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Review

Functional aspects of the somatostatinergic system in the retina and the potential therapeutic role of somatostatin in retinal disease

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Summary. The somatostatinergic system of the retina has been investigated in a variety of studies. A considerable amount of experimental evidence is available concerning the patterns of expression of somatostatin (SRIF) and its receptors in vertebrate retinas. However the functional roles of this peptidergic system in retinal physiology are far from being elucidated. Nonetheless, data have been provided concerning the regulatory action of SRIF on the excitability of different retinal cell types and on the modulation of ion channels in different vertebrate retinas. The present review is focused on recent and unpublished investigations of the mouse retina relative to the involvement of specific SRIF receptors in the regulation of ion channels and transmitter release, the transduction pathways coupled to SRIF receptors, and the mechanisms regulating the expression of SRIF and its receptors as derived from studies in transgenic animal models. In these models, altered expression levels of SRIF or of specific SRIF receptors have also been found to affect the morphology of retinal cell types (namely the rod bipolar cells) and to result in functional alterations at the level of both ion channel regulation and transmitter release. These new pieces of evidence constitute an important step forward in the understanding of the functional actions of the retinal somatostatinergic system, although our current knowledge is far from being exhaustive. The ultimate goal of understanding SRIF functional actions in the retina is concerned with the possibility of using SRIF or its analogs as therapeutic agents to cure retinal diseases. Indeed, encouraging results are being obtained in clinical investigations focused on the use of SRIF analogs to treat diabetic retinopathy, a retinal disease with high social impact and originating as a complication of diabetes. The closing part of the present paper examines the evidence supporting SRIF as a promising therapeutic agent in this disease.

Key words: Somatostatin receptors, Transgenic animals, Retinal cells, Diabetic retinopathy

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Introduction

Somatostatin (somatotropin release-inhibiting factor, SRIF) is a neuropeptide that is widely distributed in the central and peripheral nervous systems, where it plays a variety of biological roles (Blake et al., 2004; Olias et al., 2004). Two forms of SRIF have been identified: SRIF-14, the form originally discovered in the hypothalamus (Brazeau et al., 1973), and SRIF-28, a congener of SRIF-14 extended at the N-terminus that was discovered subsequently (Pradayrol et al., 1980). SRIF-14 is virtually the only form expressed in the retina (Patel, 1999). It interacts with five membrane receptors, designated sst_1-sst_5 (with two sst_2 receptor isoforms, sst_{2A} and ss_{t2B}, derived from alternative mRNA splicing) that are coupled to different transduction pathways (Reisine and Bell, 1995; Florio and Schettini, 1996; Tannenbaum and Epelbaum, 2000; Csaba and Dournaud, 2001; Lahlou et al., 2004; Olias et al., 2004).

The vertebrate retina is a widely used model of the central nervous system (see Bagnoli et al., 2003 for references), and several investigations have studied the organization of the somatostatinergic system and the functional actions of SRIF in retinas of different species. Recent papers have reviewed the expression patterns of SRIF and of its receptors in the retina, together with physiological actions of SRIF on retinal cells (Brecha, 2003; Thermos, 2003). In addition, the developmental profiles of SRIF and of SRIF receptor expression in mammalian retinas have also been summarized (Bagnoli et al., 2003). However, important results have been recently obtained in retinas of normal and transgenic

mice, and a comprehensive account of these data is still lacking. Also, the evidence concerning the transduction pathways coupled to SRIF receptors in the retina (although poorly investigated to date) deserves some attention. The present review, in addition to providing a summary of previously reviewed data, aims at filling this gap and it provides information concerning recent and unpublished data of the mouse retina, with particular attention devoted to those investigations performed using retinas of mice carrying genetic deletion of the sst_1 receptor, the sst_2 receptor, or of SRIF itself, as experimental models. Finally, the evidence will be reviewed supporting a possible role of SRIF as an important therapeutic agent in a major retinal disease such as diabetic retinopathy.

Localization of somatostatin and somatostatin receptors in the retina

The studies elucidating the expression patterns of SRIF and of its receptors in the retina have been recently reviewed by Brecha (2003) and by Thermos (2003). The following is a brief summary of such data.

SRIF immunoreactivity is localized to sparsely distributed, wide-field amacrine and displaced amacrine cells in the mouse (Cristiani et al., 2002), rat (Sagar et al., 1985; Larsen et al., 1990), guinea pig (Tornqvist et al., 1982; Spira et al., 1984) and human retina (Tornqvist and Ehinger, 1988). In the rabbit, cat and primate retina, most SRIF immunoreactive cells are displaced amacrine cells (Sagar, 1987; Sagar and Marshall, 1988; Marshak, 1989; Mitrofanis et al., 1989; White et al., 1990; Engelmann and Peichl, 1996; Rickman et al., 1996). SRIF immunoreactive cells in rabbit and human retina have been found to possess an intraretinal axon (Sagar, 1987; Sagar and Marshall, 1988), and they have been considered to be a population of the polyaxonal amacrine cell type (Völgyi et al., 2001). Confirming this classification is the recent finding of the expression of the multiphosphorilated epitope of axonal neurofilament-H in SRIF-containing processes of the rabbit retina (Völgyi and Bloomfield, 2002). A small percentage of ganglion cells has also been reported to contain SRIF immunoreactivity in the retina of the new world monkey *Tupaia belangeri* (Engelmann and Peichl, 1996) and of the cat, where SRIF-immunolabeled ganglion cells constitute a very small number of OFF-center alpha ganglion cells that are mostly localized to the inferior retina (White and Chalupa, 1991). Finally, SRIF-containing ganglion cells are transiently present during postnatal development in the rat retina (Fontanesi et al., 1997; Xiang et al., 2001).

All five sst receptor mRNAs have been detected in retinal extracts (Mori et al., 1997; Johnson et al., 1999; Cristiani et al., 2000, 2002). Both in the rat and in the mouse retina, sst_2 and sst_4 receptor mRNAs are the most abundant (Mori et al., 1997; Cristiani et al., 2002). In contrast, in the rabbit retina, sst_1 receptor mRNA is the most highly expressed, sst_2 receptor mRNA is moderate, while sst_3, sst_4 and sst_5 receptor mRNAs are low (Cristiani et al., 2000).

Immunocytochemical data show that SRIF receptors are expressed by a variety of retinal cell populations. In particular, the sst_1 receptor is predominantly expressed by SRIF-containing amacrine cells, (Helboe and Moller, 1999; Cristiani et al., 2000; Dal Monte et al., 2003b) and it functions as an autoreceptor (Mastrodimou and Thermos, 2004). In the rabbit retina, sst_1 receptors are also expressed by all the dopaminergic amacrine cells (Cristiani et al., 2000). Of the two sst_2 receptor isoforms, the sst_{2A} receptor has been immunohistochemically localized in rat, rabbit and mouse retinas (Johnson et al., 1998, 1999; Helboe and Moller, 1999; Fontanesi et al., 2000; Petrucci et al., 2001; Vasilaki et al., 2001; Cristiani et al., 2002). In rabbits, it is expressed mainly by rod bipolar and by sparse amacrine cells. These amacrine cells have been reported to lack (Johnson et al., 1998) or to partially express (Fontanesi et al., 2000) tyrosine hydroxylase (TH) immunoreactivity. In the rat retina, sst_{2A} receptor has been localized to amacrine cells, including TH-containing amacrine cells, to rod and cone bipolar cells and to horizontal cells (Johnson et al., 1999). The sst_{2B} receptor isoform in the rat retina is predominantly found on the membrane of photoreceptors, indicating SRIF actions in the outer retina (Vasilaki et al., 2001). Finally, sst_4 receptor immunolabeling in mouse retinas is localized to sparse cells in the ganglion cell layer that originate long process bundles in the nerve fiber layer and are likely to be ganglion cells (Cristiani et al., 2002). Although sst_3 and sst_5 receptor mRNAs are expressed in the retina (Mori et al., 1997; van Hagen et al., 2000; Klisovic et al., 2001; Cristiani et al., 2002), no data are available concerning sst_3 and sst_5 receptor immunoreactivities.

Somatostatin receptor coupling to transduction pathways

While numerous studies have explored signaling pathways coupled to SRIF receptors when expressed in recombinant systems, specific physiological responses of native receptor subtypes have been poorly investigated. There is evidence that distinct G proteins mediate SRIF actions in the brain (Weckbecker et al., 2003), but little is known in the retina, where G protein expression and functional coupling to SRIF receptors have been recently demonstrated: in particular, Vasilaki and colleagues (2003) have reported that sst_2 receptors couple to Go alpha in the rabbit retina. A further evidence of sst_2 receptor coupling to Go alpha comes from recent data demonstrating an increased expression of Go alpha subunits in transgenic mouse retinas with sst_2 receptor overexpression (see paragraph on "Somatostatin and its receptors in the retina of transgenic mice"; Pavan et al., 2004).

In the brain, SRIF receptors appear to influence an array of intracellular effectors including adenylyl cyclase (AC), phospholipase C, phospholipase A_2 and MAP

kinase (Csaba and Dournaud, 2001; Weckbecker et al., 2003). In addition, the involvement of nitric oxide (NO) in SRIF-evoked responses has been suggested (Lopez et al., 2001). In the retina, investigations have been concerned with the functional coupling of SRIF receptors with the AC and the NO systems.

Adenylyl cyclase/cAMP pathway

All SRIF receptors are negatively coupled to the AC/cAMP pathway in nervous and non-nervous tissues (Weckbecker et al., 2003). However, SRIF receptor coupling to AC/cAMP pathway in the retina still remains to be clarified. Indeed, contradictory results have been reported in studies investigating SRIF-induced modulation of AC/cAMP pathway in the retina. For instance, SRIF has no effects on cAMP accumulation in carp, pigeon and rabbit retinas (Schorderet et al., 1981; Watling and Dowling 1983), but it induces cAMP accumulation in the chicken retina (Firth et al., 1998). In the ovine retina, SRIF inhibits VIP-stimulated AC activity (Colas et al., 1992). Furthermore, it has been shown that AC activation, likely resulting in GABAA receptor phosphorylation, is involved in the SRIF-induced enhancement of GABAergic signaling in amacrine cells of the rat retina (Feigenspan and Bormann, 1994).

In the mouse retina, both sst_1 and sst_2 receptors, if activated individually, do not show coupling to AC activity, however sst_1 or sst_2 receptor coupling to AC inhibition may be revealed once sst_2 or $ssst_1$ receptors, respectively, are blocked by their selective antagonists (Pavan et al., 2004; Fig. 1). This finding suggests that, in the mouse retina, an interaction between sst_1 and sst_2 receptors (either directly at the receptor level or indirectly as a cross-talk between their signaling pathways) may prevent their effects on AC activity. The fact that sst_1 receptors prevent sst_2 receptor coupling to AC is confirmed by results from retinas with genetic deletion of the sst_1 receptor, in which sst_2 receptor coupling to AC inhibition becomes apparent (Pavan et al., 2004). The possibility exists that, in the mouse retina, sst_1 and sst_2 receptors exhibit hetero-dimerization, and thereby changes in their binding and functional properties, including AC regulation (Rocheville et al., 2000; Pfeiffer et al., 2001).

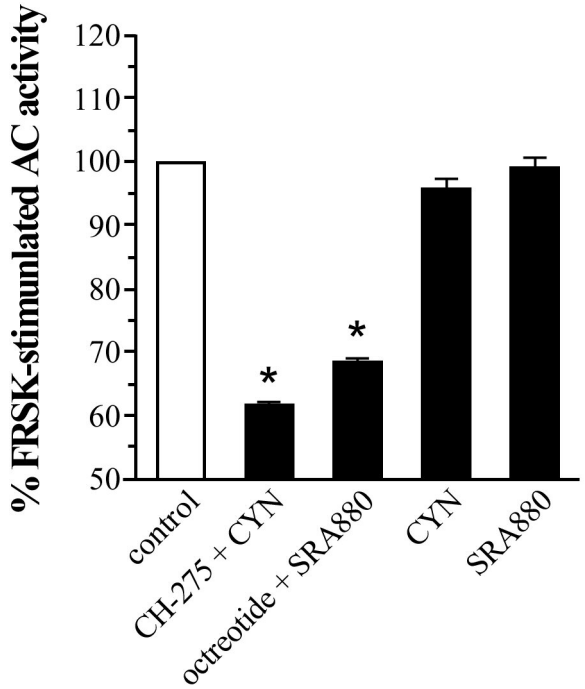

Fig. 1. Percentage of inhibition of 1 µm forskolin (FRSK)-stimulated AC activity by 1 µM of the sst_1 receptor agonist CH-275 in combination with the sst_2 receptor antagonist D-Tyr 8 Cyn 154806 (CYN; 1 µM) or 1µm of the sst_2 receptor agonist octreotide in combination with the sst_1 receptor antagonist SRA880 (1 µm) in WT mouse retinas. Control values are plotted as 100% and are similar to values obtained after 1 µm CH-275 or octreotide application in the absence of antagonists. Comparable values were also obtained after the application of 1 µm CYN or SRA880. Histograms are the means ± SEM (bars) of data from six independent experiments run in duplicate. *: p<0.001 vs. control values. From Pavan et al., 2004.

Nitric oxide pathway

The abundance of NO synthesizing enzymes identified in the vertebrate retina indicates that NO represents an important signaling molecule in this tissue (Liepe et al., 1994; Margulis et al., 1998; Cudeiro and Rivadulla, 1999; Sitaramayya, 2002), and there is evidence that it is released by amacrine and bipolar cells (Neal et al., 1998). As a membrane-permeant neuronal messenger in the central nervous system, NO produces its biological actions through distinct signal transduction pathways (Stamler et al., 1997; Ahern et al., 2002). In particular, NO initiates a signaling cascade by activating the soluble isoform of guanylyl cyclase and subsequently elevates intracellular concentration of 3',5'-cyclic guanosine monophosphate (cGMP) (Matsuoka et al., 1992; Southam and Garthwaite, 1993; Wood and Garthwaite, 1994). The main targets of cGMP are cGMP-gated channels (Zagotta and Siegelbaum, 1996), cGMP-dependent phosphodiesterases (Pineda et al., 1996; Kraus and Prast, 2002), and cGMP-stimulated protein kinase G (Jaffrey and Snyder, 1995). The NO–cGMP–protein kinase G signaling pathway may be important in the regulation of neuronal excitability and neurotransmission at pre- and postsynaptic sites. In the retina, the colocalization of sst_2 receptors with NADPH-diaphorase in rod bipolar and photoreceptor cells has been reported (Vasilaki et al., 2001), suggesting a role of SRIF in the regulation of NO production in the retina. In fact, although octreotide (a SRIF agonist mostly acting at sst_2 receptors; Siehler et al., 1998; Hannon et al., 2002a) does not ameliorate NO activity in the ischaemic retina (Celiker and Ilhan, 2002), there is conclusive

evidence that an intracellular pathway activated by SRIF in the rat retina involves the regulation of NO by an sst_2 receptor-mediated mechanism (Vasilaki et al., 2002), and recent results demonstrate that SRIF regulates NO production in human retinal pigment epithelial cell cultures by activating sst_2 receptors (Vasilaki et al., 2004).

Functional actions of somatostatin in the retina

Somatostatin modulation of ion channels

Experimental approaches using patch clamp and Ca^{2+} imaging techniques have been used to investigate SRIF modulation of ion channels expressed by retinal neurons. Low concentrations of SRIF enhance a delayed outwardly rectifying K^+ current in photoreceptor terminals in salamander retinal slices (Akopian, 2000; Akopian et al., 2000). In the same preparations, Akopian and colleagues (2000) showed that SRIF differentially modulates voltage-gated L-type Ca^{2+} currents in rod and cone photoreceptors: while it reduces Ca^{2+} current in rods, it increases Ca^{2+} current in cones. These findings are confirmed by Ca^{2+}-imaging data, showing that SRIF reduces a K^+-induced Ca^{2+} entry in rods but increases it in cones. Together, these observations in salamander retina preparations suggest that SRIF may influence transmitter release from photoreceptors through modulation of voltage-gated K^+ and Ca^{2+} currents.

In goldfish retina, SRIF, similar to other peptides including substance P and met5-enkephalin, inhibits a voltage-dependent Ca^{2+} current in rod bipolar cells (Ayoub and Matthews, 1992). In the axonal terminals of isolated rod bipolar cells of the rat retina, SRIF strongly inhibits a K^+-stimulated increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via L-type Ca^{2+} channels (Johnson et al., 2001). This effect of SRIF is likely to be mediated by sst_2 receptors. Indeed, in isolated rod bipolar cells of the rabbit retina both SRIF and the sst_2 receptor agonist octreotide inhibit Ca^{2+}- and voltage-dependent K^+ channels and reduce the $[Ca^{2+}]_i$ increase following K^+ stimulation (Petrucci et al., 2001). These effects are prevented by the sst_2 receptor antagonist L-Tyr8-Cyanamid 154806, further demonstrating that these actions of SRIF are mediated by sst_2 receptors (Petrucci et al., 2001). Recent findings obtained in our laboratory by observing Ca^{2+} dynamics in isolated rod bipolar cells from mouse retina in confocal microscopy confirm and expand the observations in rat and rabbit models. Isolated mouse rod bipolar cells were loaded with the fluorescent calcium indicator fluo-3- acetoxymethyl-ester, and changes in fluorescence intensity, indicating changes in $[Ca^{2+}]_i$, were recorded either in the cell soma or in the axon terminal following incubation with SRIF or octreotide. In basal conditions, 200 nM SRIF fails to induce changes in $[Ca^{2+}]_i$, however SRIF or octreotide at the same concentration are effective in reducing the K^+-stimulated increase in $[Ca^{2+}]_i$ both in the cell body (65.1±6%, n=8) and in the axonal terminals (92.6±4.7%, n=5) (Fig. 2). Together, these findings suggest that, similar to the SRIF effects on photoreceptor cells, also in the inner retina SRIF may influence cellular activity and transmitter release by modulating K^+ and Ca^{2+} ion channels.

Somatostatin modulation of transmitter release

Earlier investigations showed that SRIF does not change the level of light-evoked release of acetylcholine from rabbit retina (Cunningham and Neal, 1983). However, consistent with the demonstrated SRIF regulation of K^+ and Ca^{2+} currents in photoreceptors and in rod bipolar cells (see previous paragraph), SRIF has been found to affect retinal glutamate release. In particular, in explants of mouse retina, SRIF and the sst_2 receptor agonist octreotide similarly reduce the K^+-evoked release of glutamate without affecting its basal level. In retinas with targeted deletion of the sst_2 receptor, SRIF or octreotide do not affect glutamate release, indicating that they act as sst_2 receptors (Dal Monte et al., 2003a). Furthermore, the SRIF-induced inhibition of retinal glutamate release significantly increases when sst_2 receptors become over-expressed, as in sst_1 knock out (KO) retinas (see paragraph on "Somatostatin and its receptors in the retina of transgenic mice"; Bigiani et al., 2004).

Somatostatin role in visual information processing

Although Cunningham and Neal (1983) reported that, in rabbit retina in vivo, SRIF reduces the amplitude of the ERG b-wave while other peptides, such as cholecystokinin and substance P, do not affect the ERG, Zalutsky and Miller (1990), working with rabbit eyecup preparations, found that application of low concentrations of SRIF increases the amplitude of the a-, b- and c-waves. In addition, SRIF at low concentrations is excitatory on all ganglion cells. They respond changing their "signal-to-noise ratio", discharge activity and receptive field organization, with a shift in the center-surround balance towards a more dominant center. These effects exerted by SRIF are slow in onset and display long latency. In addition, SRIF has been found to also influence the activity of bipolar cells, amacrine cells, and the horizontal cell network. Finally, SRIF has been reported to increase input resistance of amacrine and bipolar cells (Zalutsky and Miller, 1990). This finding would suggest an action on ion channels, consistent with the documented effects of SRIF on K^+ and Ca^{2+} currents in photoreceptors and bipolar cells (Akopian et al., 2000; Johnson et al., 2001; Petrucci et al., 2001). Together, these data indicate SRIF as a modulatory substance which, by acting at the level of multiple retinal circuits and inducing long-lasting changes in ganglion cell physiology, may affect adaptation in the retina.

The somatostatinergic system may play a central role in the regulation of the rod pathway and interact

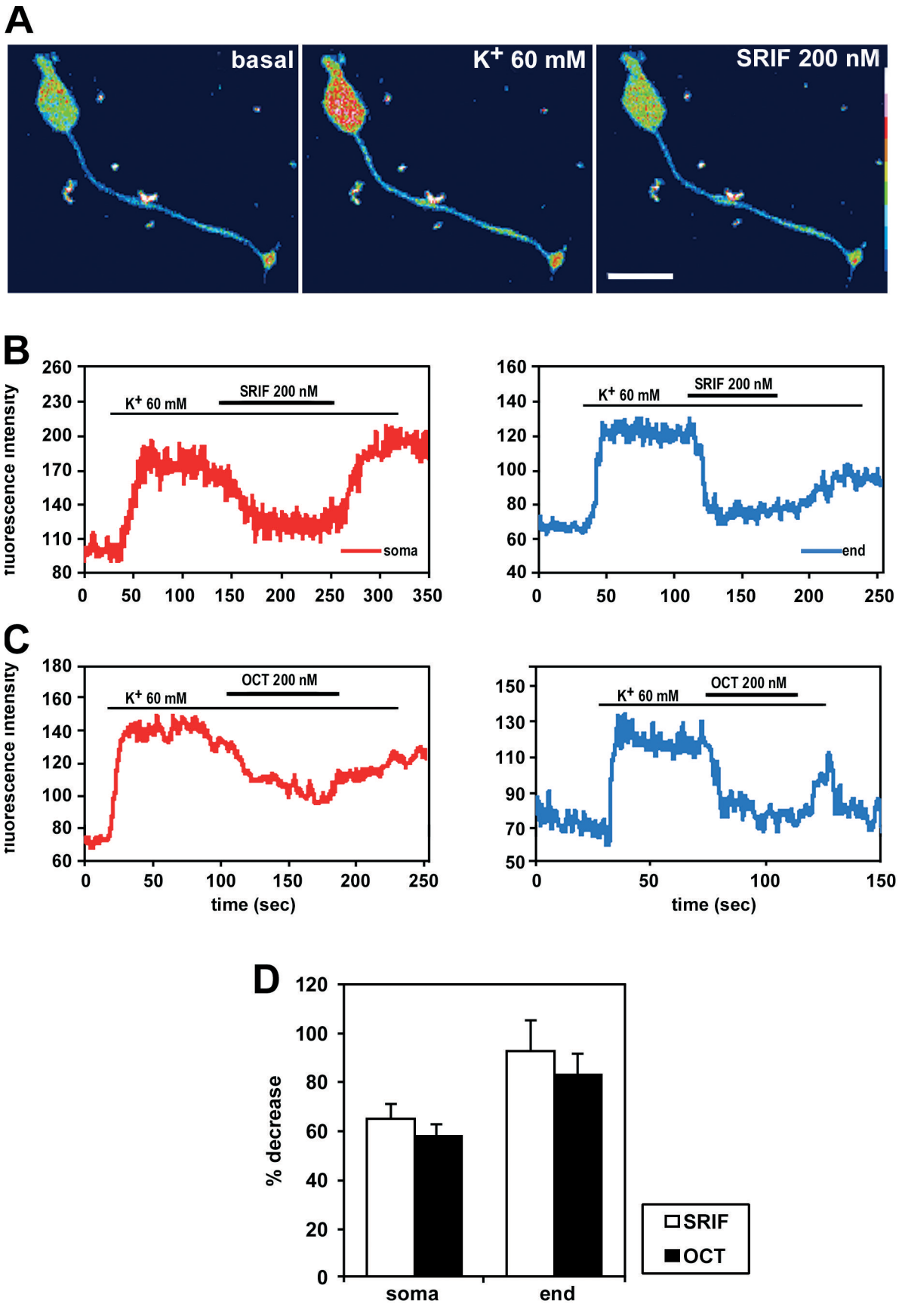

Fig. 2. SRIF and octreotide (OCT)-induced modulation of $[Ca^{2+}]_i$ in the cell body and in the axon terminal of isolated rod bipolar cells from mouse retinas. A. Pseudo-color images of an isolated mouse rod bipolar cell during a Ca^{2+} imaging experiment. B. SRIF effect on K^+-stimulated increase of $[Ca^{2+}]_i$ in the cell body (red trace) and in the terminal (blue trace). C. Similar results are obtained using OCT. D. Percent inhibition of $[Ca^{2+}]_i$ induced by SRIF and OCT at the level of the cell body (soma) and of the axon terminal (end). Data are shown as mean ± SEM and are derived from observations in 5 different rod bipolar cells. Scale bar: 10 µm.

with other neuromodulators in the processes underlying light adaptation (Fig. 3). Indeed, earlier studies have demonstrated that extracellular dopamine levels increase in the retina with increasing light intensity (Djamgoz and Wagner, 1992; Boelen et al., 1998), and the possibility exists that extracellular dopamine levels are regulated through an interplay between SRIF and substance P (Casini et al., 2002). Indeed, both SRIF receptors and the substance P receptor are expressed by dopamine-containing amacrine cells (Casini et al., 1997, 2002; Helboe and Moller, 1999; Johnson et al., 1999; Cristiani et al., 2000, 2002; Catalani et al., 2004). Furthermore, SRIF action in the rabbit eyecup preparation is consistent with a role for this peptide in light adaptation (Zalutsky and Miller, 1990), and in the chick retina SRIF acts as a dark signal (Ishimoto et al., 1986; Yang et al., 1997). On the other hand, substance P evokes dopamine release in the rabbit retina (Casini et al., 2004a) and it may act as a light signal. Based on these observations, dopamine levels may be upregulated by a stimulatory action of substance P and downregulated by an inhibitory action of SRIF on dopaminergic amacrine cells (Fig. 3). The interaction between the light signal (substance P) and the dark signal (SRIF) would therefore modulate dopamine release and light adaptation.

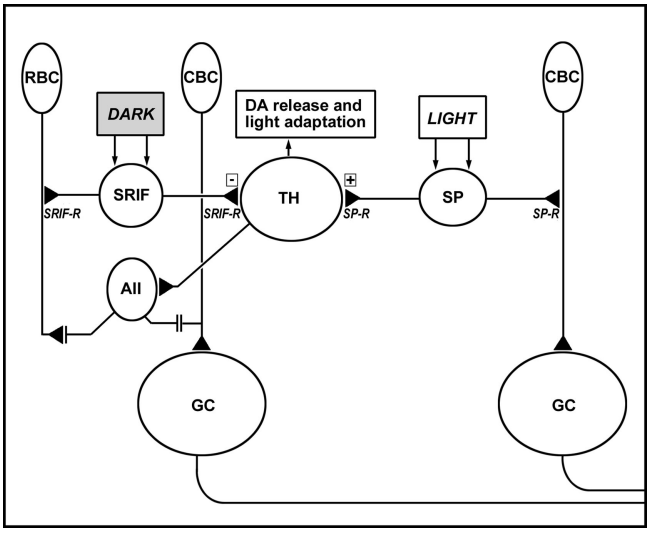

Fig. 3. Retinal circuitry mediating possible influences of SRIF on light adaptation in the rabbit retina. The peptidergic control of the rod pathway and of dopamine release, in addition to SRIF, may also include substance P (SP). SRIF released by SRIF-containing amacrine and/or displaced amacrine cells may influence rod bipolar cells and tyrosine hydroxylase (TH)-containing, dopaminergic amacrine cells acting at specific SRIF receptors (SRIF-R). The dopaminergic amacrine cells are likely to modulate AII amacrine cells, which are important interneurons in the rod pathway. They receive signals from rod bipolar cells (RBC) through chemical synapses and contact cone bipolar cells (CBC, on the left) through electrical synapses. From these cone bipolar cells, the rod signal reaches the ganglion cells. SP released by SP-containing amacrine cells acts at SP receptors (SP-R) expressed by ON type cone bipolar cells (CBC, on the right) and therefore it is likely to influence ganglion cell (GC) activity. In addition, SP-R are also expressed by dopaminergic amacrine cells and mediate SP-induced dopamine (DA) release. While SP may act as a light signal and induce dopamine release, SRIF may act as a dark signal and inhibit dopamine release. Note that, although synapses are schematically represented in this diagram, both SP and SRIF may also act in a paracrine manner. From Casini et al., 2002 (modified).

Somatostatin and its receptors in the retina of transgenic mice

Expression of somatostatin, sst_1 receptors and sst_2 receptors

Transgenic mice in which sst_1 receptors, sst_2 receptors or SRIF are knocked out have been generated and used to investigate the biological consequences of such an event (Zheng et al., 1997; Kreienkamp et al., 1999; Low et al., 2001; Allen et al., 2003). These transgenic mice do not exhibit major phenotypic defects or main behavioral impairments (Olias et al., 2004). In addition, no major compensatory regulation of SRIF or individual SRIF receptors has been described as a consequence of the genetic deletion of sst_1 or sst_2 receptors in specific brain regions (Hannon et al., 2002b). In the retina, however, we have recently demonstrated major alterations of SRIF content as a consequence of sst_1 or sst_2 receptor deletion (Dal Monte et al., 2003b; Casini et al., 2004b). In particular, although the levels of SRIF mRNA are unaltered in the retinas of transgenic mice, sst_1 KO retinas are characterized by increased levels of SRIF peptide, while sst_2 KO retinas display a significant decrease of retinal SRIF (Fig. 4). These studies in sst_1 or sst_2 receptor KO retinas suggest that the amount of retinal SRIF is likely to depend on the expression levels of the sst_1 receptor (Casini et al., 2004b), which would act as an autoreceptor (Mastrodimou and Thermos, 2004). In the absence of sst_1 receptors (as in sst_1 KO retinas) inhibitory mechanisms limiting SRIF levels in the retina would be removed, while in the presence of sst_1 receptor over-expression (as in sst_2 KO retinas, see below) such mechanisms would be strengthened.

In KO retinas, sst_1 and sst_2 receptor expressions have been found to compensate for each other. Indeed, sst_1 receptor loss causes an increased expression of sst_2 receptors, while genetic deletion of the sst_2 receptor induces an increased expression of the sst_1 receptor (Fig. 5; Dal Monte et al., 2003b; Casini et al., 2004b). Autoradiographic studies in sst_1 KO retinas clearly show a marked increase in sst_2 binding sites identified with [^{125}I]Tyr3-octreotide, indicating that the over-expressed sst_1 receptors are functional. The observation that in control wild type (WT) retinas, the total SRIF binding sites identified with [^{125}I]LTT-SRIF-28 have a density similar to that in sst_1 KO retinas suggests that the total amount of SRIF receptors does not change significantly as a consequence of sst_1 receptor deletion and indicates that, in mouse retinas, the loss of the sst_1 receptor could be totally compensated by an increase in sst_2 receptors

(Dal Monte et al., 2003b). Together, these findings indicate the presence of regulatory mechanisms between sst_1 and sst_2 receptors, leading to over-expression of one receptor in the absence of the other.

In summary, the results of SRIF and SRIF receptor expression in retinas of sst_1 or sst_2 KO mice suggest that the sst_1 receptor is important in regulating retinal SRIF and further confirm its functional role as an autoreceptor in the retina. In addition, they also show that sst_1 or sst_2 receptor expression is profoundly altered in the absence of sst_2 or sst_1 receptors, respectively, providing the first demonstration of prominent compensatory regulation in the retina as a consequence of the genetic deletion of distinct SRIF receptors.

Our recent observations in SRIF KO retinas show that all SRIF receptor mRNAs, as measured with semi-quantitative RT-PCR, are expressed at significantly higher levels than in WT retinas, with the only exception of sst_4 mRNA, whose levels are similar to those in retinas of WT animals (Fig. 6). Together with the data of sst_1 KO and sst_2 KO retinas reported above, this result indicates that all components of the somatostatinergic system in the retina are part of a complex mechanism by which the relative levels of SRIF and each of its receptors (except sst_4) are regulated depending on the abundance of other components of the system. In this mechanism, SRIF is likely to play a primary role since its absence (as in SRIF KO retinas) seems to abolish

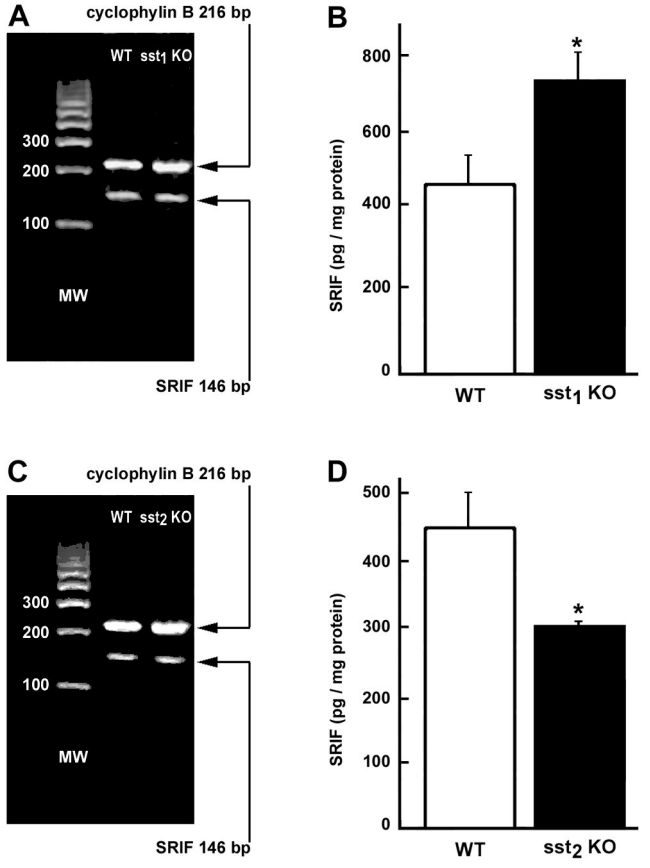

Fig. 4. SRIF mRNA and peptide in wild type (WT), sst_1 KO and sst_2 KO retinas as evaluated by RT-PCR semiquantitative analysis using cyclophyilin B mRNA as an internal standard and radioimmunoassay. **A:** in sst_1 KO retinas, the relative amount of amplified products at 146 bp corresponding to SRIF mRNA is similar to that in WT retinas. **B:** endogenous levels of SRIF in the retina of WT (open column) and sst_1 KO (filled column) mice. The amount of SRIF is expressed as pg/mg of proteins (means ± SEM). Retinal levels of SRIF in sst_1 KO mice are significantly higher than in control mice (* $p<0.001$). **C:** similar to sst_1 KO retinas, also in sst_2 KO retinas the relative amount of SRIF mRNA is similar to that in WT retinas. **D:** the levels of SRIF peptide in sst_2 KO retinas are significantly lower than those in WT retinas. Each histogram represents the mean ± SEM of the SRIF levels measured in 6 retinas. *: $p<0.001$. MW, molecular weight. A and B are from Dal Monte et al., 2003b (modified); C and D are from Casini et al., 2004b (modified).

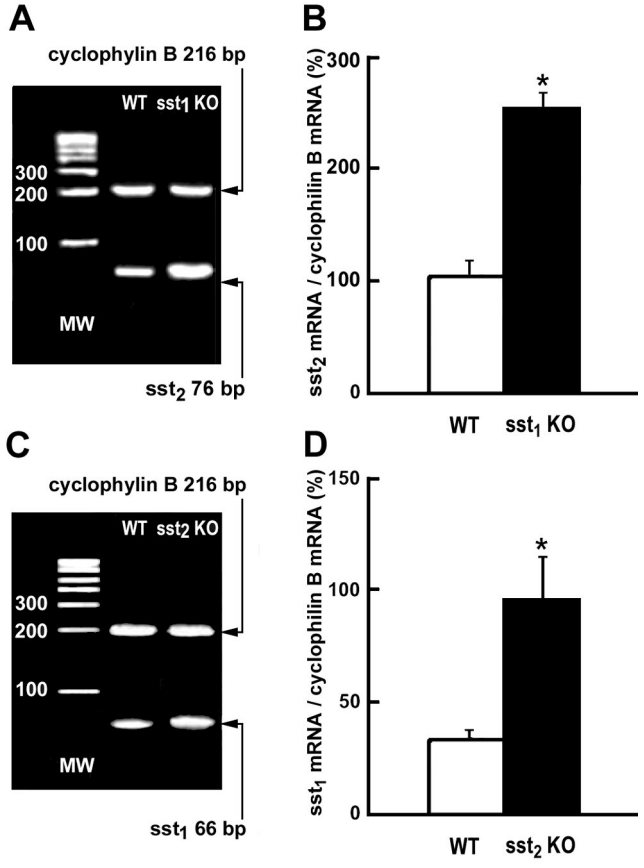

Fig. 5. A and B: sst_2 receptor mRNA as evaluated by RT-PCR semiquantitative analysis in WT and sst_1 KO mouse retinas. It can be noticed that sst_1 receptor loss causes a significant increase in the relative level of the sst_2 receptor mRNA (* $p<0.0001$). **C and D:** sst_1 receptor mRNA as evaluated by RT-PCR semiquantitative analysis in WT and sst_2 KO mouse retinas. Similar to findings in sst_1 KO retinas, the loss of sst_2 receptors is accompanied by a significant increase in the relative level of the sst_1 receptor mRNA (* $p<0.001$). A and B are from Dal Monte et al., 2003b (modified); C and D are from Casini et al., 2004b (modified).

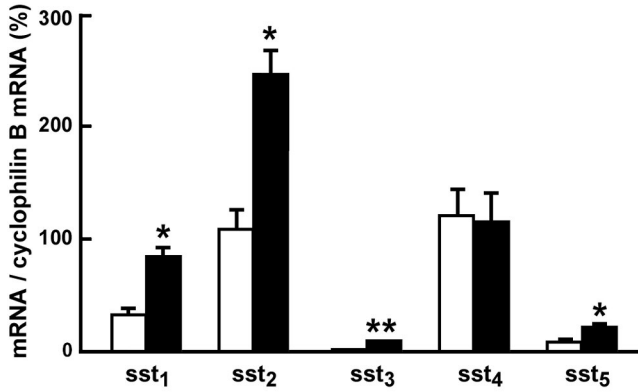

Fig. 6. Expression of SRIF receptor mRNAs in WT (open columns) and in SRIF KO (filled columns) retinas as evaluated by RT-PCR semiquantitative analysis using cyclophyilin B as an internal standard. *: p<0.01; **: p<0.0001 vs the respective control values.

compensatory mechanisms between single receptors and induces a generalized receptor over-expression.

Somatostatin function in retinas of transgenic mice

Consistent with the observation of increased sst_2 binding sites in sst_1 KO retinas (Dal Monte et al., 2003b), recent findings indicate that the over-expression of sst_2 receptors in these retinas is correlated with enhanced sst_2 receptor function (Bigiani et al., 2004). Indeed, in sst_1 KO retinas there is a significant increase of the sst_2 receptor-mediated inhibitory action of SRIF both on the calcium-dependent component of K^+ currents in isolated rod bipolar cells (Fig. 7) and on the depolarization-induced glutamate release from retinal explants (Fig. 8). The fact that an over-expression of sst_2 receptors, as in sst_1 KO retinas, correlates with an altered sst_2 receptor signaling suggests the feasibility of using

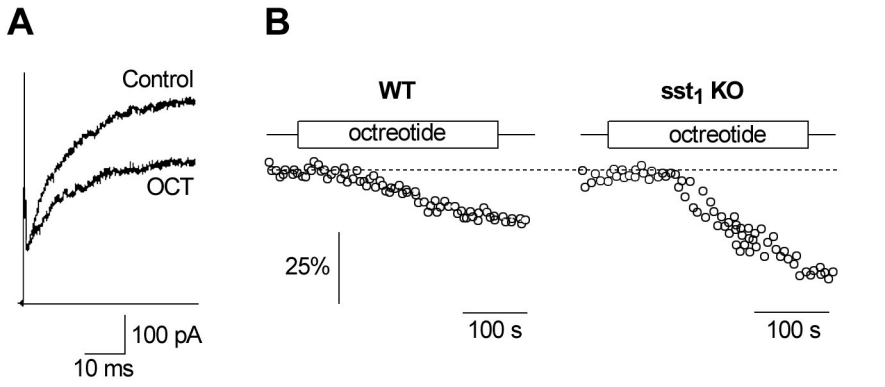

Fig. 7. Effect of octreotide (OCT) on K^+ currents (I_K) recorded from mouse rod bipolar cells. **A:** with 1 μM OCT, I_K was reduced in rod bipolar cells isolated from WT retinas. **B**: time course of the OCT effect in representative rod bipolar cells from WT and sst_1 KO retinas. The per cent reduction of I_K was more pronounced in rod bipolar cells from sst_1 KO retinas. **C:** per cent inhibition of I_K by OCT in rod bipolar cells from WT and sst_1 KO retinas. *: p<0.001. From Bigiani et al., 2004.

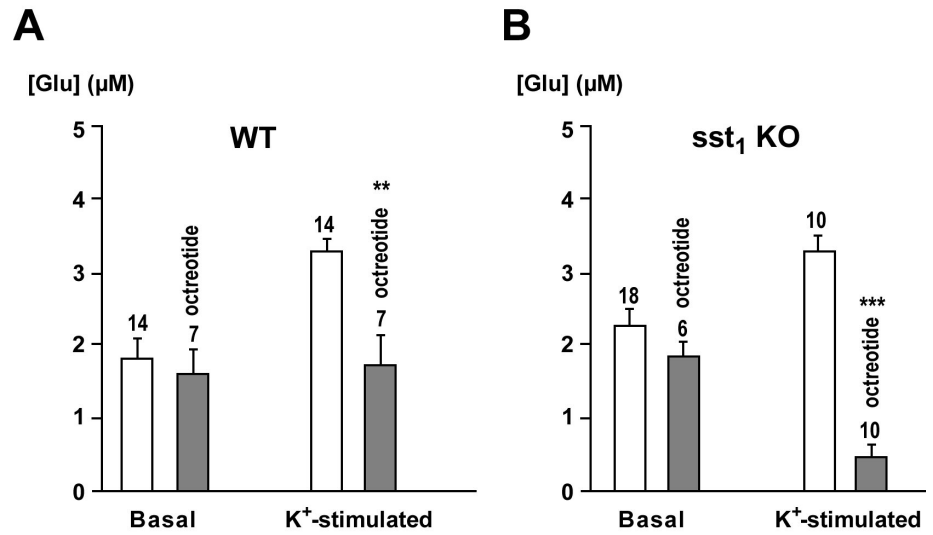

Fig. 8. Effect of octreotide on the glutamate (Glu) release in retinas from WT **(A)** and sst_1 KO **(B)** mice, as detected by HPLC. "K^+-stimulated" refers to Glu release induced by application of 50 mM K^+, which depolarizes cell membranes. Columns refer to the mean ± SEM of values of Glu concentration; numbers above the bars refer to the number of retinas used in each experiments. **: p<0.01, *** p<0.001 vs the respective control values. From Bigiani et al., 2004.

retinas with sst_1 receptor deletion to express sst_2 receptors at high densities, which may facilitate the development of therapeutical strategies based on sst_2 receptor pharmacology. Since in sst_2 receptor over-expressing retinas there is a greater inhibition of retinal release of glutamate, these retinas may constitute important experimental models to investigate therapeutic approaches in retinal diseases caused by glutamate neurotoxicity. More generally, this model would be of importance to attack those retinal diseases where SRIF is regarded as a potential therapeutic agent.

Somatostatin transduction pathways in retinas of transgenic mice

In retinas from sst_1 KO mice, in which sst_2 receptors are over-expressed (Dal Monte et al., 2003b), the level of the Go protein alpha subunit is significantly higher than in retinas of WT animals (Pavan et al., 2004; Fig. 9A). This result suggests the existence of compensatory mechanisms, evoked by alterations of SRIF transduction pathways, that result in an enhancement of G protein expression. Accordingly, SRIF and the sst_2 receptor specific agonist, octreotide, greatly inhibit forskolin-stimulated adenylyl cyclase in retinas of sst_1 KO mice (Pavan et al., 2004; Fig. 9B).

Morphological abnormalities in the inner retina of transgenic mice

Recent studies have demonstrated that specific morphological characteristics of identified cells in the mouse retina are altered by genetic deletion of distinct SRIF receptors. In particular, the somatostatinergic system seems to play a role in determining the size of the axonal terminals of rod bipolar cells, thus influencing their function in the retina (Casini et al., 2004b). These studies show that deletion of the sst_1 receptor causes individual terminal endings of rod bipolar cells measured in the lamina 5 of the inner plexiform layer (IPL) to become significantly larger than in WT retinas, whereas the deletion of the sst_2 receptor causes individual terminal endings of rod bipolar cells to become significantly smaller (Fig. 10). These alterations in the size of rod bipolar cell axonal terminals in KO retinas are already evident during postnatal maturation, indicating that these effects are likely to be related to SRIF actions during development. Despite these evident variations in size, no major ultrastructural alterations have been observed in the terminals of rod bipolar cells, suggesting that the ribbon synapses made by these cells in KO retinas are functional (Casini et al., 2004b). Furthermore, the density of synaptic vesicles within

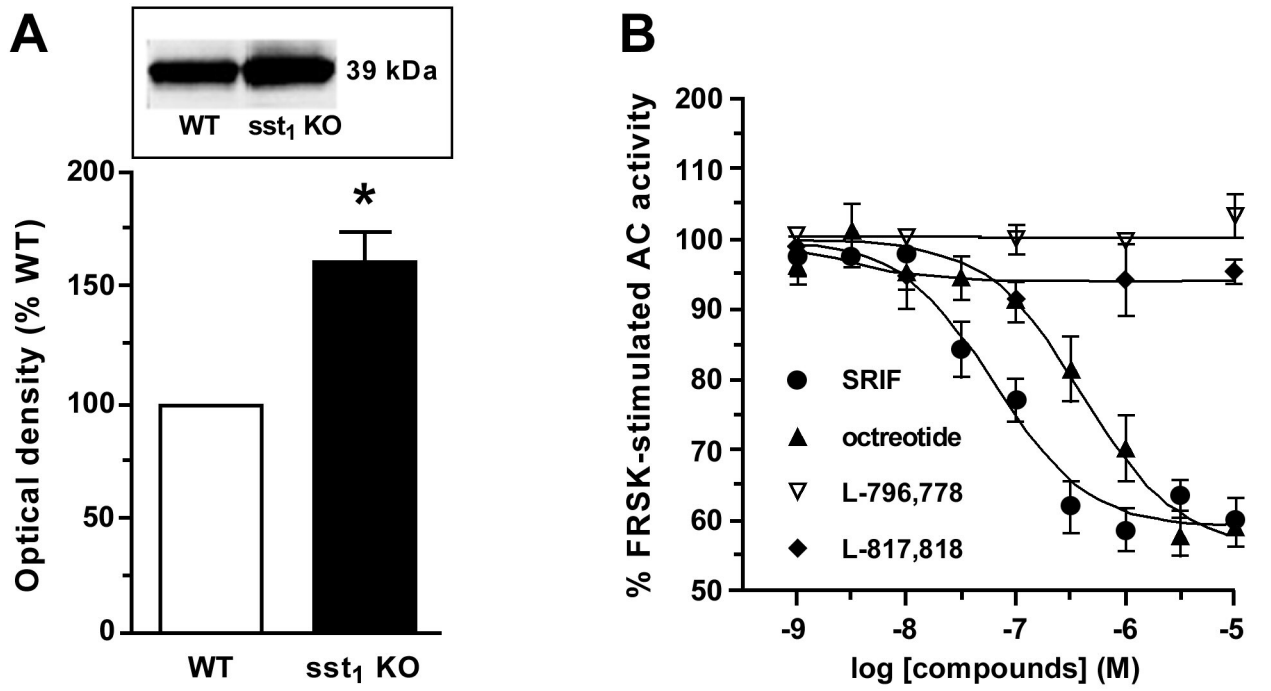

Fig. 9. A. Go alpha proteins in WT and in sst_1 KO mouse retinas as evaluated by Western blot. Bands corresponding to Go alpha proteins (39 kDa) are shown in the upper panel. Histograms are the means ± SEM (bars) of data from six independent experiments. Values of the optical density of the bands were normalized to those of α-actin and WT values plotted as 100%. *p<0.001 vs. WT values. **B.** Effects of SRIF compounds on forskolin (FRSK)-stimulated AC activity in sst_1 KO mouse retinas. Retinas were treated with FRSK in combination with increasing concentrations of SRIF, octreotide, the sst_3 receptor agonist L-796,778 or the sst_5/1 receptor agonist L-817,818. From Pavan et al., 2004 (modified).

these axonal endings is apparently similar in KO and in WT animals, suggesting that more vesicles are in the large terminals of the sst_1 KO retinas and less vesicles are in the small terminals of sst_1 KO retinas. These differences in vesicle content may affect the functional responses of rod bipolar cells in the KO retinas.

The retinas of SRIF KO mice have also been investigated recently by us. The analysis of these retinas has been conducted by measuring the size of individual terminal enlargements of rod bipolar cells (identified by protein kinase C immunoreactivity) in confocal images taken at fixed levels of the IPL. The data were collected both from whole-stained retinas (horizontal plane) and from retinal sections cut perpendicular to the vitreal surface (vertical plane). Unexpectedly, no significant alterations in the size of rod bipolar cell terminals were detected either in the horizontal or in the vertical plane (Fig. 11A-C). The overall density of the terminals (terminals/mm^2 of retinal area) was also similar in SRIF KO and in WT retinas. However, a significant difference was observed in the number of 1 µm-thick optical sections needed to span the thickness of the IPL in whole-mount preparations. With this type of analysis, we found that the IPL, and in particular the lamina containing the terminals of rod bipolar cells, is about 20% thinner in SRIF KO than in WT retinas (Fig. 11D).

Together, the studies of the morphological alterations in KO retinas indicate that the somatostatinergic system plays a role in the differentiation of the rod bipolar cell terminals, and

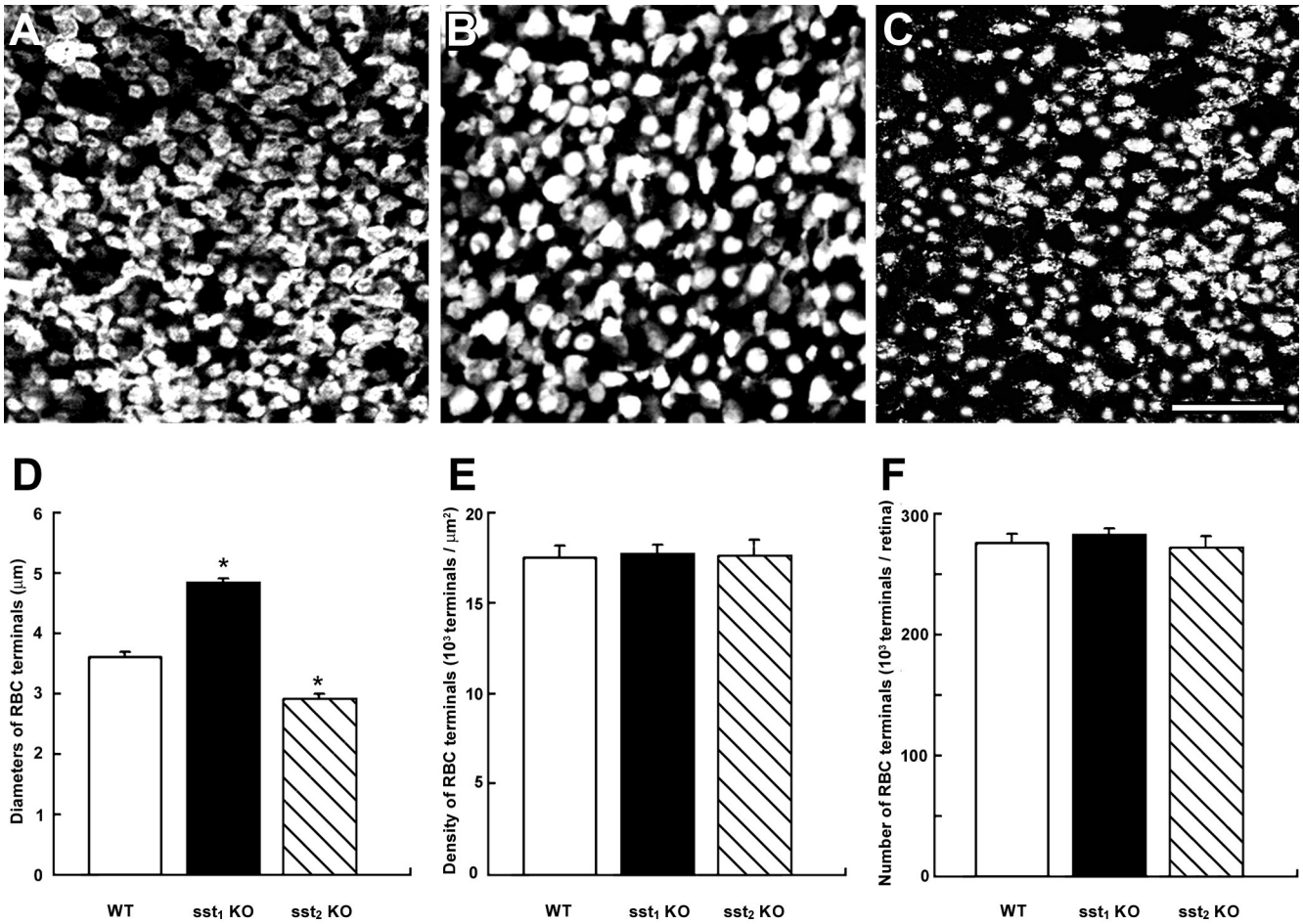

Fig. 10. A-C: confocal images from whole retinas (0.5-µm-thick optical sections) showing protein kinase C-immunoreactive rod bipolar cell axonal terminals in the IPL of WT (**A**), sst_1 KO (**B**) and sst_2 KO **(C)** retinas. Scale bar: 25 µm. **D-F:** analysis of the diameters, density and number of rod bipolar cell axonal terminals in WT (open columns), sst_1 KO (filled columns) and sst_2 KO (dashed columns) retinas. **D:** significantly larger diameters than in WT are measured in sst_1 KO retinas, whereas significantly smaller diameters are in sst_2 KO retinas. * $p<0.001$. Each histogram represents the mean ± SEM of the average diameters measured at 60 different locations in 3 retinas for each group. **E:** each histogram represents the mean ± SEM of the density of rod bipolar cell terminals measured at 90 locations in 3 retinas. **F:** each histogram represents the mean ± SEM of the product of the mean terminal density times the retinal area in 3 different retinas for each group. No significant differences between WT and KO retinas are detected in rod bipolar cell terminal density or terminal number. From Casini et al., 2004b.

therefore may affect their functions, however the mechanisms of such influence are difficult to hypothesize. As previously discussed (Casini et al., 2004b), some speculations could be derived from the observations in sst_1 KO and sst_2 KO retinas. Indeed, in sst_1 KO retinas, SRIF levels are higher than in WT retinas (Dal Monte et al., 2003b), whereas in sst_2 KO retinas, SRIF levels are lower than in WT retinas (Casini et al., 2004b). Therefore, we may speculate that the morphological changes of the rod bipolar cell axonal terminals in sst_1 or in sst_2 KO retinas are correlated to altered levels of SRIF: an increase in retinal SRIF (as in the sst_1 KO) would induce an increase of the terminal size, while a decrease in retinal SRIF (as in the sst_2 KO) would induce a decrease. The results obtained with the SRIF KO retinas only in part support this hypothesis: although a reduction of the IPL lamina containing the rod bipolar cell terminals has been observed in SRIF KO retinas, statistically significant changes in the size of these terminals have not been recorded. The picture is further complicated by the fact that, in SRIF KO retinas, sst_2 and other SRIF receptors are over-expressed. As described above, in the presence of sst_2 receptor over-expression an increase in rod bipolar terminal size should be expected. Consistently, as depicted in figure 12, the levels of protein kinase C (the rod bipolar cell marker used in immunocytochemical studies) in SRIF KO retinas are higher than in WT retinas and similar to those in sst_1 KO retinas. In summary, in SRIF KO retinas the absence of SRIF is expected to result in decreased terminal size, but the over-expression of sst_2 receptors would induce increased terminal size. These

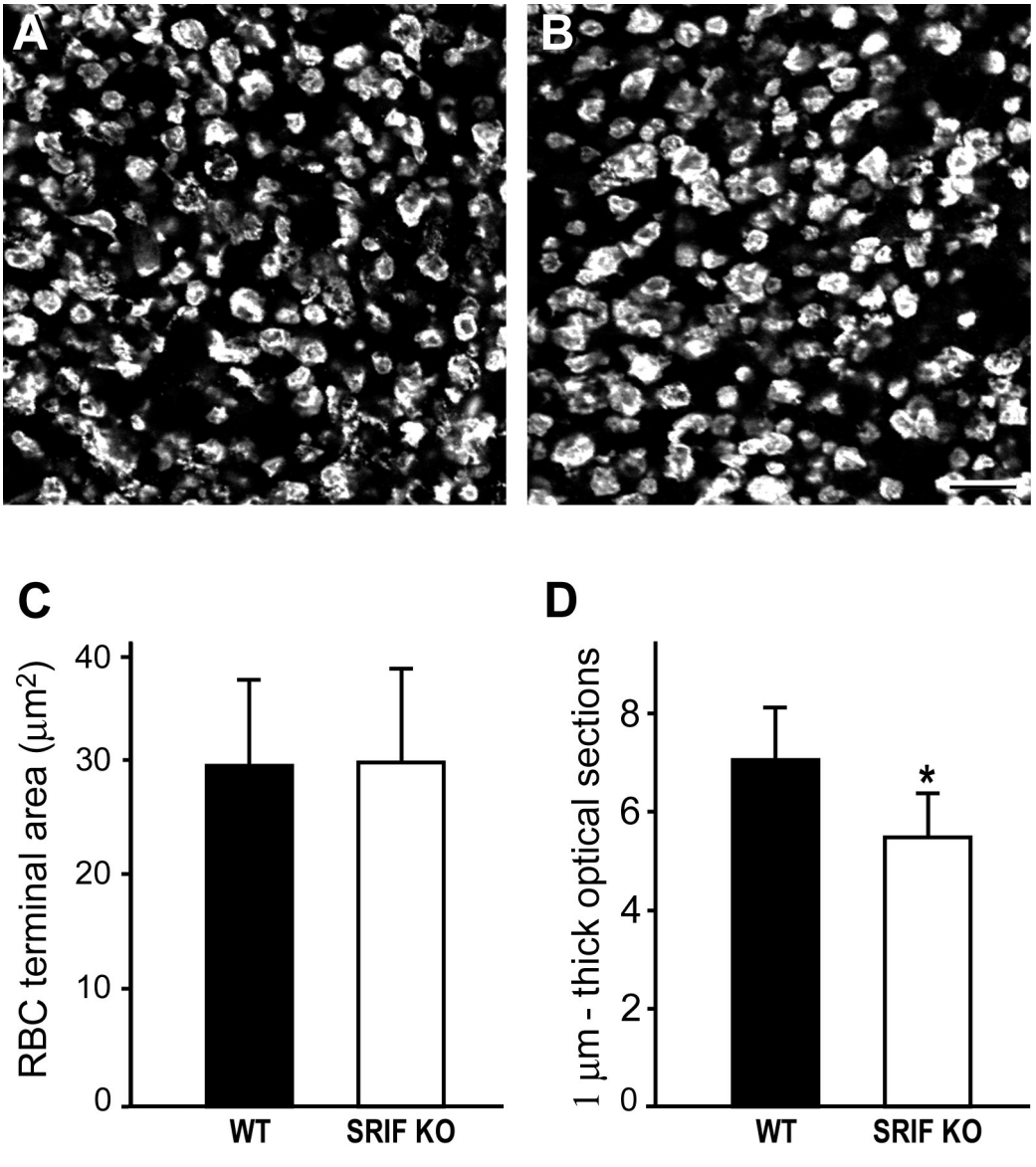

Fig. 11. A and B show optical sections (1 µm-thick) from protein kinase C-immunostained whole mounts at corresponding levels of the IPL in WT and SRIF KO retinas, respectively. Scale bar: 10 µm. **C:** the histograms represent the mean ± standard deviation of the areas of rod bipolar cell terminals measured in 2500 μm^2 fields at 11 (WT) or 10 (SRIF KO) different retinal locations. No significant difference was observed between WT and SRIF KO retinas. **D:** the histograms represent the mean ± standard deviation of the number of 1 µm-thick optical sections (similar to those shown in A and B) acquired through the IPL lamina containing the protein kinase C-immunolabeled terminals of rod bipolar cells (n=24). This IPL lamina results significantly thinner in SRIF KO than in WT retinas (* $p<0.001$).

observations indicate that a reduction of retinal SRIF per se may not be the cause of major alterations in the morphology of rod bipolar cell terminals, but a complex interplay between SRIF and its receptors is likely to take part in the morphological differentiation of these cellular structures in the retina. For instance, we may hypothesize that an increase in rod bipolar cell terminals is determined by increased levels of sst_2 receptors only in the presence of increased levels of retinal SRIF, as in sst_1 KO retinas. If this is the case, we may assume that the increase in terminal size is due to abnormally high levels of sst_2 receptors that are activated by SRIF. In SRIF KO retinas, although sst_2 receptors are over-expressed, such activation, of course, is not possible.

Role of somatostatin in retinal disease therapeutics: diabetic retinopathy

SRIF has been studied as a possible therapeutic agent in the treatment of major retinal diseases. Diabetic retinopathy (DR) is a disease causing retinal neovascularization and edema that lead to blindness. Its social impact is high, as it is the leading cause of new cases of legal blindness in working age humans in industrialized countries (Barber, 2003). Clinical management of DR relies primarily on laser ablation of the retinal vasculature. Currently, the panretinal photocoagulation treatment, although successful in causing vascular regression, is not optimal, as it may result in partial vision loss, and the disease may progress in spite of the treatment.

Diabetes

Under physiologic conditions, increased glycemia triggers beta cells for insulin secretion (Bell and Polonsky, 2001). The binding of insulin to its receptors initiates a cascade of events resulting in the uptake of glucose by the cell and its subsequent metabolism (Saltiel and Kahn, 2001). Any defects in the processes leading to glucose uptake can result in hyperglycemia, the main cause of diabetes. Two major forms of diabetes have been recognized: type 1 diabetes due to autoimmunological destruction of the pancreatic beta cells (Kelly et al., 2003); type 2 diabetes due to both inadequate insulin secretion by beta cells and insulin resistance in peripheral tissues (Gerich, 2003). DR is a complication of both types of diabetes.

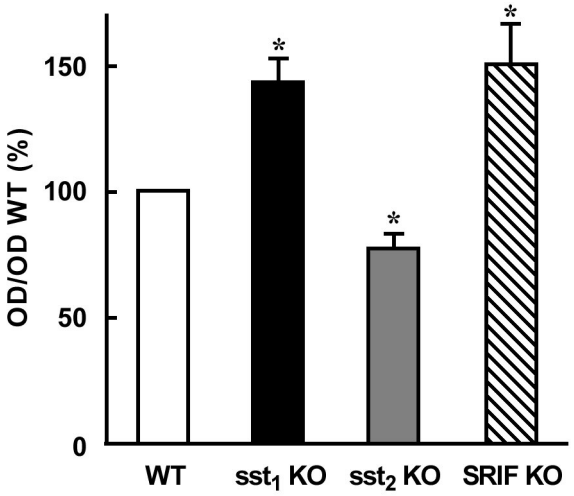

Fig. 12. Protein kinase C levels in WT and in KO mouse retinas as evaluated by Western blot. The histograms are the means ± SEM (bars) of data from six independent experiments. Values of the optical density (OD) of the bands were normalized to those of the α-actin and WT values plotted as 100%. * p<0.01 vs WT values.

Diabetic retinopathy and somatostatin therapeutic actions

In the early nineties SRIF analogues were employed in clinical studies for the treatment of DR. SRIF therapeutic use was based on the ability of SRIF to inhibit growth hormone secretion, a hormone implicated in the pathogenesis of DR (Kirkegaard et al., 1990; McCombe et al., 1991; Mallet et al., 1992; Kopchick and Okada, 2001; Gargiulo et al., 2004), and a combined treatment with SRIF analogs and retinal photocoagulation has been suggested (Spranger et al, 2001). The use of SRIF as a potential therapeutic agent in DR is hampered by its short viability in the extracellular space (Patel, 1999). Therefore, clinical investigations have focused on the use of long-acting SRIF receptor agonists (Davis et al, 2001; Grant and Caballero, 2002). In particular, the efficacy of octreotide is currently being tested for treatment of DR. Indeed, octreotide treatment may retard progression of advanced DR and may delay the point at wich laser surgery is required (Pawlikowski and Melen-Mucha, 2003; Gargiulo et al., 2004). Octreotide binds to sst_2, sst_3 and sst_5 receptors (Hannon et al., 2002a), however the retinal expression of both sst_3 and sst_5 receptors is very limited (Brecha, 2003; Thermos, 2003), indicating that the beneficial actions of octreotide are mediated by sst_2 receptors.

Diabetic retinopathy and neovascularization

DR includes a progression of disease states. Non-proliferative DR results from a series of biochemical and cellular changes causing retinal ischemia. The secretion of growth factors in response to ischemia leads to the development of proliferative DR, characterized by aberrant neovascularization of the retina and increased vascular permeability (Spranger and Pfeiffer, 2001; Wilkinson-Berka et al., 2001; Funatsu and Yamashita, 2003; Khan and Chakrabarti, 2003; Gargiulo et al., 2004). There is a loss of blood-retinal barrier integrity, with failure to control the composition of the extracellular fluid leading to neuronal loss and irreversible visual damage (Cai and Boulton, 2002). Vascular endothelial growth factor (VEGF) plays a

major role in retinal neovascularization. It is expressed by neurons and glial cells in the normal retina and acts at 3 related, high affinity receptors (VEGFR-1 to -3) (Witmer et al., 2003). In DR, VEGF levels are increased in the aqueous, vitreous and retina (Witmer et al., 2003). VEGF receptors are largely expressed on retinal microvascular endothelial cells (Bates et al., 2002). VEGFR-2 is the major mediator of the actions of VEGF, as ligand binding to VEGFR-2 increases permeability of blood vessels and proliferation of endothelial cells (Robinson and Stringer, 2001). The expression of both VEGFR-2 and 3 are increased in DR (Witmer et al., 2002). Together with VEGF, another growth factor, the Insulin-like growth factor-1 (IGF-1), is involved in the abnormal development of retinal vessels: following leaks in the blood-retinal barrier, IGF-1 enters the retina and potently stimulates endothelial cell growth (DeBosch et al., 2001). Its levels are increased in the vitreous of DR patients (Simo et al., 2002) and the levels of IGF-1 receptor mRNA are increased in the retinas of animal models of DR (Kuang et al., 2003).

Somatostatin as an antiangiogenic factor

A recent study has reported that SRIF levels measured by radioimmunoassay in the vitreous of patients with proliferative DR are significantly lower than those in the vitreous of nondiabetic control subjects (Simo et al., 2002). This result suggests that the intravitreous deficit of SRIF may contribute to the process of retinal neovascularization typical of proliferative retinopathy and supports the concept that adequate levels of SRIF are needed for the maintenance of retinal homeostasis. Several lines of clinical and experimental evidence suggest that SRIF analogs may be efficacious in inhibiting neovascularization associated with proliferative DR (Davis et al., 2001; Grant and Caballero, 2002). In particular, octreotide can very effectively suppress new bleedings and stop visual loss in patients who have failed conventional photocoagulation therapy (Grant and Caballero, 2002). The potential anti-angiogenic role of octreotide may be due to a partial correction of the systemic growth hormone and IGF-1 dysregulation and to the inhibition of the retinal secretion of VEGF (Garcia de la Torre et al., 2002). In addition, octreotide might inhibit angiogenesis directly through SRIF receptors expressed by endothelial cells. Indeed, sst_2 receptors have been localized on human endothelial cells (ten Bokum et al., 1999) and octreotide has been shown to inhibit the proliferation of human and murine endothelial cells in culture (Danesi et al., 1997; Lawnicka et al., 2000). In addition, both octreotide and the sst_2 receptor agonist BIM23027 have been found to counteract the growth factor-induced proliferation of bovine retinal endothelial cells (Baldysiak-Figiel et al., 2004).

Diabetic retinopathy and chronic neurodegeneration

One of the reasons for the only partial success of panretinal photocoagulation in the treatment of DR is that neuronal loss takes place in the diabetic retina beyond vascular changes. Indeed, functional deficits in vision are detectable soon after the onset of diabetes, before major vascular pathology has developed (Barber, 2003). However, alterations in electroretinographic responses are reversible upon pharmacological treatment, demonstrating that at early stages there is no permanent loss of neuronal function (Barber, 2003). Several neuronal alterations in the diabetic retina are characteristic of chronic neurodegenerative diseases. In particular, apoptosis, with marked loss of ganglion cells and reduction in thickness of the retina is the most evident consequence (Barber, 2003). In addition to ganglion cells, specific populations of amacrine cells may suffer damage in DR, including NADPH-containing (Li et al., 2003), dopaminergic (Nishimura and Kuriyama, 1985), substance P and vasoactive intestinal peptide containing (Troger et al., 2001), acetylcholinesterase expressing (Sanchez-Chavez and Salceda, 2001), and GABAergic (Ishikawa et al., 1996; Ambati et al., 1997) amacrine cells. An interesting possibility is that neuronal loss is caused by glutamate excitotoxicity. Indeed, there is evidence that DR is associated with an excessive synaptic concentration of glutamate (Barber, 2003). In addition, increased concentration of glutamate to levels that are potentially toxic to retinal ganglion cells has been reported in the vitreous of patients with proliferative DR (Ambati et al., 1997), and neurodegeneration in DR may occur via over-stimulation of the NMDA receptor (Smith, 2002).

Somatostatin as a neuroprotective agent

SRIF or SRIF analogs may counteract retinal damages in DR by playing a protective paracrine effect directly on retinal cells which are known to express SRIF receptors. There is experimental evidence that SRIF or its analogs may protect neurons against both natural death during development (Weill, 1991), ischemia-induced retinal damage (Celiker and Ilhan, 2002) and neurotoxicity induced by activation of the NMDA glutamate receptor (Forloni et al., 1997). This latter observation is intriguing in view of the possibility that neuronal death in diabetic retinas is caused by increased glutamate. Indeed, potential neuroprotective roles of SRIF or its analogs may be mediated by inhibition of glutamate release. As reported above, there are observations suggesting that SRIF acting at sst_2 receptors may effectively inhibit glutamate release in the retina. These observations include: (i) sst_2 receptors are expressed by rod bipolar cells (Cristiani et al., 2002), which are an important source of glutamate in the retina (Morgans, 2000); (ii) octreotide inhibits the depolarization-induced Ca^{2+} influx in isolated rod bipolar cells of the rabbit (Petrucci et al., 2001) and of the mouse retina (see paragraph "Functional actions of somatostatin in the retina"), demonstrating that SRIF acting at sst_2 receptors may inhibit a fundamental step in the process of glutamate release from rod bipolar cells;

(iii) octreotide inhibits glutamate release in normal mouse retinas while it has no effect in sst2 KO retinas (Dal Monte et al., 2003a); (iv) the effect of octreotide on glutamate release is enhanced in sst_1 KO retinas, where sst_2 receptors are over-expressed (Bigiani et al., 2004).

In summary, important therapeutic effects of SRIF or its analogs acting at sst_2 receptors are expected on the basis of the dual action that SRIF plays against neo-angiogenesis and excitotoxic neuronal death.

Concluding remarks

Although far from being fully elucidated, the functional organization of the somatostatinergic system and the roles of SRIF in retinal physiology have been the subject of a variety of studies. The seminal work of Zalutsky and Miller (1990) identified SRIF as a broad-spectrum regulatory substance in the retina affecting the activity of multiple cell types. Recent investigations have established the important influence of SRIF in the modulation of ion channels and transmitter release, have identified the sst_2 receptor as the main SRIF receptor in the retina and the sst_1 receptor as an autoreceptor. In addition, studies of the transduction pathways coupled to SRIF receptors are in progress. Important information on the somatostatinergic system in the retina has been gained by investigations using retinas of transgenic mice. These studies have revealed complex mechanisms by which the expressions of SRIF and each of its receptors are closely interrelated. Furthermore, the altered expression of a SRIF receptor (for instance the sst_2 receptor) is accompanied by alterations in function and may result in morphological abnormalities of certain retinal cell types (for instance the rod bipolar cells). Despite these recent achievements, the current knowledge of SRIF functions in the retina is largely incomplete. For instance, much has to be discovered about the intracellular pathways activated by distinct SRIF receptors, and the mechanisms regulating the expression of SRIF and of its receptors in the retina await clarification. However, the available evidence strongly suggests that SRIF may be a potent therapeutic agent in retinal disease and, in fact, clinical approaches have been initiated in the past and are continuously refined. These approaches will greatly benefit from an increased knowledge of the physiological actions of the somatostatinergic system in the retina, and appropriate SRIF-based therapies to treat retinal diseases such as DR may be envisioned in a not-so-far future.

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