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Chicken Luteinizing Hormone-Releasing hormone-I and -II are located in distinct fiber terminals in the median eminence of the quail: a light and electron microscopic study

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ABSTRACT. Using highly specific antibodies directed towards the avian luteinizing hormone-releasing hormones-I and –II (cLHRH-I and –II), we were able to show the presence of both peptides in the median eminence of the quail. In order to examine in more detail the morphological organization of both gonadotropin-releasing hormone systems in the median eminence, the present paper describes the results from a light and ultrastructural immunocytochemical double labeling study. These results demonstrate the presence of cLHRH-I and cLHRH-II in distinct fiber systems in the median eminence of the quail. Moreover, close associations between cLHRH-I or –II- immunoreactive neurons and glial cells were observed, suggesting glial-neuronal interactions.

KEY WORDS: LHRH-I, LHRH-II, immunocytochemistry, LM, EM, double staining, bird, median eminence, quail.

INTRODUCTION

In birds two luteinizing hormone-releasing hormones, cLHRH-I and cLHRH-II have been described (for a review see KING & MILLAR, 1995). MIKAMI et al. (1988) published the immunocytochemical distribution of both cLHRH variants in the hypothalamus of both chicken and quail. In 1993, Millam and co-workers used antibodies directed against the full amino acid sequences of cLHRH-I and cLHRH-II to study their distribution in the turkey hen (MILLAM et al., 1993).

In our laboratory, highly specific antibodies were developed directed against polypeptides corresponding to the C-terminal portion of cLHRH-I and –II. After exhaustive specificity testing, these polyclonal antisera were applied in an immunocytochemical study on chicken and quail (VAN GILS, ABSIL, GRAUWELS, MOONS, VANDESANDE & BALTHAZART, 1993). The most striking observation in that study was the presence of not only cLHRH-I, but also of cLHRH-II immunoreactive (ir) fibers in the median eminence (ME) of both species. This finding remained very controversial, since no other study, with any other antibody, had ever reported the presence of densely labeled cLHRH-II fibers in the ME of birds. The latter conviction has always been fundamental for ascribing cLHRH-II a neurotransmitter/neuromodulator role instead of a gonadotropin-releasing function. However, very recently, the final positive identification of cLHRH-II in the ME of birds was provided by affinity purification of the anti-cLHRH-II antiserum, in combination with nano flow quadrupole time of flight mass spectrometry (D'HONDT et al., unpublished data).

The first aim of the present study was to investigate whether cLHRH-I and –II are present in separate fiber systems or not. For this purpose, we performed immunocytochemical double stainings at the light and ultrastructural level.

Secondly, we examined the morphological features of the cLHRH-I and –II -containing fibers and terminals in

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the median eminence to obtain more information about the functioning and regulation of the LHRH system in birds. Although several physiological studies in mammals indicate that LHRH release might be regulated at the level of the ME by neuropeptides, neurotransmitters or excitatory amino acids, electron microscopic studies often fail to find synaptic contacts on LHRH neurons (KAWAKAMI, HIRUNAGI, TSUKAMURA & MAEDA, 1998). Therefore it was suggested that the LHRH release in the ME might be regulated, at least in part, in a non-synaptic manner. Since at present no information is available about the ultrastructural organization of the cLHRH-I and –II systems, the present study aimed to provide more data on this subject in birds.

MATERIAL AND METHODS

Animals and tissue preparation

Three adult female quails were used in this study. These animals were kept under a lighting schedule of 16 hours light/8 hours dark and had feed and water available ad libitum. For light microscopy, the birds were transcardially perfused with 200 ml saline solution containing 1% $NaNO_2$, followed by 200 ml Zamboni's fixative (4% paraformaldehyde + 0.2% (w/v) picric acid). For electron microscopy, median eminences were postfixed for 24 hours with modified Zamboni (Zamboni's fixative containing 0.1% glutaraldehyde). After the brains were embedded in 20% (w/v) gelatin, transverse vibratome sections (50 µm) were cut and serial sections containing the ME were collected in 12-well plates containing 0.15M phosphate buffer (pH=7.4).

Immunocytochemical procedures

Light microscopy

Basically, the immunocytochemical procedure for the double staining of cLHRH-I and –II was the same as the one described by MOONS et al. 1988. All antiserum incubations and washing steps were performed in 0.01 M Tris-HCl buffer pH 7.4, containing 0.9% (w/v) NaCl and 0.1% Triton X-100 (v/v) (TBST). After blocking of the endogenous peroxidase activity (blocking solution, Envision$^+$, DAKO, 10 min) and preincubation with 5% normal goat serum (30 min), the first primary antiserum, Ra cLHRH-II (1/10 000), was applied for an overnight incubation. After extensive rinsing, the sections were incubated with a secondary goat anti-rabbit polyclonal antiserum linked to a peroxidase-coupled dextran chain (Envision$^+$, DAKO, 30 minutes), and peroxidase activity was visualized using diaminobenzidine (20µl/ml substrate solution, Envision$^+$, DAKO) as a chromogen. The staining was stopped in distilled water. Prior to processing the sections through the second sequence, all antibodies of the first sequence were electrophoretically eluted (Moons et al. 1988). To subsequently reveal cLHRH-I immunoreactivity, the stained sections were pre-incubated in 5% normal goat serum, followed by an overnight incubation in Ra-cLHRH-I antiserum, diluted 1/15 000. Subsequent incubations steps in biotinylated goat anti-rabbit secondary antiserum (DAKO, 1/400, 30 min) and streptABComplex (DAKO, 30 min) were performed, followed by the fast blue BB staining procedure.

Electron microscopy

To detect simultaneously cLHRH-I and cLHRH-II, a pre-embedding double-labeling technique was optimized. DAB was used as the first chromogen while the second staining sequence was a silver intensified immunogold technique. Free floating vibratome sections were prepared for ICC by treatment in a solution of 0.1% sodium borohydride in 0.15M PBS, pH 7.4 for 15 minutes, followed by thorough rinsing in 0.15M PBS. To reduce aspecific staining, sections were blocked with a solution of 5% acetylated BSA (BSA-c, Aurion, the Netherlands), 0.1% CWFS (cold water fish) gelatin (Aurion, the Netherlands) and 1% normal goat serum in 0.15M PBS, pH 7.4. Unless otherwise mentioned, all subsequent incubation and rinsing steps were performed in BSA-c buffer (0.15M PBS + 0.1% BSA-c). Apart from this different buffer incubation system, all steps for the first staining sequence were the same as for light microscopy. After the DAB staining, sections were rinsed in BSA-c buffer, prior to the second staining procedure. The second primary antiserum was again applied in an overnight incubation step. After intensive rinsing in BSA-c buffer, the sections were incubated with a 1nm gold-conjugated secondary antibody (Aurion, the Netherlands). The gold was intensified with the R-Gent kit (Aurion, the Netherlands) for 15 minutes, in the dark at room temperature.

Tissue preparation for electron microscopy

Following ICC pre-embedding double labeling, sections were rinsed in 0.15M PBS buffer, pH 7.2 and fixed for 1 hour in 1% osmium tetroxide in the same buffer. Sections were then prepared for embedding in Embed 812 resin (Electron microscopy Sciences) by immersion for 10 minutes in each of a series of ethanol solutions as follows: 50%, 70%, 98%, 100%. They were infiltrated with araldite by immersion in (1) propylene oxide (PO) 100%, 10 min., (2) PO 50% + Embed 812 50% 2 hours, (3) Embed 812, overnight and (4) fresh Embed 812, 2 hours. Infiltrated sections were flat mounted between glass slides and plastic coverslips coated with liquid release agent (Electron microscopy Sciences) and cured. Pieces of the embedded ME tissue were mounted onto blocks and ultrathin sections were cut from the surface with an ultramicrotome. These sections were mounted on mesh grids and stained with lead citrate and uranyl acetate and finally viewed and photographed with a Zeiss 109 transmission electron microscope.

Immunocytochemical specificity tests

Because the two primary antisera used in this study were raised in the same species, we included a number of control experiments to prevent false positive or negative staining. For light microscopy, all antibodies of the first sequence were removed by electrophoretic elution. The efficiency of this elution step and the specificity of the double labeling procedure has been tested by omitting the DAB reaction from the first sequence and the primary or the secondary antibody from the second sequence. As a further validation of our co-localization method, two double staining sequences were performed. A first series of sections was double stained using Ra cLHRH-I as primary antibody in the first staining sequence, while a second series of sections was double stained using Ra cLHRH-II as primary antibody in the first staining sequence. In the double stainings performed for electron microscopy, no electrophoresis was performed, to obtain better preservation of the ultrastructure. To ensure the specificity of the stainings we performed two control procedures: (1): stainings omitting the DAB reaction and the primary or secondary antibody of the second sequence and (2) performance of stainings in both directions as for light microscopy.

Light microscopic data analysis

The sections stained for light microscopy were examined and photographed using a DMR-Leitz microscope. To obtain additional information, we performed some computerized image processing on the double stained vibratome sections. The Leitz microscope was connected to a personal computer, through a CCD camera (Sony ICX38AK). Several images of the same microscopic field were digitized at different focal planes. Subsequently, the brown or blue colors, respectively, were eliminated with a combination of red/green/blue optical filters on the microscope and the resulting images were digitized. These images were reversed into grey scale and optimized by means of the ADOBE Photoshop graphical software, and subsequently stored for printing.

RESULTS

Light microscopic observations

Since our anti-cLHRH-I and -II were both raised in rabbits, we included a number of control experiments to avoid unwanted false positive or negative results. The efficiency of the electrophoretic elution step has already been proven in a number of other double labeling studies (Moons, Cambré, Marivoet, Batten, Vanderhaeghen, Ollevier & Vandesande, 1988; D'Hondt, Eelen, Berghman & Vandesande, 2000). However, since the present study included a different combination of primary antisera, we included the classical control sections (omitting DAB staining as well as the primary or secondary antisera of the second sequence) to exclude definitively all assumptions of false positive results. All control sections were negative. As a second control procedure, double stainings were performed in both directions and revealed similar results.

Fig. 1 shows a typical double labeling for cLHRH-I (blue) and cLHRH-II (brown). Both peptides are quite densely stained, but cLHRH-I ir fibers are more abundant. At a higher magnification it becomes clear that cLHRH-I and cLHRH-II ir are not co-localized but are present in distinct fibers; the blue and brown reaction products do not appear to overlap.

For both peptides the staining is the most pronounced in the lateral and external zone of the median eminence. cLHRH-I and -II -ir fibers are often found perpendicular to the basal lamina of the ME.

The density of the staining makes it often difficult do distinguish double from single labeled fibers. Moreover, the thickness of the vibratome sections complicates the interpretation. To obtain additional data, we performed some computerized image processing on the stained sections. From the same microscopic field, several images at different focal plains through a vibratome section were digitized in order to get a better view of overlaying structures (Fig. 2). To distinguish single- from double- labeled fibers, we filtered one color at a time (blue or brown, respectively) from the same sections and recorded the resulting images (Fig. 3). Comparison of the three different images that were generated in this way revealed that the cLHRH-II fibers were not double stained: after elimination of the brown color from the image, the cLHRH-II fiber profiles disappeared completely (Fig. 3 D, E ,F) and vice versa, after elimination of the blue color all cLHRH-I ir fibers vanished from the image (Fig 3 A,B,C). These results indicate that cLHRH-I and –II immunoreactivities are located in different fibers in the ME.

Electron microscopic observations

Since our light microscopic observations indicated separate cLHRH-I and –II fiber terminals in the median eminence of the quail, we wanted to confirm these data at the electron microscopic level. Therefore, we conducted a pre-embedding double labeling experiment on quail ME tissue. The best results were obtained when the DAB staining for cLHRH-II was combined with the immunogold labeling for cLHRH-I.

Control sections, on which the DAB staining and the second primary or secondary antibody were omitted, showed no immunogold labeling, thereby demonstrating the specificity of the staining.

Many neuronal profiles in the quail median eminence were labeled with DAB (cLHRH-II) or immunogold (cLHRH-I). A profile was considered to be a cross-section through a cLHRH-II axon when it contained three or more densely stained vesicles and was considered cLHRH-I-

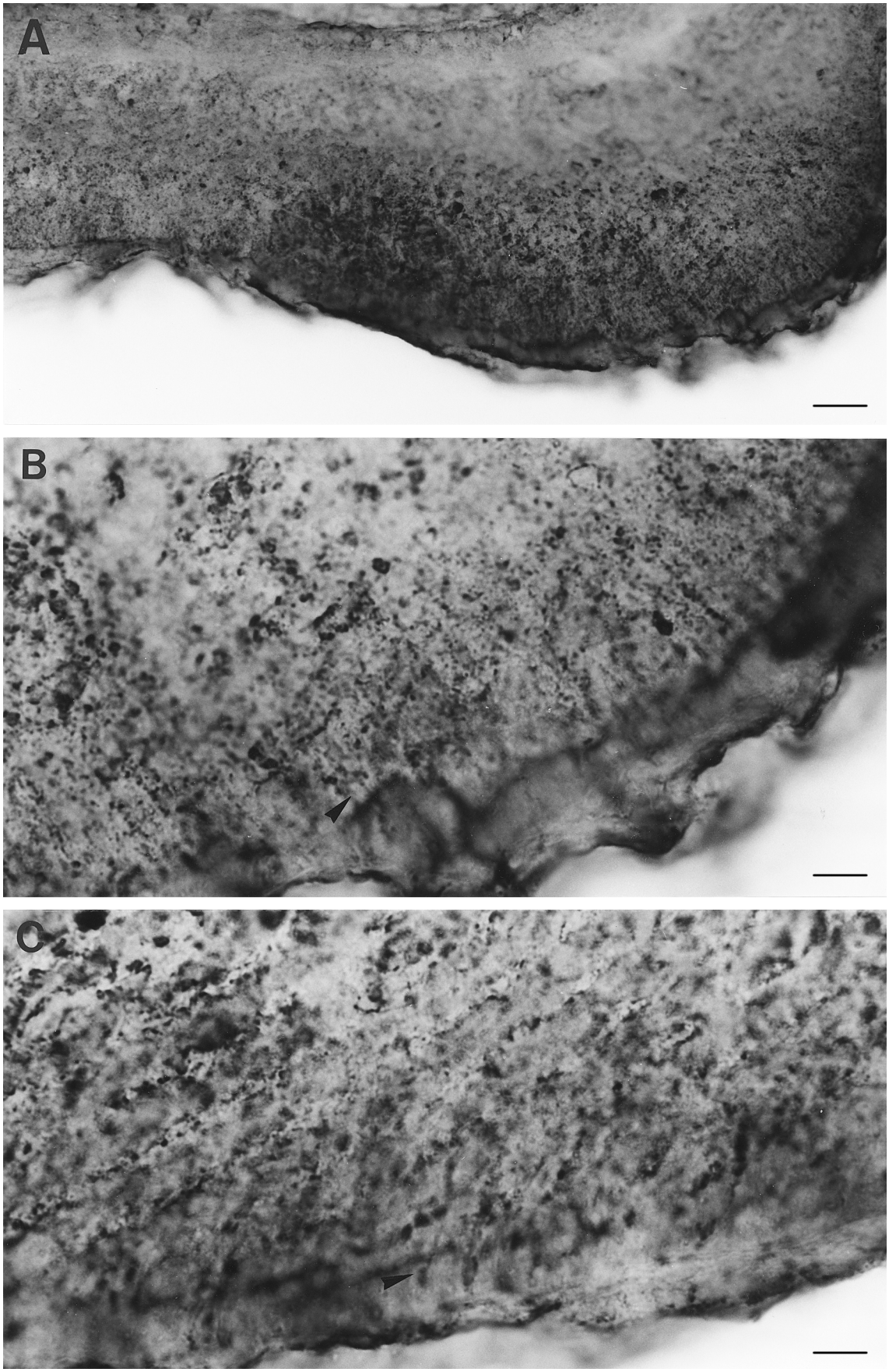

Fig. 1. – Photomicrographs showing the result of a double staining experiment between cLHRH-I (blue) and cLHRH-II (brown) in the median eminence. Both peptides appear in distinct fibers. Arrows heads indicate clearly single labeled cLHRH-II fibers. B and C are enlargements of A (magnification bars are 40 µm in A, 10 µm in B and C).

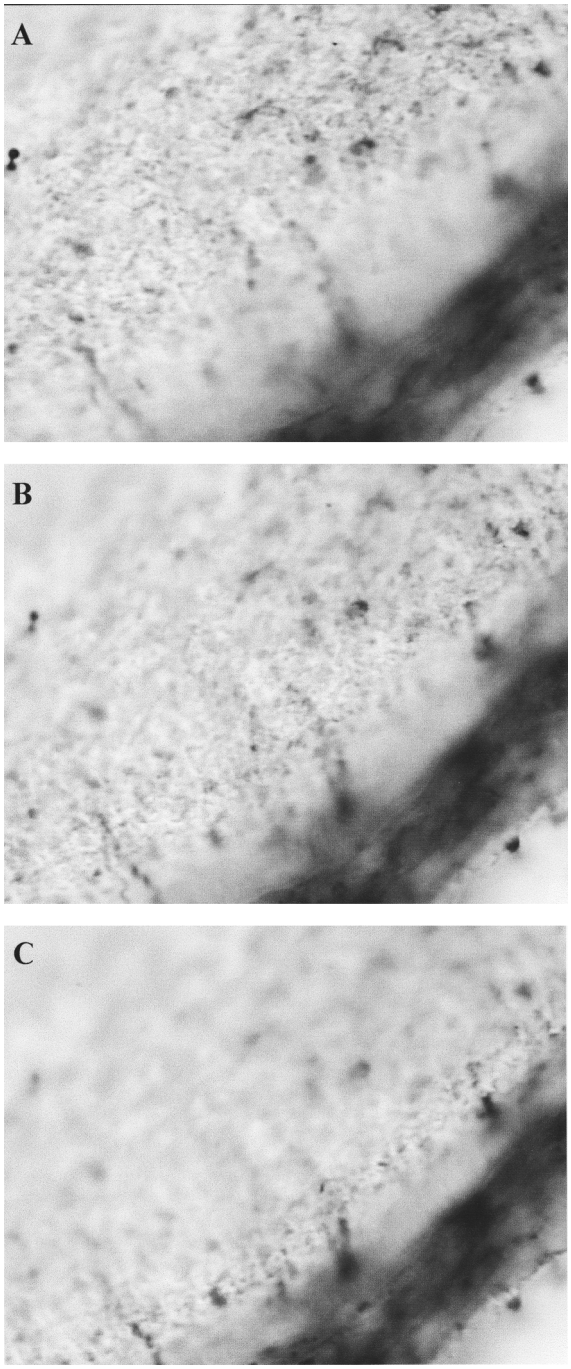

Fig. 2. – Computerized color image of a double staining between cLHRH-I (blue) and cLHRH-II (brown) in the median eminence of the chicken. A, B and C represent three images of the same microscopic field, taken at different focal planes.

positive when several gold particles were located within these fiber profiles. Double labeled profiles were never found (Fig. 4). cLHRH-I ir axons, in close contact with glial cells, were frequently observed. This was also the case for cLHRH-II ir axons but to a lesser extent (Fig 5).

Occasionally, intimate contacts were found between cLHRH-I and –II-ir axons and unlabeled axons.

DISCUSSION

The results of our double stainings at the light and ultrastructural level clearly show that cLHRH-I and –II are confined to separate fibers in the median eminence of the quail. These data together with the fact that cLHRH-I and cLHRH-II ir cell bodies are present in distinct hypothalamic nuclei, point to the existence of two separate avian gonadotropin-releasing systems.

Since the presence of cLHRH-II in the ME has always been denied, the present study is the first to provide information about its morphological characteristics. No ultrastructural data are available for cLHRH-I fibers in the ME of birds, and the morphological features of its regulation still remain to be elucidated. However, a number of studies indicate that cLHRH-I release can be regulated at the level of the ME in hens (Contijoch et al., 1992; 1993a; 1993b). This has not only been demonstrated by physiological experiments, but light microscopic double stainings have shown that dopamine- (Contijoch, Gonzalez, Singh, Malamed, Troncoso & Advis, 1992), ß-END- (Contijoch, Malamed, Sarkar, & Advis, 1993b) and NPY- (Contijoch, Malamed, McDonald & Advis, 1993a) immunoreactivities coincide with cLHRH-I ir fibers in the external layer of the ME in hens. These authors suggest the existence of synaptic contacts between these neurotransmitter/neuropeptide–containing axons and cLHRH-I nerve terminals, since dopaminergic synaptic contacts on LHRH-containing processes have been described in the ewe (Kuljis & Advis, 1989). However, more recently the possibility of a non-synaptic regulation of LHRH secretion from ME terminals has been posed by a number of ultrastructural studies which report only few synaptic contacts on mLHRH terminals in the rat median eminence (Kawakami, Hirunagi, Tsukamura & Maeda, 1998).

In the present study, performed in quail, synaptic contacts on cLHRH-I ir nerve terminals were found, although not frequently. This is in agreement with the last study and indicates that cLHRH-I release could, at least in part, be regulated in a non-synaptic manner. In contrast, cLHRH-II-positive axon profiles are more often found juxtaposed to unlabeled nerve endings, indicating that cLHRH-II release might be differently regulated.

In view of the regulation of LHRH release, local glia have been expected to play a crucial role (Ma, Berg-von der Emde, Rage, Wetsel, & Ojeda, 1997). Our ultrastructural data show that cLHRH-II as well as cLHRH-I ir axons are often found in close apposition to glial cell bodies, indicating that they may play some regulatory function.

In conclusion, the present data clearly demonstrate the neuroanatomic segregation of both gonadotropin-releasing hormone systems. The regulation of LHRH secretion and the secretion mechanisms themselves are unknown, but the present data suggest the involvement of glial-neuronal interactions.

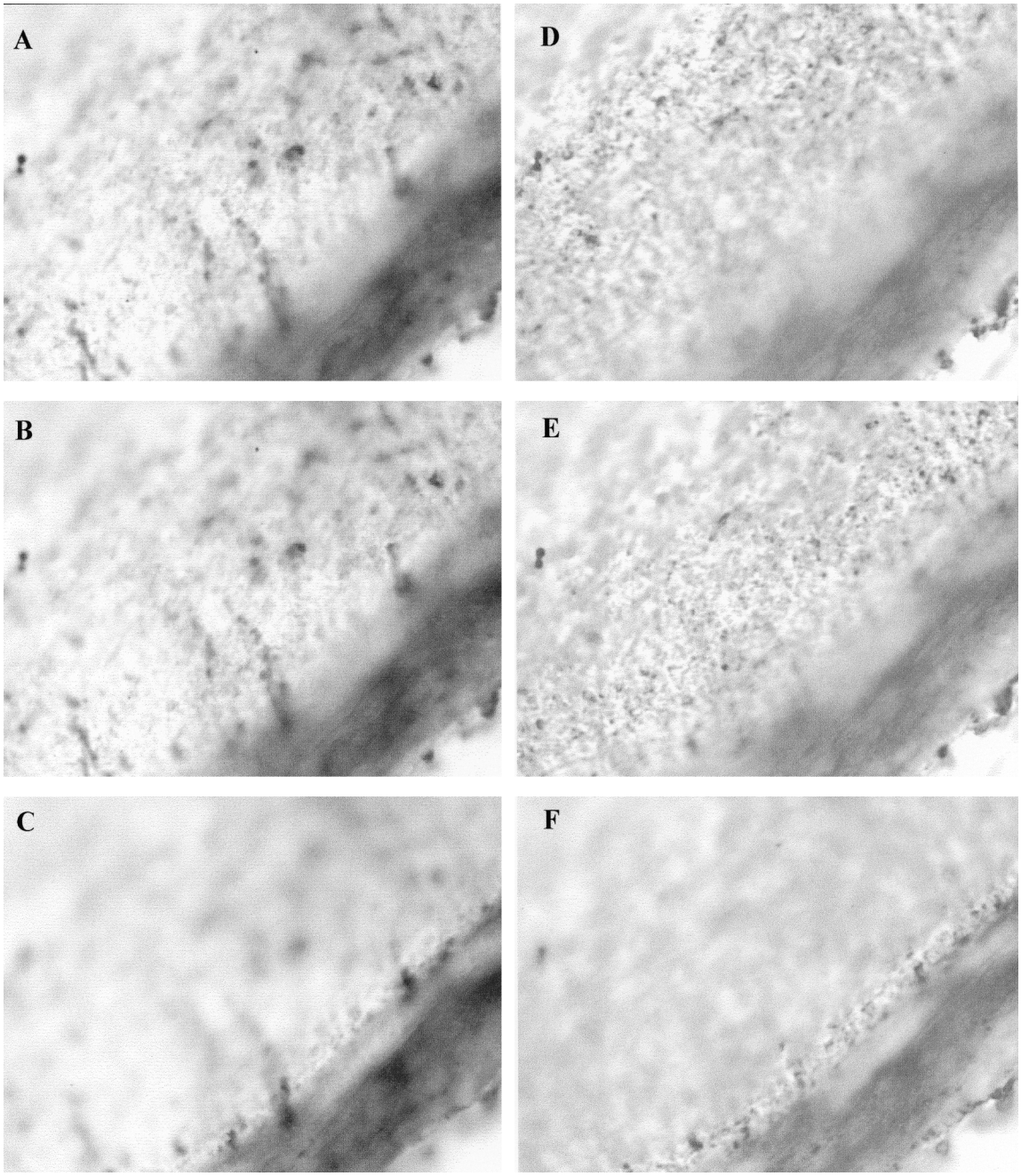

Fig. 3. – Computerized images of the cLHRH-II-ir (A, B, C) or cLHRH-I (D, E, F) immunoreactive structures of the same microscopic field, taken at different focal planes. Upon filtering of the blue color, only the brown labeled structures (cLHRH-II) remain visible and vice versa, upon filtering of the brown color, the blue (cLHRH-I)-labeled structures are left. Comparison of both sequences of images clearly indicates that cLHRH-I and cLHRH-II are present in separate fibers in the ME.

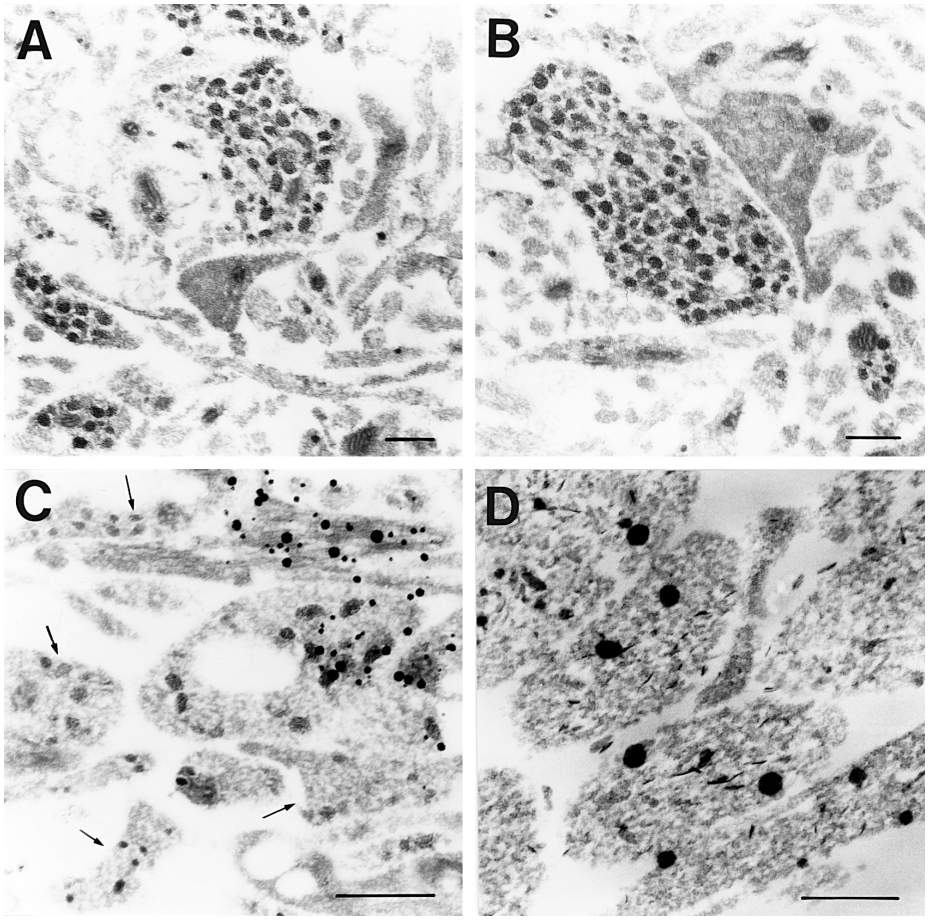

Fig. 4. – Electron micrographs demonstrating the results from a double staining for cLHRH-I and –II in the median eminence (ME) of the quail. A & B: DAB-labeled cLHRH-II-ir axons containing many secretory vesicles; C: cLHRH-I (silver-gold label) and cLHRH-II (DAB labeled, indicated by arrows) are present in distinct fiber terminals of the ME; D: high power micrograph of immunogold-stained cLHRH-I fibers in the ME (scale bars are 25 nm).

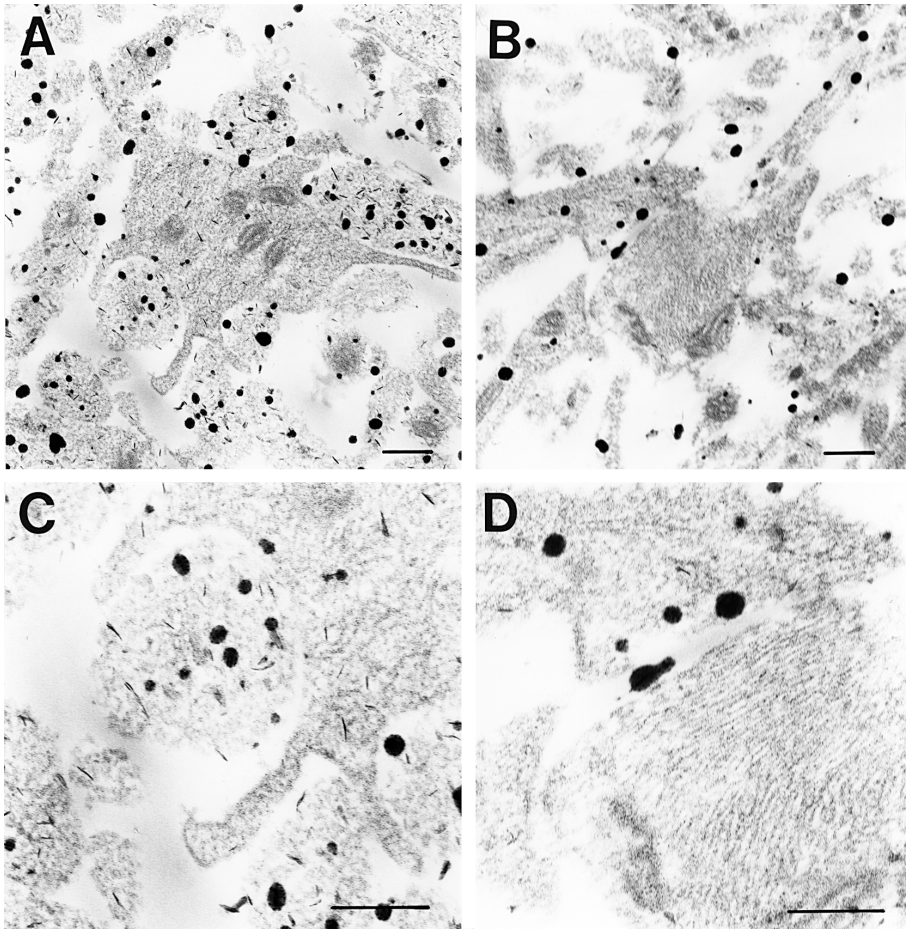

Fig. 5. – Electron micrographs demonstrating cLHRH-I (immunogold-labeled) axons in close apposition to glial cells in the median eminence (ME) of the quail. C & D are enlargements of A & B (scale bars are 25 nm).

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