Leucine-enkephalin-like Immunoreactivity in the Chicken Retina with a Special Reference to Its Fine Structures

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Leucine-enkephalin-like immunoreactivity (LEI) in the chicken retina was investigated using light and electron microscopic immunohistochemistry. A flat-mount technique for light microscopy clearly demonstrated that LEI cells were distributed widely throughout the entire retina, without any differences between the central and peripheral retinal regions. Frozen sections for light microscopy confirmed the previous findings in pigeons that LEI is localized in amacrine cells. The present electron microscopic observations confirmed the findings of light microscopy and revealed that LEI terminals make synaptic contact mainly with vesicle-containing processes that seemed to belong to the amacrine cells. The present study further suggests that LEI terminals are both pre- and postsynaptic elements onto vesicle-containing processes mentioned above. Invest Ophthalmol Vis Sci 24:879–885, 1983

Recently, the presence of leucine-enkephalin (LE) in the retina, particularly in the amacrine cells, has been demonstrated.2,10,13,16,17 However, as to the functional role of the retinal LE, little is known, although there exist some suggestions.6,7,17 In order to explore the functional role of the retinal LE, it seems necessary to elucidate the fine structure, synaptic contacts, and overall distribution of the LE cells in the retina. Unfortunately in the previous immunohistochemical studies, it was difficult to determine the overall distribution of the LE cells because the authors used frozen sections. However, we recently have succeeded in demonstrating peptide-like immunoreactivity in flat-mounted retinal tissue which make it possible for us to determine the overall distribution of LE in the retina. Therefore, in the present study, we first attempted to describe the overall distribution of LE-like immunoreactivity (LEI) using flat-mounts. We then attempted to explore the fine structure of LEI cells and terminals, little known until now, using the unlabeled antibody enzyme method of immunocytochemistry.18

Materials and Methods

Experiments were done on 30 chickens. Antiserum: Antiserum against LE was produced in response to a CDI (1-ethyl-(3,3-dimethylaminopropyl)-catbodiimide) synthetic LE (Jpn. Peptide Lab. Co. Ltd) conjugated bovine thyroglobulin (Sigma). The specificity of LE antiserum was determined by radioimmunoassay and was shown to cross-react less than 0.1% with methionine-enkephalin and less than 0.01% with dynorphine and ACTH. There was no cross-reactivity with endorphins, substance P, somatostatin, neurotensin, cholecystokinin (CCK-8), LHRH, or TRF. The specificity of immunoreactive staining also was established by preabsorption of antiserum with excess LE (0.8 µg/ml).

Tissue preparation and immunohistochemical procedure: Light microscopy: 14 chickens (each of about 50 g body weight) were used. Under nembutal anesthesia (30 mg/kg, ip) each animal was perfused via its ascending aorta with 30 ml of ice-cold saline followed by Zamboni’s fixative.18 In some cases, during the perfusion the eyes also were infused with the same fixative. After the perfusion the eyes were enucleated immediately and the anterior eye segments were removed. For frozen sections, eye cups were postfixed for one to two days at 4 C in the same fixative and then were rinsed for 24 hr in 0.1 M phosphate buffer containing 30% sucrose. Sections of the retina were cut on a cryostat either perpendicular to the vertical axis of the eye at 10 µm or tangential to the retinal...
surface at 10–15 μm. For retinal flat mounts, eye cups were prepared as above. Next the pigment epithelium was separated microsurgically from the retina. The isolated retina was postfixed in the same fixative for one to two days and rinsed in 0.1 M phosphate buffer containing 30% sucrose. Then, the isolated retina was flat-mounted on a gelatin-coated glass plate with the vitreal side facing up. All the tissues were subjected to either the indirect immunofluorescence or the unlabeled antibody-enzyme method of immunohistochemistry. The sections first were washed in 0.1 M phosphate buffer saline (PBS) [with or without 10% goat serum and 4% bovine serum albumin (BSA)] for 10 min at room temperature (15 C) prior to the beginning of the immunohistochemistry. One-half of the frozen sections and flat-mounts were incubated in the primary antiserum (which has been pretreated with thyroglobulin for 45 min) at a dilution of 1:1,000 for 24 hr at 4 C in a humid atmosphere; they then were rinsed in ice-cold PBS containing 1% Triton X-100 for 15 min and then immediately with PBS for 10 min. For the immunofluorescence procedure, the tissue was incubated with fluorescein isothiocyanate conjugated antibodies (Miles Co. Ltd.) at a dilution of 1:50 under the same conditions as described above. The tissue was washed subsequently in PBS for 10 min at 4 C and mounted in a PBS-glycerine mixture. For the unlabeled antibody enzyme technique, the tissue was incubated in goat-anti-rabbit IgG (Miles Co. Ltd.) at a dilution of 1:50 for 45 min at room temperature, and washed three times for 15 min in 0.1 M PBS. After being washed, the tissue was incubated in peroxidase-antiperoxidase complex (DAKO) at a dilution of 1:100 for 45 min at room temperature, following which it was rinsed three times, preincubated with 3,3’ diaminobenzidine HCl (DAB) (5 mg/10 ml) for 30 min, and then incubated for 2 min in DAB (5 mg/10 ml) containing 0.01% H2O2. After the reaction was completed, the materials were washed in 0.1 M PBS, dehydrated, and mounted in balsam. The remaining tissue was incubated first in the control LE-preabsorbed serum and then subjected to the immunohistochemical procedures described above.

**Electron microscopy:** Sixteen chickens (each of about 50 g body weight) were used for electron microscopy. Each animal was perfused via its ascending aorta with 30 ml ice-cold saline, followed by 200 ml modified Zamboni’s solution (0.6% glutaraldehyde, 4% paraformaldehyde, and 0.21% picric acid in 0.1 M phosphate buffer) under nembutal anesthesia (30 mg/kg, ip). During the perfusion, in some cases, the eyes also were infused with fixative. After perfusion, the eyes were enucleated immediately and fixed in the same fixative without glutaraldehyde for 24 hr. After a buffer rinse, the retinas were cut with a Vibratome® at 10 μm. One-half of the sections then were subjected to the unlabeled antibody enzyme method of immunohistochemistry described above, but without using Triton X-100. The other half of the sections were processed as control. Following the immunohistochemical processing, the sections were postfixed in 2% osmium tetroxide (OsO4) for 2 hr at 4 C, dehydrated in graded alcohol, and embedded in Epon. Serial ultrathin sections were cut and observations were made on both stained (uranyl acetate and lead citrate according to conventional methods) and nonstained sections.

**Results**

**I. Control Experiments**

The specificity of the immunoreaction was checked morphologically by comparing sections stained with LE antiserum and absorbed control serum (the specificity also was checked by radiimmunoassay; see Materials and Methods). The use of antiserum to LE absorbed with excess synthetic LE completely abolished immunostaining, indicating that structures stained by LE antiserum may be considered specifically stained. The specific property of these materials should be correctly described as LE-like immunoreactivity, but in this study, we will use the simpler term LEI.

**II. Light Microscopic Observations**

In the frozen sections, in accordance with previous studies,1,2,10,11 numerous LEI cells were observed in the proximal portion of the inner nuclear layer (INL) (Figs. 1A and 2). The majority of these LEI cell bodies were oval and about 7 μm in diameter. No immunoreactive cells were observed in other layers of the retina. The processes originating from these LEI cells entered the inner plexiform layer (IPL) where they arborized, mainly in lamina 3–4, and partly in lamina 1 of (Figs. 1A and 2C).

In the flat-mounted retina, an overall distribution of LEI cells was observed (Fig. 1B). As shown in Fig. 1B, LEI cells were widely distributed in the entire retina. The densities of the LEI cells in the central and peripheral retinal regions were almost the same (n = 1,600/mm²) and no regional differences in the distribution of the LEI cells throughout the retina were detected. Immunoreactive cell perikarya measured in the flat-mounts had a diameter of about 7 μm and the cell-to-cell spacing was approximately 45 μm.
III. Electron Microscopic Observations

Fig. 2C is a bright-field photomicrograph showing LEI in an Epon-embedded section. In Fig. 2C, as in Fig. 1A, LEI cells were located in the proximal part of the INL and LEI fibers were seen mainly in laminae 3-4 and partly in lamina 1. Fig. 2A is an electron micrograph of the perikaryon and its vitreal process of the LEI cell shown by the arrow in Fig. 2C. In agreement with the results from frozen sections, cell bodies labeled by the LE reaction product were located in the proximal part of the INL (Fig. 2A), and were oval and about 7 µm in diameter. The nucleus of these cells was surrounded by a thin rim of cytoplasm (Figs. 2A and 3), and its surface was smooth and not indented (Figs. 2A and 3). In the cytoplasm, numerous patches of LEI reaction product were identified around the endoplasmic reticulum (Fig. 3). It is noteworthy that the LEI cell surface were always ensheathed by processes of Müller cells (Figs. 2A and 3).

The processes from the LEI cells enter the IPL, being ensheathed by processes of Müller cells (Figs. 2A and B) and arborize mainly in laminae 3-4 and partly in lamina 1. In Fig. 2A, the arborization of LEI fibers in the lamina 1 is seen. In the ascending processes immunoreactive reaction products were observed along the microtubules (Figs. 2A and B). After arborization, LEI fibers are made up of varicosities interconnected by thin portions. These fibers often lacked the ensheathing Müller cell processes and showed direct apposition with neuronal elements.

The varicose terminals in the IPL measured 0.5-2.5 µm in diameter and contained numerous synaptic vesicles (Fig. 4). The fine structure of the LEI fibers was almost the same throughout the IPL, and no remarkable differences were detected between the LEI
fibers located in laminae 3–4 and 1. The LEI terminals have output synapses of the conventional kind such as (1) a presynaptic cluster of small synaptic vesicles close to the presynaptic membrane (Fig. 4B) and (2) pre- and postsynaptic membrane specialization (Figs. 4A–C) etc. These characteristic features are very similar to those of amacrine cells shown by Dowling and Boycott. The neuronal elements with which LEI terminals make synaptic contact are mainly vesicle-containing processes. These non-labeled processes are likely from amacrine cells, because they always lack synaptic ribbons and the synaptic vesicles in the non-labeled processes are distributed unevenly. The findings mentioned above suggest that LEI terminals are presynaptic in structure. However, it should be noted that LEI processes also look like postsynaptic in structure, because the cluster of vesicles was identified in the unlabeled vesicle-containing processes (Fig. 4B, shown by thin arrow) with which LEI terminals make synaptic contact as a presynaptic structure and no aggregation of vesicles were seen in the labeled process opposite the membrane density (Fig. 4B). These findings suggest that LEI terminals make a reciprocal synapse with non-labeled vesicle-containing process.

It should be noted that LEI terminals are involved frequently in complicated neuronal clusters. For example, as shown in Fig. 4C, a single LEI terminal is involved in synaptic contact with at least three unlabeled terminals, probably from amacrine cells, although in this figure it is not clear as to which polarity these contacts have.
Fig. 3. Electron micrograph showing the fine structure of LEI cells, from serial sections. Note smooth surface of the nucleus (N), small amount of cytoplasm, and en sheathment by Müller cell process (M). Note also that immunoreaction products are seen around the endoplasmic reticulum (×20,000).

Discussion

Previous immunohistochemical studies using frozen sections have demonstrated that LEI is localized in the amacrine cells. The present light microscopic observations using flat-mounts together with frozen sections have confirmed the previous findings and further elucidated that LEI cells are distributed widely in the retina, and that no regional differences were identified. This finding is in contrast with the distribution of LEI cells in the pigeon retina, in which they are more common in the red fields. This discrepancy may be attributed to the different species of birds or different kinds of antisera. Previously, we have reported that in flat mounts, substance P (SP) cells are localized mainly in the peripheral retinal region and are few in number in the central retinal region. These findings strongly suggest that LE and SP cells in the retina are functionally and structurally distinct, although both these peptides are localized in the amacrine cells. This concept is in good agreement with the suggestion by Brecha et al. that different cell types contain different peptides.

The present study also has demonstrated at the electron microscopic level that LEI is localized in amacrine cells. However, although LEI cells possess many of the well-known characteristics of amacrine cells, some of their structural features are unusual. For example, the diameter of the LEI cells is smaller, the cytoplasm of the LEI cells is much less, and the surface of the nuclei of LEI cells is smooth and not indented as in many ordinary amacrine cells, suggesting the heterogenous function of the amacrine cells as already mentioned above. The present electron mi-
C. LEI terminals often make neuronal clusters. LEI terminals make a synaptic contact simultaneously with several non-labeled vesicle-containing processes (A and B, ×43,000; C, ×40,000).

Microscopic study has shown that ascending processes from LEI cells were ensheathed by processes of Müller cells and after arborization, LEI fibers are made up of varicosities interconnected by thin portions, but these fibers often lacked the ensheathing Müller cell processes, suggesting the areas which lacked the ensheathing Müller cell process may be active sites.

The present study has shown that LEI terminals make a reciprocal synapse mainly with vesicle-containing processes, but no obvious contact with dendrites were identified. The processes with which LEI terminals make synaptic contact seem to originate from amacrine cells, because their synaptic vesicles are distributed unevenly and no synaptic ribbons were detected. Some of these amacrine cells may contain GABA, since a recent electrophysiologic study has shown that ascending processes from LEI cells were ensheathed by processes of Müller cells and after arborization, LEI fibers are made up of varicosities interconnected by thin portions, but these fiber often lacked the ensheathing Müller cell processes, suggesting the areas which lacked the ensheathing Müller cell process may be an active sites.

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has suggested an axo-axonic contact between LEI- and GABA-containing amacrine cells.

**Key words:** enkephalin, amacrine cells, flat-mounts, fine structure

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**References**