Topical administration of somatostatin prevents retinal neurodegeneration in experimental diabetes

Short running title: Eye drops of Somatostatin for Diabetic Neurodegeneration

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Word count: 4353; Number of Tables: 1; Number of Figures: 7

Online Supplemental Material: 1 Figure
ABSTRACT

Retinal neurodegeneration is an early event in the pathogenesis of diabetic retinopathy (DR). Somatostatin (SST) is an endogenous neuroprotective peptide which 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, streptozotocin-induced diabetic rats (STZ-DM) were treated with either SST eye-drops or vehicle for 15 days. Non-diabetic rats treated with vehicle served as 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 signalling pathways (Bcl-xl) were examined. Intraretinal concentrations of glutamate and its main transporter GLAST (glutamate/aspartate transporter) were also determined. Treatment with SST eye drops prevented ERG abnormalities, glial activation, apoptosis and the misbalance between proapoptotic and survival signalling detected in STZ-DM rats. In addition, SST eye drops inhibited glutamate accumulation in the retina and GLAST downregulation induced by diabetes. We conclude that topical administration of SST has a potent effect in preventing retinal neurodegeneration induced by diabetes. In addition, our findings open up a new preventive pharmacological strategy targeted to early stages of DR.
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 which 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 results 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 overexpressed in streptozotozin-induced diabetic rats (STZ-DM) (13, 14). Apart from glutamate, oxidative stress (15), AGE receptor (RAGE) upregulation (16, 17), and renin-angiotensin system (RAS) activation (18-20) play an essential role in retinal neurodegeneration induced by diabetes. Finally, recent evidence indicates that diabetes-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 hypothesise 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 Ca2+ 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 analogues 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 neovascularisation could be reduced and, consequently, the development of DME and PDR would be favoured. For all these reasons, SST replacement treatment can be considered as 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 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 present 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 signalling 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.

**METHODS**

**Experimental animal model and treatments**

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 (dose: 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 8 rats were injected with vehicle and served as a non-diabetic control group. Animals with blood glucose levels higher than 250 mg/dl the day following 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 approximately one hour prior to necropsy. Therefore, the elapsed time between the onset of diabetes 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 ARVO (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,) of $^{125}$I-somatostatin were applied in each eye with a micropipette. The animals were sacrificed at 15, 30 and 60 minutes and 4, 6, 12 and 24 hours after the application of the labeled somatostatin solution. After the sacrifice, the eyes were obtained and gently washed in a volume of 20 mL of 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 gamma 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, 3 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 to the left eye of three rats. One animal of each group was sacrificed at 10, 30 and 60 minutes after the application of the corresponding eye drop. The eyes were enucleated, fixed in 4% paraformaldehyde, submerged in optimum cutting temperature (OCT) Tissue-teck®, frozen in liquid nitrogen and stored at -80°C until microtome sectioned. The whole eyes were cryosectioned to 50µm sections, mounted with antifade-mounting medium with DAPI and visualized in fluorescent and confocal microscopes (Olympus). All images were taken with the same settings.

**Ophthalmological examinations**

Slit lamp biomicroscopy, indirect ophthalmoscopy and intraocular pressure measurement were performed in all animals at baseline and at days 7 and 13 post-treatment.

**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 hour prior to 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 being placed 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 and eosin. For each eye, a total of three sagittal sections (one section through the optic nerve; one section approximately 500 µm medially and another section approximately 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, UK). They were desparaffined in xylol, rehydrated in 100% ethanol, 90% ethanol and 75% ethanol and incubated for 10 min in phosphate buffer saline (PBS). Unspecific unions were blocked by incubating the samples for 1h in PBS 1% BSA (Bovine serum albumin), 0.05% Triton X-100. Thereafter, the primary rabbit anti-human 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 antibody anti IgG human labeled with Alexa Fluor® 568 (Invitrogen, Eugene, OR) for 1h at room temperature. The labeled sections were washed and mounted with fluorescent medium
containing 4,6-diamidino-2-phenylindole (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 1024 x 1024 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/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 ONL (score 3); Müller cell end-feet plus processes throughout with some in the ONL (score 4); Müller cell end-feet 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 was eliminated following incubation with 0.2% potassium permanganate for 20 min and 1% oxalic acid for 30 seconds before the TUNEL procedure was started. Positive and negative controls were processed at the same time. Three confocal images were recorded (40X) corresponding to a surface of 317.13 µm X 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 signalling pathways were investigated by analyzing several pro-apoptotic molecules (FasL, caspase-8, total Bid, truncated Bid, and active caspase-3), as well as anti-apoptotic markers (BclxL) by Western blot as previously described (30).

Glutamate quantification

Quantification of glutamate was performed by liquid-chromatography coupled to mass spectrometry (LC-MS/MS). Chromatographic separation was performed on an Agilent 1200 series (Waldborn, Germany) using an Ascentis Express HILIC column, 50 x 2.1 mm with 2.7 μm particle size from Supelco (Belfonte, PA) maintained at 25º C throughout the analysis, a mobile phase acetonitrile and water (50 mM ammonium acetate) with a flow rate of 0.6 mL min⁻¹. The volume injected was 10 μL. The mobile phase involved a gradient starting at 87% of ACN which was maintained for 3 minutes. 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 minutes. The mass detection system was an Agilent 6410 Triple Quad (Santa Clara, CA) using positive electrospray ionization with a gas temperature of 350º C, gas flow rate of 12 L min⁻¹, nebulizer pressure of 45 psi, capillary voltage of 3500 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). 1 μg 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, Madrid, Spain) 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, Madrid, Spain).

**Immunohistochemistry for GLAST**

Sections of 4 μm were deparaffined in xylene and hydrated in a graded ethanol series. Then, sections were antigen retrieval (sodium citrate 10 mM, 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, UK). After washing, sections were incubated with a fluorescent anti-rabbit ALEXA 488 as a secondary antibody (Life Technologies S.A., Madrid, Spain) in blocking solution for 1 h, washed and mounted in Vectashield (Vector Labs, Vector Laboratories; Burlingame, CA, USA). 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 the mean ± SD or 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 figure 1. After topical ocular administration of $^{125}$I-somatostatin, the maximum post-dose concentration (5% of applied dose per gram of tissue) was detected at 1 hour. However, at 15 minutes most of $^{125}$I-somatostatin had already reached the retina. This rapid absorption argues against the permeation of SST through the cornea. To further explore this issue a pharmacokinetic study using eye drops containing carboxyfluorescein labeled SST (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 (Figure of online-only supplemental material).

*Ocular safety of SST treatment*

No significant differences in the cornea, pupil size and IOP were observed among the three groups at baseline, and 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 (Figure 2A). 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 (Figure 2B). 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.
Neurodegeneration was prevented in diabetic rats treated with SST eye drops

Glial activation

The retinas of non-diabetic control rats revealed GFAP immunolabeling in cells and their processes in the inner layer of the retina, especially in the inner limiting membrane, and ganglion cell layer (GFAP score 0 to 1) (Figure 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 to 3). Diabetic rats treated with SST eye drops presented significantly lower GFAP score (0 to 1) than diabetic rats treated with placebo (Figure 3).

Retinal apoptosis

Representative images of the presence of apoptotic cells in the retinas of the three experimental groups are shown in Figure 4A. Two weeks after the induction of diabetes 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 to that observed in retinas from age-matched non-diabetic controls (Figure 4B). In all groups apoptosis was highest in the ganglion cell layer. 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 non-diabetic rats (Figure 4B).
Dose-response effect

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

Proapoptotic and survival molecules

The results of pro-apoptotic and survival molecules measured in the retina of the three rat groups studied are shown in figure 6. The expression of FasL, a pro-apoptotic component of the death receptor apoptotic pathway, was significantly increased in neuroretinas from diabetic rats as compared to control rats. The interaction of FasL with the death receptor Fas/CD95 assembles the death-inducing signaling complex (DISC) (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 as compared to controls. These data were reinforced by the detection of elevated levels of truncated Bid fragment (tBid) 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 these pro-apoptotic molecules.

On the other hand, a reduction in BclxL, an anti-apoptotic member of the Bcl2 family, was found in diabetic retinas in comparison with control retinas. When treated with SST eye drops BclxL expression was greatly improved (Figure 6).

Topical administration of SST prevents the increase of glutamate induced by diabetes

Glutamate levels (arbitrary units) in the diabetic retinas were higher than in non diabetic 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 (Figure 7). In diabetic rats treated with SST this downregulation was prevented and GLAST protein levels were even increased in comparison with non-diabetic rats (Figure 7).

**DISCUSSION**

In the present study we provide 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. 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 non-diabetic 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 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 non-diabetic control rats. In addition, we found a misbalance between apoptotic and survival signalling 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 significantly higher in neuroretina from STZ-induced diabetic rats than in non-diabetic 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 anti-apoptotic 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 signalling.

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 (36-38), as well as in the vitreous fluid of diabetic patients (12, 39). The reasons why diabetes 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 alpha-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 present study we provide evidence that glutamate/aspartate transporter (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, 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 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 (EGF) and platelet-derived growth factor (PDGF) (44). The potential effects of SST in abrogating microvascular impairment are beyond the scope of the present 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 hypothesise that therapeutic strategies based on the neuroprotective effects of SST would be effective in preventing or arresting DR development. The observation
that circulating GH–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 the following: 1) The paracrine effects of SST synthesized by the retina involves SSTR subtypes other than SSTR2, which mediates GH inhibition and for which octreotide presents high affinity; 2) SST analogues do not cross the blood-retinal barrier (BRB) and would have access only where there is disruption of BRB, 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 NGF 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 minimises the associated systemic effects, resulting in higher patient compliance (48). Therefore, topical therapies could revolutionise 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 by means of 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 and a significant reduction in glutamate-induced excitotoxicity are among the mechanisms by which SST exerts its beneficial actions.

**Author Contributions:** C.H. designed the project, led the analysis, wrote the manuscript, and reviewed and edited the manuscript. M.G., L.C. and A.G. led the analysis and reviewed the manuscript. J.F.-C., J.F.-S., B.P., and A.M. contributed to discussion and reviewed the manuscript. R.S. designed and coordinated the project, wrote the manuscript, and reviewed and edited the manuscript. All authors 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.

**Acknowledgments**

This study was supported by grants from the Ministerio de Ciencia e Innovación (SAF2012-35562 and SAF2009-08114), from the European Foundation for the Study of Diabetes (EFSD), and from the 7th Framework Programme (EUROCONDOR. FP7-278040). We thank Dr. Oscar Yanes (CIBERDEM) for glutamate quantification and Dr. Andrea R. Carvalho (Ophthalmologic Research Unit. VHIR) for her technical assistance in obtaining the images of pharmacokinetic analysis.

No potential conflicts of interest relevant to this article were reported.
REFERENCES


2. Lorenzi M, Gerhardinger C. Early cellular and molecular changes induced by diabetes in the retina. Diabetologia 2001 44:791-804


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;4:1496-1502


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


**Table 1.** Experimental design of the dose-efficacy study

<table>
<thead>
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<th>Group (n)</th>
<th>STZ Dose (mg/Kg)</th>
<th>Treatment</th>
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<tr>
<td>A (n=8)</td>
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<td>--</td>
</tr>
<tr>
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<td>vehicle</td>
<td>--</td>
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FIGURE LEGENDS

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

Figure 2. b-wave amplitude (A) and implicit time (B) under scotopic conditions (0 db) in both eyes at day -1 (white column) and at day 14 (black column) 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 mean and SD. * p <0.05.

Figure 3. Glial fibrillar acidic protein (GFAP) immunofluorescence. A) Images of representative samples of retina from a control rat (C), a diabetic rat treated with vehicle eye drops (D-Sham), and a diabetic rat treated with SST eye drops (D-SST). In diabetic rats (D-Sham), 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 (D-SST) prevents the glial activation. Scale bar = 50 µm. B) Quantification of glial activation based on extent of GFAP staining.

Figure 4. A) Representative images of apoptosis in the retina from a control rat (C), a diabetic rat treated with vehicle eye drops (D-Sham), and a diabetic rat treated with SST eye drops (D-SST). B) Percentage of apoptotic cells in the whole retina and in the retinal layers in each group. The following abbreviations are used in the figure: outer nuclear layer (ONL); inner nuclear layer (INL); and ganglion cell layer (GCL). Results are expressed as means ± SD. * p<0.05 in comparison with C and D-SST groups. Scale bar = 50 µm.
**Figure 5.** Dose-response effect of SST eye drops on retinal neurodegeneration. A) Glial fibrillar acidic protein (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). * p<0.05 in comparison with diabetic group treated with vehicle. ONL: Outer nuclear layer. INL: Inner nuclear layer, GCL: ganglion cell layer. Scale bar = 20 µm.

**Figure 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-8, Bid, Bim, Belx, and active caspase-3. α-tubulin 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 diabetic mice treated with the vehicle vs. diabetic mice treated with SST eye drops.
Figure 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. The following abbreviations are used in the figure: outer nuclear layer (ONL); inner nuclear layer (INL); and ganglion cell layer (GCL). Data are mean ± SD. Scale bar = 50 µm.
Figure 1
**Figure 2**

A) Amplitude (μV)

B) Implicit time (ms)
**Figure 3**

A) Images of ONL, INL, and GCL in Control, Diabetes - sham, and Diabetes - SST conditions.

B) Table showing scores and percentages for Control, D-Sham, and D-SST conditions:

<table>
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<th>Score</th>
<th>Control</th>
<th>D-Sham</th>
<th>D-SST</th>
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<tr>
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<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>
Figure 4

A) 

Control Diabetes-sham Diabetes-SST

ONL INL GCL Total

B) 

% TUNEL positive cells

Control Diabetes-sham Diabetes-SST

Figure 4
A)  

B)  

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>D-sham</th>
<th>D-SST (0.5 mg/mL)</th>
<th>D-SST (2 mg/mL)</th>
<th>D-SST (10 mg/mL)</th>
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</table>

C)  

D)  

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

Figure 5
Figure 6

Diabetes

FasL →
cleaved casp-8 (p18) →
total Bid →
truncated Bid →
cleaved casp-3 (p20) →
cleaved casp-3 (p17) →
BclxL →
αtubulin →

C  D-Sham  D-SST

BclxL/αtub (arbitrary units)

FasL/αtub (arbitrary units)

casp-8/αtub (arbitrary units)

total Bid/αtub (arbitrary units)

t-Bid/αtub (arbitrary units)

casp-3/αtub (arbitrary units)

C  D-Sham  D-SST

Figure 6
Figure 7

A) GLAST mRNA Relative Quantification (R.Q)

- C
- D-Sham
- D-SST

B) Immunofluorescence images showing ONL, INL, and GCL layers in different conditions:
- C
- D-Sham
- D-SST

C) GLAST Immunofluorescence (a.u.)

- C
- D-Sham
- D-SST
Ocular permeation of somatostatin labeled with carboxyfluorescein (CF-SST) after topical instillation in rat eyes at 10 min (A, B), 20 min (C, D) and 60 min (E, F). Anterior ocular structures are shown in A, C, E panels. Posterior ocular structures are shown in B, D, F panels. CF-SST fluorescence is green and cell nuclei were stained with DAPI (blue). At 10 minutes fluorescence is present in conjunctiva, sclera, ciliary body, choroid and photoreceptors layer. However, no fluorescence was detected passing through the cornea. At 20 minutes CF-SST was already detected in all retinal layers but this becomes more evident at 60 minutes. CB: Ciliary body; CE: corneal epithelium; CH: Choroid; CJ: Conjunctiva; CS: corneal stroma; GCL: Ganglionar cell layer; INL: Inner nuclear layer; IPL: Inner plexiform layer; I: Iris, ONL: Outer nuclear layer; OPL: Outer plexiform layer; PL: photoreceptors layer; RPE: Retinal pigment epithelium; S: Sclera. Bars: 100 µm.