NADPH-Diaphorase Colocalization with Somatostatin Receptor Subtypes sst2A and sst2B in the Retina

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PURPOSE. To investigate the differential localization of somatostatin release-inhibitory factor (SRIF) receptor subtypes (sst2A and sst2B) and their possible colocalization with reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase in the rat and rabbit retina.

METHODS. Polyclonal antibodies raised against sst2A and sst2B receptors were applied to 10- to 14-μm cryostat sections of rat and rabbit retinas fixed in parafomaldehyde. NADPH-diaphorase reactivity was assessed histochemically. Double labeling was performed for sst2A or sst2B receptors with NADPH-diaphorase, and with markers for the cell types present in the retina (protein kinase C [PKC], tyrosine hydroxylase; [TH], calbindin, and recoverin).

RESULTS. sst2A immunoreactivity was detected in rod bipolar cells and colocalized with NADPH-diaphorase in the rabbit, but not the rat, retina. sst2B was present only in photoreceptor cells of the rat and colocalized with NADPH-diaphorase.

CONCLUSIONS. These results suggest that SRIF, acting through sst2A receptors in bipolar cells and sst2B receptors in photoreceptor cells, may affect nitric oxide function in the rabbit and rat retina. (Invest Ophtalmol Vis Sci. 2001;42:1600–1609)

The neuropeptide somatostatin (SRIF) is a cyclic tetrapeptide that is widely distributed in the peripheral and central nervous systems.1,2 It mediates a diverse number of physiological actions by interacting with specific receptors in the plasma membrane.3,4 These receptors are coupled to pertussis toxin-sensitive and -insensitive G proteins and modulate the activity of adenylate cyclase, potassium and calcium channels, and other intracellular systems.5–10 Five SRIF receptor subtypes have been cloned, namely sst types 1 through 5.11 sst2 has been identified in mouse and rat to exist as two splice variants sst2A and sst2B.12,13 These receptors are expressed differentially in different tissues, are coupled to different G proteins, and modulate the actions of diverse second messengers.14–16

In the retina, SRIF was detected by immunohistochemical studies in amacrine and ganglion cells, as well as in interplexiform cells, whereas electrophysiological studies support that it may function as a neurotransmitter, neuromodulator, or trophic factor.17–22 The actions of SRIF in the retina are mediated by specific receptors. There are reports of high-affinity binding sites for SRIF in the retina of mice,23 rat,24 and rabbit.25,26 Reverse transcription–polymerase chain reaction studies, examining the differential expression of SRIF receptors in the rat eye, show sst2 to be the major subtype expressed in the retina.27 This has been substantiated with pharmacologic28 and immunohistochemical studies.29–31

sst2A receptors were shown to be localized mainly in the plasma membrane of rod bipolar cells and wide-field amacrine cells in the rabbit and in cone photoreceptors, horizontal, amacrine, and rod and cone bipolar cells in the rat.29,30 Most recently, sst2A immunoreactivity was shown to be expressed in the inner segments and terminals of rod and cone photoreceptors and in bipolar and amacrine cells of the salamander.31

In another study, immunoreactivity for both sst1 and sst2 was shown in different populations of amacrine cells of the rat retina, whereas sst2 was also found in cone photoreceptor and horizontal cells, but not in rod bipolar cells.32 Although progress has been made recently in identifying SRIF receptors and their localization in the retina, the role of SRIF in retinal circuitry remains to be elucidated.

The initial purpose of the present study was to investigate the differential localization of the two ss2 receptor subtypes, sst2A and sst2B, in the retinas of two species, rat and rabbit. The second purpose was to examine the possible association of the SRIF receptors with other known systems in the retina, to obtain more information on the functional role of somatostatin. In brain, it has been shown that SRIF colocalizes with reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (a marker for nitric oxide synthase [NOS]) in medium-sized aspiny neurons of the striatum,33 but there are no reports to suggest that the two systems interact functionally. However, in the striatum, SRIF has been shown to stimulate dopamine (DA) release.34 In the retina, SRIF, DA, and nitric oxide (NO) are considered slow modulators of the retinal circuitry, rather than fast transmitters involved in visual information transfer and appear to resemble each other.18,51,55–57

DA localizes in amacrine cells, whereas NADPH-diaphorase is present in all retinal cell types, including subpopulations of amacrine cells. Both neuroactive agents, DA and NO, have important roles in retinal physiology, through mechanisms that are still under investigation.31,37–39

In the present study, the main emphasis was given to the putative SRIF-NO interactions for the following reason: Although colocalization of SRIF with NO in amacrine cells has never been observed,40 as was the case in the striatum,33 the sst2A receptor appears to localize in most retinal cells29,30–32 that are known to express NOS or NADPH-diaphorase.37,41–45 Thus, it appeared to be of particular interest to examine whether SRIF acting through sst2A and sst2B receptors could influence NO production. Therefore, the differential localization of the SRIF receptor subtypes sst2A and sst2B and their putative coexistence with NADPH-diaphorase in the rat and rabbit retina were investigated.

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METHODS

Animals

Male albino rabbits (New Zealand), weighing 2 to 3 kg, were purchased from a local breeder and maintained in individual cages with free access to food and water. The animals were anesthetized with ketamine (100 mg/kg, intramuscularly) and killed with an endocardial injection of pentothal. Male Sprague-Dawley rats weighing 250 to 300 g were housed two to three animals per cage with free access to food and water. A 12-hour light–dark cycle was maintained. Euthanasia was performed with ether inhalation. Housing conditions and all procedures that were performed on the animals were in accordance with Greek National Laws (Animal Act, PD 160/91) and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation

After the eyes were dissected, the anterior poles were cut away and the eye cups were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer for 1 hour. After fixation, eye cups were rinsed in phosphate buffer and incubated in 30% sucrose overnight at 4°C for cryoprotection. Tissues were then frozen in isopentane at −245°C for 1 minute and kept at −80°C until further use. Eye cups were sequentially sectioned vertically at 10- or 14-μm thickness with a cryostat, thaw mounted on gelatin-coated slides, and stored at −20°C.

Immunohistochemistry

For studying SRIF receptor and tyrosine hydroxylase (TH) immunoreactivity, rabbit polyclonal antibodies against sst2A and sst2B (1:1000 and 1:2000, respectively; Gramsch Laboratories, Schwabhausen, Germany) and TH (1:1000; Chemicon International, Temecula, CA) were used. A number of mouse monoclonal antibodies were used for TH (1:250; BioTrend Chemikalien, GmbH, Cologne, Germany), protein kinase C (PKC; 1:50; Leinco Technologies, Inc., St. Louis, MO) to label rod bipolar cells, and calbindin (1:200; Sigma, Munich, Germany) to label horizontal cells. A polyclonal antibody that recognized recoverin (1:80; a gift from Eleonora Grigoryan, Institute of Developmental Biology, Moscow) was used to label photoreceptors and cone bipolar cells. After blocking in 0.1 M Tris-HCl buffer (TBS; pH 7.4) containing 3.3% normal goat serum for 30 minutes, sections were incubated with primary antibody, in 0.1 M TBS containing 0.5% normal goat serum and 0.3% Triton X-100, overnight at room temperature. Subsequently, the sections were washed in TBS and incubated in fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit IgG (1:150; Vector Laboratories, Burlingame, CA) or tetramethyl rhodamine isothiocyanate (TRITC)–conjugated goat anti-mouse IgG (1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) secondary antibody for 1 hour at room temperature. The selectivity of the polyclonal antibodies for sst2A and sst2B were tested in COS-7 cells transfected with the cDNAs of the respective receptor. In addition, their selectivity was examined in sections incubated with antibody that was preadsorbed with synthetic peptides (Gramsch Laboratories) directed to the carboxy-terminus of sst2A (ETQRTLNLNGDLQTSI; 10−6 M) and sst2B (ADNSKTGEEDTMAWV; 5 × 10−4 M).

Colocalization Studies

For the double localization of sst2A or sst2B with TH, PKC, calbindin, and recoverin, sections were incubated overnight with anti-sst2A or anti-sst2B antisera together with monoclonal anti-TH, -PKC, and -calbindin and a polyclonal anti-recoverin. Sections were then washed...
with TBS and incubated with FITC-conjugated goat anti-rabbit IgG for 1 hour, followed by a 1-hour incubation with TRITC-conjugated goat anti-mouse IgG secondary antibody for 1 hour at room temperature. The sections were mounted with fluorescent mounting medium (Vector Laboratories) and examined by light microscopy.

NADPH-Diaphorase Histochemistry

The NADPH-diaphorase histochemical technique has been used as a marker by many investigators to assess the distribution of NOS activity in the retina.\textsuperscript{35,39,42–44} We used polyclonal antibodies raised against sst2A and sst2B receptors, and double labeling was performed with NADPH-diaphorase.

For the NADPH-diaphorase studies 14-μm-thick sections were used. The protocol was slightly modified from one previously reported.\textsuperscript{45} Sections were incubated in 0.1 M Tris-HCl (pH 7.4), containing 0.8 mM \( \beta \)-NADPH, 1 mM nitro blue tetrazolium, 10 mM malic acid, and 1.5% Triton X-100 at 37°C for 1.5 hours. Sections were rinsed with 0.1 M Tris-HCl, air dried, dehydrated, and coverslipped.

![Figure 2](image2.png)

**Figure 2.** Colocalization of sst2A and PKC on rod bipolar cells of rat and rabbit retinas. All bipolar cells immunostained for sst2A (A, C) were immunofluorescent for PKC (B, D). Abbreviations defined in Figure 1. Scale bars, 50 μm.

![Figure 3](image3.png)

**Figure 3.** sst2B immunoreactivity in rat retina. sst2B immunostaining was present on photoreceptors of the rat retina (A). Control sections incubated with the sst2B antibody titrated with antigen (sst2B: ADNSKT-GEEDTMAW; 5 \( \times \) 10\(^{-5}\) M) showed no sst2B immunostaining (B). Abbreviations defined in Figure 1. Scale bar, 50 μm.
NADPH-Diaphorase and Immunofluorescence Labeling

For the double-localization studies, sections were initially enzymatically processed for NADPH-diaphorase staining and were then incubated with the different antibodies (sst2A, sst2B, TH, PKC, calbindin, and recoverin), as described. The sections were mounted with mounting medium for fluorescence and examined by light microscopy.

Microscopy

Conventional light microscopy images were taken (Axioskop with Plan-Neofluar ×20/0.50 and ×40/0.75 objectives; Carl Zeiss, Oberkochen, Germany), and immunoreactivity was also examined with a laser scanning microscope (He/Ne laser; model DM RE; Leica, Heidelberg, Germany; with Plan Fluotar ×40/1.00 or Plan Apo ×63/1.32 objectives; Carl Zeiss). Optic sections were taken with a z-axis resolution of 2 μm through the immunolabeled cells. The images shown in Figure 4 are projections of the z-axis series. Images were processed with image management software (Photoshop, ver. 5.0; Adobe, San Jose, CA).

RESULTS

sst2A and sst2B Immunoreactivity

sst2A immunoreactivity was detected in rat and rabbit retinas in cells with the morphologic and positional characteristics of rod bipolar cells (Fig. 1). These comprised a dense network in the rod-dominated rat retina (Fig. 1A), whereas they were less numerous in the rabbit (Fig. 1C). In control sections, primary antibody was preblocked with antigen, providing evidence for its specificity (Figs. 1B, 1D). Double-labeling experiments were performed with PKC antibodies to confirm the presence of the sst2A receptor on rod bipolar cells (Fig. 2).

sst2A immunoreactivity was not observed in horizontal and photoreceptor cells. To substantiate this finding, colocalization studies with sst2A and known markers were performed. In both species, no colocalization was detected with sst2A and recoverin, a marker for photoreceptors and cone bipolar cells, or with calbindin a marker for horizontal cells (data not shown).

Immunohistochemical studies with sst2B antibodies were performed in the rat and rabbit. In the rat, sst2B was found to localize only in the inner and outer segments of the photoreceptors (Fig. 3A). This staining was specific, as substantiated by the absence of immunostaining in the presence of the sst2B antigen (Fig. 3B), whereas no immunoreactivity was detected in the rabbit retina (data not shown).

Colocalization Studies

TH immunohistochemistry was performed, not only to use the TH staining as a marker for amacrine cells, but also to examine the possible coexistence of sst2A and TH, with the purpose of ascertaining whether SRIF has a role as a regulator of the DA pathway in the retina.
system in the retina. Double-labeling experiments of sst2A and TH in the rat were performed and confocal microscopy was used to examine the immunoreactivity. Confocal images are presented in Figure 4 and no colocalization of TH with sst2A was apparent (Fig. 4C). Double-labeling of sst2A and TH in the rabbit retina did not show any colocalization in the amacrine cells of the rabbit, as observed by light microscopy (Fig. 5) and confocal microscopy (data not shown).

NADPH-diaphorase Staining and sst2A and sst2B Immunoreactivity

NADPH-diaphorase staining localized in the inner segments of photoreceptors and in amacrine cells of the rat (Fig. 6A) and rabbit (Fig. 6B), and in the rabbit it also localized in cells of the inner nuclear layer (INL). Colocalization of NADPH-diaphorase staining with PKC confirmed these cells to be rod bipolar (Fig. 7). NADPH-diaphorase staining was observed on the cell bodies but not in the processes of the rod bipolar cells. In the rat, no colocalization of NADPH-diaphorase staining and PKC was observed (Fig. 8). To examine whether the cells that express NOS also express the sst2A and sst2B receptors, colocalization studies were performed. NADPH-diaphorase staining localized with the sst2A receptors in the cell bodies of rod bipolar cells of the rabbit retina (Fig. 9). The arrows in the image indicate two such cells. Although there was a population of cells that expressed both NO and sst2A, there were also cells that ex-
pressed either one or the other. sst2B receptors colocalized with NADPH-diaphorase staining in the inner segments of the photoreceptors in the rat (Fig. 10).

**DISCUSSION**

The elucidation of the role of SRIF in the neuronal circuitry of the retina is still an important target of investigation. Most recently, the presence of SRIF receptor subtypes sst1 and sst2 in distinct cells of rat and rabbit retina sheds some light on the possible role of SRIF in this tissue. The sst1 receptor was suggested to be an autoreceptor for SRIF on cells of the INL and ganglion cell layer (GCL) in the rat retina, whereas sst2 was found to be present in photoreceptors and TH-containing amacrine cells, but not on rod bipolar or horizontal cells.32 The sst2A isoform was found to be present in rod bipolar and amacrine cells in the rabbit retina30 and in rod bipolar, amacrine, horizontal, and photoreceptor cells in the rat retina.30 The discrepancy in some of the immunostaining data using the sst2A and sst2 antibodies in rabbit and rat, respectively, was believed to be species related.32

We chose to examine the immunostaining and possible differential localization of the two isoforms of the sst2 receptor, namely sst2A and sst2B, in both rat and rabbit. sst2A was found to localize in rod bipolar cells in both rabbit and rat retinas in agreement with previous reports.29,30 Confocal microscopy scanning of the sections assured us that 100% of the PKC-stained rod bipolar cells contained sst2A.

We did not detect any sst2A immunostaining in TH-containing amacrine cells in the rabbit retina, as was substantiated by the absence of colocalization of the respective antibody immunostaining. This is in agreement with observations by others.29 However, our findings do not support the presence of sst2A in TH-containing amacrine cells of the rat retina, in contrast to previous observations.30,32 These results were confirmed with confocal microscopy. In addition, no sst2A immunostaining was detected in photoreceptor or horizontal cells of the rat or rabbit, as established by the absence of colocalization of the

**FIGURE 7.** Colocalization of NADPH-diaphorase and PKC in the rabbit retina. NADPH-diaphorase staining (A) was detected in PKC (B) immunofluorescent rod bipolar cells. Abbreviations defined in Figure 1. Scale bars, 20 μm.

**FIGURE 8.** Localization of NADPH-diaphorase and PKC in the rat retina. NADPH-diaphorase (A) staining was not detected in PKC-immunofluorescent (B) rod bipolar cells. (B) Staining due to blood vessels. Abbreviations defined in Figure 1. Scale bars, 20 μm.
sst2A receptor with the known markers, recoverin and calbindin. The discrepancies in the results of this study and those previously published, such as the absence of sst2 immunoreactivity in rod bipolar cells of the rat, the absence of sst2, and the presence of sst2A in horizontal cells of the rat, could not be attributed to the species but most likely is attributable to technical differences, such as the antibodies used. Our results, however, support the presence of sst2B in the photoreceptors of the rat retina, whereas sst2B immunoreactivity was not detected in the rabbit. This could be due either to the inability of the anti-rat antibody to cross-react with the rabbit antigen or to the absence of sst2B in the rabbit.

sst2A and sst2B receptors are generated by alternative splicing of the sst2 receptor mRNA and differ only in the length and amino acid sequence of their carboxyl-termini. The third intracellular loop of the receptor has been shown to be responsible for G-protein interactions. However, the C terminus appears to play an important role in the modulation of the coupling efficiency or desensitization of the receptor. The presence of sst2A and sst2B on rod bipolar and photoreceptor cells, respectively, supports a differential role for SRIF in the regulation of glutamate release, the major neurotransmitter of these retinal cell types. Furthermore, this regulation may be mediated by different G proteins differentially coupled to the two subtypes. SRIF receptors associate with multiple G proteins, including Gia1, Gia3, and Goa, which couple to different transduction pathways, such as adenylate cyclase, phospholipase C, and membrane ion channels.

**Figure 9.** Colocalization of NADPH-diaphorase and sst2A in the rabbit retina. NADPH-diaphorase staining (A) and sst2A immunoreactivity (B) were colocalized in rod bipolar cell bodies (C). Arrows: two of the double-labeled cells. Abbreviations defined in Figure 1. Scale bars, 50 μm.
interacts selectively with Giα₃ and Goα₁₀ but not with Giα1, the G protein necessary for coupling SRIF receptors to adenylate cyclase. Pertaining to the retina, we have shown the presence of Giα1/2 and Goα in rabbit retinal membranes. Recently, it was reported that SRIF enhanced a delayed outwardly rectifying K⁺ current in both rod and cone photoreceptors in the salamander retina, whereas a differential action of SRIF was observed on rod and cone calcium currents. These effects were pertussis toxin-sensitive, even though the G proteins involved were not characterized. The receptor involved was suggested to be sst2A, because immunohistochemistry studies, in which an antibody against sst2A was used, showed immunoreactivity present in both rod and cone photoreceptors. Our data suggest that sst2B is also responsible for the actions of SRIF in retinal physiology. Therefore, SRIF synthesized and released by the amacrine cell may act at a distance in a paracrine fashion, as previously suggested.

In the retina, SRIF is one of many neuroactive substances that influence retinal circuitry. Although recent studies have improved our knowledge on the localization of the ssts in the retina, the functional role of SRIF in retinal circuitry must still be substantiated. In brain, SRIF has been shown to colocalize with NADPH-diaphorase in medium-sized aspiny neurons of the striatum and to influence DA release in the same nucleus. It was therefore of interest to examine whether SRIF

![Figure 10](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933588/)
may modulate the visual cascade by interacting with these neurochemical systems in the retina.

DA, NADPH-diaphorase, and SRIF localized in different populations of cells have important roles in retinal physiology, through mechanisms that are still under investigation. DA, NO, and SRIF are neuroactive molecules that appear to act as neuromodulators of synaptic transmission in the retina. To examine the possible regulation of the dopaminergic or NO system by TH, we studied the colocalization of sst2A and sst2B with TH and NADPH-diaphorase, respectively. Our results do not support a colocalization of sst2A and TH in amacrine cells of the rat or rabbit retina, in contrast to observations in the rat. It may be of significance to perform immunohistochemical studies using the different sst2A and sst2 antibod-

ies, under identical conditions, to ascertain the presence of sst2A or sst2 receptors on TH neurons. However, although the sst2 subtype is the most abundant sst receptor in the retina, we cannot exclude the regulation of the DA system by another sst receptor present in the retina.

NADPH-diaphorase was first found in the retina to localize in specific types of amacrine cells. In some species it is located in a specific ganglion cell type and in others in the inner segments of cone photoreceptors, with some activity also found in the outer segments. There are fewer reports showing its presence in horizontal or rod bipolar cells. In our studies, we observed NADPH-diaphorase staining in photoreceptors and amacrine cells of the rat and rabbit. No staining was detected in horizontal cells, as was substantiated by the colocalization studies with calbindin, a known marker for horizontal cells. In the rabbit, NADPH-diaphorase staining was also present in rod bipolar cells. This is the first report, to our knowledge, showing the presence of NO in rod bipolar cells of this species. In the rat, no NADPH-diaphorase staining was detected, in contrast to a previous report showing neuronal NO-like immunoreactivity to be present in some bipolar cells.

In the present study, sst2A colocalized with NADPH-diaphorase on rod bipolar cells of the rabbit retina, whereas sst2B colocalized with NADPH-diaphorase on the photoreceptors of the rat. These results support for the first time a possible functional role of SRIF in the regulation of the physiology of NO in the retina.

In conclusion, the novel finding in this work is that SRIF may differentially influence the function of retinal cells by acting through sst2A and sst2B receptor subtypes in bipolar and photoreceptor cells, respectively. The finding that both receptors colocalize with NADPH-diaphorase suggests that SRIF may play an important role in the retina by affecting NO production.

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