Opioid Pathways in an Avian Retina

II. Synaptic Organization of Enkephalin-immunoreactive Amacrine Cells¹

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Abstract

Peroxidase-antiperoxidase immunocytochemistry was utilized to investigate the synaptic organization of enkephalin-like immunoreactive amacrine (Enk-amacrine) cells in the chicken retina. An initial light microscopic analysis revealed that immunostained cell bodies were situated in either the second or third tier of cells from the border of the inner nuclear and inner plexiform layers. The processes of such cells extended into the inner plexiform layer where they ramified as a fine plexus in sublaminae 1 and 3 to 5.

At the ultrastructural level, enkephalin-positive somas exhibited a rather dense and evenly distributed peroxidase reaction product throughout their cytoplasm. Furthermore, the nucleus of Enk-amacrine cells was characterized by a round, unindented nuclear membrane. Immunoreactive processes of such cells were found to receive synaptic input from unstained amacrine and bipolar cells. Immunolabeled varicosities formed conventional synaptic contacts onto other vesicle-filled, nonimmunoreactive profiles tentatively suggested to originate from amacrine cells. Bipolar cell terminals did not receive synaptic input from stained varicosities of enkephalin-immunoreactive amacrine cells. Moreover, each of the above synaptic relationships were identified in each of sublaminae 1, and 3 to 5 of the inner plexiform layer. In addition, labeled varicosities of Enk-amacrine cells synapsed upon unstained processes which lacked synaptic vesicles and possibly arise from ganglion cells. Enkephalin-positive processes were also observed to synapse upon the vitreal pole of unstained somas situated in the innermost cell row of the inner nuclear layer. Lastly, large immunoreactive varicosities of Enk-amacrine cells were often characterized by the presence of large dense-core vesicles in addition to typical, small agranular synaptic vesicles.

Recent studies utilizing immunochemical as well as immunocytochemical techniques provide evidence for the existence of enkephalin-like substances in the vertebrate retina. In particular, opiate

receptors (Medzhiradsky, 1976; Howells et al., 1980), assayable enkephalin immunoreactivity (Humbert et al., 1979), and enkephalin-like immunoreactive neurons (Brecha et al., 1979; Stell et al., 1980, 1981; Tornqvist et al., 1981; Altschuler et al., 1982; Fukuda, 1982; Ishimoto et al., 1983; Watt et al., 1983, 1984a; Eldred and Karten, 1984) have been reported in some teleost, amphibian, avian, and mammalian retinas. A comparative analysis of previous immunocytochemical studies reveals that enkephalin immunoreactivity, when present, is localized to one or more subpopulations of amacrine cells. Moreover, enkephalin-like immunoreactive amacrine cells (Enk-amacrine cells) of different species appear to have different patterns of ramifications in the inner plexiform layer. Only limited reports have appeared which address the electrophysiological properties of enkephalins in the vertebrate retina (Dick et al., 1980; Djamgoz et al., 1981). These studies indicate that the enkephalins influence the visual responses of ganglion cells, possibly as the result of direct/indirect interactions with GABAergic amacrine cells (Djamgoz et al., 1981).

It is evident that our present understanding of the functional nature of the opiate system in the retina is still in its infancy and that future experimentation should include a careful examination of both the physiological properties and the synaptic organization of retinal opiate pathways. It is with these objectives in mind that we now report on the synaptic organization of Enk-amacrine cells in the chicken retina using electron microscopic immunocytochemistry.

Materials and Methods

Antiserum. The antiserum (batch A206) utilized in the present study was directed against Leu⁵-enkephalin and was obtained from Dr. K. J. Chang. The details of its preparation and characterization have been described previously (Miller et al., 1978; Sar et al., 1978; van Leeuwen et al., 1983).

Fixation. Nineteen 4- to 6-week-old chickens were used in the present study. Animals were exposed to ordinary room light prior to decapitation following light etherization. An eye was immediately enucleated, the anterior half was removed, and the remainder of the eyecup with the retina attached was immersed in cold oxygenated Ringer's solution (in grams per liter: NaCl, 8.5; KCl, 0.3; CaCl₂·2H₂O, 0.58; MgSO₄, 0.25; NaHCO₃, 2.75; Na₂PO₄·H₂O, 0.18; and glucose, 1.8, pH 7.3). The retina was removed from the eyecup and placed in cold fixative (4% paraformaldehyde and 0.1% glutaraldehyde in 100 mM phosphate buffer, pH 7.3) for 1 hr. The tissue was next transferred to fresh fixative (4%) without glutaraldehyde and stored overnight at 4°C.

Sectioning. On the following day, the retina was transferred to 20 mM phosphate-buffered saline (PBS, 20 mM phosphate buffer, 0.9% NaCl, pH 7.3) and brought to room temperature. The retina was diced into small trapezoid-shaped pieces (3 × 4 mm), each of which was encapsulated in low gelling temperature agarose (4% in PBS) at 37°C, allowed to cool, and vibratome sectioned in the transverse plane at a thickness of 75 μm. Sections were transferred immediately from the PBS collecting medium to normal goat serum (10% in PBS) and gently agitated on an orbital rotator for a period of 1 hr at room temperature.

Staining procedures. Immunoperoxidase and immunofluorescent staining techniques employed in the present study are modifications of protocols

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Figure 1. A, Transverse section of the chicken retina incubated in Leu⁵-enkephalin antiserum with Triton X-100. The large block arrow points to a typical immunoreactive amacrine cell body with its process entering the inner plexiform layer. Immunoreactive processes are distributed predominantly within sublaminae 1 (small block arrow), 3, and 4 (asterisk). The open block arrow denotes punctate staining deeper within sublamina 5 near the ganglion cell layer. For orientation to the multilaminated structure of the chicken retina, refer to C. B, Control transverse section of the chicken retina for electron microscopy study, incubated in preimmune serum without Triton X-100. C, One-micrometer toluidine blue-stained transverse section of the chicken retina for orientation purposes. PC, photoreceptor cell layer; OP, outer plexiform layer; IN, inner nuclear layer; GC, ganglion cell layer. Numerals 1 to 5 denote sublayers of the inner plexiform layer. D, Experimental transverse section of the chicken retina for electron microscopic study, incubated in (Leu⁵-)enkephalin antiserum without Triton X-100. The open block arrow points to a typical immunoreactive soma. Note the similarity in distribution of punctate labeling when compared with A. Magnification: A, $\times 464$; B to D, $\times 576$.

utilized elsewhere (Brecha et al., 1979; Brandon et al., 1980). Experiments for electron microscopy were performed without Triton X-100. In experiments for light microscopy only, 0.3% Triton X-100 was included in all incubation media. Additionally, both incubation and rinse times were increased in order to facilitate the penetration of antibodies deeper into the tissue. The following are the protocols for immunostaining at light and ultrastructural levels.

Immunoperoxidase (light microscopic level). Following absorption in 2% normal goat serum for 3 hr, sections were incubated in the primary antiserum (rabbit anti-Leu⁵-enkephalin, 1:1000 in PBS) overnight at 4°C. Next, sections were subjected to two 15-min rinses in PBS. The sections were subsequently incubated in goat IgG raised against rabbit IgG (1:20 in PBS) for 3 hr. The sections were then washed two times for 15 min each in PBS, incubated in rabbit peroxidase-antiperoxidase (1:50 in PBS) for 3 hr, and subsequently rinsed two times for 15 min each in PBS. The sections were then transferred to the staining solution (10 ml of PBS, 100 μ l of H₂O₂, 30 μ g of diaminobenzidine tetrahydrochloride) for 4 to 7 min. The staining reaction was terminated by rinsing the sections briefly with PBS. The sections were placed onto glass slides, coverslipped with glycerol, and examined with a Zeiss microscope.

Immunoperoxidase (electron microscopic level). Following absorption in

2% normal goat serum for 1 hr, vibratome sections were briefly rinsed in PBS and then transferred to the primary antiserum (rabbit anti-Leu⁵-enkephalin, 1:2000 in PBS) for 15 min. Next, sections were subjected to two 5-min rinses in PBS. The sections were subsequently incubated in goat IgG raised against rabbit IgG (1:30 dilution in PBS) for 10 min and subsequently rinsed two times for 10 min each in PBS. The sections were then incubated in rabbit peroxidase-antiperoxidase (1:150 in PBS) for 10 min and next rinsed two times for 5 min each in PBS. The sections were transferred to the staining solution (10 ml of PBS, 100 μ l of H₂O₂, 30 μ g of diaminobenzidine tetrahydrochloride) for 4 to 7 min. The staining reaction was terminated by rinsing the sections briefly with PBS. At this point, the sections were placed on a glass slide, coverslipped with glycerol, and examined at the light microscopic level.

Following the post-staining wash and light microscopic examination, appropriately stained sections were fixed overnight in 2% glutaraldehyde in 125 mM phosphate buffer (pH 7.3), rinsed for 30 min in 125 mM phosphate buffer, and then placed in osmication solution (2% O₃O₄ in 125 mM phosphate buffer) for 2 hr. The sections were next rinsed in 125 mM phosphate buffer for 30 min and dehydrated through increasing concentrations of alcohols to

Figure 2. *A to C*, Montage of several enkephalin-like immunoreactive amacrine cells in the chicken retina. *Arrowheads* denote staining within sublamina 5 of the inner plexiform layer near the ganglion cell layer. *D* is a control section, incubated in preimmune serum. *IN*, inner nuclear layer; *IP*, inner plexiform layer; *GC*, ganglion cell layer. Magnification (*A to D*) $\times 678$.

propylene oxide. En bloc staining in uranyl acetate (1%) for 1 hr was carried out in the 70% alcohol step. The tissue was then placed in a 1:1 mixture of propylene oxide to the embedding medium (Epon-Araldite) for 1 hr, in a 1:3 mixture overnight, and, finally, the following day, into straight monomer for 4 hr. Following equilibration with the embedding medium, the sections were diced into smaller sections suitable for electron microscopic examination. Each piece of retina was embedded in a small drop of monomer between two glass slides, one of which was wrapped in aluminum foil. Tape was used to secure the slide mold which was placed in the oven overnight at 60 to 80°C. After polymerization, the foil was peeled away to yield a 75- μ m-thick sheet of epoxy containing sections which were flatly embedded. For the ultramicrotome procedure, small areas of the epoxy sheet which contained retinal sections were removed from the glass slide with a razor blade, mounted on blank epoxy blocks with cyanoacrylate glue (Aron Alpha, Pelco), sectioned with a Sorvall MT-5000 ultramicrotome, and viewed in a JEOL Jem-100CX electron microscope.

Immunofluorescence. Immunofluorescent staining was performed on Vibratome sections (20 to 30 μ m) prepared in the same manner as described above. Sections were incubated initially in the primary antiserum (1:1000 dilution) overnight at 4°C. On the following day, sections were rinsed three times for 10 min each in PBS and subsequently incubated with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate at a dilution of 1:20 for 90 min at room temperature. The sections were then rinsed four times for 15 min each in PBS, mounted onto slides with glycerol, and viewed with a Zeiss microscope.

Control. The specificity of the anti-Leu⁵-enkephalin antiserum was tested by incubating sections in antiserum which had previously been exposed to excess antigen (either Leu⁵- or Met⁵-enkephalin, 100 μ g/ml of diluted antiserum) for 12 hr at 4°C. Sections incubated with the enkephalin-adsorbed antiserum were treated exactly as described above.

Two factors prevent us from stating with certainty that the Leu⁵-enkephalin antiserum utilized in the present study only recognizes precisely the antigen to which it was raised. First, the antiserum to Leu⁵-enkephalin used here has recently been shown to cross-react not only with Met⁵-enkephalin but also with the chemically distinct opioid peptides, dynorphins (van Leeuwen et al., 1983). All of these compounds share at least four amino acids in their primary amino acid sequence. Second, it cannot be excluded that other immunoreactive materials are present in the chicken retina which are related structurally to Leu⁵-enkephalin and are recognized by the antiserum A206 (Vandesande, 1979). In view of the apparent cross-reactive tendencies of our antiserum, as well as a general inability to determine the exact nature of the antigens being stained, we use the term "enkephalin-like immunoreactivity" to describe immunostaining observed in the present study. However, for the sake of brevity, several other terms such as "enkephalin-positive" and "enkephalin-containing" are sometimes employed in this report as synonyms for enkephalin-like immunoreactivity.

Results

Light microscopic observations. An examination of transverse vibratome-prepared sections stained in the presence of Triton X-100

Figure 3. Electron micrograph demonstrating immunoreactive soma (open block arrow). The asterisk within the unstained soma denotes the position of the stained cell in the third tier of cells from the border of the inner plexiform layer (IP). Magnification $\times 4,000$.

Figure 4. Dense-core vesicles located within an enkephalin-stained profile (open block arrow) in the chicken retina. Solid block arrows point to such well preserved vesicles. Magnification $\times 34,000$.

revealed that enkephalin-immunoreactive, pear-shaped cell bodies were situated in the vitreal half of the inner nuclear layer in the second and third tiers of cells from the border of the inner nuclear and inner plexiform layers (Figs. 1A and 2). These immunoreactive

cells sent processes into the inner plexiform layer where they formed a fine plexus of varicose fibers in sublamina 1 and a much broader band of labeling throughout sublaminae 3 and 4. In addition, immunoreactivity was observed occasionally in sublamina 5 near the ganglion cell layer (Fig. 1A, open block arrows, and Fig. 2, arrow-heads). Overall, such initial observations correspond well with previous reports on the distribution of enkephalin-like immunoreactive cells in the avian retina (see "Discussion" for details). Moreover, we have produced a monoclonal antibody specifically directed against Leu⁵-enkephalin (Tavella et al., 1985). This antibody also revealed a staining pattern quite similar to that obtained with the polyclonal antiserum A206 utilized in the present study. In retinas processed without Triton X-100 for electron microscopic analyses, the distribution of enkephalin immunoreactivity was identical to that observed in the presence of Triton X-100, although the peroxidase reaction product appeared as punctate deposits (Fig. 1D). Additionally, an examination of control vibratome sections prior to electron microscopic-embedding revealed a complete absence of enkephalin staining (Fig. 1B). Taken together, these observations indicate that a subpopulation of amacrine cells (designated here as Enk-amacrine cells) in the chicken retina (Figs. 1A and 2) probably contains enkephalins or peptides very similar to enkephalins.

Electron microscopic observations. Ultrastructural features of enkephalin-immunoreactive perikarya were studied in transverse retinal sections which were subjected to longer incubation times as used for staining at the light microscopic level. Increasing the incubation times facilitated the penetration of the antibodies deeper into the tissue to a level at which adequately preserved and stained cell bodies could be detected. An enkephalin-positive soma exhibited a rather dense peroxidase reaction product distributed throughout its cytoplasm with the possible exception of the mitochondria and tubules of endoplasmic reticulum (Fig. 3). In contrast, the nucleus of an immunoreactive cell was less intensely stained, although the density of nuclear staining was considerably greater than that observed in surrounding unstained perikarya. Furthermore, the nucleus of an Enk-amacrine cell was characterized by a round, unindented nuclear membrane.

At the electron microscopic level, the peroxidase reaction product was distributed throughout the matrix of stained processes with somewhat more dense accumulations associated with small clear vesicles and occasionally with large dense-core vesicles. In fact, large enkephalin-positive varicosities encountered in the present study were often characterized by the presence of large dense-core vesicles in addition to typical small, agranular synaptic vesicles (Fig. 4). Other morphological features of enkephalin-labeled profiles were not readily apparent due to the disruptive effect of dense accumulation of reaction product within the immunoreactive profiles. Finally, no peroxidase staining was observed upon examination of tissue from control experiments.

An examination of experimental tissue revealed that processes of Enk-amacrine cells participate in several types of synaptic relationships within the inner plexiform layer of the chicken retina. When serving as the postsynaptic element, an Enk-amacrine cell received synaptic input from other unlabeled amacrine cells (Fig. 5) or, less frequently, from bipolar cells (Fig. 6). In the latter synaptic arrangement, one member of the dyad was immunoreactive, but the second postsynaptic element was not and usually contained synaptic vesicles. Furthermore, each of these synaptic arrangements was observed in sublamina 1 as well as throughout sublaminae 3 to 5.

Enkephalin-containing amacrine cells participated in several synaptic arrangements in which they served as the presynaptic element. In most cases, the identity of the postsynaptic element was not entirely clear, an issue which is addressed further under "Discussion." First and most often, an immunoreactive varicosity was observed to form a single, conventional synaptic contact onto another unlabeled vesicle-filled profile (Fig. 7). Such postsynaptic targets were not observed to contain a synaptic ribbon although, occasionally, they did receive input from a bipolar cell (Fig. 7B). On occasion, an Enk-

Figure 5. Amacrine to amacrine cell synapses (white open block arrows) in the chicken retina. The postsynaptic element in these examples is enkephalin stained (black open block arrows). Asterisks denote the presynaptic element. Magnification (A and B) $\times 34,000$.

Figure 6. Bipolar to amacrine cell synapses in the chicken retina. In these examples one dyadic member (open block arrows) is enkephalin positive. The white asterisks denote the second element of the dyad which receives input from a bipolar cell terminal (black asterisks). Magnification $\times 34,000$.

labeled varicosity which synapsed onto a vesicle-filled profile served as the intermediate synaptic element in a serial synapse (Fig. 7C). Furthermore, in some instances, vesicle-filled profiles postsynaptic to enk-amacrine cells contained small dense-core vesicles in addition to typical agranular synaptic vesicles. In one example (Fig. 7D), the dense-core vesicle-containing profile formed a conventional synapse onto another unidentified process. Finally, enk-amacrine cells were observed to contact vesicle-filled profiles in sublamina 1 as well as throughout sublaminae 3 to 5.

Enkephalin-stained processes in sublamina 1 were found to synapse upon the soma of cells situated along the outer border of the

inner plexiform layer (Fig. 8). Enkephalin-containing amacrine cells were also observed to contact unlabeled processes which did not contain synaptic vesicles and whose origin remains uncertain (see "Discussion"). Within sublamina 5 of the inner plexiform layer, a labeled varicosity was occasionally observed to form a conventional synapse onto a large proximal process which was void of synaptic vesicles and which most likely emanated from a cell in the nearby ganglion cell layer (Fig. 9A). Furthermore, throughout the innermost half of the inner plexiform layer, an immunoreactive varicosity was noted infrequently to contact a small profile which lacked synaptic vesicles and in some instances was characterized by the presence of vesiculated endoplasmic reticulum (Fig. 9B).

Figure 7. Amacrine to amacrine cell synapses (solid block arrows) in the chicken retina. The presynaptic element in these examples is enkephalin labeled (open block arrows). In B the postsynaptic element receives a dyad synapse from a bipolar cell terminal (asterisk). In C, note the similarity in appearance between the postsynaptic element and the adjacent amacrine cell process (asterisk). In D the postsynaptic element contains a dense-core vesicle (open block arrow) and synapses onto a neighboring process (asterisk). Magnification $\times 34,000$.

Discussion

Light microscopic observations. The existence of enkephalin-like immunoreactive amacrine cells in the pigeon and chicken retina (Brecha et al., 1979; Tornqvist et al., 1981; Fukuda, 1982; Ishimoto et al., 1983; Watt et al., 1983) has recently been reported in several studies. Our light microscopic investigations are generally consistent with these reports. For instance, all studies agree on the location and density of the somas of Enk-amacrine cells and confirm that the processes of these cells project radially into the inner plexiform layer where they form a fine plexus of varicose fibers in sublamina 1. However, the pattern of immunoreactivity present in the proximal

half (sublaminae 3, 4, and 5) of the inner plexiform layer varies considerably between the various reports. In each of the previous investigations, enkephalin-immunoreactive fibers were observed in sublaminae 3 and 4 and, thus, their distribution compares favorably with the present study. However, in two of these studies (Tornqvist et al., 1981; Ishimoto et al., 1983), enkephalin-stained fibers were not found in sublamina 5 of the inner plexiform layer. This observation is in contrast to the present study in which enkephalin immunoreactivity was observed in sublamina 5 at both the light and electron microscopic levels. It is likely that these variations in the distribution of enkephalin-immunoreactive processes arise in part from factors

Figure 8. Enkephalin-stained profile (asterisk) presynaptic (open block arrow) to the vitreal pole of the soma (So) of a cell situated in the innermost cell row of the inner nuclear layer. Magnification $\times 34,000$.

such as (1) differences in the antisera used, (2) differences in experimental protocols employed, and/or (3) differences in personal interpretations of data (Childs, 1983; Petrusz, 1983).

Identification of synaptic elements. A common and recurring problem encountered in the present as well as past studies of retinal ultrastructure involves the identification of the processes participating in synaptic relationships. For instance, in the present study, we were interested in defining the synaptic interactions of enkephalin-like immunoreactive amacrine cells in the inner plexiform layer of the chicken retina. The electron microscopic immunocytochemical technique

permitted us to identify with certainty enkephalin-containing processes at the ultrastructural level. Unfortunately, the identities of synaptic elements with which enkephalinergic amacrine cells interact were not always so readily distinguished. In spite of these limitations, a number of interesting observations have emerged from our electron microscopic studies.

As the postsynaptic element, Enk-amacrine cells were observed to receive either ribbon- or conventional-type synaptic contacts. With respect to the ultrastructural organization of the inner plexiform layer, it is generally accepted that bipolar cell terminals always contain synaptic ribbons which, in turn, occur only in bipolar cells (Dowling and Boycott, 1966; Rodieck, 1973). Accordingly, the presynaptic elements which form ribbon synaptic junctions with enkephalin-stained elements most likely originate from bipolar cells. Furthermore, when an immunoreactive process received a ribbon synapse, the second member of the dyad was always unlabeled and usually vesicle filled. This finding correlates well with previous reports which indicate that, in the avian retina, both postsynaptic members at a ribbon synapse are usually of amacrine cell origin (Dubin, 1970).

Processes of enk-amacrine cells were also frequently observed to receive conventional synaptic contacts from unlabeled vesicle-filled profiles. Cumulative observations from previous studies indicate that, in the avian retina, conventional synapses originate from two sources: amacrine cells (Dubin, 1970; Rodieck, 1973) and centrifugal axons originating from the isthmo-optic nucleus (Dowling and Cowan, 1966). With respect to amacrine cells, Ramón y Cajal (see Rodieck, 1973, pp. 781–904) described several morphologically distinct types of amacrine cells in the avian retina whose processes distributed either diffusely or in a laminar fashion throughout the various levels of the inner plexiform layer. However, it is not known which of these types of amacrine cells contact Enk-amacrine cells. With respect to centrifugal fibers, Dowling and Cowan (1966) reported that, in the pigeon retina, the synaptic endings of these axons were observed most often along the outer margin of the inner plexiform layer where they contacted primarily large basal processes of amacrine cells. In the present study, immunoreactive basal processes of Enk-amacrine cells were seldom observed in sublamina 1. However, when identified, they were not found to receive conven-

Figure 9. Enkephalin-positive varicosities (open block arrows) presynaptic to processes (black asterisks) which lack synaptic vesicles. So, soma of adjacent ganglion cell layer. The solid block arrows point to the sites of synaptic contact. In B the white asterisk denotes a nearby stained varicosity. Magnification: A, $\times 21,000$; B, $\times 34,000$.

Figure 10. Summary diagram illustrating the synaptic connections of Enk-amacrine cells (ENK) in the chicken retina. In sublaminae 1, 3, 4, and 5, Enk-amacrine cells are pre- and postsynaptic to non-enkephalinergic amacrine cells (AC). Enk-amacrine cells also receive input from bipolar cells (either Ba or Bb) in sublaminae 1, 3, 4, and 5. In sublamina 1 Enk-amacrine cells contact the vitreal pole of amacrine and/or displaced ganglion cells (dashed process). In sublamina 5 Enk-amacrine cells contact large basal processes of displaced amacrine cells (DAC) and/or ganglion cells (GC). Not illustrated are Enk-amacrine cell inputs to non-vesicle-containing processes of ganglion cells and/or amacrine cells which are observed throughout the innermost half of the inner plexiform layer (IPL). Ba, off-center bipolar cell; Bb, on-center bipolar cell; INL, inner nuclear layer; GCL, ganglion cell layer. Numerals 1 to 5 denote sublaminae of the inner plexiform layer.

tional synaptic input. Moreover, small distal processes of Enk-amacrine cells were not observed to receive conventional synapses from vesicle-filled profiles displaying the characteristic features of centrifugal boutons as described by Dowling and Cowan (1966). Thus, although it is possible that the morphological characteristics as well as the synaptic relationships of centrifugal axonal boutons in the chicken retina differ from those observed in the pigeon, our findings to date indicate that non-enkephalinergic amacrine cells probably serve as the primary, if not the only, source of conventional input to Enk-amacrine cells in the chicken retina.

When identified as the presynaptic element, enkephalin-immunoreactive varicosities were most often observed to synapse onto other unlabeled vesicle-filled profiles, while occasionally contacting processes of various sizes which did not contain synaptic vesicles. Based on the following observations, vesicle-containing profiles, which were postsynaptic to Enk-amacrine cells, were presumed to be amacrine cell varicosities. First, such vesicle-filled profiles were sometimes observed to form conventional synapses, a characteristic feature of amacrine cell synaptic processes in the retina (Rodieck, 1973). Second, a vesicle-filled profile postsynaptic to an Enk-amacrine cell was occasionally found to receive a ribbon synapse from a bipolar cell terminal. Such structures were interpreted to be amacrine cell varicosities, since synaptic contacts between bipolar cells have not been reported in this retina (Rodieck, 1973). Third, vesicle-filled profiles postsynaptic to Enk-amacrine cells occasionally were noted to possess small dense-core vesicles. Within the inner plexiform layer of various species (for references, see Rodieck, 1973), such granulated vesicles have been associated with amacrine cell processes, ganglion cell dendrites, and/or centrifugal fibers. However, dense-core vesicles have not been observed in vesicle-

filled structures which contain synaptic ribbons. Finally, in the present study a synaptic ribbon was never observed in a vesicle-filled profile that was postsynaptic to an enkephalin-immunoreactive amacrine cell.

The origin of somas in the inner nuclear layer and non-vesicle-containing processes in the inner plexiform layer which both received conventional synapses from Enk-amacrine cells remains uncertain in the present study. In addition to amacrine cells, a prominent population of displaced ganglion cells has been observed in the chicken within the innermost cell row of the inner nuclear layer (Heaton et al., 1979). Therefore, it is possible that somas postsynaptic to enkephalin cells in the present study arise from amacrine and/or displaced ganglion cells. In sublamina 5 of the inner plexiform layer, enkephalin-stained varicosities were occasionally observed to contact large proximal processes in the vicinity of the ganglion cell layer. In addition to ganglion cells, displaced amacrine cells have been described in the ganglion cell layer of the avian retina (Rodieck, 1973, pp. 781-904), and it would appear that their numbers are quite substantial (Binggeli and Paule, 1969). Therefore, the processes of Enk-amacrine cells may interact with the basal processes of ganglion cells and/or displaced amacrine cells. Throughout the proximal half of the inner plexiform layer, Enk-amacrine cells were occasionally observed to contact small profiles which lacked synaptic vesicles. Such profiles were usually void of identifiable organelles, although in some instances they were characterized by the presence of vesiculated endoplasmic reticulum. Unfortunately, in the avian retina as well as in most other species, it is difficult to differentiate at the electron microscopic level small ganglion cell dendrites from small processes of amacrine cells which lack synaptic vesicles (Dubin, 1970). Therefore, it is possible that ganglion cells and/or amacrine cells give rise to such small distal processes which lack synaptic vesicles and are postsynaptic to Enk-amacrine cells.

Circuitry of enkephalinergic amacrine cells. Some of the major synaptic relationships between Enk-amacrine cells and other neurons in the chicken retina are summarized as a schematic diagram in Figure 10. First, Enk-amacrine cells are pre- and postsynaptic to other non-Enk-amacrine cells in sublaminae 1, 3, 4, and 5. Studies are presently under way to determine the identities of cells with which Enk-amacrine cells interact in the chicken retina. Our preliminary physiological studies indicate that enkephalin inhibits the K^+ -stimulated release of dopamine and GABA in the chicken retina (Su et al., 1984, 1985). Whether or not this inhibition is the result of direct interactions between Enk-amacrine cells and either dopaminergic or GABAergic amacrine cells must await future studies in which multiple histochemical and autoradiographic localizations are performed at the electron microscopic level in the same retina. Second, Enk-amacrine cells also receive synaptic input from bipolar cells in sublaminae 1, 3, 4, and 5. In this regard, it is of interest that, in most vertebrate retinas examined, the proximal region of the inner plexiform layer has been found to be associated mainly with the processing of on-center information, whereas the distal region concentrates predominantly on off-center information (Famiglietti et al., 1977; Stell et al., 1977; Nelson et al., 1978). If this aspect of retinal organization is conserved in the chicken retina, then the Enk-amacrine cells probably receive input from both on-center (Bb) and off-center (Ba) bipolar cells. Third, Enk-amacrine cells synapse upon the soma of amacrine and/or displaced ganglion cells. Also, within sublamina 5, Enk-amacrine cells also contact ganglion and/or displaced amacrine cells. Direct anatomical proof of Enk-amacrine cell input directly onto ganglion cells, either displaced or typical, may be obtained in future studies by combining enkephalin immunocytochemistry with horseradish peroxidase backfilling of ganglion cells in the same retina.

Enkephalins as retinal neurotransmitters or modulators. Although there are as yet no published reports on the effects of enkephalins and other opiates in the processing of visual information in the avian retina, in amphibian and teleost retinas, exogenously applied enkephalins have been shown to influence the responses of certain

ganglion cells (Dick et al., 1980; Djamgoz et al., 1981). Our results presented here and elsewhere show that in the chicken retina: (1) a substance immunologically and biochemically identical or very similar to enkephalin is present and synthesized (Su et al., 1983, 1985); (2) this substance is released upon stimulation of the retina in a Ca^{2+} -dependent manner (Su et al., 1983, 1985); (3) enkephalin-immunoreactive amacrine cells are localized (Watt et al., 1983; Tavella et al., 1985) and participate in several synaptic relationships (Watt et al., 1983); (4) receptors for enkephalins have been identified (Su et al., 1984); and (5) exogenously applied enkephalins and opiates inhibit the release of GABA and dopamine (Su et al., 1984; Watt et al., 1984a, b). Taken together, these studies point to enkephalins or substances very similar to enkephalins as putative neurotransmitters or modulators in the vertebrate retina. Moreover, the retina may prove to be an excellent model of the central nervous system for investigating, at cellular and molecular levels, the functional roles of these important neuroactive peptides.

References

- Altschuler, R. A., J. W. Mosinger, D. W. Hoffman, and M. H. Parakkal (1982) Immunocytochemical localization of enkephalin-like immunoreactivity in the retina of the guinea pig. *Proc. Natl. Acad. Sci. U. S. A.* 79: 2398–2400.
- Binggeli, R. L., and W. J. Paule (1969) The pigeon retina: Quantitative aspects of the optic nerve and ganglion cell layer. *J. Comp. Neurol.* 137: 1–17.
- Brandon, C., D. M. K. Lam, Y. Y. T. Su and J. -Y. Wu (1980) Immunocytochemical localization of GABA neurons in the rabbit and frog retina. *Brain Res. Bull.* 5: 21–29.
- Brecha, N., H. J. Karten, and C. Laverack (1979) Enkephalin-containing amacrine cells in the avian retina: Immunohistochemical localization. *Proc. Natl. Acad. Sci. U. S. A.* 76: 3010–3014.
- Childs, B. V. (1983) The use of multiple methods to validate immunocytochemical stains. *J. Histochem. Cytochem.* 31: 168–176.
- Dick, E., R. F. Miller, and M. M. Behbehani (1980) Opioids and substance P influence ganglion cells in amphibian retina. *Invest. Ophthalmol. Vis. Sci. (Suppl.)* 19: 32.
- Djamgoz, M. B. A., W. K. Stell, C. A. Chin, and D. M. K. Lam (1981) An opiate system in the goldfish retina. *Nature* 292: 620–623.
- Dowling, J. E., and B. B. Boycott (1966) Organization of the primate retina: Electron microscopy. *Proc. R. Soc. Lond. (Biol.)* 166: 80–111.
- Dowling, J. E., and W. M. Cowan (1966) An electron microscope study of normal and degenerating centrifugal fiber terminals in the pigeon retina. *Z. Zellforsch.* 71: 14–28.
- Dubin, M. W. (1970) The inner plexiform layer of the vertebrate retina: A quantitative and comparative electron microscopic analysis. *J. Comp. Neurol.* 140: 479–506.
- Eldred, W., and H. J. Karten (1984) Characterization and quantification of peptidergic amacrine cells in the turtle retina: Enkephalin, neurotensin and glucagon. *J. Comp. Neurol.* 221: 371–378.
- Famiglietti, E. V., A. Kaneko, and M. Tachibana (1977) Neuronal architecture of on and off pathways to ganglion cells in the carp. *Science* 198: 1267–1269.
- Fukuda, M. (1982) Localization of neuropeptides in the avian retina: An immunohistochemical analysis. *Cell. Mol. Biol.* 28: 275–283.
- Heaton, M. B., I. M. Alvarez, and J. E. Crandall (1979) The displaced ganglion cell in the avian retina: Developmental and comparative considerations. *Anat. Embryol.* 155: 161–178.
- Hokfelt, T., O. Johansson, A. Ljungdahl, J. M. Lundberg, and M. Schultzberg (1980) Peptidergic neurones. *Nature* 284: 515–521.
- Howells, R. D., J. Groth, J. M. Hiller, and E. J. Simon (1980) Opiate binding sites in the retina: Properties and distribution. *J. Pharmacol. Exp. Ther.* 215: 60–64.
- Hughes, J., H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, and H. R. Morris (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 258: 577–579.
- Humbert, J., P. Pradelles, C. Gros, and F. Dray (1979) Enkephalin-like products in embryonic chicken retina. *Neurosci. Lett.* 12: 259–263.
- Ishimoto, I., S. Shiosaka, Y. Kuwayama, M. Fukuda, S. Inagaki, H. Takagi, M. Sakanaka, A. Sasaka, E. Senba, T. Sakiyama, and M. Tohyama (1983) Leucine-enkephalin-like immunoreactivity in the chicken retina with a special reference to its fine structure. *Invest. Ophthalmol. Vis. Sci.* 24: 879–885.
- Medzihradsky, F. (1976) Stereospecific binding of etorphine in isolated neural cells and in retina, determined by a sensitive microassay. *Brain Res.* 108: 212–219.
- Miller, R. J., K. -J. Chang, B. Cooper, and P. Cuatrecasas (1978) Radioimmunoassay and characterization of enkephalins in rat tissues. *J. Biol. Chem.* 253: 531–538.
- Nelson, R., E. V. Famiglietti, and H. Kolb (1978) Intracellular staining reveals different levels of stratification for on- and off-center ganglion cells in the cat retina. *J. Neurophysiol.* 41: 472–483.
- Petrusz, P. (1983) Essential requirements for the validity of immunocytochemical staining procedures. *J. Histochem. Cytochem.* 31: 177–179.
- Rodieck, R. W. (1973) *The Vertebrate Retina: Principles of Structure and Function*, pp. 487–505; 781–904, W. H. Freeman and Co., San Francisco.
- Sar, M., W. E. Stumpf, R. J. Miller, K. -J. Chang, and P. Cuatrecasas (1978) Immunohistochemical localization of enkephalin in rat brain and spinal cord. *J. Comp. Neurol.* 182: 17–38.
- Stell, W. K., A. T. Ishida, and D. O. Lightfoot (1977) Structural basis for on- and off-center responses in retinal bipolar cells. *Science* 198: 1269–1271.
- Stell, W. K., D. Marshak, T. Yamada, N. Brecha, and H. Karten (1980) Peptides are in the eye of the beholder. *Trends Neurosci.* 3: 292–295.
- Stell, W. K., K. S. Chohan, and N. Brecha (1981) Enkephalin-immunoreactive amacrine cells in the retinas of some teleost fish. *Soc. Neurosci. Abstr.* 7: 94.
- Su, Y. Y. T., C. B. Watt, and D. M. K. Lam (1983) An enkephalin system in the chicken retina. II. Biosynthesis, release and physiological studies. *Soc. Neurosci. Abstr.* 9: 282.
- Su, Y. Y. T., S. Su, and D. M. K. Lam (1984) An enkephalin system in the chicken retina: Receptor binding and physiological studies. *Invest. Ophthalmol. Vis. Sci. (Suppl.)* 25: 292.
- Su, Y. Y. T., C. B. Watt, and D. M. K. Lam (1985) Opioid pathways in an avian retina. I. The content, biosynthesis, and release of Met⁵-enkephalin. *J. Neurosci.* 5: 851–856.
- Tavella, D., C. B. Watt, Y. Y. T. Su, K. -J. Chang, S. Handlin, V. Gaskie, and D. M. K. Lam (1985) The production and partial characterization of monoclonal antibodies against enkephalin. *Neurochem. Int.*, in press.
- Tornqvist, K., I. Loren, R. Hakanson, and F. Sundler (1981) Peptide-containing neurons in the chicken retina. *Exp. Eye Res.* 33: 55–64.
- Vandesande, F. (1979) A critical review of immunocytochemical methods for light microscopy. *J. Neurosci. Methods* 1: 3–23.
- van Leeuwen, F. W., C. W. Pool, and A. A. Sluiter (1983) Enkephalin immunoreactivity in synaptoid elements of glial cells in the rat neural lobe. *Neuroscience* 8: 229–241.
- Watt, C. B., D. Tavella, Y. Y. T. Su, Y. W. Peng, and D. M. K. Lam (1983) An enkephalin system in the chicken retina. I. Production and characterization of monoclonal antibodies: Enkephalin content and immunocytochemical localization. *Soc. Neurosci. Abstr.* 9: 282.
- Watt, C. B., K. R. Fry, S. M. Wu, and D. M. K. Lam (1984a) Immunocytochemical localization of enkephalin-like immunoreactivity in the tiger salamander retina: A light and electron microscopic study. *Invest. Ophthalmol. Vis. Sci. (Suppl.)* 25: 283.
- Watt, C. B., Y. Y. T. Su, and D. M. K. Lam (1984b) Interactions between enkephalin and GABA in an avian retina: Physiological studies and simultaneous visualization of putative GABAergic and enkephalinergic neurones. *Nature* 311: 761–763.