PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 463–471 (1983)

Somatostatin-like Immunoreactivity in the Retinae of Adult and Embryonic Chickens (41588)

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Abstract. Somatostatin, a tetradecapeptide that inhibits growth hormone release, has a widespread distribution in the central and peripheral nervous systems and other cell types. In the present investigation, the chicken neural retina was studied for the presence of structures exhibiting somatostatin-like immunoreactivity by utilizing an indirect immunofluorescence technique. Controls for specificity of staining were performed on alternate sections. Several types of distinctly labeled neurons and their processes were evident in sections of adult and late embryonic retinae. Cresyl violet staining showed that these neurons, which were scattered peripherally and more numerous centrally, occupied several strata within the inner nuclear, inner plexiform, and ganglion cell layers. Labeled neurites of immunoreactive perikarya coursed within these layers as well, often approaching other immunoreactive cells and fibers. The morphology and position of the somatostatin-containing neurons indicated that these neurons were amacrine, horizontal, or ganglion associational cells. These findings indicate that somatostatin is first detectable in the retina during the late embryonic stages of the chicken.

Somatostatin, a tetradecapeptide, was isolated from ovine hypothalamus and characterized as a somatotropin release-inhibiting factor (SRIF) by Brazeau *et al.* (1). SRIF was found to inhibit in a dose-related manner the secretion of somatotropin from dissociated cells of the adenohypohysis and to lower plasma levels of somatotropin. The highest concentration of somatostatin in the central nervous system (CNS) of the rat is found in the medial basal hypothalamus (2) with substantial levels of immunoreactive (IR) SRIF in the preoptic area (3) and circumventricular organs (4). In addition the results of iontophoretic studies indicate that somatostatin has a direct effect on neuronal activity (5). In view of these findings, it has been postulated that somatostatin functions as a neurotransmitter or neuromodulator within the CNS in addition to its role as a hypothalamic regulatory hormone. Somatostatin has been localized in peripheral tissues as well, including the endocrine pancreas (6), neurons of certain sensory and autonomic ganglia, plexuses of the gut wall (7), and the epithelia of the gastrointestinal mucosa (8).

Somatostatin-like activity has been identified in the neural retina of several species including man by techniques involving bioassay, radioimmunoassay, and immunohistochemistry (9–18). Several cell types and their processes have been shown to contain a substance having an affinity for SRIF antibodies which are specific for natural and synthetic forms of somatostatin.

Since the retina of the chicken has not been comprehensively investigated for the presence of IR-SRIF, we have determined the developmental appearance and the cellular localization of somatostatin by immunohistochemistry.

Materials and Methods. *1. Tissue preparation.* Fertile White Leghorn chicken eggs were maintained at a temperature range of 36.5°–38.5°C and a relative humidity of 55–70% saturation. Eggs were opened at specific stages of development (Hamburger and Hamilton; Table I). The embryos were removed, placed in a bath of phosphate-buffered saline (PBS, pH 7.2), and quickly decapitated. The heads of embryos less than 10 days old were left intact. Those of subsequent stages were chilled at 4°C in PBS as the eyes were removed. Eyes were opened by either a partial or complete circumferential incision near the limbus, placed in cold PBS, and refrigerated at 4°C for 1 hr. Tissues were fixed in 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH

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0037-9727/83 $1.50

TABLE I. SCHEDULE OF EMBRYOS STUDIED

N^a	2	2	2	1	3	5	5	2	1	2	1	2
Day	5	6	7	8	8.5	10	12	13	14	16	19	20
Stage	27	28	30	33	35	36	38	39	40	42	44	45

[a] N = number of animals; total = 28.

7.2) at 4°C for 12–48 hr and infiltrated with a solution of 5% sucrose in 0.1 *M* phosphate buffer (pH 7.2) at 4°C for 24 hr. The solution was then replaced with fresh 5% sucrose and the tissues were stored at 4°C until sectioning.

2. Frozen sections. Tissues were individually oriented on a microtome chuck and embedded in O.C.T. compound by freezing with liquid carbon dioxide. Each tissue block was secured in a cryostat maintained at −25°C. Sections were cut at 10 μm in thickness and collected on gelatinized slides kept at room temperature. Three consecutive sections were collected on separate slides. One section was used for control immunohistochemistry, one section for experimental immunohistochemistry, and one section for cresyl-violet staining. Two to three sections were collected per slide and allowed to air-dry for 1 to 2 min and stored at −25°C.

3. Immunohistochemistry. The indirect-immunofluorescence technique of Hokfelt *et al.* (7, 19) was used with slight modification, viz., the retinae were freeze-embedded in O.C.T. compound with liquid carbon dioxide, and the concentrations of antisera were decreased. Slides were brought to room temperature, and areas containing the sections were encircled with enamel-based nail polish to limit spreading of the antisera. Sections were covered with PBS and allowed to stand for 15–30 min. The excess PBS was drawn off with a Pasteur pipet. The moistened sections were covered with rabbit anti-somatostatin serum (primary antiserum, obtained from Immuno Nuclear Corporation, Stillwater, Minn. and reconstituted from the lyphilized state with sterile water and diluted 1:100 with 0.3% Triton X-100 in PBS). This antiserum which recognizes the central portion of the somatostatin molecule, was generated against a CDI conjugate with keyhole limpet hemocyanin and synthetic somatostatin. It has RIA-proven specificity and potent staining ability for somatostatin-containing neurons of the pyriform cortex and fibers and terminals in the arcuate and ventral nuclei as well as the median eminence of the hypothalamus. The antiserum was applied so that the sections were completely covered. The slides were placed horizontally in an air-tight chamber containing PBS-moistened towelling, incubated at 4°C for 12–24 hr, rinsed, and immersed in a bath of fresh PBS for 15 min at room temperature. The excess PBS was drawn off and fluorescein-conjugated goat anti-rabbit immunoglobulins (secondary antiserum, obtained from Cal Biochem-Behring, San Diego, California, reconstituted, and diluted 1:40 as above) was applied to these sections. Slides were placed horizontally in a second incubation chamber at 37°C for 30–60 min. A stream of PBS was used to remove excess antiserum and the slides were immersed in fresh PBS at room temperature for 15 min. Excess

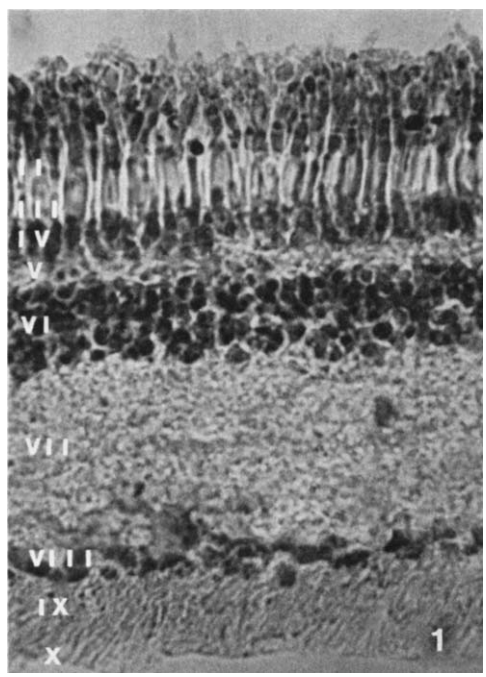

FIG. 1. Adult neural retina, illustrating layers II–X. Cresyl violet, ×1350.

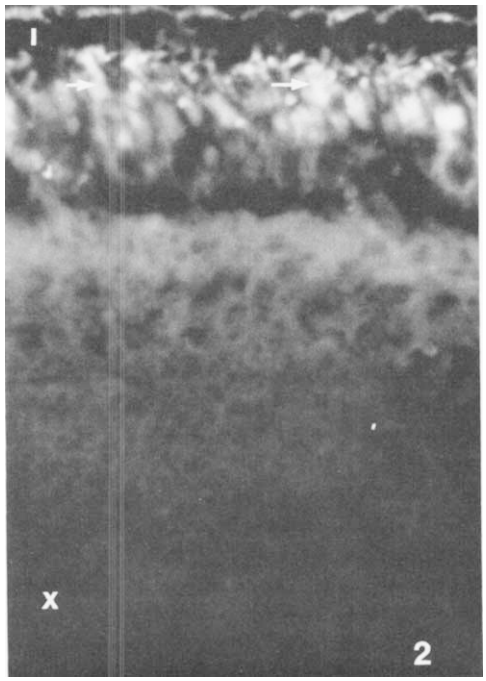

FIG. 2. Adult neural and pigmented retinae, section exposed to primary antiserum pretreated with excess somatostatin demonstrating layers I–X. Specific fluorescence is absent, whereas autofluorescence associated with the outer segments remains (arrows). ×1400.

PBS was removed and the sections were mounted in 3:1 glycerin:PBS solution and coverslipped.

For control immunohistochemistry, the primary antiserum was preabsorbed with synthetic SRIF in some cases and in other cases normal rabbit antiserum was substituted for the primary antiserum. In the remaining control slides no primary antiserum was used. These sections were then processed as above. Slides containing sections for correlative morphology were stained with cresyl violet. Slides were examined with a Leitz Wetslar Sm-Lux fluorescence microscope.

Results. *1. SRIF immunoreactivity in the adult retina.* Tissue processing techniques maintained normal retinal morphology (Fig. 1). Distinctively labeled neurons and nerve processes were evident in sections of adult retinae exposed to nonpreabsorbed antisera. Fluorescent structures exhibiting somatostatin-like immunoreactivity were observed in several layers of the neural retina and presented various morphological profiles. Immunoreactive cells and fibers were relatively sparse in the periphery and more numerous centrally. Labeled structures were absent in control sections although a nonspecific yellow–orange autofluorescence associated with the outer segments remained (Fig. 2). A dull green background fluorescence over tissue sections treated with absorbed primary antiserum during the first incubation was observed. This was slightly but appreciably intensified when normal rabbit serum was used during the first incubation. The background fluorescence over tissue sections was brighter

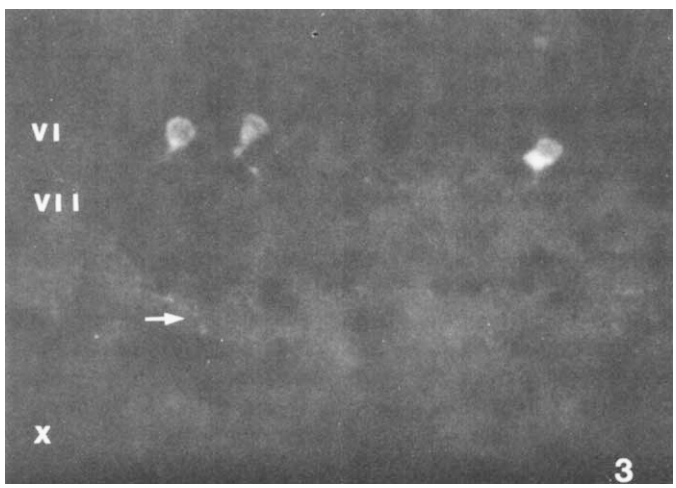

FIG. 3. Adult neural retina with several smaller IR-SRIF cells within layer VI and fibers directed into layer VII (arrow). ×1500.

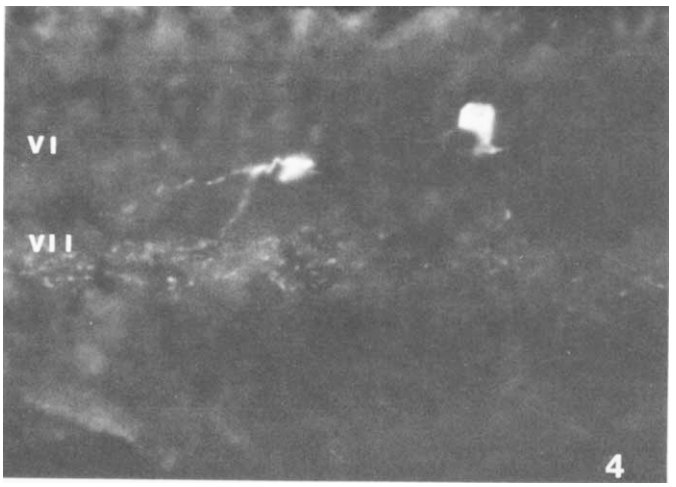

FIG. 4. Adult neural retina, central region, with SRIF-containing multipolar somata and their processes directed into layers VI and VII. Note that fine processes are directed laterally while the major fibers are oriented inwardly. ×1500.

still—but not excessive—when anti-somatostatin immunoglobulins were utilized.

The majority of immunoreactive cells observed in experimental sections occupied the inner nuclear layer (layer VI). Smaller cells with labeled processes were more often observed deeply placed within this layer, whereas larger multipolar cells were situated near the inner and outer margins of this layer. The somata of the smaller cells were round to ovoid or droplet shaped (Fig. 3). These cells were the most frequently observed type of associational cell exhibiting somatostatin-like immunoreactivity. Inwardly directed fibers of these neurons entered the inner plexiform layer (layer VII) and ramified within the outer strata near the interface with layer VI (sublamina a) or occasionally within the inner strata (sublamina b) nearer the ganglion cell layer (layer VIII).

Immunoreactive somata of larger multipolar neurons of layer VI were located nearer its inner and outer margins (Fig. 4). These cells were not as numerous as the smaller cells.

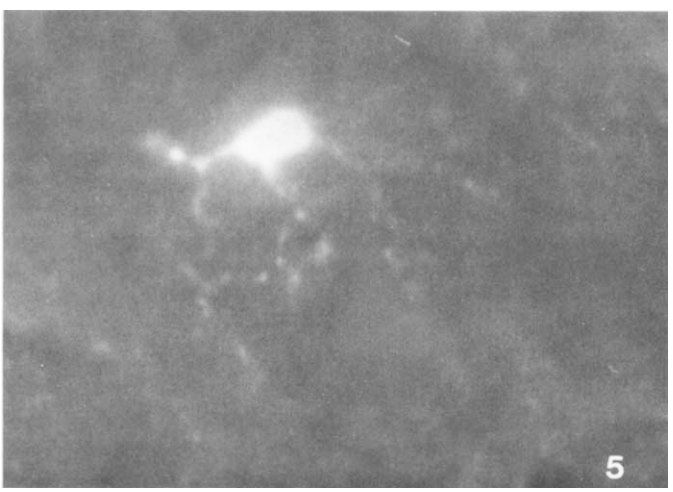

FIG. 5. A large, ovoid-shaped, SRIF-positive neuron at the inner margin of layer VI with fibers projecting into vicinal regions of layers VI and VII. ×2300.

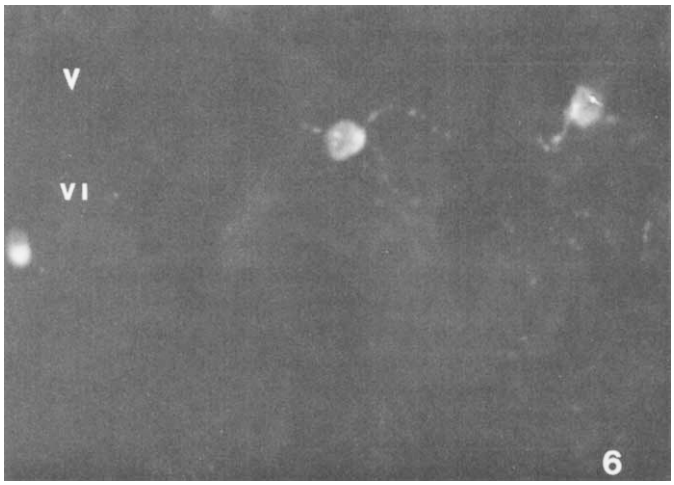

FIG. 6. Adult neural retina, peripheral region. Large, round immunoreactive neurons at the outer margins of layer VI direct processes into layers V and VI. ×2000.

Labeled processes arising from these neurons were oriented at various angles from the cell body. Some of these fibers coursed into layer VI, whereas other labeled fibers entered layer VII.

The third type of neurons present were among the innermost cells of layer VI (Fig. 5). These neurons possessed immunoreactive fibers exiting from a flattened surface of the cell body and its poles, with arborizations into both layers VI and VII. These cells were few in number and observed only in central retinal areas. The fourth type of neurons were larger multipolar cells at the outer border of layer VI resembling horizontal cells (Fig. 6). Some fibers of these neurons remained in layer VI, whereas others were directed into the outer plexiform layer (layer V).

In central areas of the retina, labeled multipolar neurons were observed occupying layer VII. Some of these displaced cells directed single processes inwardly into layer VIII (Fig. 7), whereas other cell processes coursed into the surrounding plexiform layer (Fig. 8). Labeled

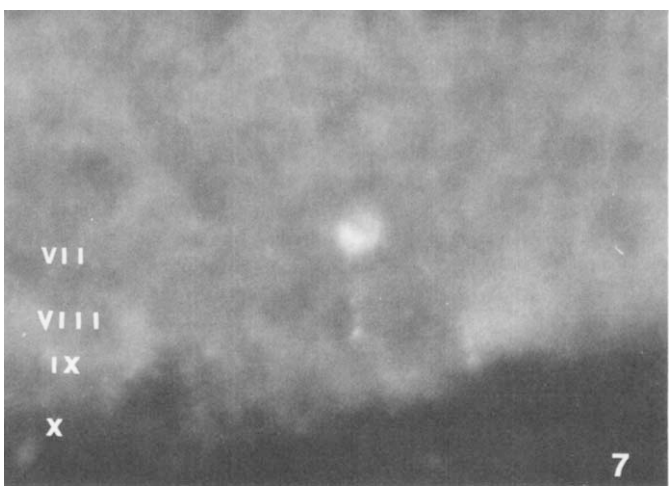

FIG. 7. Adult neural retina, inner layers, showing a displaced cell of layer VII directing a labeled process into layer VIII. ×2150.

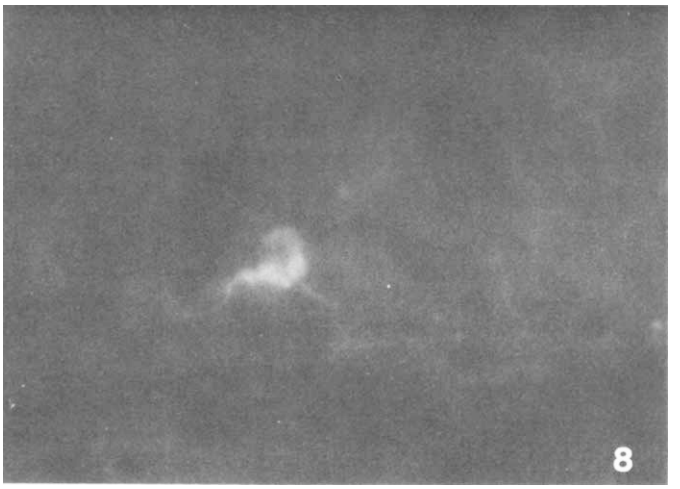

FIG. 8. Adult neural retina, illustrating a displaced neuron of layer VII possessing laterally oriented cell processes. ×2000.

neurites of displaced cells were observed intermingling with other immunoreactive fibers.

Immunoreactive ganglion cells of various shapes were observed within layer VIII. Some of these cells were round and directed stout immunoreactive hillocks inwardly toward the optic nerve fiber layer (layer IX) (Fig. 9). Other ganglion cells were larger with highly immunoreactive fibers diverging into layers VII and IX (Fig. 10).

Somatostatin-containing cells of the retina were observed only in its sensory portion. Labeled perikarya were not observed in the lenticular (nonsensory) portion of the retina. However, immunoreactive fibers were occasionally observed occupying the inner layers in some areas of the lenticular retina.

2. Immunoreactivity in the embryonic retina. Embryonic chicken retinae showed a few scattered cells and fibers exhibiting somatostatin-like immunoreactivity. These were detected as early as stage 38 of embryonic development. A common feature of these cells was a paucity of labeled processes. Specifically stained structures were not observed in con-

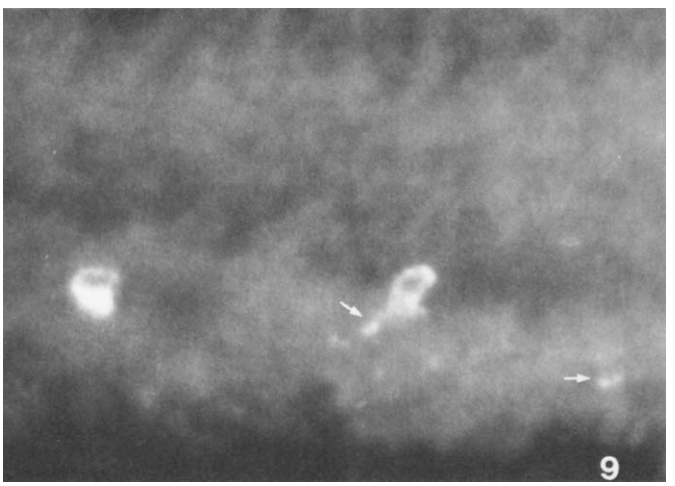

FIG. 9. Adult neural retina, inner layers, illustrating two labeled ganglion cells with inwardly oriented hillocks and processes entering layer IX (arrows). ×1850.

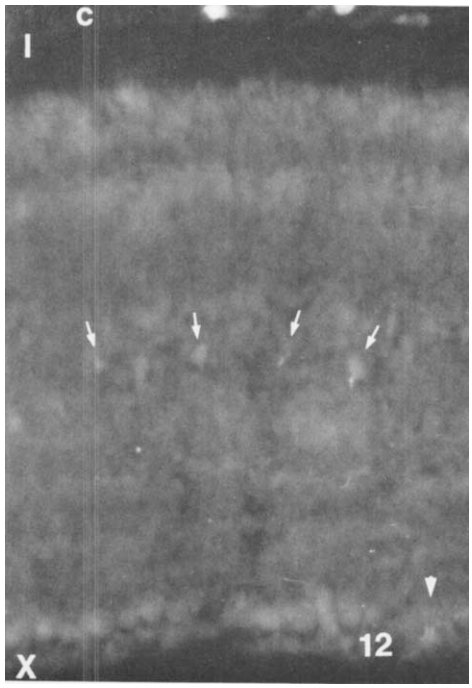

FIG. 12. Embryonic neural and pigmented retinae, stage 45, illustrating faintly labeled perikarya of layer VI (arrows) and the hillock of an immunoreactive ganglion cell (arrowhead). c = choroid. ×1100.

the cell boundaries in all neural tissues studied (20, 21). Appearance of an immunological reaction product over cellular elements of tissue sections was indicative of the presence of a substance which is chemically similar or identical to somatostatin. The presence of somatostatin-like immunoreactivity was verified in that the reaction product was absent from control sections. Thus, the presumably functional cell types of embryonic and adult chicken neural retinae, having selective affinity for the primary antiserum used in the present study, probably contained the active form of somatostatin.

Levels of somatostatin in the rat retina vary depending on the type and amount of extraneous tissue included in the radioimmunoassay (9, 13, 15). If specific tissues are to be evaluated for somatostatin-like immunoreactivity, then the selected tissue must be free of heterogenous tissues. Since the chicken neural retina is avascular (22), blood-borne somatostatin would not react to primary antibodies over sections of the neural retina; therefore, the reaction product over blood vessel walls reported by Krisch and Leonhardt (10) in the rat retina was not found in the chicken retina. Thus the neural retina of the chicken lends itself to a clear study of somatostatin-containing neurons.

Based on localization and morphological cell types, the immunoreactive cells observed in the chicken neural retina were associational neurons. These cells resembled horizontal, amacrine, and ganglion cells. Immunoreactive amacrine cells varied in size, shape, and position within layer VI. These neurons occupied several cellular rows within this layer, whereas, in the rat retina they are confined to the inner row of layer VI (10). In studies of the young chick (17) and pigeon (18) IR-SRIF-containing amacrine cells were observed only within the inner rows of layer VI. The round perikarya located in the outer rows of layer VI in the adult chicken (horizontal cells) were not observed in the young pigeon (18) or chick (17). This seems to be an age related phenomenon. Immunoreactive neurons occupying layer VII in the adult chicken retina appeared to be displaced amacrine cells. They were similar in size and shape to amacrine cells in layer VI. Krisch and Leonhardt (10) did not report labeled neurons occupying layer VII in the rat retina, nor were they described in the young chick (17) or pigeon (18) retinae. Somatostatin-containing neurites of layer VII in the chicken retina originated predominantly from the perikarya of labeled amacrine cells of layer VI. The nerve fiber layer of the chicken retina contained a paucity of labeled neurites. Gallego and Cruz (23) reported the presence of large ganglion cells in the mammalian retina, axons of which remain intraretinal. These were considered to function as associational neurons. It is plausible to assume that at least some of the somatostatin-containing ganglion cells observed in the present study, rat (10), and goldfish (11) are intraretinal neuromodulatory or association neurons. Labeled ganglion cells were not observed in previous investigations of young birds (17, 18), thus some types of cellular immunoreactivity may depend on age as well as on species differences.

Apparently, most somatostatin-containing neurons of the chicken retina do not become immunoreactive until after hatching. Only sparsely distributed, lightly stained cells of layer VI were clearly labeled in late embryonic retinae. Labeled processes of these cells did not ramify extensively as seen in the adult. Waggoner *et al.* (24) reported that significant

FIG. 10. Adult neural retina, inner layers, demonstrating a large ganglion cell with laterally oriented processes, one of which is approached by a labeled fiber from layer VII (arrow). ×2000.

trol sections. IR-SRIF was apparently lacking in the retina prior to embryonic Day 10 (stage 36). Fluorescent fiber-like structures were observed in experimental sections from stage 36 and all subsequent stages studied. These structures were most common in layers VI and VII. Immunoreactive cell somata were more apparent by stage 42 (Fig. 11). The ovoid cell bodies appeared to be confined to layer VI. Each labeled neuron possessed a single labeled fiber oriented inwardly toward layer VII. The retina of stage 44 contained labeled neurons which were similar to those observed at stage 42. The retina of stage 45 embryos resembled the adult pattern in morphology and topographical distribution of labeled cells (Fig. 12).

Discussion. Published reports confirming the presence of somatostatin-like immunoreactivity in the neural retina have provided significant data concerning the morphology of cellular elements containing this neuropeptide. Physiological roles of immunoreactive structures have been postulated, and phylogenetic comparisons have been described (10–12). The results of this study in the chicken supplement the accumulated data in this and other species with emphasis on the morphology and topographical location of immunoreactive cellular elements. Complementary radioimmunological studies have shown that significant amounts of somatostatin-like material are present in the neural retina of a number of vertebrate species (9, 11–18). Labeled structures in the present study are consistent with the proposal that several types of neurons in the chicken retina contain SRIF.

Microscopically, the majority of the reaction product has been observed within or on

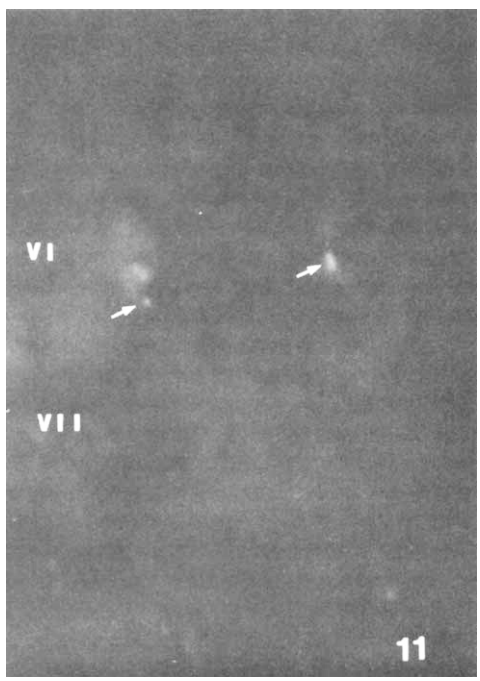

FIG. 11. Embryonic neural retina, stage 42, showing faintly labeled perikarya of layer VI and their inwardly directed fibers (arrows). ×2250.

differences existed between the retinae of embryonic Day 17 and post-hatching Day-7 chicks, and the similarity between the latter and the adult chicken on the basis of soluble proteins present in these tissues. Somatostatin in the developing retina appeared to follow this pattern by becoming a major neuronal peptide after hatching.

The immunoreactive neurons in the embryonic retina resembled amacrine and ganglion cells in position and orientation of neurites. It remains to be determined when immunoreactive neurons of other types observed in the adult chicken become immunoreactive. This is probably a post-hatching phenomenon as the conditions employed in this experiment were very reliable for determining the presence of immunoreactive somatostatin.

The authors wish to thank Mrs. Jill Witterschein for assistance with manuscript preparation.

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Received September 2, 1982. P.S.E.B.M. 1983, Vol. 172.