somatostatin expressed as the percentage of applied dose for each gram of tissue (percent dose/g) as a function of time. Uptake of the radioactivity in the eye content was clearly observed ($n$ = 1 for each time point).**

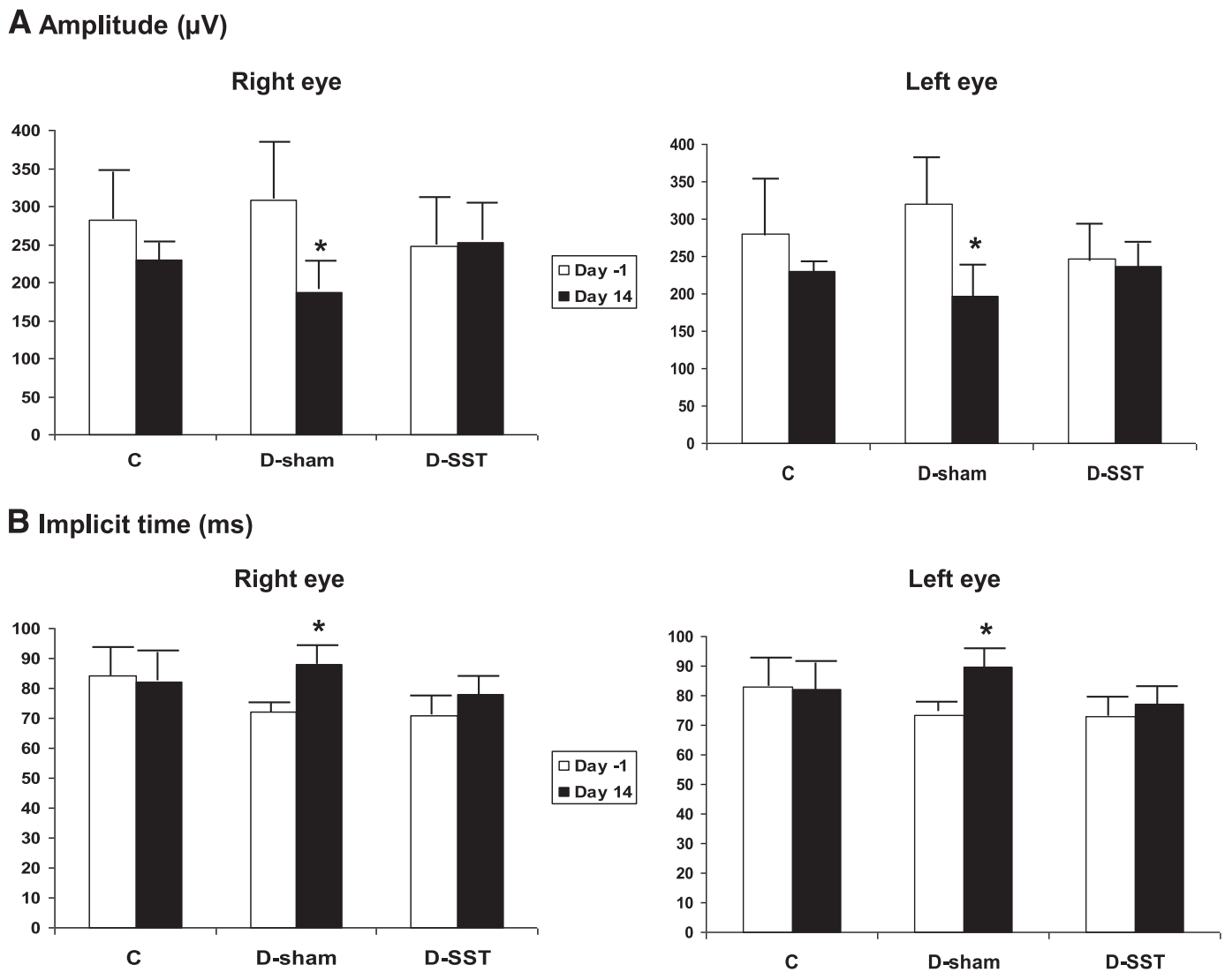



**FIG. 2. The b-wave amplitude (*A*) and implicit time (*B*) under scotopic conditions (0 db) in both eyes at day −1 (□) and at day 14 (■) in the studied groups. C, control rats; D-sham, diabetic rats treated with vehicle eye drops; D-SST, diabetic rats treated with SST eye drops. Data are means ± SD. **P* < 0.05.**

**Ophthalmological examinations.** Slit lamp biomicroscopy, indirect ophthalmoscopy, and intraocular pressure measurement were performed in all animals at baseline and at days 7 and 13 posttreatment.

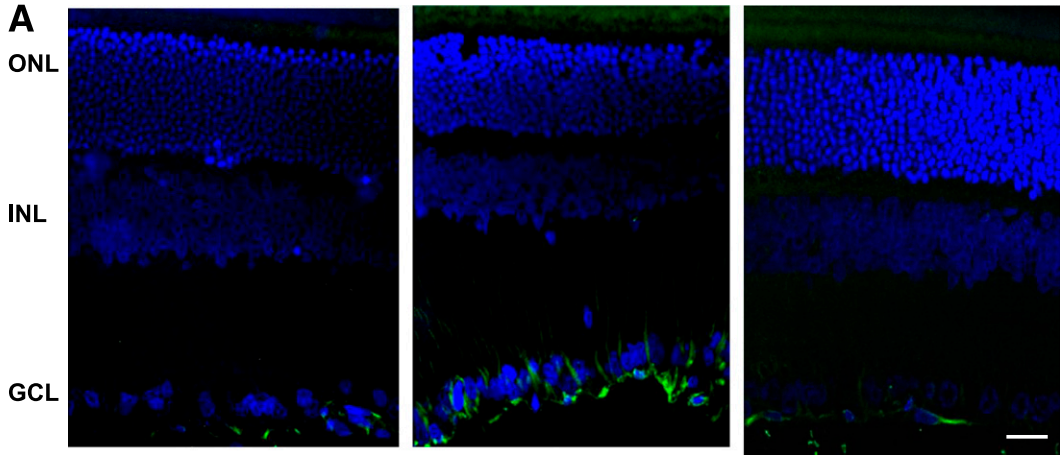
**Electroretinography.** Electroretinogram evaluations were performed on day −1 before treatment and on day 14 as follows: the animals were dark adapted for at least 1 h prior to electroretinography (ERG) recording and then anesthetized with isoflurane to maintain the anesthesia prior to and during the procedure. Tropicamide (1%) was applied to each eye prior to the test. A contact lens electrode was placed on the surface of each eye, and a needle electrode was placed cutaneously on the head between the two eyes. A cutaneous ground electrode was placed near the base of the tail. Carboxymethylcellulose (1%) drops were applied to the interior surface of the contact lens electrodes prior to their placement on the eyes. A topical anesthetic (proparacaine, 0.5%) was applied to the eyes. The amplitude and implicit time of the b-wave after scotopic flash stimuli were measured. b-wave amplitude and implicit time were measured as defined by the International Society for Clinical Electrophysiology of Vision (28).

**Histopathology.** Eyes were prepared for histopathological examination by being embedded in paraffin wax, sectioned, and stained with hematoxylin-eosin. For each eye, a total of three sagittal sections (one section through the optic nerve, one section ~500 μm medially, and another section ~500 μm laterally from the optic nerve) were evaluated.

***Assessment of glial activation.*** Sections of 7-μm thicknesses were obtained from the paraffin blocks and fixed on highly adherent slides (Visionbiosystems; Newcastle upon Tyne, U.K.). They were desparaffined in xylol; rehydrated in 100% ethanol, 90% ethanol, and 75% ethanol; and incubated for 10 min in PBS. Unspecific unions were blocked by incubating the samples for 1 h in PBS 1% BSA and 0.05% Triton X-100. Thereafter, the primary rabbit anti-human glial fibrillar acidic protein (GFAP) antibody (Sigma, Madrid, Spain) diluted in the blocking buffer (1:100) was incubated for 36 h at 4°C. After three washings for 5 min with PBS, the sections were incubated with a secondary human IgG antibody labeled with Alexa Fluor 568 (Invitrogen, Eugene, OR) for 1 h at room temperature. The labeled sections were washed and mounted with fluorescent medium containing DAPI for identifying cell nuclei (Vector Laboratories, Burlingame, CA). Positive sections for GFAP were captured in a confocal microscope (FV1000; Olympus, Hamburg, Germany). Optical sections were obtained with a 568-nm laser for Alexa Fluor 568 and a 405-nm laser for the DAPI (image resolution 1,024 × 1,024 pixels). The degree of glial activation was evaluated by using a score system based on the extent of GFAP staining (29). This scoring system is as follows: negligible staining (score 0), Müller cell endfeet region/ganglion cell layer (GCL) only (score 1), Müller cell endfeet region/GCL plus a few proximal processes (score 2), Müller cell endfeet plus many processes but not extending to outer nuclear layer (ONL) (score 3), Müller cell endfeet plus processes throughout with some in the ONL (score 4), and Müller cell endfeet plus a lot of dark processes from GCL to the outer margin of ONL (score 5).

***Assessment of apoptosis.*** The In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany) was used to evaluate the presence of apoptotic cells. Sections were deparaffined in xylol; rehydrated in 100% ethanol, 90% ethanol, and 75% ethanol; and incubated for 10 min in PBS. Autofluorescence

A




B

| | Control | D-Sham | D-SST |
|---|---|---|---|
| Score 0 | 50% | 0% | 50% |
| Score 1 | 50% | 25% | 50% |
| Score 2 | 0% | 50% | 0% |
| Score 3 | 0% | 25% | 0% |
| Score 4 | 0% | 0% | 0% |
| Score 5 | 0% | 0% | 0% |

**FIG. 3. GFAP immunofluorescence. *A*: Images of representative samples of retina from a control rat (Control), a diabetic rat treated with vehicle eye drops (D-Sham), and a diabetic rat treated with SST eye drops (D-SST). In diabetic rats, the endfeet of the Müller cells showed abundant GFAP immunofluorescence (green), and the radial processed stained intensely throughout the inner retina. The topical treatment with SST prevents the glial activation. Scale bar = 50 μm. *B*: Quantification of glial activation based on extent of GFAP staining. INL, inner nuclear layer.**

was eliminated after incubation with 0.2% potassium permanganate for 20 min and 1% oxalic acid for 30 s before the TUNEL procedure was started. Positive and negative controls were processed at the same time. Three confocal images were recorded (×40) corresponding to a surface of 317.13 × 317.13 μm for each section. Staining with propidium iodide was done to examine nucleus morphology and discard false positives. The total number of nuclei and nuclei positive for TUNEL were counted with the ImageJ program.

***Proapoptotic and survival signaling.*** Proapoptotic signaling pathways were investigated by analyzing several proapoptotic molecules (FasL, caspase-8, total Bid, truncated Bid, and active caspase-3), as well as antiapoptotic markers (BclxL), by Western blot as previously described (30).

**Glutamate quantification.** Quantification of glutamate was performed by liquid chromatography coupled to mass spectrometry. Chromatographic separation was performed on an Agilent 1200 series (Waldbronn, Germany) using an Ascentis Express HILIC column, 50 × 2.1 mm with 2.7 μm particle size from Supelco (Belfonte, PA), maintained at 25°C throughout the analysis, a mobile phase acetonitrile (ACN), and water (50 mmol/L ammonium acetate) with a flow rate of 0.6 mL/min. The volume injected was 10 μL. The mobile phase involved a gradient starting at 87% of ACN, which was maintained for 3 min. Then, from min 3 to 10 the ACN content was decreased to 20% and increased again to 87% at min 12.5. Glutamate was eluted at 6.5 min. The mass detection system was an Agilent 6410 Triple Quad (Santa Clara, CA) using positive electrospray ionization with a gas temperature of 350°C, gas flow rate of 12 L/min, nebulizer pressure of 45 ψ, capillary voltage of 3,500 V, fragmentor of 135 V, and collision energy of 10 V.

**GLAST expression**

***RNA isolation and GLAST mRNA quantification.*** Total RNA was extracted from retinas with the Rneasy Mini kit with DNAase digestion (Qiagen; IZASA, Madrid, Spain). One microgram of total RNA was used for reverse transcription with TaqMan and random primer reagents (Applied Biosystems, Madrid, Spain). Real-time PCR was performed with the GLAST specific assay for exon 3–4 boundary Rn00570130_m1 (Applied Biosystems), and results were normalized to the expression level of β-actin (Rn00667969_m1) as a housekeeping gene. Relative quantification values were obtained using an ABI Prism 7000 SDS software (Applied Biosystems).

***Immunohistochemistry for GLAST.*** Sections of 4 μm were deparaffined in xylene and hydrated in a graded ethanol series. Then, sections were antigen retrieved (sodium citrate 10 mmol/L, pH 6.0) and washed in PBS. Next, sections were incubated in blocking solution (2% BSA, Tween 0.05% PBS) for 1 h at room temperature followed by incubation with primary antibody anti-GLAST (1:200; Abcam, Cambridge, U.K.). After washing, sections were incubated with a fluorescent anti-rabbit Alexa Fluor 488 as a secondary antibody (Life Technologies, Madrid, Spain) in blocking solution for 1 h, washed, and mounted in Vectashield (Vector Laboratories). DAPI was used for nuclear staining. Quantification of fluorescence intensity of images was performed as previously explained for GFAP.

**Statistical analysis.** Normal distribution of the variables was evaluated using the Kolmogorov-Smirnov test. Data were expressed either as means ± SD or as median (range). Comparisons of continuous variables between groups were performed using the unpaired Student $t$ test or Mann-Whitney $U$ test. For comparisons within groups (ERG of day 14 vs. ERG of day −1), the paired Student $t$ test was used. Levels of statistical significance were set at $P < 0.05$.

## RESULTS

**SST contained in eye drops reached the retina.** The results of pharmacokinetic study are displayed in Fig. 1. After topical ocular administration of $^{125}$I-somatostatin, the maximum postdose concentration (5% of applied dose per gram of tissue) was detected at 1 h. However, at 15 min most of the $^{125}$I-somatostatin had already reached the retina. This rapid absorption argues against the permeation of SST through the cornea. For further exploration of this issue, a pharmacokinetic study using eye drops containing CF-SST was performed. This study clearly showed that SST contained in the eye drops reached the retina not through the cornea but by the trans-scleral route, thus bypassing the anterior chamber (Supplementary Fig. 1).

**Ocular safety of SST treatment.** No significant differences in the cornea, pupil size, or intraocular pressure were observed among the three groups at baseline or during follow-up.

**SST treatment prevents ERG abnormalities.** ERG was performed to assess retinal function. b-wave amplitude was significantly reduced at day 14 in the diabetic group treated with vehicle (Fig. 2*A*). By contrast, in the control group and in the diabetic group treated with SST eye drops no reduction of b-wave amplitude was observed. b-wave implicit time increased at day 14 in diabetic rats treated with vehicle (Fig. 2*B*). However, in the control group and in the diabetic group treated with SST eye drops the implicit time did not increase at day 14. Therefore, SST treatment prevented ERG abnormalities caused by diabetes mellitus.

**Neurodegeneration was prevented in diabetic rats treated with SST eye drops**

***Glial activation.*** The retinas of nondiabetic control rats revealed GFAP immunolabeling in cells and their processes in the inner layer of the retina, especially in the inner limiting membrane, and GCL (GFAP score 0–1) (Fig. 3). According to their location and morphology, these GFAP-positive cells were interpreted as being retinal astrocytes. However, a slight GFAP immunofluorescence in Müller cells could be detected. In the retina of diabetic rats treated with placebo, GFAP expression was prominent along the inner limiting membrane, in Müller cell endfeet, and in Müller cell radial fibers extending through both the inner and outer retina (GFAP score 1–3). Diabetic rats treated with SST eye drops presented significantly lower GFAP score (0–1) than diabetic rats treated with placebo (Fig. 3).

***Retinal apoptosis.*** Representative images of the presence of apoptotic cells in the retinas of the three experimental groups are shown in Fig. 4*A*. Two weeks after the induction of diabetes mellitus with STZ, the whole percentage of retinal apoptotic cells, as well as the percentage of apoptotic cells in retinal layers, was significantly higher in comparison with that observed in retinas from age-matched nondiabetic controls (Fig. 4*B*). In all groups, apoptosis was highest in the GCL. Diabetic rats treated with SST eye drops presented a significantly lower ratio of apoptosis in all retinal layers than diabetic rats treated with placebo and similar to nondiabetic rats (Fig. 4*B*).

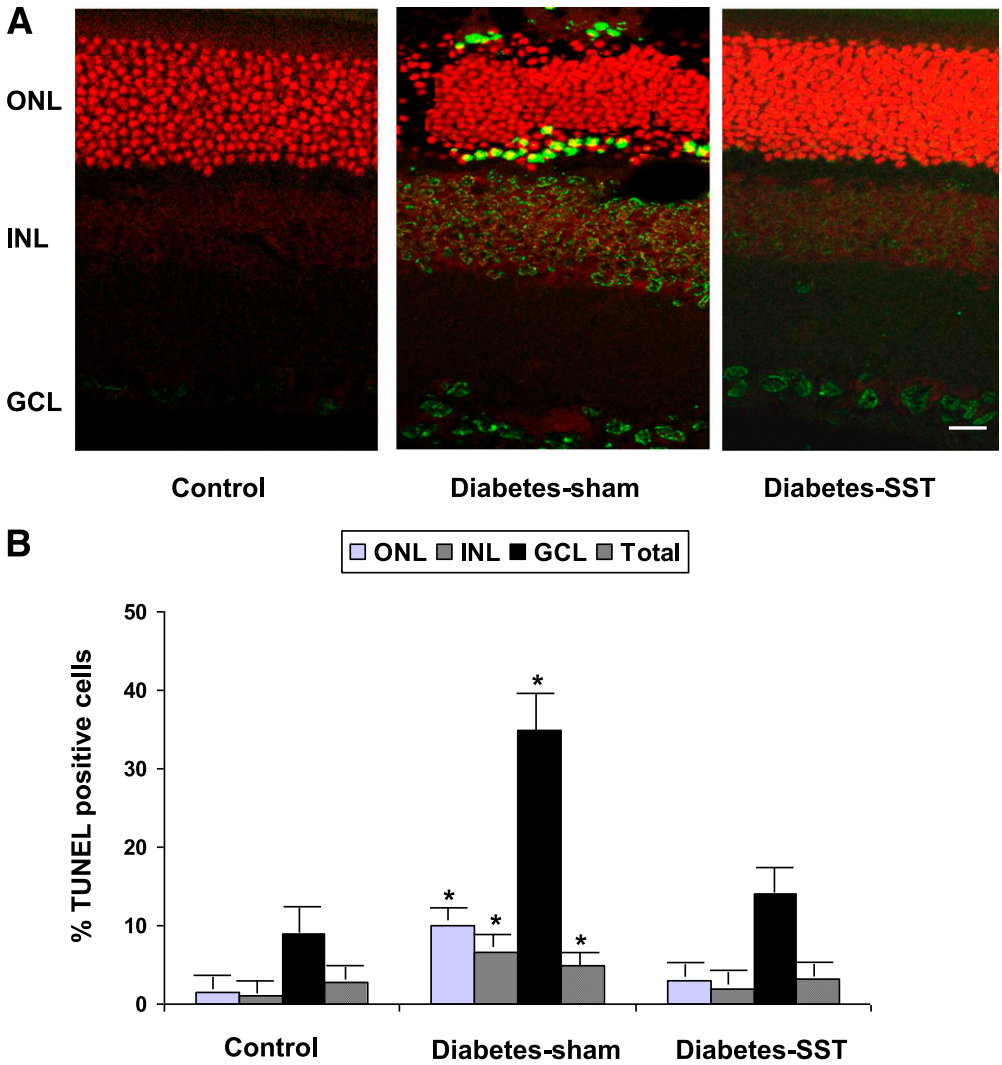


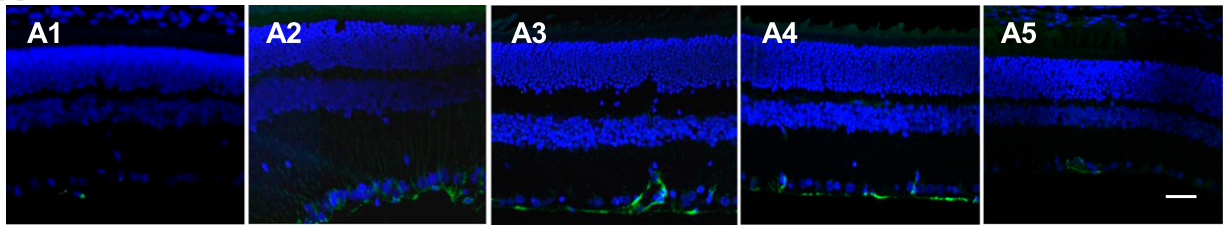

**FIG. 4.** *A*: Representative images of apoptosis in the retina from a control rat, a diabetic rat treated with vehicle eye drops (Diabetes-sham), and a diabetic rat treated with SST eye drops (Diabetes-SST). *B*: Percentage of apoptotic cells in the whole retina and in the retinal layers in each group. Results are expressed as means ± SD. INL, inner nuclear layer. **P* < 0.05 in comparison with control and diabetic SST treatment groups. Scale bar = 50 μm.

***Dose response effect.*** The effectiveness of SST eye drops at concentrations of 2 mg/mL was similar to that of eye drops at 10 mg/mL concentrations in preventing glial activation and apoptosis. However, the effect of 0.5 mg/mL was significantly lower than that obtained using either 2 or 10 mg/mL (Fig. 5).

***Proapoptotic and survival molecules.*** The results of proapoptotic and survival molecules measured in the retina of the three rat groups studied are shown in Fig. 6. The expression of FasL, a proapoptotic component of the death receptor apoptotic pathway, was significantly increased in neuroretinas from diabetic rats compared with control rats. The interaction of FasL with the death receptor Fas/CD95 assembles the death-inducing signaling complex (31). This includes the recruitment of caspase-8. Activation of caspase-8, monitored by the presence of its 18-kDa active fragment, was significantly increased in neuroretinas of diabetic rats compared with controls. These data were reinforced by the detection of elevated levels of truncated Bid fragment in diabetic samples. As a result, neuroretina from diabetic rats displayed a significant activation of the executer caspase-3, monitored by the presence of its 17-kDa active fragment. Treatment with SST eye drops prevented the upregulation of all of these proapoptotic molecules.

On the other hand, a reduction in BclxL, an antiapoptotic member of the Bcl2 family, was found in diabetic retinas in comparison with control retinas. When treated

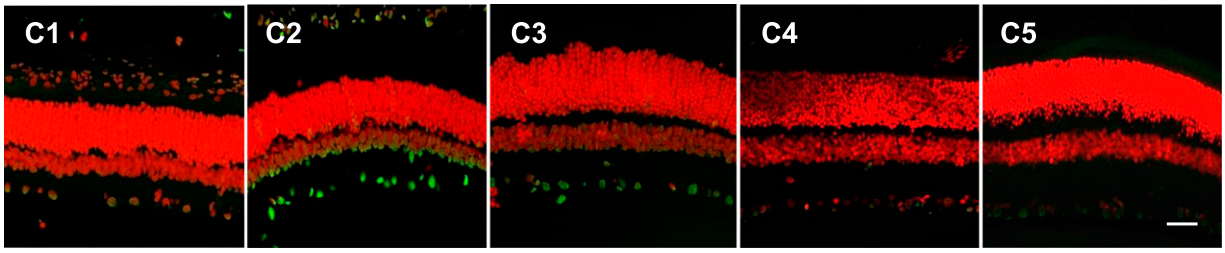
A




B

| | Control | D-sham | D-SST (0.5 mg/mL) | D-SST (2 mg/mL) | D-SST (10 mg/mL) |
|---|---|---|---|---|---|
| Score 0 | 62.5% | 0% | 37.5% | 50% | 50% |
| Score 1 | 37.5% | 25% | 37.5% | 50% | 50% |
| Score 2 | 0% | 50% | 12.5% | 0% | 0% |
| Score 3 | 0% | 25% | 12.5% | 0% | 0% |
| Score 4 | 0% | 0% | 0% | 0% | 0% |
| Score 5 | 0% | 0% | 0% | 0% | 0% |

C




D

| | Control | D-sham | D-SST (0.5 mg/mL) | D-SST (2 mg/mL) | D-SST (10 mg/mL) |
|---|---|---|---|---|---|
| ONL | 0 (0-1.2)* | 6.9 (0.4-12.7) | 0.9 (0-4.2)* | 1.2 (0-6.7)* | 0.2 (0-4.3)* |
| INL | 2.7 (0-23.6)* | 25.5 (2.6-57.3) | 9.9 (0-14.1)* | 2.6 (0-16.3)* | 1 (0-17.6)* |
| GCL | 29.9 (0-48.9)* | 49.8 (33-78) | 48 (8.4-56.2) | 26.6 (0-45)* | 22.5 (6.4-47.5)* |

**FIG. 5. Dose-response effect of SST eye drops on retinal neurodegeneration. *A*: GFAP immunofluorescence (green) corresponding to representative samples of retina from a control rat (A1), a diabetic rat treated with vehicle eye drops (A2), and diabetic rats treated with SST eye drops at doses of 0.5 mg/mL (A3), 2 mg/mL (A4), and 10 mg/mL (A5). *B*: Quantification of glial activation based on extent of GFAP staining corresponding to all rats ($n$ = 8) of each group. *C*: Images of apoptosis assessed by TUNEL assay (green) in representative samples of retina from a control rat (C1), a diabetic rat treated with vehicle eye drops (C2), and diabetic rats treated with SST eye drops at doses of 0.5 mg/mL (C3), 2 mg/mL (C4), and 10 mg/mL (C5). *D*: Percentage of apoptotic cells in the retinal layers in each group ($n$ = 8). Results are expressed as median (range). Control, control rats; D-sham, diabetic rats treated with vehicle eye drops; D-SST, diabetic rats treated with SST eye drops; INL, inner nuclear layer. *$P$ < 0.05 in comparison with diabetic group treated with vehicle. Scale bar = 20 μm.**

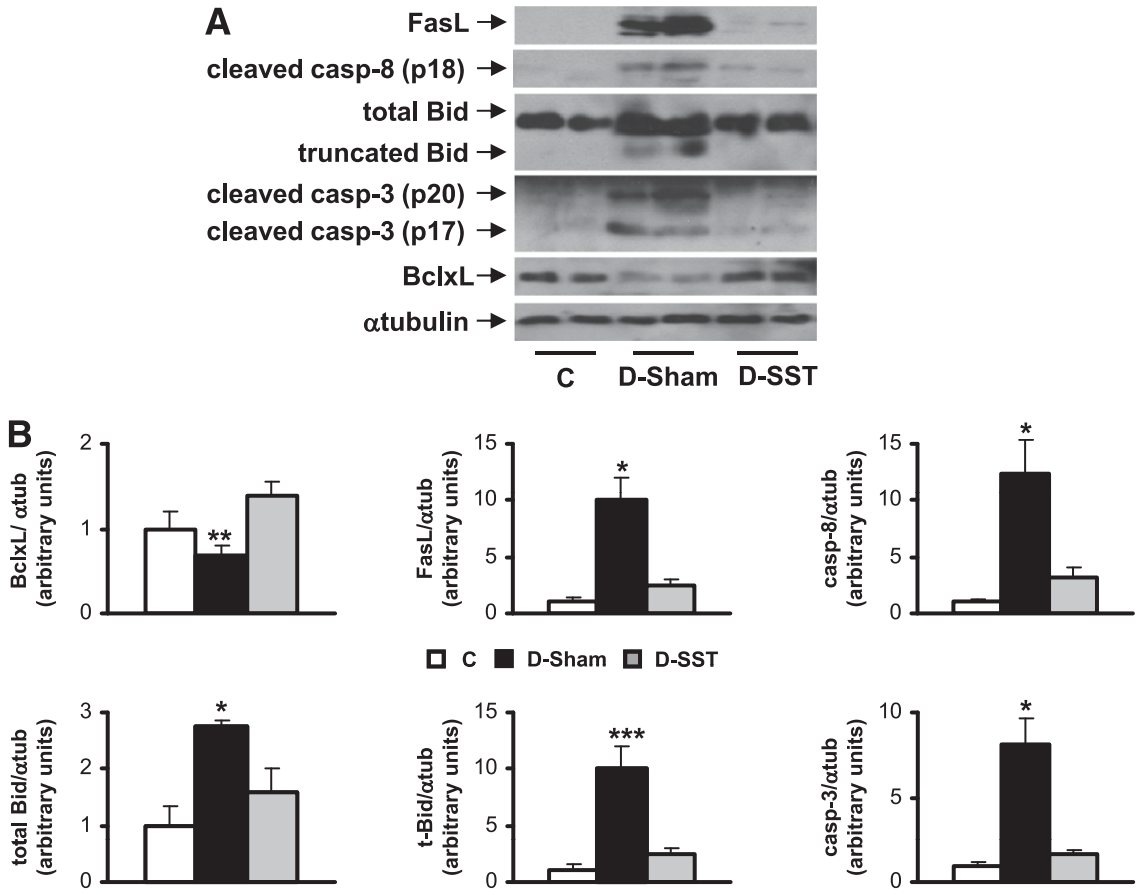



**FIG. 6. Apoptotic signaling pathways in the neuroretina of three groups. *A*: Protein extracts were prepared from neuroretina. Total protein (50 μg) was used for Western blot analysis with the antibodies against FasL, caspase (casp)-8, Bid, Bim, BclxL, and active caspase-3. α-Tubulin (αtub) antibody was used as a loading control. *B*: Autoradiograms were quantified by scanning densitometry. C, control rats; D-Sham, diabetic rats treated with vehicle eye drops; D-SST, diabetic rats treated with SST eye drops. Results are expressed as arbitrary units of protein expression and are means ± SD. **P* < 0.05, ***P* < 0.01, and ****P* < 0.005 for diabetic mice treated with the vehicle vs. diabetic mice treated with SST eye drops.**

with SST eye drops, BclxL expression was greatly improved (Fig. 6).

**Topical administration of SST prevents the increase of glutamate induced by diabetes mellitus.** Glutamate levels (arbitrary units, median [range]) in the diabetic retinas were higher than in nondiabetic retinas (117 [57–412] vs. 47 [27–300]; $P = 0.012$). In diabetic rats treated with SST eye drops, glutamate concentration (40 [21–127]) was significantly decreased in comparison with diabetic rats treated with vehicle ($P = 0.021$) and similar to control rats ($P = 0.51$).

**Upregulation of GLAST expression after SST treatment.** Gene and protein expression of GLAST were downregulated in retinas of diabetic rats (Fig. 7). In diabetic rats treated with SST, this downregulation was prevented and GLAST protein levels were even increased in comparison with nondiabetic rats (Fig. 7).

## DISCUSSION

In the current study, we provide the first evidence that topical administration of SST prevents retinal neurodegeneration in STZ-DM rats. SST eye drops prevented b-wave abnormalities in the ERG (reduction in amplitude and increase in implicit time), which are considered sensitive indicators of DR (32). In addition, topical administration of SST abrogated the characteristic hallmarks of neurodegeneration (glial activation and apoptosis) caused by short-term diabetes mellitus. In this regard, it should be noted that the degree of glial activation and apoptosis in diabetic rats treated with SST eye drops was very similar to that found in nondiabetic rats.

Glial activation is a prominent event in retinal neurodegeneration. Retinal astrocytes normally express GFAP, while Müller cells do not. However, during the course of diabetes mellitus there is an aberrant expression of GFAP by Müller cells (33). Because Müller cells produce factors capable of modulating blood flow, vascular permeability, and cell survival and because their processes surround all the blood vessels in the retina, it seems that these cells play a key role in the pathogenesis of retinal microangiopathy in the diabetic eye (33,34). In this paper, we have found that SST eye drops were able to significantly prevent glial activation or, in other words, most of the aberrant expression of GFAP in Müller cells.

Apart from glial activation, apoptosis is the other hallmark of retinal neurodegeneration. As previously reported (35), we found a significant rate of apoptosis in retinas from STZ-induced diabetic rats in comparison with nondiabetic control rats. In addition, we found a misbalance between apoptotic and survival signaling pathways. In this regard, we demonstrated that the activation of the death receptor pathway, monitored by the elevation in the expression of FasL and the activation of caspase-8, was

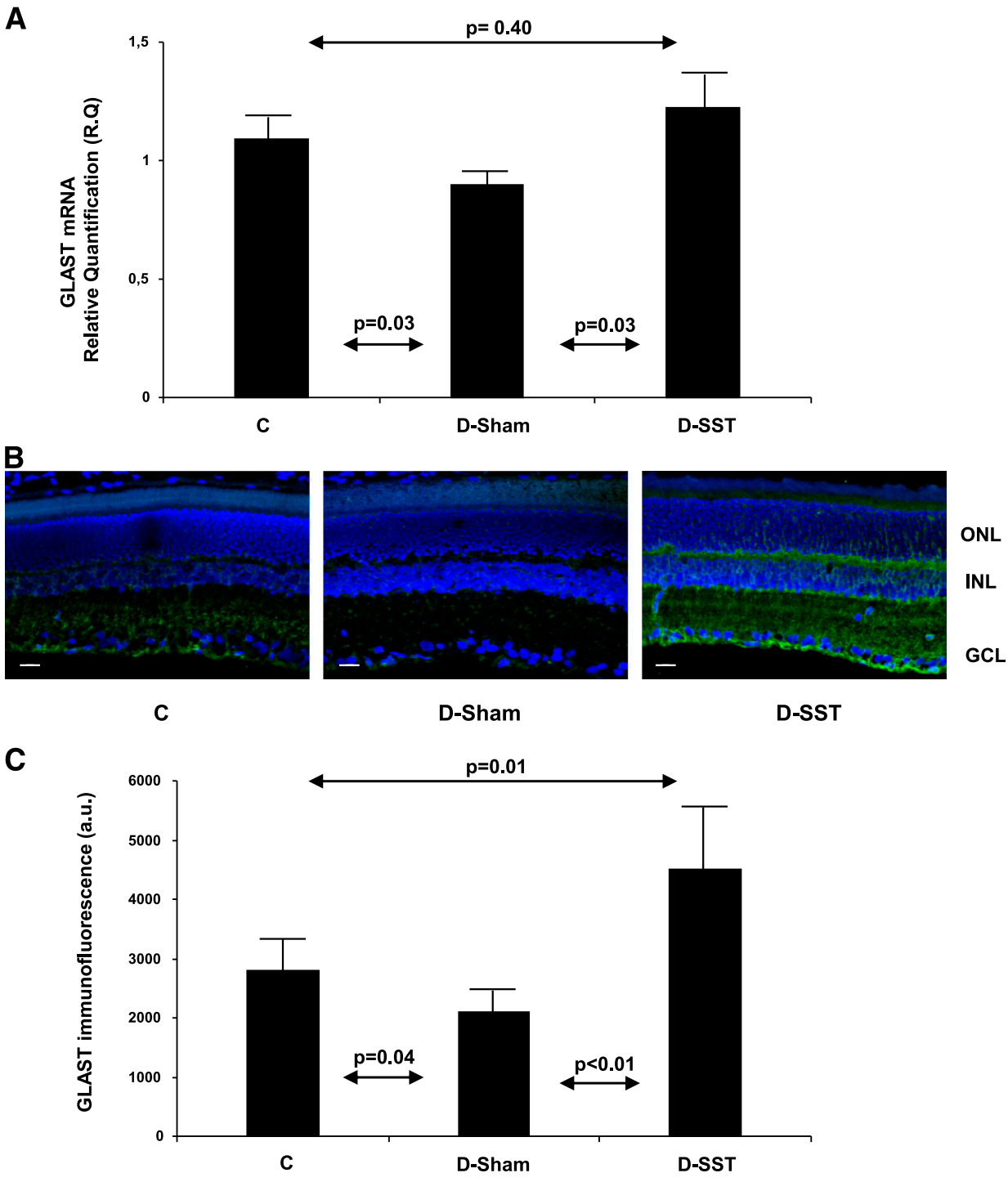



**FIG. 7.** *A*: GLAST mRNA in control rats (C), diabetic rats treated with vehicle eye drops (D-Sham), and diabetic rats treated with SST eye drops (D-SST). *B*: Immunofluorescence of GLAST in representative samples of retina from each group. *C*: Quantification of GLAST immunofluorescence in the three studied groups. Data are means ± SD. a.u., arbitrary units; INL, inner nuclear layer. Scale bar = 50 μm.

significantly higher in neuroretina from STZ-induced diabetic rats than in nondiabetic controls. Importantly, our data clearly show increased Bid cleavage in the neuroretina of diabetic rats. This result suggests that in the diabetic neuroretina, the apoptotic signals emerging from the death receptor pathway are amplified to additional activation of the intrinsic apoptotic signaling pathway, resulting in a stronger activation of the executer caspase-3. On the other hand, a significant downregulation of BclxL (an antiapoptotic molecule belonging to the Bcl2 family) was found in diabetic retinas. Notably, topical administration of SST was able to prevent all these abnormalities in proapoptotic/survival signaling.

Apart from the prevention of apoptosis and glial activation, SST eye drops significantly reduced glutamate retinal levels. Rapid removal or inactivation of glutamate is necessary to maintain the normal function of the retina and for avoiding excitotoxicity. Elevated levels of glutamate in the retina have been found in experimental models of diabetes mellitus (36–38), as well as in the vitreous fluid

of diabetic patients (12,39). The reasons why diabetes mellitus facilitates extracellular accumulation of glutamate include *1*) an increase of glutamate production by glial cells due to the loss of the Müller cell–specific enzyme glutamine synthetase, which converts glutamate to glutamine (36,37); *2*) a reduction in the retinal ability to oxidize glutamate to α-ketoglutarate (37); *3*) the death of neurons with subsequent release of intracellular contents; and *4*) the impairment of glutamate removal from extracellular space by the glial cells (12). Regarding this later point, in the current study we provide evidence that GLAST, the main glutamate transporter expressed by Müller cells (40), is significantly decreased in STZ-induced diabetic rats. More importantly, SST eye drops not only completely prevent GLAST downregulation induced by diabetes mellitus but even lead to a significant increase in GLAST expression (mRNA and protein). These findings can contribute to our understanding of the underlying mechanisms involved in the retinal reduction of AMPA-induced neurotoxicity reported for SST and SST analogs administered intravitreally (25).

The study of the mechanisms linking neurodegeneration with early vascular abnormalities is crucial for understanding how neuroprotection could also exert beneficial effects in diabetic microangiopathy. SST may inhibit angiogenesis directly through SST receptors present on endothelial cells (41) and also indirectly through the downregulation of vascular endothelial growth factor (VEGF) (42,43) or by the inhibition of postreceptor signaling events not only of VEGF but also of other peptide growth factors such as IGF-1, epidermal growth factor, and platelet-derived growth factor (44). The potential effects of SST in abrogating microvascular impairment are beyond the scope of the current study, and specific studies on this issue are urgently needed.

Given the essential role of neurodegeneration in the pathogenesis of DR, it is reasonable to hypothesize that therapeutic strategies based on the neuroprotective effects of SST would be effective in preventing or arresting DR development. The observation that circulating growth hormone–IGF-1 production is reduced by SST has been the basis for proposing systemic administration of SST analogs for treating DR. However, this concept has not been supported by clinical intervention trials. The main concerns of systemic administration of SST are as follows: *1*) the paracrine effects of SST synthesized by the retina involve SSTR subtypes other than SSTR2, which mediates growth hormone inhibition and for which octreotide presents high affinity, and *2*) SST analogs do not cross the blood-retinal barrier and would have access only where there is disruption of the blood-retinal barrier, thus limiting the amount of the drug reaching the retinal target tissues. For these reasons and given that a downregulation of retinal production of SST occurs in diabetic retina, a replacement using local administration of the natural peptide seems a reasonable approach. In this regard, the neuroprotective effects of topical administration of brimonidine and nerve growth factor have already been reported in experimental models (45–47). These findings open up the possibility of developing a topical therapy targeting the early stages of DR in which the use of the only currently established therapies such as laser photocoagulation or intravitreal injections of corticosteroids or anti-VEGF agents are inappropriately invasive. In addition, topical administration of drugs limits their action to the eye and minimizes the associated systemic effects, resulting in higher patient compliance (48). Therefore, topical therapies could revolutionize the care of diabetic patients (49). In this regard, a phase II-III, randomized controlled clinical trial (EUROCONDOR-278040) to assess the efficacy of SST and brimonidine administered topically to prevent or arrest DR has been approved by the European Commission in the setting of the FP7-HEALTH-2011.

Finally, the route by which SST contained in the eye drops reaches the retina deserves a brief comment. The pharmacokinetic study revealed a rapid absorption of $^{125}$I-SST, which was unexpected for a compound that reached the retina diffusing through the cornea. The study performed using CF-SST showed that the absorption of SST eye drops was mainly by the trans-scleral route.

In summary, we provide first evidence that topical administration of SST has a potent effect in preventing the retinal neurodegenerative process that occurs in the early stages of DR. A preventive effect on the impairment of survival/apoptotic signaling induced by diabetes mellitus and a significant reduction in glutamate-induced excitotoxicity are among the mechanisms by which SST exerts its beneficial actions.

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C.H. designed the project, led the analysis, wrote the manuscript, reviewed and edited the manuscript, and approved the final version of the manuscript. M.G.-R. and L.C. led the analysis, reviewed the manuscript, and approved the final version of the manuscript. J.F.-C., J.F.-S., and B.P. contributed to discussion, reviewed the manuscript, and approved the final version of the manuscript. A.G.-R. led the analysis, reviewed the manuscript, and approved the final version of the manuscript. A.M.V. contributed to discussion, reviewed the manuscript, and approved the final version of the manuscript. R.S. designed and coordinated the project, wrote the manuscript, reviewed and edited the manuscript, and approved the final version of the manuscript. C.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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## REFERENCES

1. Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest 1998;102:783–791
2. Lorenzi M, Gerhardinger C. Early cellular and molecular changes induced by diabetes in the retina. Diabetologia 2001;44:791–804
3. Barber AJ. A new view of diabetic retinopathy: a neurodegenerative disease of the eye. Prog Neuropsychopharmacol Biol Psychiatry 2003;27:283–290
4. Asnaghi V, Gerhardinger C, Hoehn T, Adeboje A, Lorenzi M. A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat. Diabetes 2003;52:506–511

5. Antonetti DA, Barber AJ, Bronson SK, et al.; JDRF Diabetic Retinopathy Center Group. Diabetic retinopathy: seeing beyond glucose-induced microvascular disease. Diabetes 2006;55:2401–2411
6. Fletcher EL, Phipps JA, Ward MM, Puthussery T, Wilkinson-Berka JL. Neuronal and glial cell abnormality as predictors of progression of diabetic retinopathy. Curr Pharm Des 2007;13:2699–2712
7. Barber AJ, Gardner TW, Abcouwer SF. The significance of vascular and neural apoptosis to the pathology of diabetic retinopathy. Invest Ophthalmol Vis Sci 2011;52:1156–1163
8. Antonetti DA, Klein R, Gardner TW. Diabetic retinopathy. N Engl J Med 2012;366:1227–1239
9. Carrasco E, Hernández C, Miralles A, Huguet P, Farrés J, Simó R. Lower somatostatin expression is an early event in diabetic retinopathy and is associated with retinal neurodegeneration. Diabetes Care 2007;30:2902–2908
10. Carrasco E, Hernández C, de Torres I, Farrés J, Simó R. Lowered cortistatin expression is an early event in the human diabetic retina and is associated with apoptosis and glial activation. Mol Vis 2008;14:1496–1502
11. Garcia-Ramírez M, Hernández C, Villarroel M, et al. Interphotoreceptor retinoid-binding protein (IRBP) is downregulated at early stages of diabetic retinopathy. Diabetologia 2009;52:2633–2641
12. Pulido JE, Pulido JS, Erie JC, et al. A role for excitatory amino acids in diabetic eye disease. Exp Diabetes Res 2007;2007:36150
13. Ng YK, Zeng XX, Ling EA. Expression of glutamate receptors and calcium-binding proteins in the retina of streptozotocin-induced diabetic rats. Brain Res 2004;1018:66–72
14. Santiago AR, Gaspar JM, Baptista FI, et al. Diabetes changes the levels of ionotropic glutamate receptors in the rat retina. Mol Vis 2009;15:1620–1630
15. Silva KC, Rosales MA, Biswas SK, Lopes de Faria JB, Lopes de Faria JM. Diabetic retinal neurodegeneration is associated with mitochondrial oxidative stress and is improved by an angiotensin receptor blocker in a model combining hypertension and diabetes. Diabetes 2009;58:1382–1390
16. Zong H, Ward M, Madden A, et al. Hyperglycaemia-induced pro-inflammatory responses by retinal Müller glia are regulated by the receptor for advanced glycation end-products (RAGE). Diabetologia 2010;53:2656–2666
17. Berner AK, Brouwers O, Pringle R, et al. Protection against methylglyoxal-derived AGEs by regulation of glyoxalase 1 prevents retinal neuroglial and vasodegenerative pathology. Diabetologia 2012;55:845–854
18. Wilkinson-Berka JL. Angiotensin and diabetic retinopathy. Int J Biochem Cell Biol 2006;38:752–765
19. Kurihara T, Ozawa Y, Nagai N, et al. Angiotensin II type 1 receptor signaling contributes to synaptophysin degradation and neuronal dysfunction in the diabetic retina. Diabetes 2008;57:2191–2198
20. Downie LE, Pianta MJ, Vingrys AJ, Wilkinson-Berka JL, Fletcher EL. AT1 receptor inhibition prevents astrocyte degeneration and restores vascular growth in oxygen-induced retinopathy. Glia 2008;56:1076–1090
21. Simó R, Hernández C; European Consortium for the Early Treatment of Diabetic Retinopathy (EUROCONDOR). Neurodegeneration is an early event in diabetic retinopathy: therapeutic implications. Br J Ophthalmol 2012;96:1285–1290
22. Simó R, Lecube A, Sararols L, et al. Deficit of somatostatin-like immunoreactivity in the vitreous fluid of diabetic patients: possible role in the development of proliferative diabetic retinopathy. Diabetes Care 2002;25:2282–2286
23. Hernández C, Carrasco E, Casamitjana R, Deulofeu R, García-Arumí J, Simó R. Somatostatin molecular variants in the vitreous fluid: a comparative study between diabetic patients with proliferative diabetic retinopathy and nondiabetic control subjects. Diabetes Care 2005;28:1941–1947
24. Cervia D, Casini G, Bagnoli P. Physiology and pathology of somatostatin in the mammalian retina: a current view. Mol Cell Endocrinol 2008;286:112–122
25. Kiagiadaki F, Savvaki M, Thermos K. Activation of somatostatin receptor (sst 5) protects the rat retina from AMPA-induced neurotoxicity. Neuropharmacology 2010;58:297–303
26. Simó R, Carrasco E, Fonollosa A, García-Arumí J, Casamitjana R, Hernández C. Deficit of somatostatin in the vitreous fluid of patients with diabetic macular edema. Diabetes Care 2007;30:725–727
27. Eljarrat-Binstock E, Pe'er J, Domb AJ. New techniques for drug delivery to the posterior eye segment. Pharm Res 2010;27:530–543
28. Marmor MF, Holder GE, Seeliger MW, Yamamoto S; International Society for Clinical Electrophysiology of Vision. Standard for clinical electroretinography (2004 update). Doc Ophthalmol 2004;108:107–114
29. Anderson PJ, Watts H, Hille C, et al. Glial and endothelial blood-retinal barrier responses to amyloid-beta in the neural retina of the rat. Clin Ophthalmol 2008;2:801–816
30. Valverde AM, Miranda S, García-Ramírez M, González-Rodriguez A, Hernández C, Simó R. Proapoptotic and survival signaling in the neuroretina at early stages of diabetic retinopathy. Mol Vis 2013;19:47–53
31. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001;104:487–501
32. Holopigian K, Seiple W, Lorenzo M, Carr R. A comparison of photopic and scotopic electroretinographic changes in early diabetic retinopathy. Invest Ophthalmol Vis Sci 1992;33:2773–2780
33. Mizutani M, Gerhardinger C, Lorenzi M. Müller cell changes in human diabetic retinopathy. Diabetes 1998;47:445–449
34. Wang J, Xu X, Elliott MH, Zhu M, Le YZ. Müller cell-derived VEGF is essential for diabetes-induced retinal inflammation and vascular leakage. Diabetes 2010;59:2297–2305
35. Robinson R, Barathi VA, Chaurasia SS, Wong TY, Kern TS. Update on animal models of diabetic retinopathy: from molecular approaches to mice and higher mammals. Dis Model Mech 2012;5:444–456
36. Lieth E, Barber AJ, Xu B, et al.; Penn State Retina Research Group. Glial reactivity and impaired glutamate metabolism in short-term experimental diabetic retinopathy. Diabetes 1988;47:815–820
37. Lieth E, LaNoue KF, Antonetti DA, Ratz M; Penn State Retina Research Group. Diabetes reduces glutamate oxidation and glutamine synthesis in the retina. Exp Eye 2000;70:723–730
38. Kowluru RA, Engerman RL, Case GL, Kern TS. Retinal glutamate in diabetes and effect of antioxidants. Neurochem Int 2001;38:385–390
39. Ambati J, Chalam KV, Chawla DK, et al. Elevated gamma-aminobutyric acid, glutamate, and vascular endothelial growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. Arch Ophthalmol 1997;115:1161–1166
40. Rauen T, Rothstein JD, Wässle H. Differential expression of three glutamate transporter subtypes in the rat retina. Cell Tissue Res 1996;286:325–336
41. Wilson SH, Davis MI, Caballero S, Grant MB. Modulation of retinal endothelial cell behaviour by insulin-like growth factor I and somatostatin analogues: implications for diabetic retinopathy. Growth Horm IGF Res 2001;11(Suppl. A):S53–S59
42. Cervia D, Catalani E, Dal Monte M, Casini G. Vascular endothelial growth factor in the ischemic retina and its regulation by somatostatin. J Neurochem 2012;120:818–829
43. Mei S, Cammalleri M, Azara D, Casini G, Bagnoli P, Dal Monte M. Mechanisms underlying somatostatin receptor 2 down-regulation of vascular endothelial growth factor expression in response to hypoxia in mouse retinal explants. J Pathol 2012;226:519–533
44. Simó R, Carrasco E, García-Ramírez M, Hernández C. Angiogenic and antiangiogenic factors in proliferative diabetic retinopathy. Curr Diabetes Rev 2006;2:71–98
45. Saylor M, McLoon LK, Harrison AR, Lee MS. Experimental and clinical evidence for brimonidine as an optic nerve and retinal neuroprotective agent: an evidence-based review. Arch Ophthalmol 2009;127:402–406
46. Lambiase A, Aloe L, Centofanti M, et al. Experimental and clinical evidence of neuroprotection by nerve growth factor eye drops: Implications for glaucoma. Proc Natl Acad Sci USA 2008;106:13469–13474
47. Dong CJ, Guo Y, Agey P, Wheeler L, Hare WA. Alpha2 adrenergic modulation of NMDA receptor function as a major mechanism of RGC protection in experimental glaucoma and retinal excitotoxicity. Invest Ophthalmol Vis Sci 2008;49:4515–4522
48. Aiello LP. Targeting intraocular neovascularization and edema—one drop at a time. N Engl J Med 2008;359:967–969
49. Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. Lancet 2010;376:124–136