Somatostatin in Rat Retina : Localization by In Situ Hybridization Histochemistry and Immunocytochemistry

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GAUR, V. P., YAMAGUCHI, K. and TURNER, J.E. *Somatostatin in Rat Retina : Localization by In Situ Hybridization Histochemistry and Immunocytochemistry.* Tohoku J. Exp. Med., 1990, **162** (2), 121-126 — In order to analyse the mRNA-protein relationships of somatostatin containing neurons in the rat retina, we have used a combined method with in situ hybridization histochemistry using radio-labeled oligodeoxyribonucleotides complementary for rat somatostatin mRNA and immunocytochemistry using antiserum for somatostatin protein. We found double labeled amacrine cells in the innermost and outermost laminae of the inner nuclear layer as well as the displaced amacrine cells in the ganglion cell layer in the retina. Some cells in the inner nuclear layer had high levels of somatostatin mRNA. Radially immunostained interplexiform fibers were observed adjacent to the amacrine cells with high level of somatostatin mRNA. These data suggest the site of synthesis of somatostatin and post-translational migration of this neuropeptide within the rat retinal tissue ——— somatostatin ; retina ; in situ hybridization ; immunocytochemistry

Somatostatin-14 peptide has been detected in rat retina by radio-immunoassays (Rorstad et al. 1979). Availability of polyclonal and monoclonal antibodies to somatostatin has permitted immunolocalization of this neuropeptide to specific cell types in the retina (Tornqvist et al. 1982 ; Negishi et al. 1985 ; Ferriero and Sagar 1987). There is, however, a disagreement in reports concerning cellular localization of somatostatin-like reactivity in the rat retina. For example, Krisch and Leonhardt (1979) reported specific immunostaining for somatostatin in horizontal and amacrine cells, and large neurons of the ganglion cells layer. Lin et al. (1983) found immunostained cell bodies in the outer nuclear layer, and Sagar et al. (1985) reported specific somatostatin staining in some cells in the innermost lamina of the inner nuclear layer and in the ganglion

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cell layer. In order to resolve the observed disparities, we have used a combined method which allows *in situ* hybridization histochemistry and immunocytochemistry in the same tissue as proposed by Shivers et al. (1986). Using this technique, we have found that while a subpopulation of amacrine cells is responsible for somatostatin synthesis, some amount of the neuropeptide is transported to the interplexiform fibers in the rat retina.

MATERIALS AND METHODS

Adult (200–300) g male Sprague-Dawley rats were anesthetized by administration of sodium pentobarbital (6 mg/100 g intraperitoneal injection), and perfused through ascending aorta with 20 ml phosphate buffered saline (PBS: 0.15 M NaCl in 10 mM sodium phosphate, pH 7.4) followed by 400 ml fixative (4% paraformaldehyde in 10 mM PBS, pH 7.4). Immediately after fixation the eyes were removed and immersed in the same fixative for additional 30 min. Then cornea and lens were removed under surgical microscope. Tissues were immersed for another 2 hr in the fixative and then placed in PBS containing 30% sucrose and 0.02% diethylpyrocarbonate (DEPC; RNase inhibitor) for overnight at 4°C. Following sucrose infiltration, tissue blocks were rapidly frozen on crushed dry ice and 12 μ m sections were cut on a cryostat. Sections were thaw-mounted onto microscope slides coated with 0.5% gelatin and 0.05% chrome alum and stored with desiccant at –80°C until use.

Gloves were worn when handling the microscope slides to protect against RNase contamination of the sections. All glass and plastic-ware were treated with DEPC water, then rinsed with distilled, deionized water before use. Cryosections were incubated in primary rabbit antiserum to somatostatin (ImmunoNuclear Corp., Stillwater, MN, USA) diluted 1:400 in 10 mM PBS containing 0.1% Triton-X-100 and 0.02% DEPC, for overnight at 4°C. The tissues were washed twice for 15 min in PBS with 0.02% DEPC. Secondary incubations were in goat anti-rabbit IgG, diluted 1:500 in 10 mM PBS containing 0.1% Triton-X-100 and 0.02% DEPC for 2 hr at room temperature. Tissues were washed twice for 15 min and peroxidase antiperoxidase rabbit IgG (ICN Biomedicals, Costa Mesa, CA, USA) (diluted 1:500 with 0.1% Triton-X-100 and 0.02% DEPC) was applied for 1 hr at room temperature. Following the conventional diaminobenzidine reaction, sections were washed in deionized water pretreated with 0.02% DEPC. Sections were air dried and stored with a desiccant at room temperature.

The 23 mer (5'TTCTTGCAGCCAGCTTTGCGTTC3'), that is complementary to the rat mRNA coding for residues 12–19 of somatostatin 28, was synthesized on an Applied Biosystems Automated DNA Synthesizer (Preistley et al. 1988). Probes were labeled at the 5' end with 32 P by use of T4 polynucleotide kinase and γ - 32 P-ATP. The isotope was always used within 14 days (one half life) of the manufacturer's activity date and this gave probe specific activities of about 10^7 cpm/ μ g.

Immunostained sections were incubated in pre-hybridization buffer containing 50% deionized formamide, 6X standard saline citrate (SSC). 2X Denhardt's solution, 50 mM Tris-HCl pH 7.0, 5 mM EDTA pH 7.0, 0.1% sodium dodecyl sulphate (SDS), 100 μ g/ml denatured salmon testis DNA. Prehybridization buffer was prepared as a 2X solution without formamide and immediately before use, an aliquot was boiled for 2 min, quenched on ice and mixed 1:1 with deionised formamide. Small volumes of prehybridization buffer were pipetted onto the slides and spread to cover sections using a pipette tip. Slides were prehybridized without coverslips for 1–1.5 hr while supported horizontally in humidified chambers at 42°C. Following the prehybridization step, sections were dehydrated through 50%, 70%, 95% and 100% ethanol series each containing 0.33 M ammonium acetate and air dried for 30 min before hybridization.

Hybridization buffer was the same as pre-hybridization buffer. Radiolabeled probe was dissolved in 2X hybridization buffer, heat denatured at 100°C for 5 min and then diluted to give 1X hybridization buffer by addition of an equal volume of formamide. Probe was immediately pipetted onto the slides and a glass coverslip was applied to distribute probe evenly over sections and to prevent sections from drying out. Slides were incubated in humid chambers at 42°C for 48 hr. Specificity of the in situ hybridization histochemistry was checked by the use of sections pretreated with RNase and by the use of an excess of unlabeled probe in the hybridization buffer.

After hybridization each slide was dipped in 4X SSC and the cover slip was carefully removed. Slides were washed successively in 4X SSC for 2 hr (four changes of 250 ml), 2X SSC (two changes of 250 ml) and 0.1X SSC for 2 min. The 4X SSC and 2X SSC washes were done at 37°C with constant shaking and the final wash was done at room temperature as suggested by Priestley et al. (1988). Sections were then dehydrated through 50%, 70%, 95% and 100% ethanol solutions each containing 0.33 M ammonium acetate and were air dried. The sections were dipped in Kodak NTB2 emulsion, exposed at 4°C for 6 days, and developed in Kodak D-19 developer and Kodak Fix.

RESULTS AND DISCUSSION

Results of the combined immunocytochemistry and in situ hybridization technique are summarized in Fig. 1. Availability of the 23-mer synthetic oligo-probe made it possible to study the localization of somatostatin mRNA in the immunostained cell bodies in retina. In this study, DEPC concentration was kept to a minimum and the primary antibody (anti-somatostatin) was used at higher concentration than used for routine application. Use of DEPC during tissue fixation, embedding, and various steps for immunocytochemistry did not cause any denaturation of the antigen nor the immunoglobulin. Diaminobenzidine (DAB) reaction product did not create a nonspecific binding of silver grains in in situ hybridization, although longer exposure of the sections for this chromogen. This was perhaps due to the prehybridization step which caused acetylation of the tissue, a requirement to prevent the nonspecific binding (Brahic et al. 1984). In this regard, our observations are consistent with those of Shivers et al. (1986). Our conclusion was based on the good preserved sections with whole thickness of the retina. As criteria for evaluation, only regions showing cell bodies with reaction products with whole thickness of the retina preserved were chosen as representative for identification of cell type. As shown in Fig. 1A, B, and C, we found specific double labeling in some cell bodies in the inner nuclear layer and the ganglion cell layer. Labeled cell bodies in the inner nuclear layer were found distributed on the inner (Fig. 1A) and the outer (Fig. 1B) lamina. Somatostatin immunostain positive cells located in the ganglion cell layer had fewer silver grains (mRNA label) in comparison with the cells in the inner nuclear layer (Fig. 1C). We are confident that these cells represent a subpopulation of conventional and displaced amacrine cells. It is important to note that, with the technique employed in this study, the immunostaining was restricted to the amacrine cell bodies and no immunostaining was seen associated with their processes, while routine somatostatin immunostaining shows labeling of the

amacrine cell bodies and their processes in the IPL. We are unable to explain this change. In addition to the labeling of these cell bodies, we occasionally observed immunostained fibers which were oriented radially across the retina between the two plexiform layers. These fibers although immunoreactive with the somatostatin antibody were always without any silver grains and were seen in contact with heavily silver grain bearing amacrine cells (Fig. 1A and B). When the anti-somatostatin serum and/or oligoprobe were omitted from the incubation, it resulted in a virtual absence of any staining in the retina (Fig. 1D) thus indicating a specific reactivity of our antiserum and the probe.

Somatostatin-like immunoreactivity has been detected in retinas of widely distributed species. While data from gel chromatography and radioimmunoassays suggested occurrence of two forms of somatostatin (SS-14 and SS-28) in the vertebrate retina, rat retina contains only SS-14 form (Rorstad et al. 1979). The issue of identification of somatostatin labeled soma in the ganglion cell layer has been addressed by previous studies (Kirsch and Leonhardt 1979; Lin et al. 1983; Sagar et al. 1985). Our observations are consistent with the assertion that amacrine cells and a subpopulation of cells in the ganglion cell layer (displaced amacrine cells) contain somatostatin. Some cells in the inner nuclear layer had high levels of somatostatin mRNA. It suggests that the amacrine cell (both classical and displaced type) soma are the exclusive site for the somatostatin mRNA localization, and hence, site of synthesis for this neuropeptide. Radially oriented interplexiform fibers immunostained but lacking any somatostatin mRNA activity were, however, observed adjacent to the amacrine cells containing large amount of somatostatin mRNA. These data suggest that the translated gene product is transported to other regions such as interplexiform fibers. Such a possibility, although needs further verification, is not totally unexpected since neuronal fibers often span larger areas to serve as receptors of communication from other neurons.

Fig. 1. Double staining with in situ hybridization histochemistry for somatostatin mRNA and immunocytochemistry. $\times 480$

A. Autoradiographic localization of somatostatin mRNA using ^{32}P labeled complementary oligomer. Micrograph shows a silver grain bearing cell body in the innermost lamina of the INL (arrow). Adjacent to this cell, a radially oriented immunostained interplexiform fiber is seen (arrow heads). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

B. In the outermost lamina of the INL, a double stained cell soma is observed (arrow). Adjacent to this cell, a radially oriented immunostained interplexiform fiber is seen (arrowheads).

C. Micrograph shows double stained displaced amacrine cell in the ganglion cell layer (arrow). Note the autoradiographic labeling is limited.

D. Negative control. Somatostatin antiserum and the specific oligoprobe were omitted from the incubation as a check of the specificity of the double labeling technique. Note the absence of immunostaining and the silver grains associated with any particular structure of the retina.

In conclusion, a combined use of in situ hybridization histochemistry and immunocytochemistry for somatostatin in retinal tissue has demonstrated the site of somatostatin synthesis in amacrine cells and suggests its post translational migration. As evident from this study, this technique is particularly useful for studies on the retina as well as on central nervous system, since neurons extend processes and localization of proteins might be different from that of their mRNA. Investigation regarding the localization of mRNA and resulting proteins could be useful to determine structure-function relationships of neurons.

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