Journal of Neurochemistry JNC

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Journal:	*Journal of Neurochemistry*
Manuscript ID:	JNC-E-2008-0306.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
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Modulation of the neuronal response to ischemia by somatostatin analogues in wild-type and knock-out mouse retinas

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Abbreviations used: CT, cycle threshold; DAPI, 4'-6-diamidino-2-phenylindole; ESI-MS, electrospray tandem mass spectrometry; GCL, ganglion cell layer; GPCRs, G protein-coupled receptors; GRKs, GPCR kinases; INL, the inner nucleat layer; KO, knock-out; ONL, outer nuclear layer; PB, phosphate buffer; PBS, phosphate-buffered saline; QPCR, Real-Time RT-PCR; RGSs, regulators of G protein signalling; RT, reverse transcriptase; SRIF, somatotropin release-inhibiting factor/somatostatin; $sst_{1\text{-}5}$, SRIF receptor 1 through 5; WT, wild type.

Abstract

Somatostatin acts at five G protein-coupled receptors, sst_1-sst_5. In mouse ischemic retinas, the over-expression of sst_2 (as in sst_1 knock-out mice) results in reduction of cell death and glutamate release. Here, we reported that, in wild-type retinas, somatostatin, the multireceptor ligand pasireotide and the sst_2 agonist octreotide decreased ischemia-induced cell death and that octreotide also decreased glutamate release. In contrast, cell death was increased by blocking sst_2 with cyanamide. In sst_2 over-expressing ischemic retinas, somatostatin analogues increased cell death, and octreotide also increased glutamate release. To explain this reversal of the anti-ischemic effect of somatostatin agonists in the presence of sst_2 over-expression, we tested sst_2 desensitisation due to internalisation or altered receptor function. We observed that: i) sst_2 was not internalised, ii) among G protein-coupled receptor kinases (GRKs) and regulators of G protein signalling (RGSs), GRK1 and RGS1 expression increased following ischemia, iii) both GRK1 and RGS1 were down-regulated by octreotide in wild-type ischemic retinas, iv) octreotide down-regulated GRK1 but not RGS1 in sst_2 over-expressing ischemic retinas. These results demonstrate that sst_2 activation protects against retinal ischemia. However, in the presence of sst_2 over-expression sst_2 is functionally desensitised by agonists, possibly due to sustained RGS1 levels.

Key words: somatostatin receptors, cell death, glutamate release, G protein-coupled receptor kinases, regulators of G protein signalling

Running title: Somatostatin analogues in ischemic retinas

Introduction

The primary cause of neuronal death in retinal diseases is ischemia, a condition that can be considered a final common pathway for injury in different retinal pathologies (Quigley *et al.* 1985; Quigley *et al.* 1995; Levin and Louhab 1996; Osborne *et al.* 1999; Barber 2003; Osborne *et al.* 2004). Glutamate excitotoxicity, occurring during retinal ischemia (Osborne *et al.* 2004), is likely to be a major cause of neuronal damage following an ischemic insult (Lipton 1999; Won *et al.* 2002; Arundine and Tymianski 2003; Camacho and Massieu 2006). The peptide somatostatin (somatotropin release-inhibiting factor, SRIF) plays important physiological roles, mostly inhibitory, which have formed the basis for the clinical use of SRIF compounds (Weckbecker *et al.* 2003; Cervia and Bagnoli 2007), and it may protect the retina against ischemia in a variety of retinal diseases (Cervia *et al.* 2008). SRIF binds to its 5 G protein-coupled receptors (GPCRs) which have been named sst_1-sst_5 (Hoyer *et al.* 1995) and are expressed in the retina (Bagnoli *et al.* 2003; Thermos 2003; Casini *et al.* 2005; Cervia *et al.* 2008). SRIF has a protective role in the retina, as shown in studies in guinea pigs and rats where sst_2 agonists protect, to a certain extent, retinal cells from the ischemic damage (Celiker and Ilhan 2002; Mastrodimou *et al.* 2005; Mastrodimou *et al.* 2008).

Recently, we have developed an *in vitro* model of the ischemic mouse retina, in which possible positive effects exerted by the somatostatinergic system against ischemia-induced damages have been investigated (Catalani *et al.* 2007). In particular, we demonstrated that an increased presence of functional sst_2, consequent to the genetic deletion of sst_1 (Dal Monte *et al.* 2003b; Casini *et al.* 2004), results in significant reduction of both ischemia-induced glutamate release and cell death, with respect to the wild type (WT) (Catalani *et al.* 2007). In the present study, we asked whether these protective effects observed in sst_1 knock-out (KO) retinas could be modulated by agonists or antagonists of sst_2. In particular, we expected that sst_2 agonism on over-expressed sst_2 (as in sst_1 KO retinas) could result in significant potentiation of protection against ischemia, while a sst_2 antagonist should abolish the protection given by sst_2 over-expression. To investigate this, SRIF, the $sst_{1,2,3,5}$ peptidyl agonist pasireotide, the sst_2-preferring peptidyl agonist octreotide and the sst_2-selective peptidyl antagonist cyanamide (Lewis *et al.* 2003; Nunn *et al.* 2003; Weckbecker *et al.* 2003) were added to the ischemic solution in order to determine the effects of the pharmacological modulation of SRIF receptors on retinal cell survival. In addition, the effects of octreotide on glutamate release were also studied. The data obtained in these experiments indicated the possibility that desensitisation of sst_2 occurs in the presence of SRIF agonists. Desensitisation of GPCRs, defined

as a reduction in receptor signalling after pre-exposure to agonist, is a complex process that may involve either a loss of plasma membrane receptors due to receptor endocytosis or a reduced coupling efficiency between cell surface receptors and the intracellular signalling machinery. To study these mechanisms in our model, we i) investigated the possibility that octreotide causes internalisation of sst_2 and, ii) investigated the expression of different regulators of GPCRs (Jean-Baptiste et al. 2006; Moore et al. 2007; Ribas et al. 2007).

Materials and methods

Animals and *in vitro* treatments of retinas

Experiments were performed on mouse retinas of WT (C57BL/6 strain) and sst_1 KO of both sexes at 6-8 weeks after birth (20-30 g body weight). The generation of sst_1 KO mice has been detailed in Kreienkamp *et al.* (1999). To reduce genetic background variability, the sst_1 null allele was backcrossed for five successive generations onto the C57BL/6J strain to produce N_5 incipient-congenic mice. Animals were kept in a regulated environment (23 ± 1°C, 50 ± 5% humidity) with a 12 h light/dark cycle (lights on at 08.00 a.m.) with food and water ad lib. In all experiments, mice were anaesthetized by i.p. injection of Avertin (1.2% tribromoethanol and 2.4% amylene hydrate, 0.02 ml/g body weight) and killed by cervical dislocation. Using published protocols (Catalani *et al.* 2007), the retinas were rapidly dissected and incubated for 1 h at 37°C in air-saturated phosphate-buffered saline (PBS) containing 6 mM glucose in open vials (non ischemic, control conditions) or in N_2-saturated PBS containing 250 mM of iodoacetic acid in airtight vials (ischemic treatment). SRIF compounds were applied for 1 h during the incubation period. All experiments were performed at the same time of the day (between 09:00 and 11:00 a.m.) in order to exclude possible circadian influences. The data were collected from both male and female WT and sst_1 KO mice; the results were combined as there were no apparent gender differences. Experiments were performed in compliance with the Italian law on animal care N° 116/1992 and in accordance with the European Community Council Directive (EEC/609/86). All efforts were made to reduce both animal suffering and the number of animals used.

TUNEL staining

At the end of the incubation period, the retinas were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 45 to 60 min at room temperature and stored overnight in 25% sucrose in 0.1 M PB at 4°C. Retinal sections were cut perpendicularly to the vitreal surface at 10 µm with a cryostat, mounted onto gelatine-coated slides and stored at -20°C. All retinas were cut with the same temporal-to-nasal orientation. Consecutive sections were alternately put onto a series of five slides, so that on each slide the sections were spaced every 50 µm. Five sections were put on each slide, and at least 2 series of slides (10 sections) were prepared and analysed from each retina. Corresponding series of sections were used in each experiment. An *in situ* cell death detection kit (Roche Diagnostics, Basel, Switzerland) was used to identify apoptotic profiles in the nuclear layers of the retina by TUNEL technique according to the manufacturer's instructions. The slides were coverslipped in a 0.1 M PB-glycerine mixture containing 0.5 µg/ml of 4'-6-diamidino-2-phenylindole (DAPI). To calculate an "index of cell death" (Catalani *et al.* 2007), images of both the TUNEL and the DAPI stainings were acquired with a digital photocamera (Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany) connected to a Zeiss microscope equipped with epi-fluorescence. The measurements were obtained with a system of computer-assisted image analysis using Axiovision 4 and KS-300 software (Zeiss), as previously described (Catalani *et al.* 2007). Briefly, in the outer nuclear layer (ONL) and in the inner nucleat layer (INL), both the TUNEL and the DAPI images relative to the same field were visualized. The areas of the ONL and of the INL in the DAPI image were measured. Subsequently, the TUNEL image was thresholded and the labelled areas in the ONL and in the INL were measured. The ratio between the area covered by the TUNEL staining and the area of the retinal layer seen with the DAPI counterstain was taken as an "index of cell death". In the ganglion cell layer (GCL), the "index of cell death" was calculated as the ratio between the number of the TUNEL-labelled and that of the DAPI-stained cells. After statistics, the raw data (arbitrary units) from the same experiment were calculated as a percentage of the cell death in untreated retinas since basal values among different experiments may vary. Then, data belonging from different experiments were represented and averaged in the same graph.

Glutamate release

At the end of the incubation period, the vials containing supernatants of each retinas were stored at -80°C. The rate of glutamate release was measured using HPLC electrospray tandem mass spectrometry (ESI-MS), according to previous studies (Catalani *et al.* 2007; Timperio *et al.* 2007). Briefly, the samples were lyophilised and reconstituted in 100 µl of 10% acetonitrile. For chemical

derivatisation, they were incubated for 30 min in darkness at 60°C in a water bath with 20 µl of 10 M KOH and 500 µl of 0.1 mM dansyl chloride. HPLC was performed using a Beckman Model 126 Programming Solvent Module pump chromatograph (Beckman Instruments, Brea, CA, USA). The chromatographic separations were carried out with Waters ODS2 Spherisorb 250 mm x 4.6 mm i.d. 5 µm packed column (Teknokroma, Barcelona, Spain) as stationary phase, preceded by a guard column of Spherisorb RPC18, 5 µm, 4 mm x 4 mm. The mobile phase was double-distilled water 0.5% formic acid (mobile phase A), and acetonitrile 0.5% formic acid (mobile phase B). ESI-MS was performed on an ion trap mass spectrometer (Esquire 3000 plus, Bruker Daltonik, Bremen, Germany) in the positive mode. For the analysis with pneumatically assisted ESI, an electrospray voltage of 4 kV and a Nebulizer 35 psi and Dry Gas 10 l/min were employed. The temperature of dry gas was set to 300°C. Full-scan spectra were acquired over the range 50-1500 m/z (scan duration, 1 s). After statistics, the raw data (ng/ml) from the same experiment were calculated as a percentage of the glutamate release in untreated retinas since basal values among different experiments may vary (WT: 20.29-41.27 ng/ml; sst_1 KO: 21.83-38.51 ng/ml). Then, data belonging from different experiments were represented and averaged in the same graph.

Immunohistochemistry

Antibodies directed to sst_{2A} isoform (Vanetti *et al.* 1992; Patel *et al.* 1993) were purchased from Gramsch (Gramsch Laboratories, Schwabhausen, Germany). A rabbit antiserum (cat. # SS-800) or a guinea pig antiserum (cat. # SS-870) were used at 1:500 and 1:5000 dilution, respectively. The sections were washed in 0.1 M PB and incubated overnight at 4 °C in primary antibody diluted in 0.1 M PB containing 0.5% Triton X-100. Following washes in 0.1 M PB, the sections were incubated with the appropriate secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) at a dilution of 1:200 in 0.1 M PB containing 0.5% Triton X-100 for 1 h at room temperature. Finally, the slides were coverslipped in a 0.1 M PB-glycerine mixture and observed with a Zeiss microscope equipped with epi-fluorescence. Digital images were acquired using a 40x plan-NEOFLUAR objective, a digital Axiocam MRC photocamera and Axiovision 4 software (Zeiss). The digital images were optimised for contrast and brightness using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA) and saved at a minimum of 300 dpi.

PCR experiments

Total RNA from 4 retinas for each experimental condition was extracted with the RNeasy Mini Kit with DNAse digestion (Qiagen, Valencia, CA, USA), according to the manufacturer's

recommended procedure. After solubilization in RNase-free water, total RNA was quantified by Bio-Rad SmartSpec 3000 spectrophotometer (Hercules, CA, USA). First-strand cDNA was generated from 3 µg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad). As show in Table 1, a set of primer pairs amplifying at 102-195 bp fragment have been designed by Primer3 software (Rozen and Skaletsky 2000) or have been obtained from the Real Time PCR Primer and Probe Database, RTprimerDB (Pattyn *et al.* 2006) to hybridise to unique regions of the appropriate gene sequence.

The reverse transcriptase (RT)-PCR reactions were carried out using 1 µl of cDNA in a 15 µl total volume of PCR buffer (Invitrogen, Milano, Italy), containing 3 mM $MgCl_2$, 300 µM dNTPs, and 300 nM of appropriate primers. Taq polymerase (0.35 U) was also added. The amplification reactions were carried out in a thermal gradient cycler (Bio-Rad) for 40 cycles. Each cycle consisted of denaturation for 30 sec at 94°C, annealing for 30 sec at 58°C, and an extension for 30 sec at 72°C. A final extension step at 72°C for 5 min terminated the amplification. For each amplification, two types of controls were performed: (i) RT-PCR mixture with no reverse transcriptase to control for genomic DNA contamination, and (ii) PCR mixture with no cDNA template, to check for possible external contamination. A 5 µl sample of the PCR reaction was electrophoresed on an ethidium bromide-containing 2% agarose gel by the use of the Bio-Rad Sub-cell GT system.

Real-Time RT-PCR (QPCR) was performed using SYBR Green on MiniOpticon Two-Color Real-Time PCR detection System (Bio-Rad). The final reaction mixture contained 1 µl of cDNA, 300 nM of each primer, 7.5 µl of iQ SYBR Green Supermix (Bio-Rad), and RNase-free water to complete the reaction mixture volume to 15 µl. All reactions were run as triplicates. The QPCR was performed with hot-start denaturation step at 95°C for 3 min, and then was carried out for 40 cycles at 95°C for 10 sec and 58° for 20 sec. The fluorescence was read during the reaction by the Opticon Monitor 3 software (Bio-Rad), allowing a continuous monitoring of the amount of PCR products. Primers have been initially used to generate a standard curve over a large dynamic range of starting cDNA quantity which allows to calculate the amplification efficiency (a critical value for the correct quantification of expression data) for each of the primer pairs (Table 1). The melt-curve analysis was performed at the end of each experiment to verify that a single product per primer pair was amplified **(data not shown)**. As to control experiments, gel electrophoresis was also performed to verify the sizes of the amplified QPCR products. RPL13a, a stable mRNA during ischemia (Tian *et al.* 2007), was used in our experiments as an internal standard. As previously described, samples were compared using the relative cycle threshold (CT) method (Livak and Schmittgen 2001). The fold increase or decrease was determined relative to a control after normalizing to ribosomal protein

L13a (RPL13a) (internal standard). The formula $2^{-\Delta\Delta CT}$ was used, where ΔCT is: (gene of interest CT) - (RPL13a CT), and ΔΔCT is (ΔCT experimental) - (ΔCT control). After statistics, data belonging from different experiments were represented and averaged in the same graph.

Statistics

Upon verification of normal distribution, statistical significance of raw data between the groups in each experiment was evaluated using unpaired Student's *t*-test or ANOVA followed by multiple comparison Newman-Keuls Multiple Comparison post-test. The GraphPad Prism software package (Graph Software, San Diego, CA, USA) was used. The results were expressed as means ± SEM of the indicated n values.

Chemicals

SRIF (SRIF-14) was purchased from Bachem (Bubendorf, Switzerland). Octreotide (also known as SMS 201-995 or sandostatin), pasireotide (also known as SOM230) and cyanamide ($D\text{-}Tyr^8$ cyanamide 154806) were a gift of Prof. D. Hoyer and Dr. H. Schmid (Novartis Pharma, Basel, Switzerland). CH-275 (Weckbecker *et al.* 2003) was purchased from NeoMPS (Strasbourg, France). Acetonitrile and Taq polymerase were purchased from Carlo Erba (Milano, Italy) and Invitrogen, respectively. The forward and reverse primers as well as agarose were purchased from Eurobio (Les Ulis, France). Where not specified, chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Results

Effects of SRIF compounds on ischemia-induced cell death

All compounds were applied for 1 h at 1 µM, a concentration giving maximal receptor occupancy in different systems (Weckbecker *et al.* 2003; Cervia *et al.* 2005), including mouse and rat retina (Bigiani et al. 2004; Pavan et al. 2004; Mastrodimou et al. 2005; Mastrodimou et al. 2006a; Mastrodimou et al. 2006b; Mastrodimou et al. 2008). As shown in Fig. 1 and 2, treatments with SRIF, pasireotide or octreotide resulted in overall protection of WT ischemic retinas. In particular,

SRIF, pasireotide and octreotide decreased the index of cell death calculated with the TUNEL staining in the ONL of WT ischemic retinas by 38 ± 5%, 39 ± 6% and 28 ± 3%, respectively. Similarly, pasireotide and octreotide, but not SRIF, decreased the index of cell death in the INL (by 40 ± 4% and 33 ± 5%, respectively) and in the GCL (by 18 ± 3% and 38 ± 6%, respectively) of WT ischemic retinas. In contrast to our expectations, however, treatments with SRIF, pasireotide or octreotide resulted in significant increases of the index of cell death in sst_1 KO ischemic retinas. Indeed, SRIF, pasireotide and octreotide increased the index of cell death in the ONL (by 34 ± 6%, 74 ± 15% and 68 ± 10%, respectively), in the INL (by 122 ± 14%, 52 ± 11% and 73 ± 17%, respectively), and in the GCL (by 159 ± 40%, 214 ± 86% and 184 ± 42%, respectively) of sst_1 KO ischemic retinas. The administration of the sst_2 antagonist cyanamide had comparable effects in both strains. In particular, it increased the index of cell death in the ONL, INL and GCL by 40 ± 20%, 102 ± 33% and 199 ± 27%, respectively, in the WT ischemic retinas and by 92 ± 12%, 65 ± 17% and 71 ± 32%, respectively, in the sst_1 KO ischemic retinas.

SRIF, pasireotide or octreotide at a concentration of 10 nM (1 h) did not affect the index of cell death either in WT or in sst_1 KO ischemic retinas (n = 3, data not shown). This observation seems to exclude the possibility of protective effects of low agonist concentrations in sst_1 KO mice. In addition, as shown in Fig. 3, no apparent variations in the TUNEL staining were detected in the absence or in the presence of the sst_1 agonist CH-275 (1 h at 1 μM) in WT ischemic retinas, thus excluding a major role of sst_1 in our system, in agreement with recent results in the rat retina (Kiagiadaki and Thermos 2008).

Effects of octreotide on ischemia-induced glutamate release

We have previously demonstrated that glutamate release significantly increases in WT retinas incubated in the ischemic solution and that this increase is significantly reduced, in parallel with the index of cell death, in sst_1 KO retinas (Catalani *et al.* 2007). Similarly, in the present study, we observed that the rates of glutamate release paralleled the changes of the index of cell death following treatment with a sst_2 agonist. Indeed, octreotide (1 h at 1 μM) decreased glutamate release in ischemic WT retinas by 22 ± 3%, while it increased glutamate release in ischemic sst_1 KO retinas by 45 ± 14% (Fig. 4).

sst_2-ligand complex internalisation during ischemia

The observations of increased ischemic damage and glutamate release in sst_1 KO ischemic retinas in the presence of SRIF, pasireotide and/or octreotide may be explained by sst_2 desensitisation, possibly through the endocytosis of plasma membrane sst_2-ligand complex in retinal cells. We

therefore tested possible ligand-mediated sst_2 internalisation in cells of WT or sst_1 KO ischemic retina, by means of sst_{2A} immunofluorescence. In both WT and sst_1 KO control retinas, the pattern of sst_{2A} immunostaining was consistent with that described previously (Cristiani *et al.* 2002; Casini *et al.* 2004). As recently reported by us (Catalani *et al.* 2007), after 1 h ischemia sst_{2A} immunofluorescence was reduced in rod bipolar cells, however this reduction was less pronounced in sst_1 KO than in WT retinas (Fig. 5A-D). For this analysis we only concentrated on sst_{2A} expressing amacrine cells; indeed, sst_{2A} immunostaining in amacrine cells did not appear to be affected by 1 h ischemic treatment either in WT or in sst_1 KO retinas. As shown in Fig. 5E-H, both large and small amacrine cell somata were labelled at or near their plasma membrane. In addition, some sst_{2A} immunostaining was also observed in the cytoplasm of large-sized amacrine cell bodies, but no differences were observed between untreated and octreotide-treated ischemic retinas (1 h at 1 μM), either in the WT or in the sst_1 KO animals.

Effects of octreotide on GPCR regulatory proteins during ischemia

The desensitisation of the sst_2-agonist complex in sst_1 KO retinas may occur through a reduced coupling efficiency between cell surface receptors and the intracellular signalling machinery. For instance, GPCR regulatory proteins may induce the inhibition of signalling when sst_2 is continuously occupied by the agonist. To substantiate this hypothesis, we first investigated the expression of: i) GPCR kinases (GRKs, including GRK1 and GRK2) and, ii) regulators of G protein signalling (RGSs, including RGS1, RGS2, RGS5 and RGS9). As shown in Fig. 6A, by means of RT-PCR, we found that GRK1, GRK2, RGS1, RGS2, RGS5 and RGS9 mRNAs are indeed expressed in the mouse retina. Then, in another set of experiments, QPCR using SYBR Green chemistry was used to quantify the relative levels of these mRNAs in different models/experimental conditions. As shown in Fig. 6B, GRK1, GRK2, RGS1, RGS2, RGS5 and RGS9 mRNAs were similarly expressed in retinas of WT and sst_1 KO mice. In addition, the incubation of WT retinas in the ischemic solution elicited a marked induction of GRK1 and RGS1 mRNA levels, being 18 ± 7- and 33 ± 7-fold enriched, respectively, with respect to non ischemic retinas (Fig. 6C). The incubation of sst_1 KO retinas in the ischemic solution caused a limited increase of GRK1 and RGS1 mRNA levels by 2.0 ± 0.06- and 2.8 ± 0.06-fold, respectively (Fig. 6D). In contrast, ischemic treatment did not change the mRNA levels of GRK2, RGS2, RGS5 and RGS9 in both WT and sst_1 KO retinas. Finally, we investigated the mRNA levels of the two isoforms responding to ischemia (GRK1 and RGS1) in ischemic retinas treated with octreotide (1 h at 1 μM). In ischemic retinas of WT mice, octreotide decreased by 47 ± 5% and 70 ± 5% the mRNA levels of GRK1 and RGS1,

respectively. In contrast, in ischemic retinas of sst_1 KO mice, while octreotide decreased by 74 ± 7% the mRNA levels of GRK1, it did not affect the mRNA levels of RGS1 (Fig. 7).

Discussion

Among pharmacological targets for the treatment of ischemia-based retinal diseases, the SRIF system is a candidate that is being subjected to intensive investigation (Cervia *et al.* 2008). In the present study, we demonstrated that the pharmacological activation of SRIF receptors expressed in retinas of WT mice protects against ischemia. In particular, SRIF application decreased cell death in the ONL, although no effects of SRIF were observed in the INL and GCL. The only partial effect of SRIF can be easily explained taking into account that the native peptide is likely to be subjected to rapid enzymatic degradation and subsequent poor access to the retina of mouse, as previously suggested in the rat retina (Mastrodimou *et al.* 2005; Kiagiadaki and Thermos 2008). On the other hand, we cannot exclude that other factors may influence SRIF's action (i.e., absorption). The powerful activity of SRIF in all retinal layers of sst_1 KO mice is probably due to the over-expression of sst_2, an event that affects the ligand efficacy.

Because of their long-acting properties both *in vitro* and *in vivo*, low molecular weight/metabolically stable compounds are more suitable for clinical application or experimental investigations than SRIF (Weckbecker *et al.* 2003; Cervia and Bagnoli 2007). Consistently, we showed that in WT ischemic retinas both the multireceptor ligand pasireotide and the sst_2-preferring agonist octreotide decreased cell death in all retinal layers, suggesting that SRIF receptor activation in the retina can be strongly influenced by the ligand used, in agreement with results obtained in different systems (Siehler and Hoyer 1999a, b; Giannetti *et al.* 2000; Cervia *et al.* 2003a; Cervia *et al.* 2003b; Cervia *et al.* 2003c; Nunn *et al.* 2004; Tulipano and Schulz 2007). In addition, the observations that pasireotide effects (and its efficacy) were mimicked by the selective activation of sst_2 (by octreotide), but not of sst_1 (by CH-275), and that the selective blocking of sst_2 (by cyanamide) increased cell death are consistent with a major role of sst_2 against retinal ischemia in mice. This conclusion is also supported by previous data reporting that octreotide protects against ischemia-reperfusion injury in guinea pig retinas (Celiker and Ilhan 2002) and that different sst_2 agonists (including octreotide) attenuate cell death in an *in vitro* model of the ischemic rat retina (Mastrodimou *et al.* 2005; Mastrodimou *et al.* 2008).

We have observed that, at nanomolar concentrations octreotide is not active in mouse ischemic retinas. Accordingly, in the rat retina, sst_2 agonists have been recently demonstrated to be neuroprotective in a micromolar dose-dependent manner (Kiagiadaki and Thermos 2008). It is important to note that, at the concentration of 1 μM (used in the present work), octreotide may be active on SRIF receptors other than sst_2, i.e., sst_3 and sst_5, according to its affinity values *in vitro* (Cervia et al. 2005). Although it is difficult to conjecture about the actual drug concentration reaching the retina (likely much smaller than that administered in the incubation solution), we cannot completely exclude an involvement of sst_3 and/or sst_5 in the observed effects. On the other hand, evidence of sst_3 and sst_5 presence/localization in the retina are still elusive (Cervia *et al.* 2008). In addition, selective agonists for sst_3 and sst_5 have been previously found to elicit no effects in the mouse retina (Pavan *et al.* 2004). In contrast, the expression and the localisation of sst_2 have been extensively demonstrated in a variety of retinal cell populations of mammals (Thermos 2003; Casini *et al.* 2005; Cervia *et al.* 2008). In particular, in the mouse retina, sst_{2A} immunoreactivity has been reported in rod bipolar cells, horizontal cells, and in amacrine cells, including glycinergic amacrine cells and the population of tyrosine hydroxylase-containing, dopaminergic amacrine cells (Cristiani *et al.* 2002; Casini *et al.* 2004). Functionally, in mouse retinal explants, sst_2 has been shown to inhibit K^+-induced glutamate release (Dal Monte *et al.* 2003a), and in general a prominent role of sst_2 in the retina involves the regulation of glutamate release (Cervia *et al.* 2008). This function is likely to represent an important contribution to the regulation of the glutamatergic transmission along the vertical retinal visual pathway. By limiting the amount of glutamate available to glutamate receptors, sst_2 may also exert an important neuroprotective function against glutamate neurotoxicity that characterises many retinal diseases, including ischemic retinal injury (Osborne *et al.* 2004). Consistently, the SRIF-induced inhibition of glutamate release is significantly stronger when sst_2 is over-expressed, as in sst_1 KO retinas (Bigiani *et al.* 2004), and ischemic sst_1 KO retinas display a marked reduction of both cell death and glutamate release with respect to WT retinas (Catalani *et al.* 2007). In agreement with these observations, in the present study we found that sst_2 activation decreases glutamate release in ischemic WT retinas.

As previously suggested (Bigiani *et al.* 2004; Cammalleri *et al.* 2004; Pavan *et al.* 2004; Cammalleri *et al.* 2006), the density of sst_2 may represent a rate-limiting factor for the sst_2-mediated functions, and sst_2 over-expression may overcome this limitation. This interpretation may also help to explain the fact that, in untreated sst_1 KO ischemic retinas, a protective activity occurred (present results; Catalani *et al.* 2007) indicating that a critical concentration of active sst_2 for the creation of protective effects against ischemia can result from an increase in the number of expressed receptors

(as in the sst_1 KO retinas). In addition, the fact that sst_2 blocking results in the reversal of sst_2 protective effects suggests a constitutive activity of the receptor under ischemic conditions. In agreement with our hypothesis, a recombinant truncated form of the rat sst_2 has been previously shown to display agonist-independent, constitutive activity (Schwartkop *et al.* 1999) and constitutive activation of sst_2 has been recently demonstrated in mouse pituitary corticotroph cells (Ben-Shlomo *et al.* 2007).

Surprisingly, we observed that in sst_1 KO retinas the pharmacological activation of SRIF receptors under ischemic conditions does not protect the retina, but instead it increases both cell death and glutamate release: both the pharmacological activation (agonism) and blocking (antagonism) of sst_2 exert similar functional effects. These results are not in line with our expectation of ameliorating effects of octreotide in retinas over-expressing sst_2 (as the sst_1 KO retinas) as compared to WT retinas. The simplest explanation for this effect is that in sst_1 KO retinas sst_2 may be rapidly desensitised by agonists, thus resulting in a decrease of its functional activity. For instance, desensitisation may involve a loss of plasma membrane sst_2 due to receptor endocytosis. Indeed, there is a general agreement that, in a variety of differentiated cells and in model cell lines, as well as in tissues samples, interactions of SRIF agonists with sst_2 (after a few minutes of agonist exposure) may result in internalisation of receptor-agonist complexes (Sarret *et al.* 1999; Petrucci *et al.* 2000; Stroh *et al.* 2000; Csaba *et al.* 2001; Csaba *et al.* 2002; Elberg *et al.* 2002; Csaba *et al.* 2003; Hofland and Lamberts 2003; Liu *et al.* 2005; Cescato *et al.* 2006; Csaba *et al.* 2007; Duran-Prado *et al.* 2007; Liu *et al.* 2008). In our system, the consequence of such internalisation would be a decrease of available sst_2 on cell membranes of sst_1 KO retinas and reversal of the anti-ischemic effect. However, our data are not consistent with this possibility since we demonstrated that sst_2 was not internalised as a consequence of the treatment with octreotide. Alternatively, desensitisation of the sst_2-agonist complex in sst_1 KO retinas may occur through a reduced coupling efficiency between cell surface receptors and the intracellular signalling machinery. In this respect, recent results in recombinant systems demonstrate the occurrence of sst_2 uncoupling through receptor phosphorylation distinct from sst_2 internalisation (Liu *et al.* 2008). GRKs are key participants in the pathways leading to phosphorylation of serine/threonine residues in many different types of ligand-occupied GPCR. The phosphorylation of GPCRs by GRKs promotes receptor desensitisation through the uncoupling of GPCRs from G proteins as well as through the modulation of intracellular signalling cascades (Moore et al. 2007; Ribas et al. 2007). In addition to GRKs, RGSs have been also found to regulate GPCR responses by binding to and stimulating the GTPase activity of the receptor-activated GTP-bound Gα (Jean-Baptiste *et al.* 2006). Previous results demonstrated

the presence of GRK1 and RGS9 in the mouse retina (Weiss *et al.* 2001; Krispel *et al.* 2006; Song *et al.* 2007). In addition, GRK2 was detected in the chicken retina (de Almeida Gomes and Ventura 2004). In this paper, we have demonstrated that GRK1, GRK2, RGS1, RGS2, RGS5 and RGS9 are all expressed in the retina, with no difference among WT and sst_1 KO mice. In addition, only the expression of GRK1 and RGS1 is increased by retinal ischemic damage but with much more efficacy in WT mice than in sst_1 KO mice. These results indicate that low levels of these proteins correlate with the protective effects exerted by sst_2, as for instance in untreated sst_1 KO retinas. In line with this finding we also found that sst_2 agonism in WT retinas down-regulates both GRK1 and RGS1 probably unmasking sst_2 protective effects against ischemic damage. If that is the case, the fact that the activation of over-expressed sst_2 (as in sst_1 KO retinas) did not affect RGS1 levels may explain the desensitisation of sst_2-agonist complex: sst_2 is occupied by the agonists but its protective functions are inhibited by its uncoupling to RGS1 inhibition, thus resulting in the observed detrimental effects of sst_2 agonism on ischemia. The existence of possible interactions between GRKs/RGSs, including RGS1, and SRIF receptors (Kong *et al.* 2002; Young Shim *et al.* 2006) suggests that coordinate regulation of SRIF system and these GPCR regulatory proteins may occur.

Conclusion

The results of the present study confirm and expand previous observations suggesting important protective actions exerted by SRIF acting at the sst_2 against retinal ischemia. Together, these data constitute a solid background for SRIF-based therapies of retinal diseases in the future. In particular, in WT mouse retinas, sst_2 agonism resulted in a protective effects against ischemia while sst_2 antagonism had a detrimental effect. On the other hand, the investigation of the functional role of the over-expressed sst_2 in sst_1 KO retinas appears to be more complex than expected, since both the activation and the blocking of sst_2 had detrimental effects on ischemia. Our hypothesis is that the desensitisation of the sst_2-agonist complex in sst_1 KO retinas occurs through a reduced coupling efficiency between cell surface receptors and the intracellular signalling machinery. In different systems, including the retina, many mechanisms are involved in the amplification occurring in the signalling cascade of SRIF receptors (Cervia and Bagnoli 2007; Cervia *et al.* 2008). However, whether sst_2 expressed in WT or sst_1 KO retinas are coupled to different machineries of signalling mechanisms during ischemia, and the possibile implications in pharmacological/clinical studies, needs to be further investigated.

Acknowledgements

This work has been supported by the Italian Ministry of University and Research (PRIN2005, grant # 2005052312). We are greatful to Dr. M. Fagioni (Department of Environmental Sciences, University of Tuscia) for the excellent assistance in the HPLC ESI-MS assay. We also thank Dr. C. Papeschi for animal care at the University of Tuscia and G. Bertolini for assistance with mouse colonies at the University of Pisa.

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Table 1 Primers used for PCR analysis

Gene	Primer sequence	product length (bp)	QPCR: amplification efficiency[a]	Gene Bank Accession No.
RPL13a	F: 5'-cactctggaggagaaacggaagg-3' R: 5'-gcaggcatgaggcaaacagtc-3'	182	96.7%	NM_009438
GRK1	F: 5'-ttgacaaagcggacacagag-3' R: 5'-tggtgattcccttcatgtca-3'	153	96.0%	NM_011881
GRK2	F: 5'-gccatggagaagagcaagac-3' R: 5'-cactgcttccccaatttcat-3'	195	96.8%	NM_177078
RGS1	F: 5'-gccaaagacttttggaatgg-3' R: 5'-tggttggcaaggagtttttc-3'	153	99.2%	NM_015811
RGS2	F: 5'-gctacatgagaccaggagtc-3' R: 5'-cctaggttgctgacttcctg-3'	187	95.6%	NM_009061
RGS5	F: 5'-cggagaaggcaaagcaaat-3' R: 5'-ccaggttcttcatggtgatgt-3'	102	100.0%	NM_009063
RGS9	F: 5'-gaggatggcatttctccaaa-3' R: 5'-ggttctgtgcctccaggtta-3'	182	99.5%	NM_011268

[a] as calculated by the Opticon Monitor 3 software (Bio-Rad)

Legends

Fig. 1. TUNEL staining in non ischemic (control) or ischemic retinas of WT **(A-D)** and sst_1 KO **(E-H)** mice. The ischemic treatment was performed in the absence or in the presence of octreotide (OCT) or cyanamide (CYN) (1 h at 1 µM). Each panel is representative of 10 sections from 8 retinas (independent experiments). Calibration bar: 20 µm.

Fig. 2. Cell death in ischemic retinas of WT and sst_1 KO mice. Quantitative evaluation of cell death in the ONL **(A)**, INL **(B)** and GCL **(C)** after ischemic treatment in the presence of different SRIF compounds (1 h at 1 µM). In the ONL and INL, the ratio between the area covered by the TUNEL staining and the area of the retinal layer seen with the DAPI counterstain was taken as an "index of cell death". In the GCL, the "index of cell death" was calculated as the ratio between the number of the TUNEL-labelled and that of the DAPI-stained cells. After statistics, the raw data (arbitrary units) from the same experiment were calculated as a percentage of the cell death in untreated retinas since basal values among different experiments may vary. Then, data belonging from different experiments have been represented and averaged in the same graph. Each histogram represents the mean ± SEM of data from 8 retinas (independent experiments). *$p < 0.05$ vs the respective control values (i.e., untreated WT or sst_1 KO ischemic retinas) set as the 100% (ANOVA followed by Newman-Keuls post test on raw data).

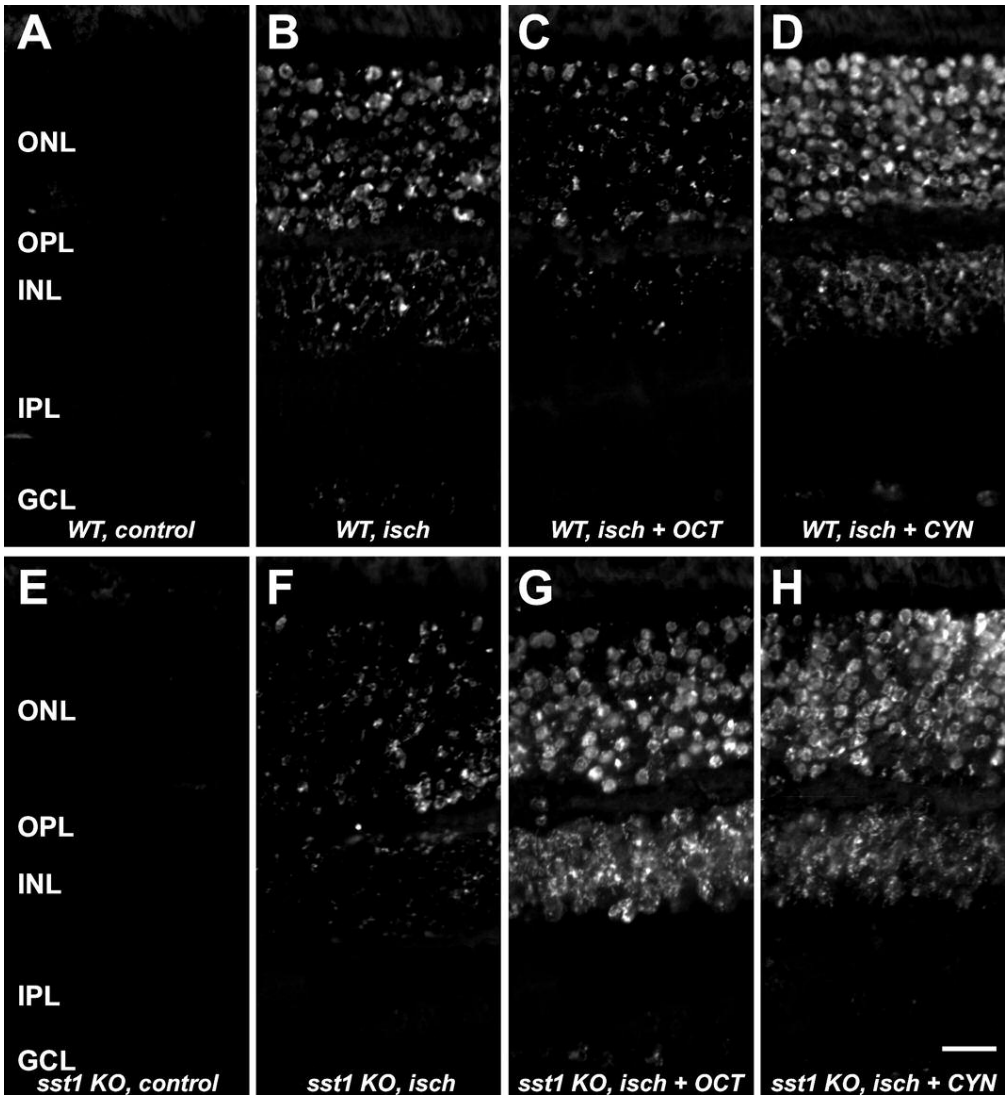
Fig 3. Representative TUNEL staining in untreated WT ischemic retinas **(A)** or in WT ischemic retinas treated with CH-275 (1 h at 1 µM) **(B)**. Calibration bar: 20 µm.

Fig 4. Glutamate release in retinas of WT and sst_1 KO mice after ischemic treatment in the presence of octreotide (1 h at 1 µM). After statistics, the raw data (ng/ml) from the same experiment were calculated as a percentage of the glutamate release in untreated retinas since basal values among different experiments may vary (WT: 20.29-41.27 ng/ml; sst_1 KO: 21.83-38.51 ng/ml). Then, data belonging from different experiments have been represented and averaged in the same graph. Each histogram represents the mean ± SEM of data from 6-7 retinas (independent experiments). *$p < 0.05$ vs the respective control values (i.e., untreated WT or sst_1 KO ischemic retinas) set as the 100% (ANOVA followed by Newman-Keuls post test on raw data).

Fig 5. **A-D**: sst_{2A} immunostaining patterns in WT and sst_1 KO retinas in control or in ischemic conditions. **E-H**: sst_{2A} immunostaining in retinas of WT and sst_1 KO mice after ischemic treatment in the absence or in the presence of octreotide (1 h at 1 µM). Each panel is representative of 8 retinas (independent experiments). In all cases, large-sized sst_2 immunostained amacrine cells displayed some immunoreactivity inside their cell bodies, while the small-sized sst_2 immunostained amacrine cells (insets) were only labelled at or near their plasma membrane. Calibration bars: 20 µm.

Fig 6. Expression levels of GPCR regulatory proteins in mouse retina. **(A)** RT-PCR products of RPL13a and of different GRKs/RGSs in WT retinas. The panel is representative of 3 independent experiments. QPCR evaluation of different GRKs/RGSs in WT and sst_1 KO non ischemic retinas **(B)**, in WT non ischemic and ischemic retinas **(C)** and, in sst_1 KO non ischemic and ischemic retinas **(D)**. Data were analysed by the formula $2^{-\Delta\Delta CT}$ using RPL13a as internal standard. Each histogram represents the mean ± SEM of data from 3 independent experiments. *$p < 0.05$ vs the respective control values (i.e., WT or sst_1 KO non ischemic) (ANOVA followed by Newman-Keuls post test on raw data).

Fig 7. QPCR evaluation of GRK1 **(A)** and RGS1 **(B)** in WT and sst_1 KO retinas after ischemic treatment both in the absence and in the presence of octreotide (1 h at 1 µM). Data were analysed by the formula $2^{-\Delta\Delta CT}$ using RPL13a as internal standard. Each histogram represents the mean ± SEM of data from 3 independent experiments. *$p < 0.05$ vs the respective control values (i.e., untreated WT or sst_1 KO ischemic retinas) (unpaired Student's *t*-test on raw data).



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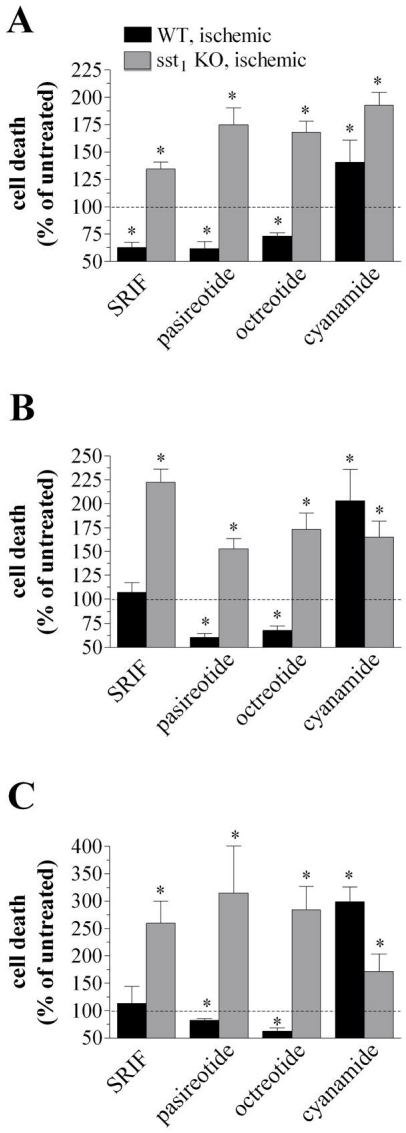

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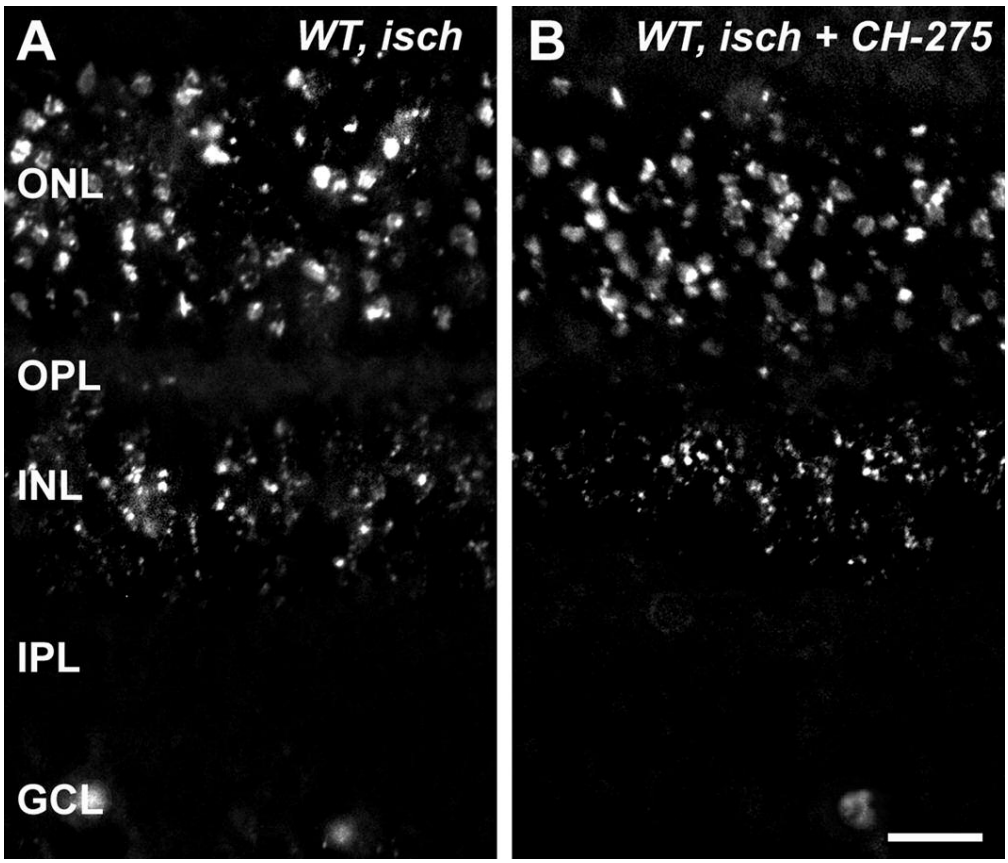

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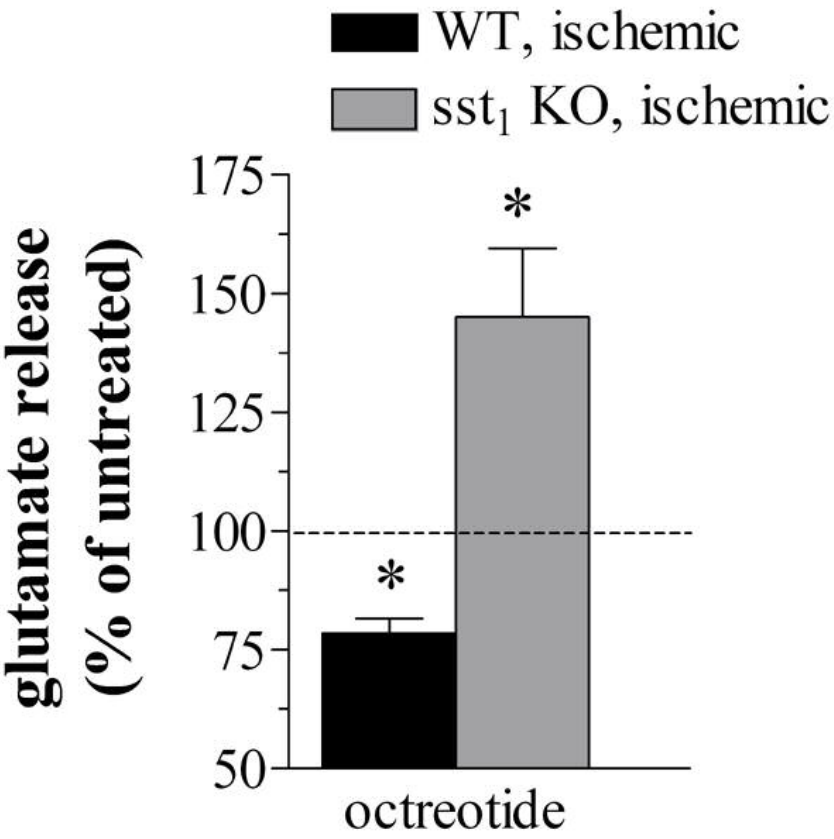

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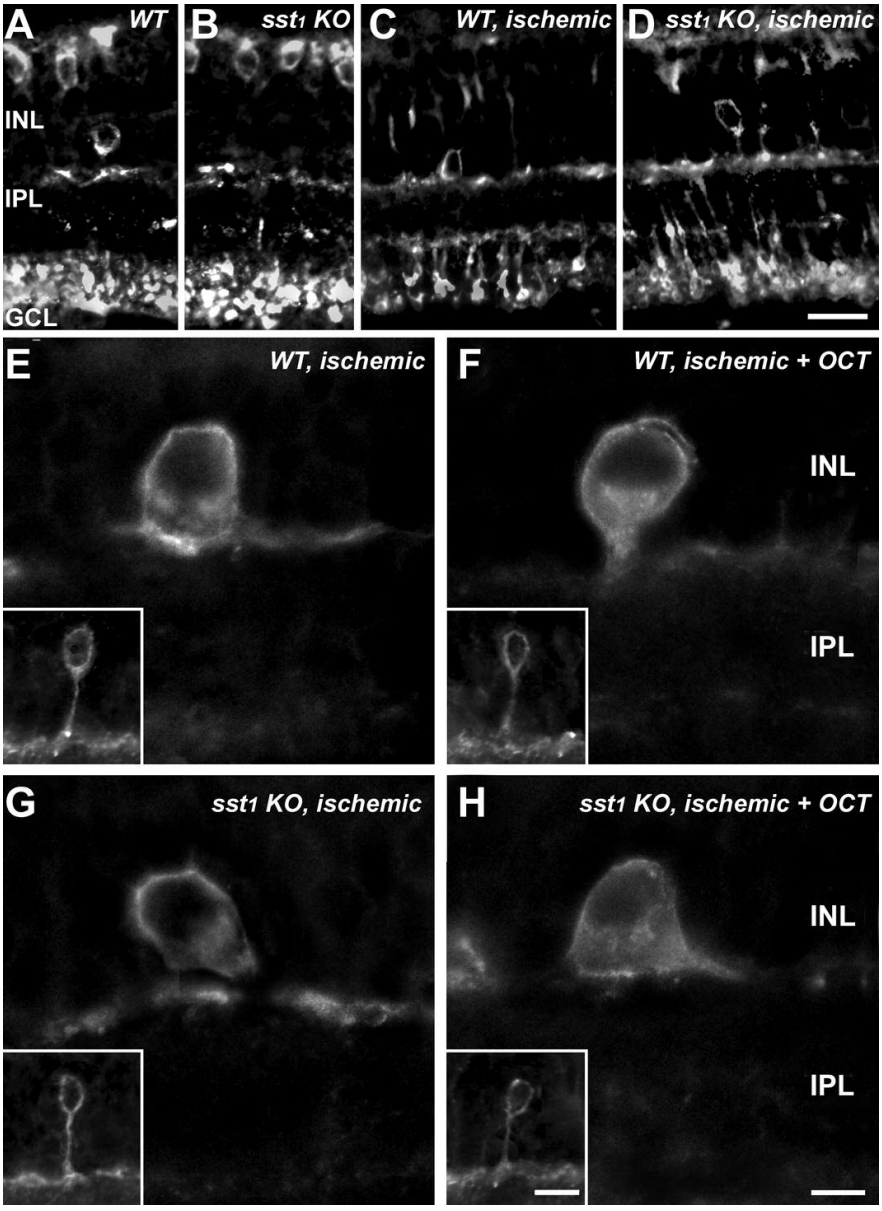

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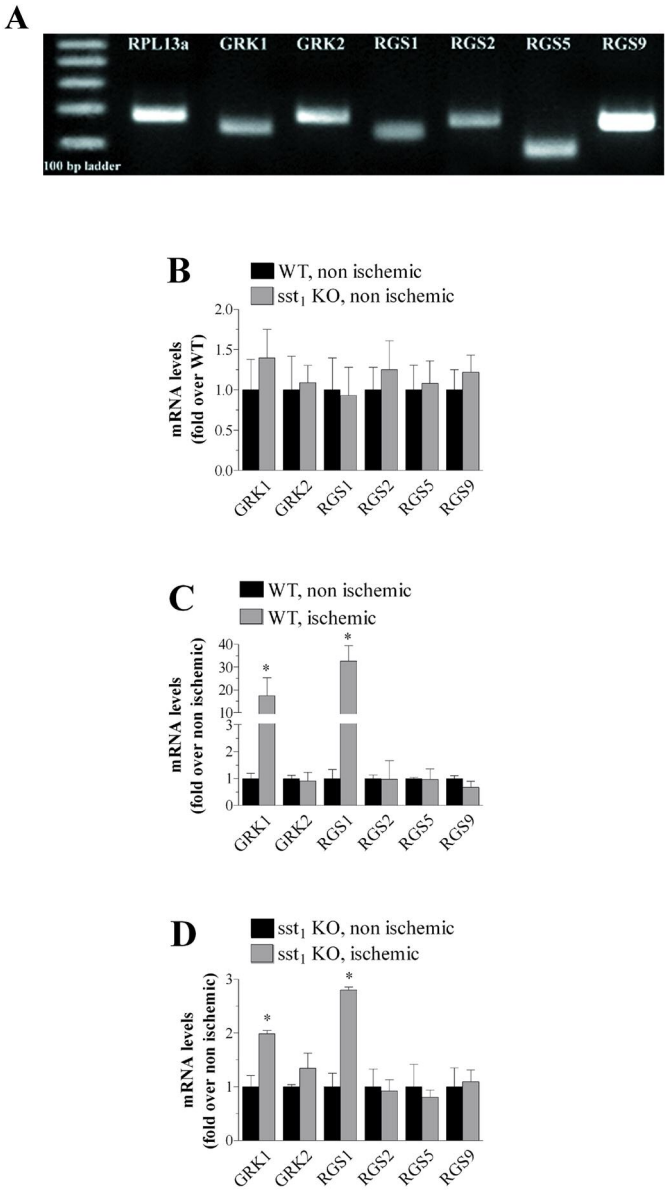

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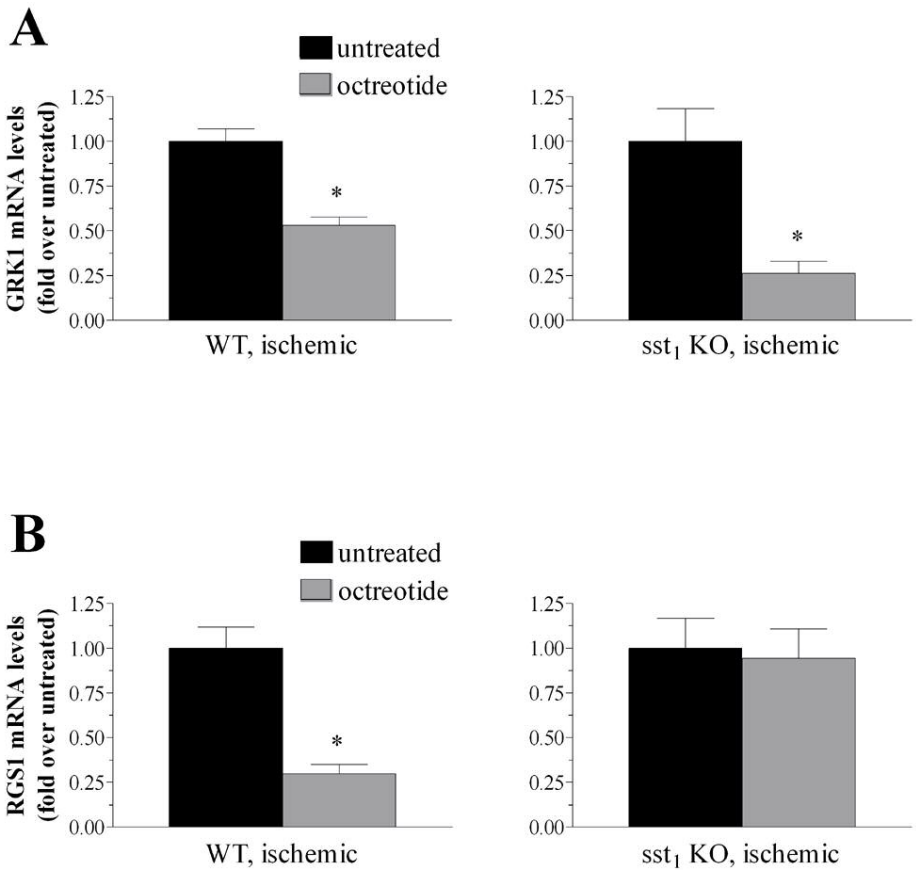

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