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Wavy Multistratified Amacrine Cells in the Monkey Retina Contain Immunoreactive Secretoneurin

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Abstract

The goals of this study were to describe the morphology, neurotransmitter content and synaptic connections of neurons in primate retinas that contain the neuropeptide secretoneurin. Amacrine cells were labeled with antibodies to secretoneurin in macaque and baboon retinas. Their processes formed three distinct plexuses in the inner plexiform layer: one in the outermost stratum, one in the center and one in the innermost stratum. In light microscopic double immunolabeling experiments, GABA was colocalized with secretoneurin in these cells, but glycine transporter 1 and Substance P were not. ON bipolar cell axon terminals labeled with antibody to the cholecystikinin precursor, G6-gly, have ON responses to stimulation of short wavelength sensitive (S) cones. Axons of these bipolar cells made contacts with amacrine cell dendrites containing secretoneurin. Secretoneurin-IR amacrine cells also made contacts with retinal ganglion cell dendrites labeled with antibody to the photopigment melanopsin, which have OFF responses to stimulation of S cones. Using electron microscopic immunolabeling, 436 synapses from macaque retina were analyzed. Axons from bipolar cells were identified by their characteristic synaptic ribbons; their synaptic densities were asymmetric like those of excitatory synapses in the brain.

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Contributors

Andrea S. Bordt carried out immunolabeling experiments, acquired and analyzed light microscopic images and wrote the manuscript.

Jens Hannibal made and characterized the melanopsin antiserum.

Nobuo Kouyama analyzed light microscopic images.

Ye Long carried out immunolabeling experiments and acquired and analyzed light microscopic images.

David W. Marshak designed the experiments, acquired and analyzed the electron microscopic images and wrote the manuscript.

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Amacrine cells made and received conventional synapses with symmetric synaptic densities, like those of inhibitory synapses in the brain. Ganglion cell dendrites were identified by their absence of presynaptic specializations; they received inputs from both amacrine cells and bipolar cells. The majority of inputs to the secretoneurin-IR amacrine cells were from other amacrine cells, but they also received 21% of their input from bipolar cells. They directed most of their output, 54%, to amacrine cells, but there were many synapses onto bipolar cell axons and ganglion cell dendrites, as well. The synaptic connections were very similar in the three plexuses with one notable exception; output synapses to bipolar cells were significantly less common in the innermost one, where the S-ON bipolar cells terminate. Taken together, these findings suggest that the secretoneurin-IR amacrine cells in primates receive excitatory input from S-ON bipolar cells and, in turn, inhibit intrinsically photosensitive retinal ganglion cells.

Graphical abstract

In the primate retina, a population of inhibitory local circuit neurons (red) contains the neuropeptide secretoneurin (SN). These play an important role in color vision. They receive excitatory input from bipolar cells (blue) that contact blue cones. The bipolar cells have depolarizing (S-ON) responses to short wavelength stimuli, and they make excitatory synapses onto the amacrine cells (triangles, blue arrow). The amacrine cells, in turn, make inhibitory synapses (circles, red arrow) onto intrinsically photosensitive retinal ganglion cells (green) that express the photopigment melanopsin (MOPS+). The ganglion cells convey this information to the brain (green arrow) through their axons in the optic nerve.

Keywords

Neuropeptide; GABA; Primate; Melanopsin; Color Vision

1. Introduction

Secretogranin II is a protein found in large dense-core vesicles throughout the nervous and endocrine systems. In the brain, over 90% of secretogranin II is processed by proteolytic enzymes into smaller peptides, including the 33 amino acid neuropeptide secretoneurin [1]. Secretoneurin is widely distributed in the brain and spinal cord, and it has modulatory

actions typical of neuropeptides [2]. For example, secretoneurin releases dopamine from slice preparations of the rat striatum [3].

Immunoreactive secretoneurin in the human retina has been characterized using liquid chromatography and radioimmunoassay. None of the immunoreactive material was secretogranin, and less than 6% was an intermediate sized form. The remainder was secretoneurin, itself, indicating that it was, by far, the major form [4]. In human retinas, secretoneurin has been localized to amacrine cells, inhibitory local circuit neurons of the inner retina [4]. Dendrites of the secretoneurin-immunoreactive (IR) amacrine cells branched in the same stratum of the inner plexiform layer (IPL) as the axons of bipolar cells that are selectively excited by signals from short wavelength sensitive (S) cones [5]. Dendrites of intrinsically photosensitive retinal ganglion cells also branch in that stratum, and these cells are inhibited by signals from S cones [6]. Taken together, these findings suggest that the secretoneurin-IR amacrine cells convey inhibitory signals from the S-cone bipolar cells to the retinal ganglion cells, as observed previously in the ground squirrel retina [7, 8]. To test this hypothesis, we studied secretoneurin-IR amacrine cells in the retinas of Old World Monkeys using both light and electron microscopic immunolabeling techniques. Based on these results, we proposed that the secretoneurin-IR amacrine cells play a role in processing short wavelength information.

2. Materials and Methods

Eye tissue from 3 baboons (*Papio anubis*) and 5 macaques (4 *Macaca mulatto*, 1 *Macaca fascicularis*) was used for the experiments reported here. Tissue was provided by The Washington Regional Primate Research Center (Seattle, WA, USA) and the Southwest National Primate Research Center (San Antonio, TX, USA). Animals were euthanized according to approved care and use standards for humane reasons or else after experiments that did not involve the eyes. The eye was cut open behind the ora serrata, the vitreous humor was removed, and the tissue was incubated in Ames medium (Sigma-Aldrich) equilibrated with 95% oxygen and 5% carbon dioxide at 20°C before fixation. All of the fixatives included paraformaldehyde, and in some instances, glutaraldehyde or picric acid was added to enhance preservation of the ultrastructure. The primary antibodies used in this study are described in Table 1.

2.1 Antibody Characterization

The rabbit antiserum to secretoneurin was described previously; it recognizes the precursor, secretogranin II, as well as the neuropeptide in human retinal extracts [9]. In the human retina, the labeling was blocked when the antiserum was preincubated with synthetic secretoneurin (10 µM, 24 hours, 4°C) [4]. The goat antibody to secretoneurin was prepared from antiserum against human secretoneurin; it also recognizes both secretoneurin and secretogranin II (information from Santa Cruz Biotechnology).

The 16 N-terminal amino acids of rat secretogranin II were synthesized by standard t-BOC chemistry and purified by HPLC. The abbreviation for the resulting peptide (AR-16) indicates the first and last amino acid according to the single letter code plus the total number of amino acids. The peptide structures were verified by amino acid analysis and

mass spectrometry. AR-16 was coupled via an additional C-terminal cysteine to maleimide-activated keyhole limpet hemocyanin, and the conjugate was injected into rabbits following a standard immunization protocol to generate a polyclonal peptide antiserum. The antiserum recognizes secretogranin II and smaller peptides containing the N-terminus generated by prohormone convertase [10]. Both of the antisera to secretoneurin and the antiserum to AR-16 yielded the same results. The pattern of labeling was the same as described previously in the human retina [4].

All the other antisera used in this study have been used in primate retinas, and the labeling observed here was identical to that reported previously. The antiserum to C-terminal Binding Protein-2 (CtBP-2) labels synaptic ribbons [11]. Antiserum to GABA labels a subset of amacrine cells and, to a lesser extent, retinal ganglion cells [12]. The monoclonal antibody to Substance P labels a population of axon-bearing amacrine cells in macaque and human retinas [13, 14]. The antibody to Glycine Transporter 1 (Gly-T1) labels glycinergic amacrine cells in macaque retinas [15]. The antiserum to the glycine-extended precursor of cholecystokinin and gastrin (G6-gly) labeled amacrine cells and bipolar cells that selectively contact blue cones in macaque retina [16]. The antibody to melanopsin labeled intrinsically photosensitive retinal ganglion cells in macaque and human retinas [17].

2.2 Immunoperoxidase

An isolated retina from a *Papio anubis* was fixed overnight in picric acid formaldehyde, 2% paraformaldehyde, 0.1% picric acid in 0.1M sodium phosphate buffer pH 7.4 (PB). After this and all subsequent steps in the labeling protocols, the retina was washed in 3 changes of phosphate buffered saline (PBS); all incubations with antibodies were done at 4°C in PBS, as described previously [18]. Briefly, the retina was incubated in 1% NaBH₄ at room temperature for 1 hour, followed by 10% normal goat serum and 0.3% Triton X-100 (Triton) for 2 hours at 4°C. The retina was incubated in 1:1,000 rabbit anti-secretoneurin antibody with 0.3% Triton, washed, and incubated with biotinylated secondary antibody (1:100 overnight) and avidin-biotin peroxidase (1:100 overnight; ABC Standard Kit, Vector Laboratories). The peroxidase was visualized with diaminobenzidine tetrahydrochloride. The retina was treated for 10 min with 0.1% OsO₄ to stabilize the reaction product and to enhance contrast. It was then dehydrated and embedded flat in epon.

Peripheral retina from 1 *Macacca mulatta* was processed similarly. The tissue was fixed for 1 hour in 0.1% glutaraldehyde and 4% paraformaldehyde in PB pH 7.4 at 38°C and postfixed overnight with 4% paraformaldehyde in PB pH 10 at 4°C. The tissue was labeled and processed as described above, and 30 µm sections were cut using a sliding microtome.

Retinas from 2 macaques were used for electron microscopy. A retina from a *Macaca mulatta* was fixed for 30 min in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M PB pH 7.4 at 38°C and postfixed overnight in 4% paraformaldehyde in 0.1M PB pH 10 at 4°C. A retina from a *Macaca fascicularis* was fixed for 30 min at 20°C with 0.05% glutaraldehyde and 4% paraformaldehyde in 0.1M PB. The tissue was postfixed as described above, except that the duration was 8 days. Both retinas were treated with 1% NaBH₄ in PBS and a series of graded ethanol solutions. Sections were cut at 500 µm using a tissue chopper and labeled as described above. The *M. mulatto* tissue was incubated in 1:1,000 rabbit anti-secretoneurin

for 10 days. The *M. fascicularis* tissue was incubated for 12 days in either rabbit anti-AR-16 or rabbit anti-secretoneurin, both at 1:1,000. The retinas were processed as described above, and the tissue was treated with 1% OsO₄ for 1 hour, dehydrated and embedded in epon. Ultrathin sections were cut, collected on formvar-coated, single hole grids and stained with 1% aqueous uranyl acetate for 20 min followed by 0.2% aqueous lead citrate for 10–15 min [18]. Labeled profiles were imaged at 2,000 X and at 10–20,000 X in a JEOL 100CX electron microscope.

2.3 Immunofluorescence

A *Papio anubis* retina was used for the double labeling experiments using anti-secretoneurin and anti-melanopsin. The tissue was fixed in 4% paraformaldehyde in 0.1M PB for 1 hour at room temperature. The tissue was incubated with Antigen Retrieval Solution pH 9 (Dako) for 1.5 hours at 80°C, cooled and incubated with the two primary antisera sequentially. The first step was 11 days with 1:10,000 rabbit anti-melanopsin, 20% ChemiBLOCKER (Millipore) and 0.3% Triton, and the second was 14 days with 1:10,000 goat anti-secretoneurin in the same diluent. Immunoreactive melanopsin was detected with 1:100 biotinylated anti-rabbit IgG for 5 days followed by 1:200 Alexa Fluor 488 Streptavidin for 4 days. To label the immunoreactive secretoneurin, 1:200 Cy3 donkey anti-goat IgG was included with the streptavidin.

Retina from another *Papio anubis* was used for the secretoneurin/GABA and secretoneurin/Glyt-1 double labeling experiments and also for the stratification analysis. It was fixed in 4% paraformaldehyde in 0.1M PB, pH 7.4, for 1 hour at 20°C, embedded in agarose and sectioned using a vibratome. Sections were incubated with both primary antibodies simultaneously (1:1,000 rabbit anti-secretoneurin, 1:1,000 rat anti-GABA or 1:1,000 goat anti-Glyt-1), and the antibodies were detected with affinity-purified secondary antibodies conjugated with Cy3 (secretoneurin) or Alexa 488 (GABA, Glyt-1).

Two *Macaca mulatta* retinas were used for the triple labeling experiments. One retina was isolated and fixed for 1 hour in 4% paraformaldehyde in 0.1M PB, pH 7.4 at 20°C. A small piece was embedded in agarose, and 50 µm sections were cut using a vibratome. The sections were incubated for 8 days in 1:1,500 rabbit anti-G6-gly, 1:1,000 mouse anti-CtBP2, and 1:10,000 goat anti-secretoneurin in PBS with 20% ChemiBLOCKER and 0.3% Triton at 4°C. After incubation for 6 days in affinity-purified, labeled secondary antibodies (1:200) at 4°C, tissue was mounted in Antifade Mounting Medium with DAPI (Vector Laboratories). A retina from another *M. mulatta* was fixed for 1 hour with 4% paraformaldehyde and 0.05% glutaraldehyde in PB at 20°C. After treatment for 60 min with 1% NaBH₄ in PBS, the tissue was labeled as described above, except that the Mouse anti-CtBP2 was 1:200, the primary antibody step was 4 days long, and the secondary antibody step was 2 days long.

2.4 Quantitative Analyses

To calculate stratification depths for secretoneurin-IR profiles, a section 30 µm thick and 72 µm long from the baboon retina described above was imaged. Immunoreactive profiles were counted and their intensities and positions were measured using the Plot Profile function in ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland,

USA, <https://imagej.nih.gov/ij/>, 1997–2016.). The analysis was done in 25 consecutive optical sections, 0.4 μm each. The boundary between the inner nuclear layer (INL) and the IPL was designated as 0% and the IPL- ganglion cell layer (GCL) boundary as 100% depth. The resulting values were grouped in bins of 5% using Microsoft Excel to generate the histogram in Figure 2. The data was fitted by 3 normal curves using R (<https://www.r-project.org/>).

The data on the synaptic connections of the labeled amacrine cells shown in Table 2 was analyzed to test the hypothesis that the frequency of each type of synapse was the same in each of the 3 strata of the IPL. A Chi-squared test was performed using SAS software.

2.5 Imaging and Illustrations

Fluorescent images were collected with Zeiss LSM 410, 510 and LSM 780 confocal laser scanning microscopes. Brightness, contrast and levels of confocal images were adjusted using both Zen software (Carl Zeiss Microscopy) and Photoshop (Adobe Systems). Photoshop was used to adjust the brightness and contrast of electron microscopic images and also to arrange and to annotate figures for publication [18].

3. Results

3.1 Secretoneurin-IR amacrine cells

Labeled somas were observed in both the inner nuclear layer (INL) and the ganglion cell layer (GCL). However, the cells with somas in the GCL did not have labeled dendrites; these were not studied further. The labeled dendrites in the inner plexiform layer (IPL) typically originated from amacrine cells with somas in the INL. These were classified as amacrine cells based on the size and location of their somas. The labeled amacrine cell somas were approximately 9 μm in diameter. They had 2–3 primary dendrites that branched and gave rise to curving, varicose terminal dendrites. An example of an amacrine cell with its soma in the INL labeled using the immunoperoxidase method is illustrated in Figure 1A. These dendrites were found in the outermost stratum (S1), the middle (S3) and the innermost stratum (S5) of the IPL (Fig. 1). Like the wavy, multistratified type 1 cells described by Mariani [19], the descending dendrites originated from those in S1. Vertical sections labeled using immunofluorescence were analyzed in order to more completely describe the distribution of secretoneurin-IR varicosities within the IPL. Depth was defined as a percentage of the distance from the INL to the GCL. Three distinct peaks were seen at 16.7%, 54.8% and 89.6% of the IPL depth (Fig. 2).

Both of the antisera to secretoneurin recognized intact secretogranin II in large dense core vesicles in addition to the processed peptide. In experiments using highly sensitive methods to visualize the primary antibody, somas in the ganglion cell layer were also labeled. The processes of these cells were barely visible and they were not studied further. This additional labeling was not seen when fixative contained glutaraldehyde and the primary antiserum was visualized using the avidin-biotin peroxidase method, as in the electron microscopic immunolabeling experiments.

3.2 Secretoneurin-IR amacrine cells contain GABA

Double immunolabeling experiments were carried out to investigate whether secretoneurin-IR amacrine cells contained GABA. All secretoneurin-IR cells were GABA-positive (Fig. 3). To determine whether secretoneurin-IR cells use glycine as a neurotransmitter, another set of experiments was carried out using an antibody to the glycine transporter Glyt-1. There was no co-localization, but there did appear to be contacts between secretoneurin-IR and Glyt1-IR dendrites (Fig. 4). Immunoreactive secretoneurin also did not colocalize with immunoreactive Substance P (Fig. 5).

3.3 Synaptic connections of secretoneurin-IR amacrine cells

Like the secretoneurin-IR dendrites, the axons of bipolar cells that depolarize in response to increments in light intensity (ON) receiving input from short wavelength sensitive (S) cones terminate in S5. To determine whether the two types of cells interact, double label experiments were done using antibody to the glycine-extended precursor of cholecystinin (G6-Gly) to label the bipolar cells [16]. The axon terminals of S-ON bipolar cells labeled with anti-G6-gly contacted secretoneurin-IR dendrites in S5 (Fig. 6). In triple label experiments using the synaptic ribbon marker C-terminal binding protein 2 (CtBP2), all three markers were present at these contacts, indicating that the bipolar cells were presynaptic. In other strata of the IPL, the axons of the labeled bipolar cells also made contacts that we interpreted as synapses en passant to secretoneurin-IR dendrites (Fig. 7).

Dendrites of intrinsically-photosensitive retinal ganglion cells containing the photopigment melanopsin are found in both S1 and S5 [17, 20]. Contacts with dendrites of secretoneurin-IR amacrine cells were observed in double immunolabeling experiments using antibody to melanopsin. All of the melanopsin cells labeled in this study were the outer stratifying type. Although the positions of their somas varied, as described previously [17], the vast majority of their dendrites were found at the boundary between the INL and the IPL. There were contacts between secretoneurin-IR dendrites and the somas and dendrites of the melanopsin cells (Fig. 8). Although the melanopsin cells are presynaptic within the retina, the terminals of their axon collaterals make these synapses, not their dendrites [21]. In a recent electron microscopic immunolabeling study of macaque retina, no presynaptic specializations were observed in the dendrites of melanopsin cells [17]. Based on these findings, we concluded that the secretoneurin-IR amacrine cells were presynaptic to the dendrites of retinal ganglion cells expressing melanopsin.

The synaptic connections of secretoneurin-IR amacrine cells were also studied by electron microscopy (Fig. 9). The rabbit antibodies to secretoneurin and AR-16 yielded the same pattern of labeling, and therefore, results with the 2 antibodies were considered together. The labeled profiles were subdivided according to their depth in the IPL. They contained clear synaptic vesicles and also, in some instances, large, dense-core vesicles, which were very well labeled. The unlabeled profiles were identified by their characteristic ultrastructure [22]. The criteria for evaluating labeled synapses were described in detail recently [23]. The sample included 436 synapses, including approximately equal numbers from each of the strata of the IPL in which labeled dendrites were found (Table 2).

In all 3 strata, synaptic inputs were relatively uncommon; only 21% (91/436) of the synapses were from unlabeled processes onto secretoneurin-IR dendrites. Synapses from other amacrine cells greatly outnumbered those from bipolar cells; 81% (74/91) of the input to the secretoneurin-IR dendrites came from amacrine cells. Other amacrine cells were also the most common types postsynaptic to the secretoneurin-IR dendrites; they received 54% (185/345) of the output, on average. There were also a considerable number of synapses from secretoneurin-IR dendrites onto retinal ganglion cells; these comprised 38% (131/345) of the output, on average. The pattern of synaptic connections was very similar in all three strata, with one exception. Based on a Chi-squared analysis, the only statistically significant deviation from the pattern was the small number of labeled synapses from secretoneurin-IR dendrites onto bipolar cell axons in S5. Overall, the distributions in the 3 strata were similar ($X^2(8) = 8.889$, $p = 0.352$). There was only one table cell significantly different from the expected frequency (cell $X^2(1) = 4.518$, $p = 0.03$), indicating fewer synapses from the labeled cells onto bipolar cells in stratum 5.

The distribution of synaptic connections was similar in all three strata, and there were essentially the same number of secretoneurin-IR synapses in each one. The majority of the synapses (345/436, 79%) were from labeled processes onto unlabeled processes. Amacrine cells were the most common postsynaptic cells (185/345, 54% of output), but there were a considerable number of synapses onto ganglion cells (131/345, 38% of output) and a smaller number of synapses onto bipolar cells (29/345, 8% of output). Amacrine cells provided the major input (74/91, 81% of input), but the secretoneurin-IR cells received a substantial number of synapses from bipolar cell axons (17/91, 19% of input). Based on a Chi-squared test, the only statistically significant deviation (*) from the hypothesis that each type of synaptic connection is equally distributed among the 3 strata was the small number of output synapses onto bipolar cells in stratum 5 ($p = 0.03$).

4. Discussion

4.1 Localization of immunoreactive secretoneurin in monkey retinas

One morphological type of amacrine cell was well labeled with the antibodies to secretoneurin and its precursor, secretogranin II. These had somas approximately 9 μm in diameter located primarily in the INL. They had delicate dendrites with a curving trajectory that ramified at three distinct levels in the IPL, peaking at 16.7%, 54.8% and 89.6% of the distance from the INL to the GCL. They closely resembled the wavy multistratified type 1 cells described previously in the macaque retina using the Golgi method [19]. They were also very similar to the tristratified amacrine cells described in the human retina [24]. However, in double labeling experiments, we showed that they were different from the tristratified amacrine cells containing immunoreactive Substance P [4, 13, 25]. In the human retina, immunoreactive secretoneurin and Substance P were found in the same 3 strata of the IPL, but the distribution of labeled processes was quite different. In those single label studies, immunoreactive Substance P was most abundant in S3 [13, 25], while immunoreactive secretoneurin was least abundant in that stratum [4].

One set of secretoneurin-IR dendrites co-stratified with the axon terminals of S-ON bipolar cells [26], and they made contacts that were interpreted as synaptic inputs from those axon

terminals. The secretoneurin-IR amacrine cell dendrites also made contacts with dendrites of retinal ganglion cells containing melanopsin, which have OFF responses to selective stimulation of S-cones [6]. However, the secretoneurin-IR amacrine cells were morphologically different from the other amacrine cells associated with the S-cone system in primate retinas. In macaque retinas, small bistratified ganglion cells have S-ON responses to light. One population of amacrine cells is labeled after intracellular injection of small tracer into small bistratified cells. These tracer-coupled amacrine cells have smaller somas than the secretoneurin-IR amacrine cells, and they are bistratified, having fine, varicose dendrites ramifying at depths of 15–20% and 85–90% of the distance from the INL to the GCL [27, 28]. The secretoneurin-IR amacrine cells were also different in several respects from the amacrine cells that are tracer-coupled to the inner-stratifying melanopsin cells, which have OFF responses to S-cone stimulation. Those amacrine cells have larger somas, 12 μm in diameter, which are located mainly in the GCL. The tracer-coupled amacrine cells are also tristratified, but the stratification pattern is slightly different from that of the secretoneurin-IR amacrine cells. The dendrites of the amacrine cells coupled to the melanopsin cells ramify closer to the inner and outer boundaries of the IPL in macaque retina [17].

Two amacrine cells described in other mammalian retinas are somewhat similar to the secretoneurin-IR amacrine cells, but they do not appear to be homologous. In rabbits, diffuse multistratified amacrine cells have dendrites that ramify in three or more strata of the IPL. However, the dendrites of the multistratified cells originate from a single primary dendrite and do not reach the innermost strata of the IPL [29, 30]. In these respects, they are different from the secretoneurin-IR amacrine cells whose descending dendrites originate from higher order dendrites and extend to S5. The amacrine cells that generate S-OFF responses of retinal ganglion cells in the ground squirrel retina are different from the secretoneurin-IR amacrine cells in several ways. Their thin, varicose dendrites descend through the IPL, but they do not branch in three distinct strata like the secretoneurin-IR cells. The amacrine cells from the ground squirrel also have bulbous terminals in S5 that were not seen on the secretoneurin-IR cells. A third difference is that the amacrine cells from ground squirrels are glycinergic [7], but the secretoneurin-IR amacrine cells are GABAergic.

4.2 Synaptic connections of amacrine cells containing immunoreactive secretoneurin

In the electron microscopic immunolabeling study described here, the secretoneurin-IR dendrites received a significant proportion of their input, about 20%, from bipolar cells at ribbon synapses. These synapses from bipolar cells were found in all 3 strata of the IPL in which secretoneurin-IR dendrites ramified. In the light microscopic double label experiments, one of the presynaptic bipolar cells was tentatively identified as the S-ON bipolar cell. Some of those synapses appeared to be made by the bipolar cell axon terminals in S5, and others appeared to be made by the axons en passant. In triple label experiments, synaptic ribbons were associated with both types of contacts. Both types of synapses have been observed previously in studies of S-ON bipolar cells in macaque retina. In an electron microscopic immunolabeling study using antibody to G6-gly to label S-ON bipolar cells, the axon terminals directed half of their output to unidentified amacrine cells, but they received very little input from amacrine cells, typically not in a reciprocal configuration [16]. In a study of serial sections from central macaque retina using conventional transmission electron

microscopy, ribbon synapses made by axons of S-ON bipolar cells were found in the most distal 10% of the IPL [31]. The results of the Chi-squared analysis of the synaptic connections of secretoneurin-IR amacrine cells also support this conclusion. There were significantly fewer synapses from secretoneurin-IR amacrine cells onto bipolar cell axons in S5 than in other strata of the IPL. This is consistent with the findings that the S-ON bipolar cell axons devote approximately half of their output to amacrine cells and receive very little reciprocal inhibition [10], and it suggests that the bipolar cells that contact the secretoneurin-IR dendrites are presynaptic in the double immunolabeling experiments described above. Taken together, these 3 lines of evidence strongly suggest that the S-ON bipolar cells are presynaptic to the secretoneurin-IR amacrine cells.

The secretoneurin-IR dendrites also directed about 40% of their output to dendrites of retinal ganglion cells in all three strata. In light microscopic double labeling experiments, one type of retinal ganglion cell whose dendrites made contacts with the secretoneurin-IR dendrites was identified as the outer-stratifying melanopsin cell [17, 20]. Additional subtypes of human retinal ganglion cells containing melanopsin have been described recently [32], but they were not identified in this study. Several lines of evidence suggest that these contacts onto the dendrites of the melanopsin cells are synaptic inputs from the secretoneurin-IR amacrine cells. The outer-stratifying melanopsin cells receive inputs from amacrine cells at conventional synapses [17], and they express the inhibitory synapse marker, gephyrin [33]. They also express receptors for the amacrine cell neurotransmitters GABA and glycine [34]. These findings suggest that the outer-stratifying melanopsin cells have OFF responses to stimulation of S-cones, like the inner-stratifying melanopsin cells [6]. These responses may originate from amacrine cell inputs, as they do in the ground squirrel retina [8]. There is also psychophysical evidence suggesting that S-OFF responses arise from amacrine cells in humans. Responses to increments in the intensity of S-cone isolating stimuli are significantly faster than those to decrements in the same stimulus [35]. The presynaptic amacrine cells have not been identified, but they are expected to have S-ON responses, like those described previously in ground squirrel retina [7] and those expected from the secretoneurin-IR amacrine cells described here.

The majority of synapses of the secretoneurin-IR amacrine cells were with other, unlabeled amacrine cells. In the double label experiments using antibody to GlyT-1, the secretoneurin-IR amacrine cells were not labeled, themselves, but they made frequent contacts with labeled profiles. This finding suggests that many of the amacrine cells that interact with the secretoneurin-IR cells are glycinergic. In primate retinas, these are narrow-field amacrine cells that branch in two or more of the five strata of the IPL. The best-known narrow-field amacrine cells in primates are AII cells, whose morphology, synaptic connections and light responses are very similar to those of other mammals [36–38]. Several other types of glycinergic amacrine cells have been described in primate retinas, as well, and they are all candidates to interact with the secretoneurin-IR cells. These include: diffuse cells [39], knotty type 2 cells [40], A8 cells [11] and several other knotty amacrine cell types that are known only from retinas labeled with the Golgi method [19]. Amacrine cells that contain vesicular glutamate transporter 3 are also glycinergic, but because they are not labeled by antisera to GlyT-1 [23], they did not account for any of the profiles observed in this study.

4.3 Conclusions

The wavy multistratified type 1 amacrine cells in macaque and baboon retina express the neuropeptide secretoneurin, and based on their synaptic contacts, they are expected to depolarize in response to increments in short wavelength light. They also express GABA, and among their postsynaptic targets are the retinal ganglion cells containing melanopsin. These inhibitory inputs might mediate responses of these ganglion cells to decrements in the intensity of short wavelength light. It is likely that the multistratified type 1 amacrine cells have additional functions because many of the neurons they interact with remain unidentified. In addition, there are other retinal neurons that are labeled less intensely with antibodies to secretoneurin, and these should be characterized in future experiments.

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HIGHLIGHTS

- In the primate retina, a population of inhibitory local circuit neurons contains the neuropeptide secretoneurin. These play an important role in color vision.
- They receive excitatory input from bipolar cells that contact blue cones. The bipolar cells have depolarizing (S-ON) responses to short wavelength stimuli, and they make excitatory synapses onto the amacrine cells.
- The amacrine cells, in turn, make inhibitory synapses onto intrinsically photosensitive retinal ganglion cells that express the photopigment melanopsin. The ganglion cells convey this information to the brain through their axons in the optic nerve.

Figure 1.

Secretoneurin-IR cells labeled with peroxidase in primate retina. A. The soma in the center is located in the INL of a baboon retina. It gives rise to 3 large-diameter primary dendrites and one thin primary dendrite (arrows). The thin primary dendrite and the higher-order dendrites are varicose and have a curving trajectory. B. A soma in the INL and primary descending dendrites (arrows) of a secretoneurin-IR cell in macaque retina. Secretoneurin cells are tristratified, ramifying in the outermost, middle, and innermost strata of the IPL (arrowheads).

Figure 2.

Stratification of secretoneurin-IR profiles in the IPL. Baboon retina was labeled with rabbit anti-secretoneurin antibody and imaged with confocal microscopy. Secretoneurin-IR profile depths in the IPL were quantified as described in Methods; the INL is 0% and the GCL is 100%. The peaks roughly correspond with strata 1, 3 and 5 of the IPL.

Figure 3.
GABA immunoreactivity in a secretoneurin-IR amacrine cell from baboon retina.
Secretoneurin immunoreactivity (red) is observed in some GABA-IR somas (green,
arrowhead). Single optical section. Scale bar = 10 μ m.

Figure 4. Secretoneurin and Glyt-1 immunoreactivity are not colocalized in baboon retina. However, there were appositions between secretoneurin-IR (red) and glycinergic dendrites in the IPL (green, arrowheads). Single optical section. Scale bar = 10 μ m.

Figure 5. Secretoneurin and Substance P are not colocalized. The dendrites containing immunoreactive secretoneurin (red) and the processes containing immunoreactive Substance P (green) were both found in S3. A soma containing immunoreactive Substance P (arrowhead) was also found here. Single optical section. Scale bar = 20 μ m.

Figure 6.

Secretoneurin-IR dendrites (red) contact G6-gly-IR bipolar cells (green) in a macaque retina. Anti-G6-gly labels bipolar cells that receive input from short wavelength-sensitive cones. Note that many of the dendrites in the outer plexiform layer (OPL) extend obliquely. Their axons terminate in S5 of the IPL, and three appositions with secretoneurin-IR dendrites (arrowheads, insets) are shown here. The main panel is an orthogonal projection of 9 optical sections, z step = 0.37 μm. Each inset is a single optical section. Main panel scale bar = 20 μm; inset scale bar = 5 μm.

Figure 7.

Both the axons and the axon terminals of G6-gly-IR bipolar cells (green) contact secretoneurin-IR dendrites (red) in a macaque retina. Nuclei of somas in the outer nuclear layer (ONL), INL, and GCL were labeled with DAPI (blue). A. A descending axon of a G6-gly-IR bipolar cell contains a synaptic ribbon labeled with anti-CtBP2 (blue) apposed to a secretoneurin-IR dendrite (arrowhead, inset). The main panel is an orthogonal projection of 3 optical sections, z step = 1 μm, scale bar = 5 μm. The inset is a single optical section, scale bar = 1.25 μm. B. Secretoneurin-IR dendrites are closely apposed to the descending axon of a G6-gly-IR bipolar cell (filled arrowhead) and an axon terminal (hollow arrowhead). The main panel is an orthogonal projection of 3 single optical sections, z step = 0.26 μm, scale bar = 10 μm; the insets are both single optical sections, scale bars = 2.5 μm.

Figure 8.

Interactions between dendrites of secretoneurin-IR amacrine cells (red) and melanopsin-IR (green) retinal ganglion cells. Secretoneurin-IR dendrites are closely apposed to dendrites of outer-stratifying melanopsin cells (arrowheads). A. Note that a secretoneurin-IR dendrite also contacts the soma of an outer melanopsin cell. The main figure is an orthogonal projection of 6 optical sections showing only the melanopsin signal (green), z step = 0.5 μm , scale bar = 20 μm . Insets are single optical sections displaying both melanopsin (green) and secretoneurin (red) signals, scale bars = 2 μm . B. Note the co-fasciculation of the 2 dendrites. The top figure is an orthogonal projection of 10 optical sections, z step = 0.31 μm . The others are consecutive single optical sections. Scale bar = 5 μm .

Figure 9.

The synaptic connections of secretoneurin cells were analyzed in 2 macaque retinas using electron microscopy. A. The secretoneurin-IR profile is presynaptic to a bipolar cell axon terminal (b) at a conventional synapse (arrowheads). Note the synaptic ribbon (arrow); these are electron dense structures located at the presynaptic terminal and surrounded by a halo of tethered synaptic vesicles. B. The secretoneurin-IR profile is postsynaptic to an unlabeled amacrine cell process (a) at a conventional synapse (arrowheads). C. The secretoneurin cell is presynaptic to a ganglion cell dendrite (g) at a conventional synapse (arrowheads).

Table 1

Antibodies used in this study

Antibody/Kit	Antigen	Manufacturer, catalog number, species	Dilution used
Primary Antibodies			
Secretoneurin	Rat secretogranin II aa 154–186 + N-terminal cysteine	Dr. Reiner Fischer-Colbrie, Rabbit	1:1,000
Secretoneurin	Human secretoneurin	Santa Cruz Biotechnology, sc-54326, Goat	1:10,000
AR-16	16 N-terminal amino acids of rat secretogranin II	Dr. Reiner Fischer-Colbrie, Rabbit	
GABA	Gamma-amino butyric acid (GABA)	Dr. David V. Pow, Rat	1:1,000
Glycine Transporter I	Rat glial GlyT-1, aa 614–633	Biogenesis, Catalog No. 4710-8050, Goat	1:1,000
G6-gly	Tyr-Gly-Trp-Met-Asp-Phe-Gly	Dr. John Del Valle, R6B6, Rabbit	1:1,500
CtBP2	aa 361–445 of mouse CtBP2	BD Biosciences, 612044, Mouse	1:1,000
Melanopsin	C-terminal region of human melanopsin	Dr. Jens Hannibal, 5J68-7, Rabbit	1:10,000
Substance P	C-terminal region of substance P	Accurate Chemical & Scientific Corporation, MAS 035, Rat	1:1,000
Secondary Antibodies			
Biotin-SP (long spacer) AffiniPure Donkey Anti-Rabbit IgG (H+L)		Jackson ImmunoResearch Laboratories, Inc., 711-065-152	1:100
Alexa Fluor® 488 Streptavidin		Jackson ImmunoResearch Laboratories, Inc., 016-540-084	1:200
Alexa Fluor® 488 AffiniPure F(ab') ₂ Fragment Donkey		Jackson ImmunoResearch Laboratories, Inc., 711-546-152	1:200
Anti-Rabbit IgG (H+L)			
Alexa Fluor® 647 AffiniPure F(ab') ₂ Fragment Donkey Anti-Mouse IgG (H+L)		Jackson ImmunoResearch Laboratories, Inc., 715-606-150	1:200
Cy TM 3 AffiniPure Donkey Anti-Goat IgG (H+L)		Jackson ImmunoResearch Laboratories, Inc., 705-165-147	1:200
Cy TM 3 AffiniPure F(ab') ₂ Fragment Donkey Anti-Mouse IgG (H+L)		Jackson ImmunoResearch Laboratories, Inc., 715-166-150	1:200
Alexa Fluor® 594 AffiniPure F(ab') ₂ Fragment Donkey Anti-Goat IgG (H+L)		Jackson ImmunoResearch Laboratories, Inc., 705-586-147	1:200
Kits			
ABC Kit		VECTASTAIN ABC Kit, Peroxidase Standard Kit, PK-4000	

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Table 2

Synaptic contacts of secretoneurin-IR amacrine cells.

Stratum	Inputs from		Outputs to			Total
	Amacrine	Bipolar	Bipolar	Amacrine	Ganglion	
1	26	6	12	63	38	145
3	24	6	14	59	44	147
5	24	5	3*	63	49	144
Total	74	17	29	185	131	436