MODULATION OF VOLTAGE-GATED ION CHANNELS IN RAT RETINAL GANGLION CELLS

MEDIATED BY SOMATOSTATIN RECEPTOR SUBTYPE 4

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Running Title: Sst₄ receptor mediated inhibition of rat retinal ganglion cells

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Abstract

Somatostatin (SRIF) is known to modulate the excitability of retinal ganglion cells, but the membrane mechanisms responsible and the extent to which intracellular calcium signaling is affected have not been determined. We show that somatostatin receptor subtype 4 (sst₄) is expressed specifically in rat ganglion cells and that the generation of repetitive action potentials by isolated ganglion cells is reduced in the presence of L-803,087, a selective sst₄ agonist (10 nM). Under voltage clamp, L-803,087 increased outward K⁺ currents by 51.1 ± 13.1 % at 0 mV and suppressed Ca²⁺ channel currents by 32.5 ± 9.4 % at -10 mV in whole-cell patch clamped ganglion cells. The N-type Ca²⁺ channel blocker ω-conotoxin GVIA (CTX, 1 µM) reduced I₉Ca in ganglion cells by 43.5 ± 7.2 % at -10 mV, after which addition of L-803,087 further reduced I₉Ca by 28.0 ± 16.0 %. In contrast, ganglion cells treated first with nifedipine (NIF, 10 µM), which blocked 46.1 ± 3.5% of the control current at -10 mV, did not undergo any further reduction in I₉Ca in the presence of L-803,087 (-3.5 ± 3.8 % vs. NIF), showing that stimulation of sst₄ reduces Ca²⁺ influx through L-type Ca²⁺ channels. To assess the effects of sst₄ stimulation on intracellular Ca²⁺ levels ([Ca²⁺]ₗ) in ganglion cells, fura-2 was used to measure changes in [Ca²⁺]ₗ in response to depolarization induced by elevated [K⁺]₀. [Ca²⁺]ₗ was increased to a lesser extent (86%) in the presence of L-803,087 compared to recordings made in the absence of the sst₄ agonist, and this effect was blocked by NIF (10 µM). Suppression of spiking and Ca²⁺ signaling via sst₄ may contribute to the reported neuroprotective actions of somatostatin and promote ganglion cell survival following ischemia and axonal trauma.
Introduction

Somatostatin (SRIF) is a neuropeptide transmitter in many brain regions including the retina. Somatostatin is a cyclic tetradecapeptide with multiple cellular actions mediated by five G-protein-coupled receptors designated sst1, sst2A/sst2B, sst3, sst4 and sst5 (Hoyer et al. 1995; Møller et al. 2003; Vanetti et al. 1992). Somatostatin signal transduction cascades may lead to activation of voltage-gated ion currents (Akopian et al. 2000; Wang et al. 1989; Ikeda and Schofield 1989; Inoue et al. 1988; Ishibashi and Akaike 1995; Mihara et al. 1987) and modulation of glutamate-induced currents in neurons (Viollet et al. 1997; Moneta et al. 2002).

In mammalian retina, somatostatin is predominantly, if not exclusively, expressed by a limited number of widely ramifying and sparsely distributed amacrine cells (Brecha 1983; 2003; Cristiani et al. 2002). In contrast, the sst receptors, all subtypes of which are expressed in the retina, are distributed to specific retinal cell populations, including subtypes of photoreceptor, bipolar, amacrine and ganglion cells (Brecha 1983; 2003; Thermos 2003). Sst4 receptors are found only in ganglion cells of the rat retina (Vasilaki et al. 2002) potentially making them a selective therapeutic target in retinal diseases.

Somatostatin has several known actions on retinal neurons. In photoreceptor terminals, somatostatin elicits an increase in a delayed rectifier K⁺ current (I_KV), a decrease in L-type Ca²⁺ current (I_Ca) in rods, and an increase in I_Ca in cones (Akopian et al. 2000). Somatostatin also inhibits calcium-activated K⁺ (BK) channels in rabbit bipolar cells via inhibition of L-type Ca²⁺ channels (Petrucci et al. 2001), and high K⁺-evoked increases of [Ca²⁺], in rat rod bipolar cells (Johnson et al. 2001). Ganglion cells recorded in rabbit eyecup show that somatostatin evokes long-lasting increases in both spontaneous and light-evoked spike activity, increases the signal-
to-noise ratio of light-evoked responses, and produces a shift in the center-surround balance
toward a more dominant center for all ganglion cell receptive fields (Zalutsky and Miller 1990).

Studies have shown that somatostatin analogues may be effective therapeutic agents in
retinal diseases (Vasilaki and Thermos 2009). Excessive Ca^{2+} influx into cells is thought to be a
major contributor to cell death in ischemic and excitotoxic models of neuronal injury (Smaili et
al. 2009). Therefore, mechanisms of Ca^{2+} entry are prime candidates as targets for potential
neuroprotective effects. Most sst subtypes have been shown to modulate voltage-gated Ca^{2+}
channels as well as glutamate receptor channels in several organ systems (reviewed by Cervia
and Bagnoli 2007). For example, sst_2 has been most extensively studied and has been shown to
interact with G_{i/o} proteins, resulting in a reduction in intracellular Ca^{2+} levels in a variety of cell
types (Cervia and Bagnoli 2007). However, relatively little is known about the signaling
pathways and Ca^{2+} modulatory effects of the other sst receptor subtypes. In particular, there
have been no prior reports, to our knowledge, of sst_4 receptor stimulation influencing voltage-
gated Ca^{2+} channels.

The objective of this study was to investigate the actions of sst_4 signaling on membrane
excitability in rat retinal ganglion cells in terms of the involvement and modulation of specific
ionic currents and to define effects of sst_4 signaling on intracellular calcium signaling.

Methods

Experimental protocols for electrophysiological and imaging experiments were approved
by the Dalhousie University Committee on Laboratory Animals and performed in accordance
with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals
Purified retinal ganglion cell culture

The two-step panning procedure to purify the ganglion cells has been previously described (Barres et al. 1988; Hartwick et al. 2004). Briefly, litters of Long-Evans rats (Charles River, Montreal, Quebec) were sacrificed at postnatal days 7 by overexposure to halothane and decapitation. Dissected eyecups were immersed in Hibernate-A (BrainBits, Springfield, IL) with 2% B27 supplements and 10 μg/ml gentamicin. Retinas were removed and treated as described in Hartwick et al. 2004. Purified ganglion cells were plated onto poly-D-lysine/laminin-coated Biocoat glass coverslips (12 mm round; BD Biosciences, Bedford, MA) in 4-well tissue culture plates at a density of 2.5x10^4 cells per well. Cultures were maintained at 37 °C in a humidified 5% CO₂-air atmosphere. Patch recordings were made on the second day following cell dissociation and panning.

Immunohistochemical procedures

Sprague–Dawley rats (150–250 g; Harlan, Indianapolis, IN) of either sex, were maintained on a 12-h/12-h light–dark schedule. Following an overdose of isofluorane, the eyes were removed, the anterior segment dissected and the posterior eye-cup containing the retina fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) for 1 hr at room temperature. The eyecup was then stored in 25% sucrose in 0.1 M PB at 4°C. Sections of the retina were cut perpendicular to the vitreal surface with a cryostat at 12 μm, mounted on gelatin-coated slides,
air dried and stored at $-20^\circ$C. Purified ganglion cells were fixed in PFA (0.01 M PB, pH 7.4) for
10 min at room temperature and stored in 0.01 M PB (4°C) until immunostaining.

The polyclonal antibody used in the present study was developed using a synthetic
peptide directed to the C-terminus of the rat sst$_4$ receptor (amino acids 362-384), as previously
described by Vasilaki et al. (2002). Specificity of the antibody used here was evaluated by direct
comparison with immunostaining by an antibody used previously (Vasilaki et al. 2002; Brecha et
al. 2002). Sst$_4$ immunostaining of rat retinal sections using either antibody produced an identical
pattern of localization.

Retinal sections and purified ganglion cells were washed in 0.1 M PB, incubated for 12–
36 h in sst$_4$ primary antibody (1:3000) with 0.5% Triton X-100 and 10% normal goat serum at
4°C, then washed in 0.1 M PB. Samples were incubated in Alexa 488 goat anti-rabbit antibodies
(Invitrogen, Carlsbad, CA) for 2 hrs at room temperature and then coverslipped using the
ProLong Antifade Kit (Vector Laboratories, Burlingame, CA).

Electrophysiological recordings

To record outward K$^+$ channel currents and action potentials in isolated ganglion cells, the
extracellular bathing solution contained (mM): 137 NaCl, 5 KCl, 1 MgCl$_2$, 2.5 CaCl$_2$, 22.2
glucose, and 5 HEPES, adjusted to pH 7.2 with NaOH. The intracellular pipette solution
contained (mM): 140 KCl, 2 MgCl$_2$, 1 CaCl$_2$, 1.5 EGTA, 10 HEPES, adjusted to pH 7.2 with
KOH. To isolate Ca$^{2+}$ channel currents the extracellular solution contained (in mM): 115 NaCl,
2.5 KCl, 5 CsCl, 10 BaCl$_2$, 15 TEACl, 10 glucose, and 15 HEPES, adjusted to pH 7.6 with
NaOH, while the intracellular pipette solution contained (mM): 140 CsCl, 0.8 MgCl$_2$, 0.1 CaCl$_2$,
1 EGTA, 10 HEPES, adjusted to pH 7.2 with CsOH. Tetrodotoxin (TTX; 1 $\mu$M) was added to
block Na channels. Room temperature (21-25°C) solutions were superfused via a fast perfusion system (VC8-S, ALA Scientific, Albany, NY). Patch electrodes with 5-10 MΩ tip resistance were pulled from fire polished borosilicate glass capillary tubes using a micro-pipette puller (Sutter Instruments, Novato, CA). The bath reference electrode consisted of an agar bridge with an AgCl wire. Cell voltage was clamped with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) using whole-cell capacitance and series resistance compensation. The current signal was filtered at 0.5 Hz (Ithaco 4302 Dual 24dB/octave filter, Ithaca, NY) and digitized at 1 kHz with an Indec Systems interface (Sunnyvale, CA) for storage on the hard disk of a computer running BASIC-FASTLAB acquisition software. Holding potential was set at -60 mV. In current-clamp experiments, a 150 pA step for 150 ms elicited action potentials. In voltage-clamp experiments, voltage steps (150 ms, 1 Hz) were made in 10 mV increments from -60 mV to +40 mV. Steady-state currents, measured as the mean of the last 10 ms of each voltage step, were used to construct current-voltage (I-V) relations. All recordings made in the presence of drugs were taken after cells had been exposed to the drug for 2.5 minutes.

Ca²⁺ imaging in retinal flat-mounts

Fura-2 pentasodium solution (2 μL, 60 μM) was injected into the vitreous via the optic nerve stump of eyes removed from Long-Evans rats (100-150 g; Charles River Laboratories, QC) sacrificed via cervical dislocation following isofluorane anaesthesia. The fura-2 was electroporated into cells of the ganglion cell layer with 50 ms, 40 mV pulses at 1Hz for 5 seconds with the anode on the cornea and the cathode at the optic nerve stump. The retina was flat-mounted ganglion cell side up on nitro-cellulose paper and superfused at a rate of 1 ml/min with oxygenated Ringers solution containing (mM) 145 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 10
glucose, and 10 HEPES (adjusted to pH 7.4 with NaOH). The flat-mounts were imaged using a SenSys cooled CCD camera on a Nikon UM-2 microscope with a 40X water immersion objective. Cells were depolarized by superfusing high K⁺ solution (50 mM added KCl, NaCl reduced by the same) for 30 s. Fura-2 fluorescence was produced by excitation from a 100W Xenon arc lamp with filter sets for excitation at 340 and 380 nm, and emission at 510 nm (Lambda 10, Sutter Instruments, Novato, USA), and ratio measurements were performed every 5 or 10 s. Ganglion cell fluorescence was converted to ratiometric values by Imaging Workbench 5.1 (Indec BioSystems, Santa Clara, CA, USA), and saved to a hard disc. The mean fura-2 ratio at each time point was calculated over a large area of each ganglion cell body.

Drugs and Chemicals

All chemicals and reagents, unless otherwise noted, were obtained from Sigma Aldrich (Oakville, ON). L-803,087 was purchased from Tocris Biosciences (Ellisville, MO). Tetrodotoxin (TTX), charybdotoxin (ChTX) and ω-conotoxin GVIA (CTX) were obtained from Ascent Scientific (Princeton, NJ). Fura-2 was purchased from Invitrogen (Burlington, ON). L-803,087 (0.001%) and nifedipine (0.1%) were prepared as DMSO stock solutions, frozen at -20°C and thawed immediately before experiments (numbers in parenthesis indicate percentage of DMSO in the final working solution). Vehicle control experiments (0.1% DMSO) were used where appropriate for comparisons between treatments. TTX was prepared as a stock in 0.4 mM citrate buffer solution and frozen at -20°C until use. All other drugs and reagents were prepared in double distilled water either as stock solutions (frozen at -20°C) or prepared fresh before performing experiments.
**Data analysis**

All data are reported as the means ± SEM. Data analysis for electrophysiology and Ca$^{2+}$ imaging experiments was performed using Clampfit 10 software (Molecular Devices, CA). Graphing and statistical analyses were performed using SigmaPlot 11.0 software (Systat Software Inc, San Jose, CA). Specific statistical tests used are noted in the figure legend in the appropriate results sections. P values of less than 0.05 were considered statistically significant. All pair-wise multiple comparison procedures were performed in SigmaPlot, using the Holm-Sidak method.

**Results**

**Localization of sst4 to the ganglion cells of rat retina**

Since the aim of the present study was to determine the effects of selective sst4 stimulation on voltage-gated ion channels in mammalian ganglion cells, we first examined the expression pattern of sst4 in the mammalian retina using an affinity-purified polyclonal antibody directed to the C-terminus of rat sst4 (see Methods). Figure 1A shows sst4 immunoreactivity in a rat retinal section. Sst4 is localized to multistratified dendrites in the IPL and to numerous cell bodies in the ganglion cell layer (GCL), similar to a previous report (Brecha et al. 2002). Soma staining in the GCL is characterized by a granular appearance in the cytoplasm suggesting association with the Golgi complex and endoplasmic reticulum. Robust immunostaining was observed in the optic nerve fiber layer and optic nerve (not shown). Preadsorbing the antibody with the synthetic peptide ($10^{-6}$M sst4(362-384)) produced no immunostaining in the retina (Fig. 1B). In purified ganglion cell cultures, immunostaining was evident over the somata and dendrites (Fig. 1C).
Also, 100% of purified RGCs stained positive for sst4. Preadsorption control experiments in cultures showed weak secondary staining of only the ganglion cell somata (Fig. 1D).

Sst4 was expressed by many ganglion cells in rat retina, the identity of which was based on the size of immunoreactive cells. Immunostained cells ranged from 12 to 25 μm in diameter in the rat retina and in general, matched the known spectrum of ganglion cell diameters, although the smallest ganglion cells are under-represented compared to the general population of ganglion cells (Raymond et al. 2008). In retinal sections, the primary dendrites were seen to branch at several levels in the IPL and an immunoreactive axon was often observed at the base of the cell body. These axons formed fascicles within the nerve fiber layer.

*Patch clamp analysis of somatostatin and a selective sst4 agonist, L-803,087, on ion channels in isolated rat ganglion cells*

In preliminary experiments, we determined the effects of non-selective sst receptor stimulation using the endogenous agonist, somatostatin, in purified retinal ganglion cell cultures. Figure 2A shows representative voltage-clamp recordings of outward K⁺ current (I_K) in the absence (left) and presence (right) of SRIF (1 μM). Administration of somatostatin increased I_K compared to the control recording. Figure 2B shows examples of inward Ca²⁺ channel current (I_{Ca}) recorded in the presence of K⁺-channel blockers in the absence (left) and presence (right) of somatostatin. Mean current-voltage relations (Figures 2C and 2D) show that administration of somatostatin to ganglion cells increased I_K, while I_{Ca} was decreased compared to control recordings. No TTX was included in these experiments so large unclamped sodium currents appear at the initiation of the depolarizing steps.
Next, the selective sst$_4$ agonist, L-803,087 (10 nM), was administered to ganglion cells. Figure 3 shows examples of action potentials recorded from a ganglion cell under current-clamp conditions. In the absence of drug (Fig. 3A), repetitive action potentials were generated when current was injected through the microelectrode. Administration of L-803,087 reduced the amplitude and frequency of these action potentials (Fig. 3B) and eventually, only a single action potential was generated in response to the current injection (Fig. 3C). This inhibitory effect on action potential generation was partially reversible upon removal of the drug. In 7 cells, the resting potential was unchanged by L-803,087, being $-64.9 \pm 1.4$ mV in control and $-63.9 \pm 1.9$ mV after administration of the drug. The amplitude of action potentials (second in train) was reduced from $35.9 \pm 11.4$ mV before drug to $19.9 \pm 10.5$ mV in L-803,087 ($p<0.05$, paired t-test, $n=6$). The rate of action potential repolarization was reduced in 5 cells by $68\% \pm 9.3\%$ by the addition of L-803,087.

To examine the effects of sst$_4$ stimulation on membrane currents, ganglion cells were voltage-clamped in the absence and presence of L-803,087. Figure 4A shows representative recordings of I$_K$ in the absence (left) and presence (right) of L-803,087. Mean data show that the administration of L-803,087 significantly enhanced I$_K$ at positive membrane potentials (Figure 4B). Next, to determine which K$^+$ channels were involved in this increase in I$_K$, known K$^+$ channel blockers were applied in the absence and presence of L-803,087. Figures 4C and D show the percentage of I$_K$ blocked when different K$^+$ channel blockers were applied to ganglion cells in the absence and presence of L-803,087 at 0 mV. In the absence of L-803,087, TEA$^+$ blocked 49.6 $\pm$ 6.6 % of the steady-state I$_K$, while 4-aminopyridine (4-AP) and charybdotoxin (ChTX) blocked 40.8 $\pm$ 7.8 % and 14.8 $\pm$ 6.5 %, respectively (Figure 4C). However, when the same blockers were applied after L-803,087 administration, the majority of I$_K$ was sensitive to 4-
AP (50.5 ± 5.8%), while TEA⁺ and ChTX blocked only 25.2 ± 5.2 % and 3.0%, respectively (Figure 4D).

Next, Ca²⁺ channel currents were isolated and recorded in the absence and presence of L-803,087 in ganglion cells. Figure 5A shows examples of Ca²⁺ channel currents in the absence (left) and presence (right) of L-803,087. Mean data show that L-803,087 significantly reduced Ca²⁺ channel currents (Figure 5B). Next, selective Ca²⁺ channel blockers were administered in the absence and presence of L-803,087 to determine which Ca²⁺ channel subtypes may be involved in the sst₄ signaling pathway. Figure 5C shows the percentage of Ca²⁺ channel current blocked by the administration of nifedipine (NIF, L-type Ca²⁺ channel blocker), ω-conotoxin GVIA (CTX, N-type Ca²⁺ channel blocker) and L-803,087. Mean data show that NIF and CTX blocked 46.1 ± 3.5 % and 43.5 ± 7.2 % of the Ca²⁺ channel current, respectively, while L-803,087 blocked 32.5 ± 9.4 % of the Ca²⁺ channel current. Figure 5D shows that when L-803,087 was administered after NIF, there was no additional block of the Ca²⁺ channel current (-3.5 ± 3.8 %); however, when L-803,087 was applied after CTX, there was a further 28.0 ± 16.0 % block of the Ca²⁺ channel current.

**Calcium signal imaging in retinal flatmounts**

Application of high extracellular K⁺ solution (52.5 mM K⁺, see Methods for full composition) for 30 s produced transient increases in intracellular Ca²⁺ levels that declined in amplitude with each subsequent high K⁺ application. As seen in Figure 6A, in the absence of drug (left) the second of two high K⁺ evoked Ca²⁺ transients was smaller than the first. However, when L-803,087 (100 nM) was administered during high K⁺ application (right), the second Ca²⁺ transient was reduced more than the control example. Mean data showed that under control conditions the second Ca²⁺ transient was routinely smaller than the first (Figure 6B; second transient/ first
transient: 78.2 ± 2.3 %). Administration of L-803,087 reduced Ca²⁺ transients compared to the first recorded Ca²⁺ transient in the absence of drug (67.9 ± 1.4 %; p<0.05 vs. control). To test whether the results of the patch clamp experiments would be supported in Ca²⁺ imaging, we applied nifedipine (NIF) before and during the treatment with L-803,087. When NIF (10 µM) was applied during the second high K⁺ application, mean data show that NIF reduced Ca²⁺ transient amplitudes to 62.2 ± 1.8 % compared to the first transient (p<0.05 vs. control).

However, when NIF and L-803,087 were applied together, there was no further reduction in Ca²⁺ transient amplitude compared to either drug alone (66.9 ± 1.8%; p<0.05 vs. control; NS vs. NIF or L-803,087 alone). Hence, blockade of L-type Ca²⁺ channels by NIF occluded further block by L-803,087.

### Discussion

#### Summary of key findings

Results of the present study show that in the rat retina, ganglion cells are the only cell type expressing sst₄. Thus, the aim of this study was to investigate the effects of an sst₄ agonist (L-803,087) on voltage-gated ion channels as potential therapeutic avenues to selectively target retinal ganglion cells. This is the first study, to our knowledge, showing that selective activation of sst₄ in isolated retinal ganglion cells enhanced outward K⁺ current and reduced inward Ca²⁺ current. In addition, we show that administration of L-803,087 inhibited L-type, but not N-type Ca²⁺ channels, suggesting a preferential signaling pathway interaction between sst₄ and L-type Ca²⁺ channels in retinal ganglion cells.
As shown in other studies of mammalian retina, sst4 immunoreactivity was found in many rat retinal ganglion cell bodies, but in no other retinal cell somata (Thermos 2003; Cristiani et al. 2002; Brecha et al. 2002; Dal Monte et al. 2003; Vasilaki et al. 2002). The dendrites of immunopurified ganglion cells showed strong punctate staining, suggesting the possibility of localized sst4 signaling in the dendrites. Since all purified retinal ganglion cells exhibited strong immunostaining over the cell somata and all dendrites, these results suggest an important role for sst4 in a significant population of ganglion cells. Further studies are warranted to determine the extent of sst4 expression in ganglion cells of the rat retina.

Previous work showing localization of somatostatin to wide-field amacrine cells suggests that this peptide has a broad modulatory influence via the activation of different sst receptors. As we know, somatostatin acts on specific cell types via distinct sst receptors. Thus, results of this study, together with previous studies, suggest that sst4 is a target that is found only on ganglion cells (Cristiani et al. 2002; Brecha et al. 2002; Dal Monte et al. 2003; Vasilaki et al. 2002).

Electrophysiological results of this study showed that selective stimulation of sst4 receptors by L-803,087 in rat ganglion cells most prominently enhanced steady-state I_K and reduced L-type Ca^{2+} channel current. Generation of repetitive action potentials in ganglion cells was inhibited by L-803,087 and Ca^{2+} imaging showed that intracellular levels of Ca^{2+} were increased to a lesser extent in response to depolarization in the presence of L-803,087. Together, these data suggest that stimulation of sst4 reduces the excitability of ganglion cells and limits
Ca\textsuperscript{2+} influx. Thus, the present study on the effects of sst\textsubscript{4} stimulation in isolated ganglion cells motivates elucidation of a potential role of sst\textsubscript{4} as a therapeutic target.

The inhibition of spiking shown in the present study could be due to the enhancement of K\textsuperscript{+} channel activity produced by sst\textsubscript{4} receptor activation. Indeed, patch-clamp studies of whole cell currents showed that application of L-803,087 greatly enhanced outward currents. In addition, the K\textsuperscript{+} channels involved appear to be sustained, 4-AP sensitive voltage gated K\textsuperscript{+} channels, the activity of which is known to be involved in repolarizing the membrane after the upstroke of an action potential. Depending on the exact activation range of these K\textsuperscript{+} channels; their modulation may also affect cell resting potential, which in turn can affect steady-state levels of sodium channel inactivation. In the present study, resting potential, which was set to a value near -60 mV with the injection of hyperpolarizing current under control conditions, was not affected by L-803,087. However, as this study did not address the potential of sodium channel modulation by sst\textsubscript{4} stimulation, this would be an important topic for future investigation.

Results of the present study showed that administration of L-803,087 reduced Ca\textsuperscript{2+} currents in isolated retinal ganglion cells. In addition, we show that this effect of L-803,087 was unique to L-type (and not N-type) Ca\textsuperscript{2+} channels. While the reduced Ca\textsuperscript{2+} channel activity induced by L-803,087 likely had little effect on ganglion cell excitability, lowering intracellular Ca\textsuperscript{2+} is generally accepted as a mechanism to reduce cellular toxicity and death. As the primary aim of this study was to determine the effects of sst\textsubscript{4} stimulation on voltage-gated ion channels, we did not investigate cell death or apoptosis. Direct evaluation of the effects of specific sst\textsubscript{4} agonists on cell death will be an important area to pursue in future studies.

The actions of somatostatin on voltage-gated ion channels in ganglion cells, shown here, share some features of somatostatin action mediated by stimulation of other sst receptor subtypes
in other retinal neurons: In rod photoreceptors, somatostatin elicits an increase in delayed
rectifier K⁺ current (I_{KV}) and decreases L-type Ca^{2+} current (but not in cones, where it produces
increases in both K⁺ and Ca^{2+} currents; Akopian et al. 2000). In bipolar cells, somatostatin
inhibits L-type Ca^{2+} channels which leads to inhibition of calcium-activated K⁺ channels
(Petrucci et al. 2001). In isolated rod bipolar cells somatostatin inhibits high K⁺-evoked
increases of [Ca^{2+}]_i, presumably by inhibiting Ca^{2+} currents (Johnson et al. 2001). In ganglion
cells in the intact retina, however, somatostatin increases spontaneous and light-evoked spike
activity (Zalutsky and Miller 1990). The results of the present study, from isolated ganglion
cells, neither replicate nor provide an explanation for this action. Rather, it is likely that in the
eyecup, under dark adapted conditions, somatostatin increased the excitatory drive provided to
ganglion cells from bipolar cells, possibly by suppressing inhibitory circuits, and/or by directly
suppressing inhibitory input onto ganglion cells. Indeed, amacrine cell inhibitory signaling was
shown to be suppressed by somatostatin as the antagonistic surround of ganglion cells was
reduced (Zalutsky and Miller 1990). Thus, somatostatin signaling pathways in the mammalian
retina are complex and require further studies to fully elucidate and understand their
mechanisms.

*Potential neuroprotective uses of sst₄ agonists*

The results of the present study show that sst₄ stimulation reduced L-type Ca^{2+} channel
current, reduced depolarizing membrane potential excursions and suppressed intracellular Ca^{2+}
levels. These findings suggest that sst₄ agonists have the potential to act as selective
neuroprotective agents by targeting ion channels via sst₄ signaling pathways to reduce
excitability and Ca^{2+} influx. The results are consistent with previous work suggesting that, in
general, somatostatin may prevent pro-apoptotic signaling events (Cervia and Bagnoli 2007).
The targeting of sst4 receptors to suppress Ca^{2+} signaling provides a potential new therapeutic avenue in the treatment of neurodegenerative diseases of the retina. In spite of potentially interfering with the normal actions of somatostatin released endogenously in the retina, it is possible that selective sst4 receptor activation could reduce ganglion cell excitability sufficiently to counteract disease-induced elevations in [Ca^{2+}]_i and delay or prevent cell degeneration.

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Figure Legends

Figure 1: Sst₄ immunoreactivity in ganglion cells of the rat retina. A. Immunostaining was found in medium to large cell bodies in the ganglion cell layer and in dendrites that ramify throughout the inner plexiform layer. B. Staining was not seen in sections incubated with sst₄ antibody preadsorbed with the sst₄(362-384) peptide (10⁻⁶ M). Scale bar for A and B is 20µm. C. Purified retinal ganglion cells exhibited strong immunostaining over cell somata and all dendrites. D. Preadsorption experiments showed weak staining of the secondary antibody confined to the somata of panned cells, with no staining detectable over the dendrites. Scale bar for C and D is 50µm.

Figure 2: Effects of non-selective sst stimulation, using somatostatin, on transmembrane currents in isolated ganglion cells. A. Representative Iᵦ recordings from a ganglion cell in the absence (left) and presence (right) of 1 µM somatostatin (SRIF). Voltage command protocol shown below. B. Examples of Ca²⁺ channel currents in the absence (left) and presence (right) of somatostatin (SRIF). Voltage command protocol shown below. The recordings were made without TTX. C. Steady-state current-voltage (I-V) relationships for Iᵦ in the absence (filled symbols) and presence of 1 µM somatostatin (hollow symbols). D. Steady-state Ca²⁺ channel current-voltage relationships in the absence (filled symbols) and presence of 1 µM somatostatin (hollow symbols). N=2 cells/group, statistics not performed.
Figure 3: Action potential generation was inhibited by selective sst₄ stimulation in isolated ganglion cells.

A. Example of a current-clamp recording, in which injection of depolarizing current (150 pA, timing per trace shown below) generated repetitive action potentials in an isolated ganglion cell. The cell’s resting potential was set near -60 mV with continuous injection of hyperpolarizing current. B. Following administration of L-803,087 (10 nM) for 30 seconds, the frequency of action potentials was reduced in the same cell. C. Continued treatment with L-803,087 inhibited the repetitive action potentials all together.

Figure 4: K⁺ currents in isolated ganglion cells were enhanced following L-803,087 administration.

A. Representative examples of Iₖ recordings from an isolated ganglion cell under voltage-clamp conditions in the absence (left) and presence (right) of L-803,087 (10 nM). Voltage command protocol shown below. B. Mean I-V curve data show that administration of L-803,087 significantly increased Iₖ at more positive membrane potentials (n=12; *p<0.05 two way repeated measure ANOVA vs. control). C. In the absence of L-803,087, administration of K⁺ channel blockers, TEA⁺ (20 mM), 4-AP (5 mM) and ChTX (100 nM) reduced Iₖ compared to control recordings (recorded at 0 mV, n=7-10 cells/group). D. Following administration of L-803,087, TEA⁺ and ChTX blocked smaller proportions of Iₖ compared to the control conditions, while 4-AP blocked a larger proportion of Iₖ compared to control (recorded at 0 mV, n=1-5 cells/group).
Figure 5:  

Ca\textsuperscript{2+} channel currents in isolated ganglion cells were reduced by L-803,087.  

A.  Examples of Ca\textsuperscript{2+} channel currents recorded from an isolated ganglion cell under voltage-clamp in the absence (left) and presence (right) of L-803,087 (10 nM).  Voltage command protocol shown below.  

B.  Mean data show that administration of L-803,087 significantly reduced Ca\textsuperscript{2+} channel current at intermediate membrane potentials (n=4; *p<0.05 two way repeated measure ANOVA vs. control).  

C.  In the absence of L-803,087, administration of Ca\textsuperscript{2+} channel blockers, NIF (10 µM) and CTX (1 µM) reduced Ca\textsuperscript{2+} channel currents compared to control recordings (recorded at -10 mV, n=3-4 cells/group).  

D.  Following administration of NIF, L-803,087 had no additional blocking effect on Ca\textsuperscript{2+} channel current, while following administration of CTX, L-803,087 further reduced Ca\textsuperscript{2+} channel current (-10 mV, n=3-7 cells/group; **p<0.05 ANOVA vs. L-803,087).

Figure 6:  Intracellular Ca\textsuperscript{2+} signaling was reduced by L-803,087 in ganglion cells in retinal flatmount.  

A.  Applications of 52 mM K\textsuperscript{+} solution for 30 s depolarized ganglion cells and produced transient increases in intracellular Ca\textsuperscript{2+} concentration, measured with the dye fura-2.  

Traces (left) show that, in the absence of drug, the second of paired K\textsuperscript{+} applications produced a smaller peak Ca\textsuperscript{2+} signal.  On the right, the paired K\textsuperscript{+} pulses, with L-803,087 (100 nM) applied 30 seconds prior to the second K\textsuperscript{+} application, show greater reduction of the Ca\textsuperscript{2+} signal compared to control.  Panel on far right shows a fluorescent image (stimulated at 380 nm) of a fura-2 containing ganglion cell in the flatmount retina.  

B.  Mean data show amplitudes of the second high K\textsuperscript{+} response expressed as a percentage of first peak in control, L-803,087 (L-803, 100 nM), nifedipine (NIF, 10 µM) and nifedipine plus L-803,087.  The mean Ca\textsuperscript{2+} transient amplitudes of all three drug treatment groups (L-803, NIF, L-803 + NIF) were significantly
smaller than those of the control group but they did not differ among themselves (n=21-89 cells/group; *p<0.05, ANOVA).

References


**Cristiani R, Petrucci C, Dal Monte M, Bagnoli P.** Somatostatin (SRIF) and SRIF receptors in the mouse retina. *Brain Res* 936: 1-14, 2002.

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A
Control

B
L-803.087
30 seconds

C
L-803.087
2.5 minutes

25 mV
25 ms
Figure 4

A

Control

L-803,087

25 ms

250 pA

Voltage (mV)

-60 -40 -20 0 20 40

IK (pA)

0 200 400 600 800 1000 1200 1400

Figure 4

B

Control

L-803,087

Block of IK (% Control)

0 20 40 60 80 100

Block of IK (% L-803 Response)

0 20 40 60 80 100

C

Block of k ( % Control)

TEA 4-AP ChTX

D

Block of k ( % L-803 Response)

TEA 4-AP ChTX
Figure 5

A

Control L-803,087

B

Voltage (mV)

C

Block of ICa (% Control)

D

Further Block of ICa by L-803,087 (%)