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Immunocytochemical Localization of LHRH in Axons and Nerve Terminals of the Rat Median Eminence (38294)

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(Introduced by C. H. Sawyer)

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Immunocytochemical identification of nerve terminals reacting specifically with anti-LHRH antisera has recently been reported in various species, like the guinea pig (1) and the duck (2), employing a double antibody labeling technique, with the second antibody bound to fluorescein isothiocyanate (3). However, visualization of such terminals has not yet been described in the rat; on the other hand, a double labeling with a second, peroxidase bound, antibody does not seem to have been successfully applied to that material as yet. In the present paper, we report identification of fibers containing an antigen reacting with anti-LHRH in the rat median eminence, using both fluorescein and peroxidase labels.

Methods. The antibodies were obtained from rabbits injected with synthetic LHRH (Hoffman-LaRoche, Basle) coupled to guinea pig gamma globulin (4), according to the technique described by Avraméas (5). In a radioimmunoassay system (6), the titer of the antiserum is approximately 1/100,000; it can detect as little as 1–5 pg of LHRH. Its specificity was tested against oxytocin, lysin-vasopressin and TRH, as well as against LH, FSH and Prolactin; it does not cross react with these hormones at doses up to 500 μg. With this antibody, endogenous levels of LHRH were determined in the rat hypothalamus; values ranged between 1 and 3 ng/hypothalamus of normal male rats. Injected to cyclic female rats (200 μl ip at 1200 noon of the day of proestrus), this antiserum inhibited the preovulatory surge of LH and FSH and blocked ovulation (7).

Male rats were perfused by aortal cannulation with various fixatives (200 ml total volume) under light chloral hydrate anesthesia. The fixatives used were either Bouin–Hollande without acetic acid (4% formalin, 4% picric acid, 2.5% copper acetate and 10% of a saturated solution of Hg sublimate), 4% paraformaldehyde in saline solution, or picroformol. After dissection, the hypothalamus was postfixed for 4 hr at 4°. The tissues were dehydrated in graded butanol series followed by a mixture of paraffin and butanol and embedded in paraffin. Five micrometers serial sections were rehydrated, washed in water overnight, immersed in 0.01 *M* saline phosphate buffer solution (PBS) for 30 min and incubated with the antiserum against LHRH at various dilutions for 90 min. Adjacent sections were incubated for the same time with (a) normal rabbit serum, (b) serum of a rabbit immunized against guinea pig gamma globulins, (c) anti-LHRH serum absorbed previously with an excess of LHRH by overnight incubation at 4° of 100 μl of pure serum with 20 μl of a 3.10^{-4} *M* solution of synthetic LHRH, (d) anti-LH serum (8), (e) anti-LHRH serum incubated overnight with a cytoplasmic fraction from cerebral cortex and subsequently centrifuged (17,000*g* for 30 min) in an attempt to eliminate background staining. Some sections were treated for 5 min with 0.5 *M* HCl, or with 1% trypsin in PBS, prior to incubation with the antisera. After thorough washing in PBS, a second incubation with either peroxidase– or fluorescein–labeled sheep gamma globulin anti–rabbit gamma globulins (Institut Pasteur, Paris), was carried out for 90 min; the peroxidase was revealed by 3–3′ diaminobenzidine (DAB) (9).

Results and Discussion. Nerve terminals showing a clear-cut reaction with the peroxidase (Figs. 1A and 2A) or fluorescein–labeled antibody (Fig. 1B) can be seen in the median emi-

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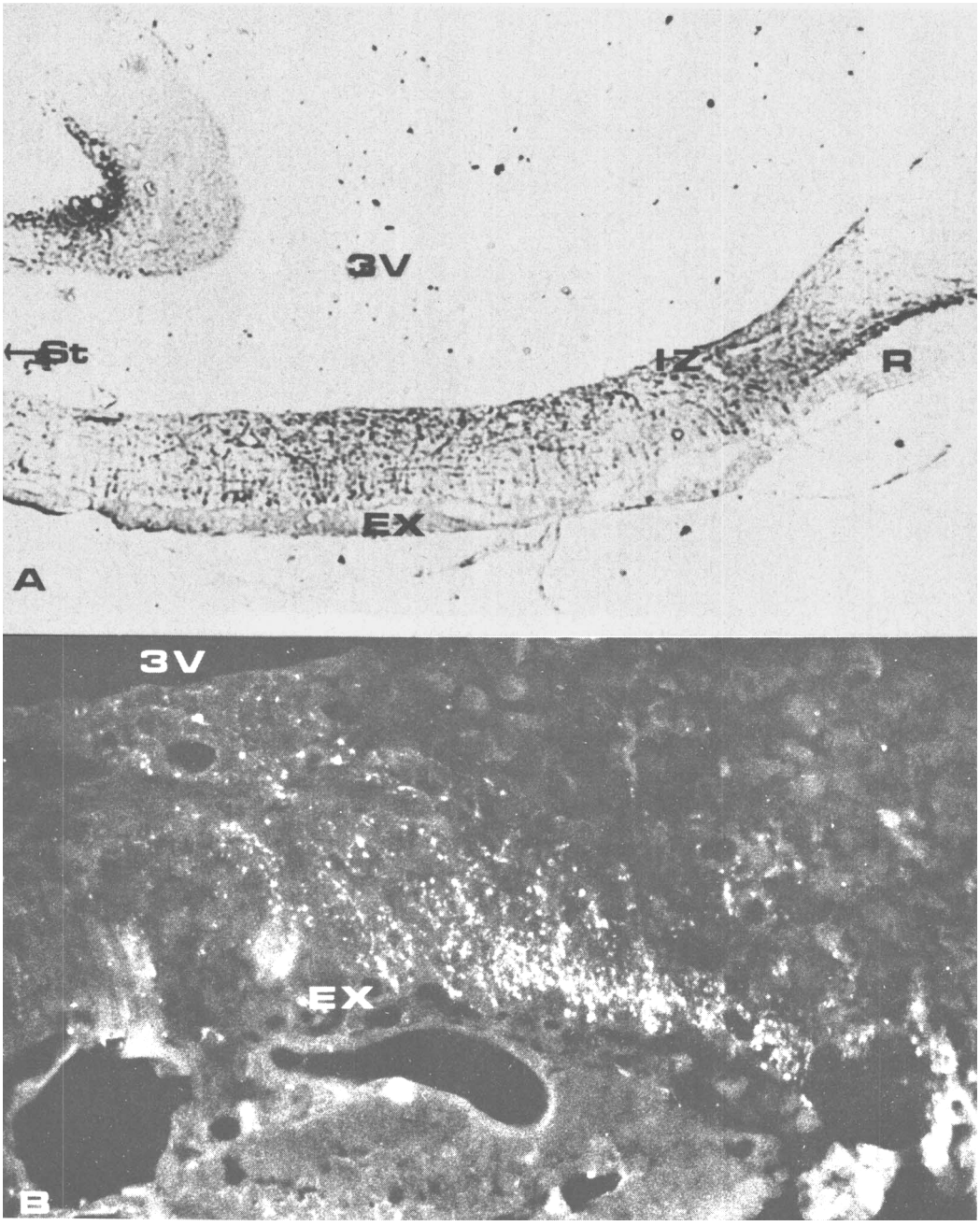

FIG. 1. Peroxidase or fluorescein revelation of LHRH containing axons and nerve terminals in the median eminence of normal rats. A, medial sagittal section of the median eminence. R, antigen containing terminals in the rostral portion of the external zone; ST, pituitary stalk; 3V, third ventricle; IZ, internal zone; EX, external zone of the median eminence. Incubation with anti-LHRH (1/100) and subsequent labeling with peroxidase, × 93. B, frontal section across the rostral part of the median eminence. EX, external zone; 3V, third ventricle. Incubation with anti-LHRH (1/100) and labeling with fluorescein, × 430.

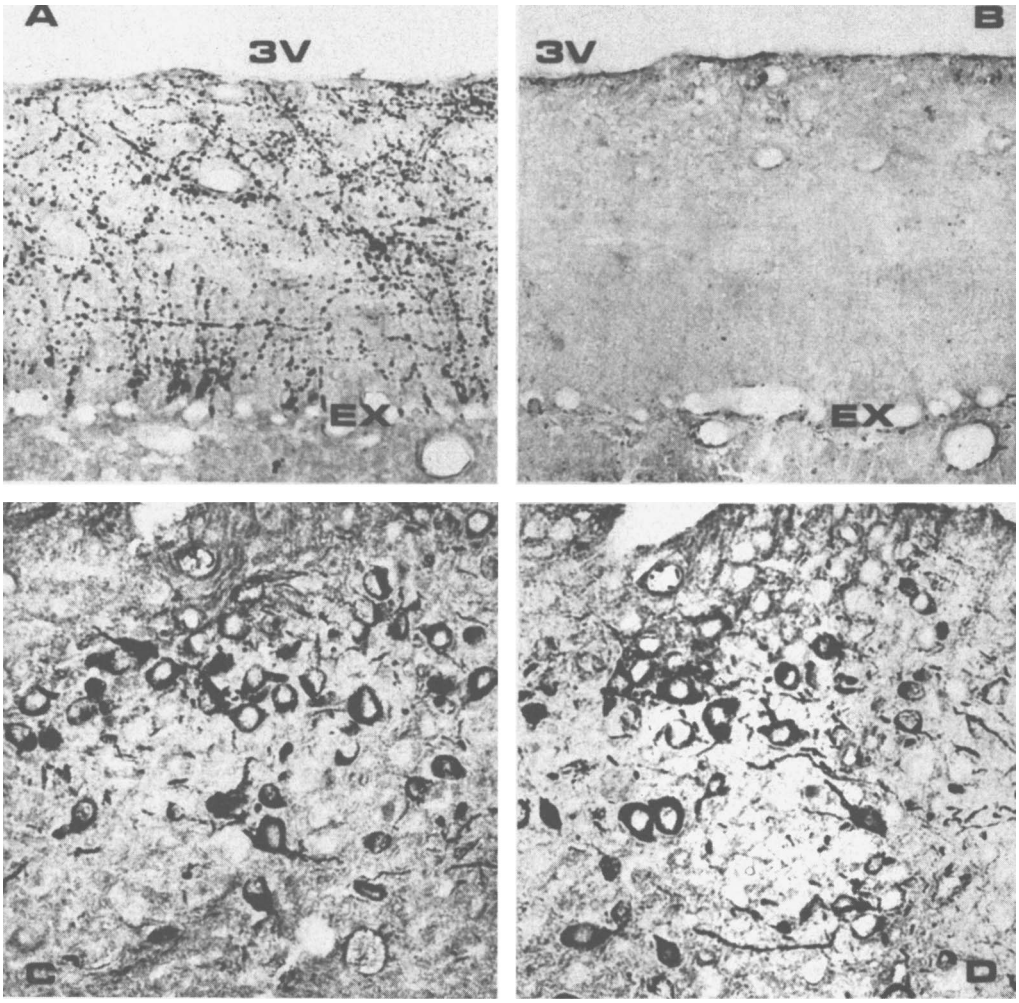

FIG. 2. A and B. Sagittal sections. EX, external zone; IZ, internal zone; 3V, third ventricle. A was incubated with 1/100 anti-LHRH, and B with 1/50 anti guinea pig gamma globulins; both were labeled with peroxidase, × 325. Note the complete disappearance of labeled material in the control section. C and D. Artefactual labeling of axons and cell bodies in the arcuate nucleus of male rats. C. Incubation with 1/50 anti-LHRH and peroxidase revelation. D. Incubation with 1/50 anti guinea pig gamma globulins; revelation as under C, × 200.

nence of normal male rats. On sagittal sections, fibers first appear in the most cephalic part of this region, as if they were coming from the mediobasal retrochiasmatic area. They progressively diverge as they enter the anterior median eminence. Further backwards, the portion of the median eminence located in front of the stalk appears to contain scattered labeled fibers; several of them can be traced over a certain distance, running either parallel to the base of the brain, or radially from the segment adjacent to the third ventricle towards the external layer of the median eminence (Fig. 2A), where they seem to terminate at the level of the portal plexus. Finally, a dense population of antibody labeled granules can be seen at the beginning of the posterior median eminence back to the pituitary stalk (Fig. 1A).

At higher magnifications (Fig. 2A), stained granules of two different sizes can be distinguished. The most anterior tuberal fibers and the portion located close to the stalk contain rather large granules, with a marked accumulation close to blood vessels. These elements look like

axon terminals. In the middle portion, on the contrary, smaller, lined up granular elements produce regularly spaced thickenings in the course of antigen-containing axons.

A surprisingly high quantity of fibers seem to run very close to the third ventricle, in the internal zone of the median eminence (Fig. 2A).

On frontal cuts, the distribution of labeled material is quite medial in the retrochiasmatic region; at the level of the dorsal lip of the third ventricular recess, it appears predominantly paramedial (about 500 μm lateral to the midline) (Fig. 1B).

The specificity of our cytoimmunological technique appears fairly satisfactory, since no labeling of immunoreactive fibers or terminals in the median eminence was induced in adjacent sections treated either with DAB alone (to check a possible interference of endogenous peroxidase activity), or with normal serum (Table I, Group 1) or with an anti-LH antiserum (Table I, Group 10). Prior absorption of the anti-LHRH activity by incubation of the antiserum with the synthetic antigen prevents labeling of the polypeptide containing axons (Table I, Group 8). In addition, incubation of alternate sections with rabbit anti guinea pig gamma globulin has proved a very valuable way to check eventual artefactual reactions. Under our experimental conditions, we felt that this countercheck was imperative, since our anti-LHRH antibody was obtained by immunizing rabbits with synthetic LHRH bound to guinea pig gamma globulin in order to increase its antigenicity. Median eminence fibers treated with such anti-gamma globulins have proved totally unreactive (Fig. 2B and Table I, Groups 2 and 3). However, after this treatment, reactions can be shown to occur at other levels of the hypothalamus or even other brain areas, both in fiber tracts and in cell bodies (Fig. 2, C and D, and Table I, Groups 2 and 3). The probability of obtaining artefacts of that kind increases with the concentration of the antiserum (Table I, Groups 4–5 and 6–7). It is further enhanced upon pretreatment of the animals with colchicine (Table I, Group 12) (50 μg infused into the lateral ventricle 24 or 48 hr before sacrifice), possibly as a consequence of

TABLE I. Specific and Background Peroxidase Labeling of Hypothalamic Neurons after Different Pretreatments of Histological Sections and Incubations with Various Antisera[a].

Group	Pretreatment	Treatment	Dilution of serum	Specific labeling of median eminence axons	Occurrence of non specific labeling[b]	Background labeling[c]
1	0	Normal rabbit serum	1/50	0	0	0
2	0	A-αG	1/50	0	+	+
3	HCl	A-αG	1/50	0	±	±
4	0	A-LHRH	1/50	++	+	++
5	0	A-LHRH	1/100	+	±	++
6	HCl	A-LHRH	1/50	+++	±	+
7	HCl	A-LHRH	1/100	++	0	±
8	HCl	A-LHRH Absorbed with LHRH	1/50	0	±	+
9	HCl	A-LHRH Absorbed with cortex	1/50	++	0	0
10	HCl	Anti-LH	1/200	0	0	0
11	Trypsin	A-LHRH	1/50	++	+	+
12	Colchicine[d]	A-LHRH	1/50	±	+	+

[a] See text for description of experimental procedure.

[b] This refers to eventual nonspecific labeling of fiber tracts or perikarya within various brain structures.

[c] Overall background staining of the sections.

[d] Castrated males.

upstream accumulation of nonspecific protein material induced by the drug, which has been shown to inhibit axonal transport (10). Under such conditions, the antigenic material present in the median eminence almost completely disappeared, a result which could be expected on the basis of the above mentioned impairment of axonal flux. In contrast to this and to other reports from the literature(11)no specific immunoreactive material could be seen within perikarya of any hypothalamic structure in colchicine treated animals; labeling of fibers and cell bodies obtained under such conditions (Table I), in particular in the arcuate nucleus (Fig. 2C), the septum and around the anterior commissure, could in all cases be rejected as nonspecific, since similar labeling resulted from incubation of the slides with anti-gamma globulins only (Fig. 2D). On the basis of these data, we think that the localization of postulated LHRH cell bodies recently reported by other authors (12) in anterior and dorsal hypothalamic regions has to be considered with caution. In our opinion, this reservation is further substantiated by the fact that, in that study, cell bodies could only be detected by applying relatively high concentration of the antiserum to sections from colchicine-treated animals, and that no systematic specificity countercheck with antibodies directed against the carrier protein seems to have been carried out.

That immunoreactive material could not be visualized in cell bodies under our experimental conditions is probably due to insufficient concentration of the antigen at the level of the perikaryon. LHRH is very likely present at much higher concentrations within terminals of peptidergic neurons than in the perikaryal or axonal cytoplasm (6, 13). If this were the case antibodies against LHRH much more potent than those now available would probably permit identification and localization of the hypothalamic cell bodies involved in the biosynthesis of LHRH.

In an attempt to delineate the origin of nonspecific binding or background staining, we have observed that prior absorption of the antibody with the 17,000*g* supernatant obtained from cerebral cortex fractionated according to the technique described by Whittaker (14) was effective in decreasing both nonspecific labeling of various cell bodies and background staining (Table I, Group 9). In contrast, the specific reaction within the median eminence was only slightly decreased in this experimental group, probably as a consequence of a minor loss of antigenic potency of the serum in the course of the absorption procedure. This suggests that the background and the nonspecific labeling observed on sections incubated with the crude antiserum are likely to result from cross reactions of brain proteins with undetermined binding sites present in our gamma globulin population. Similar cross reactions have also been observed in crude subcellular fractions of the brain (Ramirez, Gautron, Epelbaum, Pattou and Kordon, unpublished observations). In that case HCl pretreatment of such fractions obtained from basomedial hypothalamic fragments precipitated the cross reacting material and yielded a better correlation between radioimmunoassay and bioassay data. In the present study, a similar explanation is likely to account for the decrease in nonspecific binding obtained by preincubation with 0.05 *M* HCl (Table I, Groups 6 and 7 as compared to Groups 4 and 5), an effect also reported in other studies (12).

Fixation of the tissues with Bouin–Hollande, even in the absence of acetic acid, does not seem a favorable condition for cytoimmunological visualization of LHRH; under our experimental conditions, we did not observe any significant labeling with this technique. Paraformaldehyde and picroformol appear, on the contrary, equally fit for this determination. Long postfixation periods have an impairing effect on the penetration of the antibody or the availability of the antigenic sites, but this is mainly due to an excess of precipitated proteins at the surface of the cells, since binding to specific antibodies can be reactivated upon preincubation of the slides with trypsin (Table I, Group 11). However, the cells are markedly damaged by this procedure and the specificity of the immunological reaction is not as good as in the absence of enzymatic hydrolysis.

In addition, the intensity of both peroxidase and fluorescence revelation of LHRH is enhanced by prior incubation with HCl. This suggests that, in median eminence terminals, the polypeptide is either partially bound to proteic material or to membranes, or that its antigenicity is masked by HCl-extractable material.

Summary. Cytoimmunological localization of LHRH-containing axons and terminals in the median eminence of male rats was achieved with a potent anti-LHRH antiserum and subsequent

labeling with a second, peroxidase– or fluorescein isothiocyanate-bound, anti-gamma globulin antibody. The distribution of peptidergic fibers extends from the rostral part of the median eminence, immediately behind the retrochiasmatic area, to the region of the pituitary stalk. In the external zone of the rostral median eminence and close to the stalk, the antigenic material appears in the form of large granules accumulated around blood vessels. This distribution suggests that such granules are likely to represent axon terminals, in contrast to axonal processes containing smaller granular elements lined up radially or longitudinally underneath the external zone.

Specific LHRH labeling could not be visualized at the level of perikarya, even in castrated and colchicine-treated animals; however, nonspecific staining was eventually observed within cell bodies or fiber tracts throughout the hypothalamus as well as other brain structures, particularly after colchicine treatment and incubation with relatively high concentrations of antisera. It thus appears that, under our experimental conditions, only distal portions of LHRH producing neurons can be detected by immunocytochemistry.

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