

Localization of Luteinizing Hormone-Releasing Hormone (LHRH) Neurons That Project to the Median Eminence

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The neuropeptide, luteinizing hormone-releasing hormone (LHRH), is released from nerve terminals in the median eminence and carried via the hypophysial portal system to the anterior pituitary, where it stimulates the release of gonadotropins. LHRH-containing neurons are located in many different regions of the rodent brain, including olfactory, septal, preoptic, and hypothalamic structures. Since those LHRH neurons that project to the median eminence form the final common pathway for the regulation of the pituitary/gonadal axis, we wished to determine which of these cell groups are afferent to this structure. A retrograde tracer, the lectin wheat germ agglutinin (WGA), was placed directly on the exposed surface of median eminence. Following survival times of 8–13 hr, brains were prepared for the dual immunocytochemical detection of WGA and LHRH. Approximately 50% of the LHRH neurons from the level of the septal nuclei caudalward were found to contain WGA immunoreactivity and therefore to project to the median eminence. The remaining single-labeled LHRH neurons were intermingled with the double-labeled cells. The 2 populations were not distinguishable from each other on either cytological or cytoarchitectonic criteria. Those LHRH neurons that were not retrogradely labeled following an injection of tracer into the median eminence are presumed to project to other regions of the central nervous system. We conclude that the LHRH neurons that are directly involved in the regulation of reproductive function are very heterogeneous, widely scattered in telencephalic and diencephalic regions.

The decapeptide, luteinizing hormone-releasing hormone (LHRH), when released from neurosecretory terminals in the median eminence, stimulates the release of both gonadotropins LH and FSH from the anterior pituitary (Schally et al., 1971). The distribution of neurons within the central nervous system that are immunoreactive for LHRH has been described for many species, including the rat (for reviews, see Setalo et al., 1976; Sternberger and Hoffman, 1978; Witkin et al., 1982; Krey and Silverman, 1983; Shivers et al., 1983a; Liposits et al., 1984). The majority of LHRH-immunoreactive cells are located in

septal, preoptic, and hypothalamic regions, forming a loose continuum from the level of the diagonal band of Broca, including both its vertical and horizontal limbs, the medial and triangular septal nuclei, and periventricular, medial, and lateral preoptic and anterior hypothalamic areas. Some LHRH cells are found rostral to the supraoptic nucleus, others in the retrochiasmatic zone, just medial to the optic tract. A few LHRH neurons are seen within the circumventricular organs, i.e., the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ. Finally, LHRH neurons are associated with the accessory olfactory bulb and other olfactory-related structures, including the nervus terminalis. Although the several pathways that LHRH fibers take from anterior structures to the neurosecretory terminus in the median eminence have been identified (Merchen-thaler et al., 1980, 1984; Kawano and Daikoku, 1981; Hoffman and Gibbs, 1982; King et al., 1982), precisely which LHRH cells contribute to the innervation of this structure is not known.

Since release of LHRH from terminals in the median eminence is the source of this neurohormone in the portal vasculature and hence the final common pathway for the regulation of the pituitary/gonadal axis (see Krey and Silverman, 1983), it is critical to our understanding of the CNS regulation of reproductive physiology to determine the location of the LHRH-containing median eminence afferents. To this end, we placed a tracer protein, the lectin wheat germ agglutinin (WGA), onto the median eminence and combined its immunocytochemical localization with that for LHRH in the same tissue section, using a double-label procedure. A preliminary report of this study has appeared (Silverman and Renaud, 1985).

Materials and Methods

Ten adult male Sprague-Dawley rats weighing 250–300 gm received injections of WGA. Of these, 5 injections were made into the median eminence. To control for spread into the adjacent brain tissue, 3 injections were made into the arcuate nucleus. Since neurosecretory neurons might have access to material in either the cerebrospinal fluid or peripheral blood, injections of relatively large quantities were made into the lateral ventricle and peripheral vasculature. For those injections onto the ventral surface of the CNS, animals were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and the median eminence region exposed using a transpharyngeal surgical approach (Renaud, 1976). WGA (EY Labs; 10% wt/vol in saline) was administered directly into the external zone of the median eminence by pressure injection of nanoliter amounts using glass micropipettes of 20–40 μ m outer tip diameter and a picospritzer (General Valve, NJ). Animals survived under continuing intravenous anesthesia for 8–13 hr and were then perfused transcardially with Zamboni's fixative (buffered picric acid and paraformaldehyde). Brains were dissected and shipped in buffer or fixative by overnight airmail from Montreal to New York.

An intraventricular injection of 1 μ l of the WGA solution was made under pentobarbital anesthesia into the left lateral ventricle, using ste-

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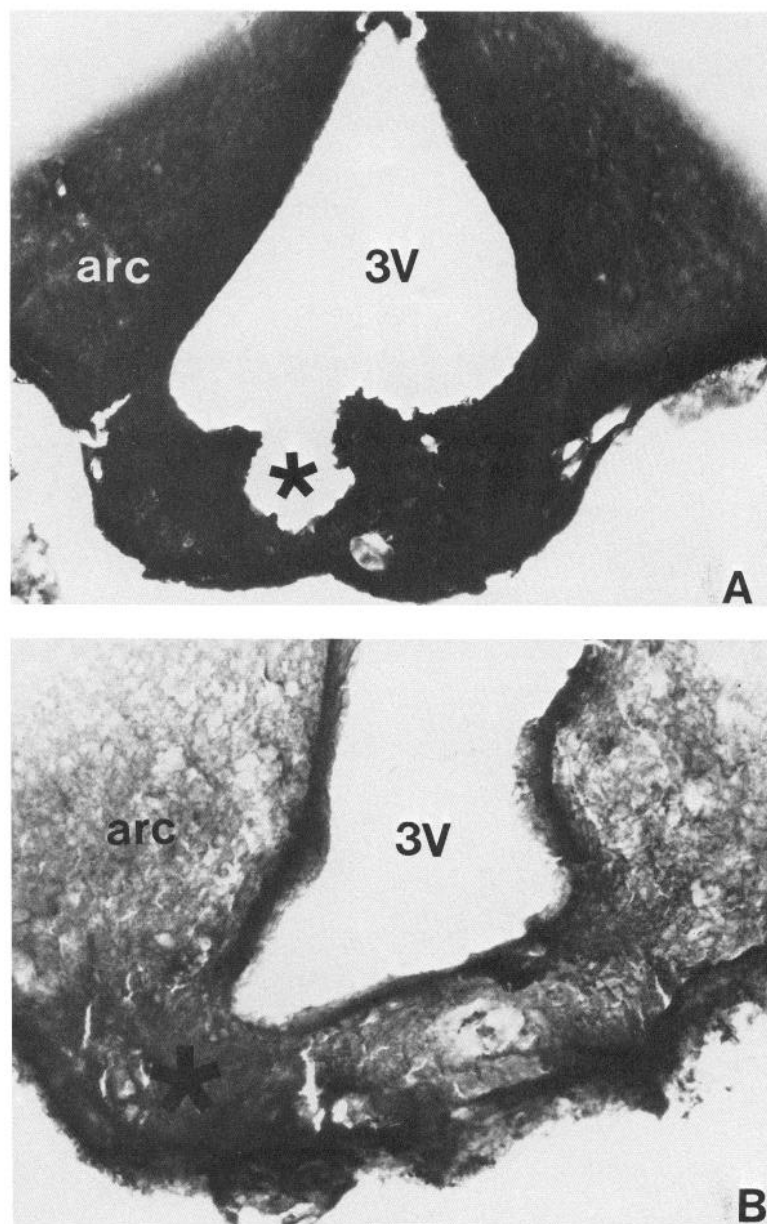


Figure 1. Low-power photomicrographs of 2 different injection sites into the median eminence, as demonstrated by immunocytochemical localization of the WGA. In *A*, the lectin has spread throughout the median eminence and ventral arcuate nucleus (*arc*), although the injection was made in the central median eminence at the site marked by the asterisk. The reaction was so intense that the tissue is disrupted. In *B*, a smaller injection was made at a slightly more lateral site. 3*V*, Third ventricle. $\times 50$.

reotaxic coordinates from Pellegrino et al. (1979); these were AP -1.2 , ML $+1.0$, and DV (from dura) -4.0 . An injection of $100 \mu\text{l}$ of WGA (1% wt/vol in saline) was made into the jugular vein under methoxyflurathane (Metaflane; Pittman Moore) anesthesia. Two animals survived for 24 hr after administration of the lectin before perfusion with fixative.

Fixation by immersion was continued for 48–96 hr and sections were then cut with a Vibratome (Lancer) at a thickness of $50\text{--}60 \mu\text{m}$. Section thickness varied slightly from brain to brain and within a particular brain because of limitations of the Vibratome itself and the degree to which the brain had hardened following fixation. Vibratome sections were used because they provided a relatively easy means of surveying the entire forebrain of each animal. They also produced excellent preservation of neuronal morphology. Sections were washed in several changes of buffer (0.1 M phosphate buffer, pH 7.3, used throughout) and then exposed to 0.5% hydrogen peroxide for 30 min to reduce endogenous peroxidase activity. This was followed by extensive buffer washes, the final one containing 1% normal goat serum and 0.1% Triton X-100. All sections of every animal were then incubated in a rabbit antiserum to WGA (1:1500; EY Labs) for 48 hr and the antigen localized using a biotinylated goat anti-rabbit IgG, an avidin-biotin-HRP conjugate (Vectastain), and 3,3' diaminobenzidine (Sigma) as the chromogen. Af-

ter several washes in buffer, sections were placed in the antibody to LHRH (LR-1, the gift of Robert Benoit, McGill University, diluted 1:5000; see Silverman, 1984) for 48 hr. The second antigen was detected using a biotinylated goat anti-rabbit IgG and avidin-fluorescein (Vector Lab). Sections from the most rostral level of the diagonal band through to the median eminence were processed and examined with a Leitz fluorescent microscope with epifluorescent optics and appropriate filters.

To test for the possibility that the fluorescence was due to the binding of the avidin-fluorescein to the first application of the biotinylated second antibody, control sections were incubated in normal rabbit serum rather than in antiserum to LHRH. Although the remaining steps were carried out, no fluorescence was observed in these sections.

All LHRH neurons were counted and mapped, using the atlas of Pellegrino et al. (1979). Photomicrographs were made with Kodak Tri X for fluorescence and Kodak Pan X for bright field.

Results

Injection sites: median eminence

In 5 animals, the WGA immunoreactivity covered the entire median eminence, including the lateral zone above the tubero-

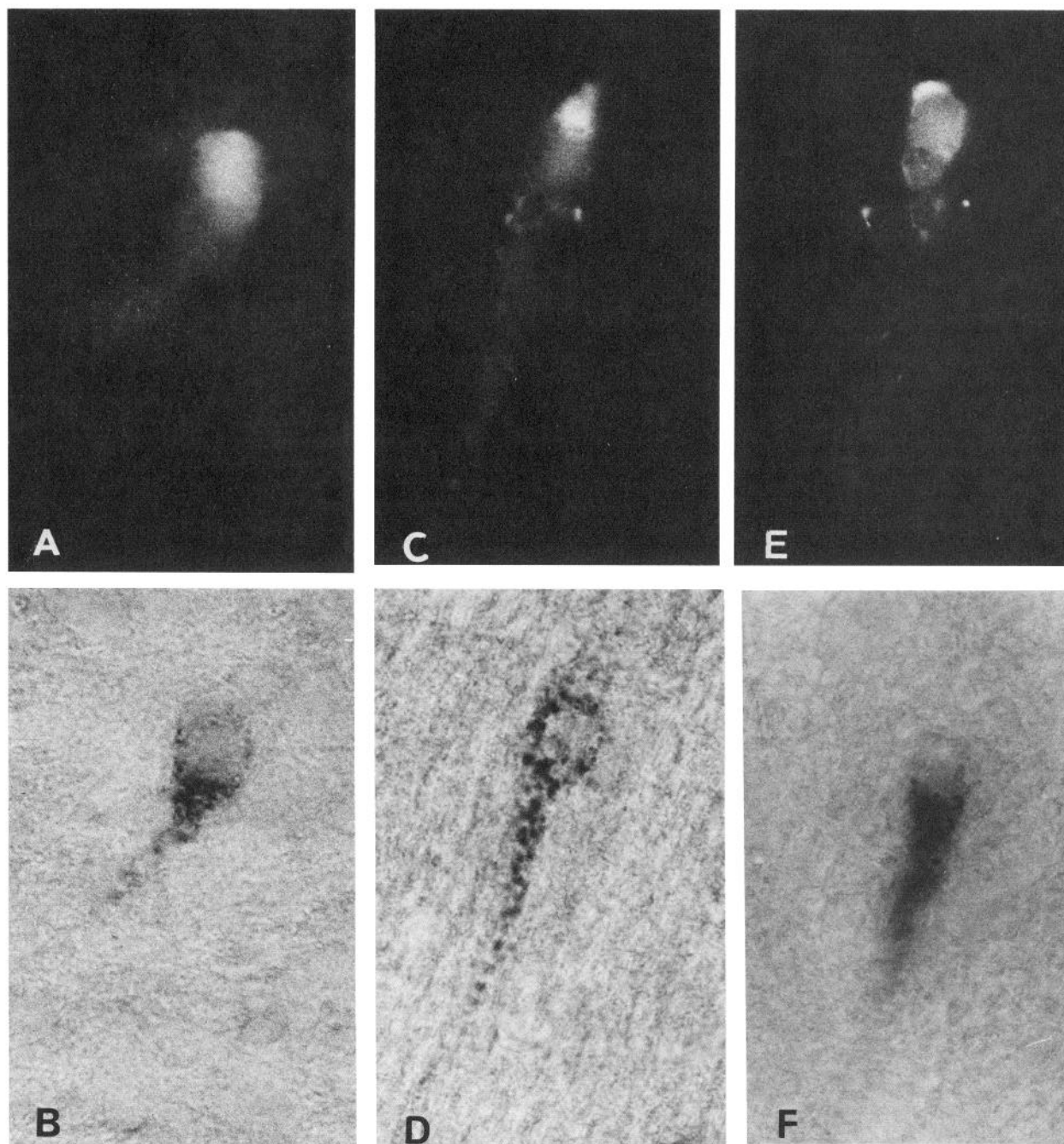


Figure 2. *A–F*, Photomicrographs of LHRH neurons that also contain retrogradely transported WGA. *Top panel (A, C, E)* shows immunofluorescent LHRH neurons; *bottom panel (B, D, F)* shows the WGA immunoreactivity in each of the same cells. Cells in *A* and *E* are smooth in contour, while those in *C* are more spinous. The spines are not well illustrated in the photomicrograph, as they were not as intensely fluorescent as the cell body. $\times 400$.

infundibular sulci, where most of the LHRH fibers terminate (Setalo et al., 1976) (Fig. 1, *A, B*). In most animals there was obvious diffusion of lectin into the ventral portions of the arcuate nucleus (Fig. 1*A*), but no WGA was detected in the posterior pituitary.

Distribution of median eminence afferents

WGA immunoreactivity appeared in neuronal perikarya, proximal dendrites, and some axons as distinct brown dots indicative of the vesicular structures within which the lectin is transported from the terminal (Fig. 2, *A, C, E*). These labeled vesicles were

distributed quite uniformly in the cytoplasm of the perikaryon and proximal dendrites. The immunofluorescence for the neuropeptide filled the cytoplasmic compartments (Fig. 2, *B, D, F*), leaving the nucleus unlabeled. The distribution of the immunofluorescence within the cell was broader than is indicated in the photomicrographs.

Double-labeled as well as single-labeled LHRH cells were easily detectable and showed no preferential distribution through their septal and diencephalic range. This is shown diagrammatically in Figure 3, *A–D*. For the animal represented in the diagram, 431 LHRH cells were counted, and of these 230 were

double-labeled. The number of LHRH cells visualized per brain varied from 139 to 431 but in all cases approximately 50% (range, 45–59%; see Table 1) of the LHRH cells were double-labeled, i.e., they contained WGA immunoreactivity.

As can be seen from Figure 3, neurons immunoreactive for both LHRH and the lectin and those containing only the neuropeptide shared all of the cytoarchitectonic divisions from the level of the diagonal band caudalward to the retrochiasmatic zone, and were frequently in close proximity to one another. LHRH neurons can be separated into 2 major morphological categories: smooth and thorny (Wray and Hoffman, 1987). Double-labeled LHRH cells were of either type (Fig. 2, *A*, *C*) and thus could not be distinguished from LHRH cells that did not innervate the median eminence on the basis of their cellular morphology.

Control injections

In the 2 animals with the largest injections of WGA into the arcuate nucleus and medial basal hypothalamus, only a small proportion of the total number of LHRH cells also contained the lectin (7 out of a total of 300 and 15 of a total of 230 LHRH-containing neurons). These double-labeled neurons were found predominately in the medial and ventral aspects of the preoptic area. In the third animal with an arcuate nucleus injection, some of the lectin solution also entered the ventricular space. In this animal, LHRH cells that also contained WGA were more widely dispersed but were similarly few in number (24 out of 391). Following an injection of WGA into the jugular vein, which retrogradely labeled neurons in the supraoptic and paraventricular nuclei (presumably via their terminals in the neural lobe), no LHRH neurons contained detectable levels of WGA immunoreactivity (out of a total of 247 LHRH-containing neurons). Following a WGA injection into the lateral ventricle, one double-labeled cell was found in the diagonal band.

Discussion

The results presented here indicate that a minimum of 50% of LHRH neurons in septal, preoptic, and hypothalamic structures innervates the median eminence. Judging from injections made into the contiguous arcuate nucleus, only a small percentage of these lectin-containing LHRH cells could be afferent to other medial basal hypothalamic structures. Similar results have been obtained in both intact and castrated male mice following injection of large quantities of HRP into the vascular system, using a combination of HRP histochemical detection and the immunofluorescent localization of LHRH (Jennes and Stumpf, 1986). These latter experiments would identify LHRH cells that terminate in any structure outside of the blood-brain barrier, including, but not exclusively, the median eminence. In earlier studies that analyzed afferent projections to the median eminence without regard for their chemical identity, Wiegand and Price (1980), using applications of HRP or I^{125} WGA, identified retrogradely labeled neurons in several periventricular and medial hypothalamic cell groups and, in small numbers, in the ventral preoptic area dorsal to the OVLT. A more extensive distribution, very similar to that reported here, was found by Lechan et al. (1982) following application of WGA to the median eminence and the immunocytochemical detection of the lectin. These authors reported retrogradely filled neurons present in many hypothalamic, preoptic, and septal architectonic regions.

In our current experiments, LHRH neurons that were double-labeled and, therefore, innervated the median eminence were

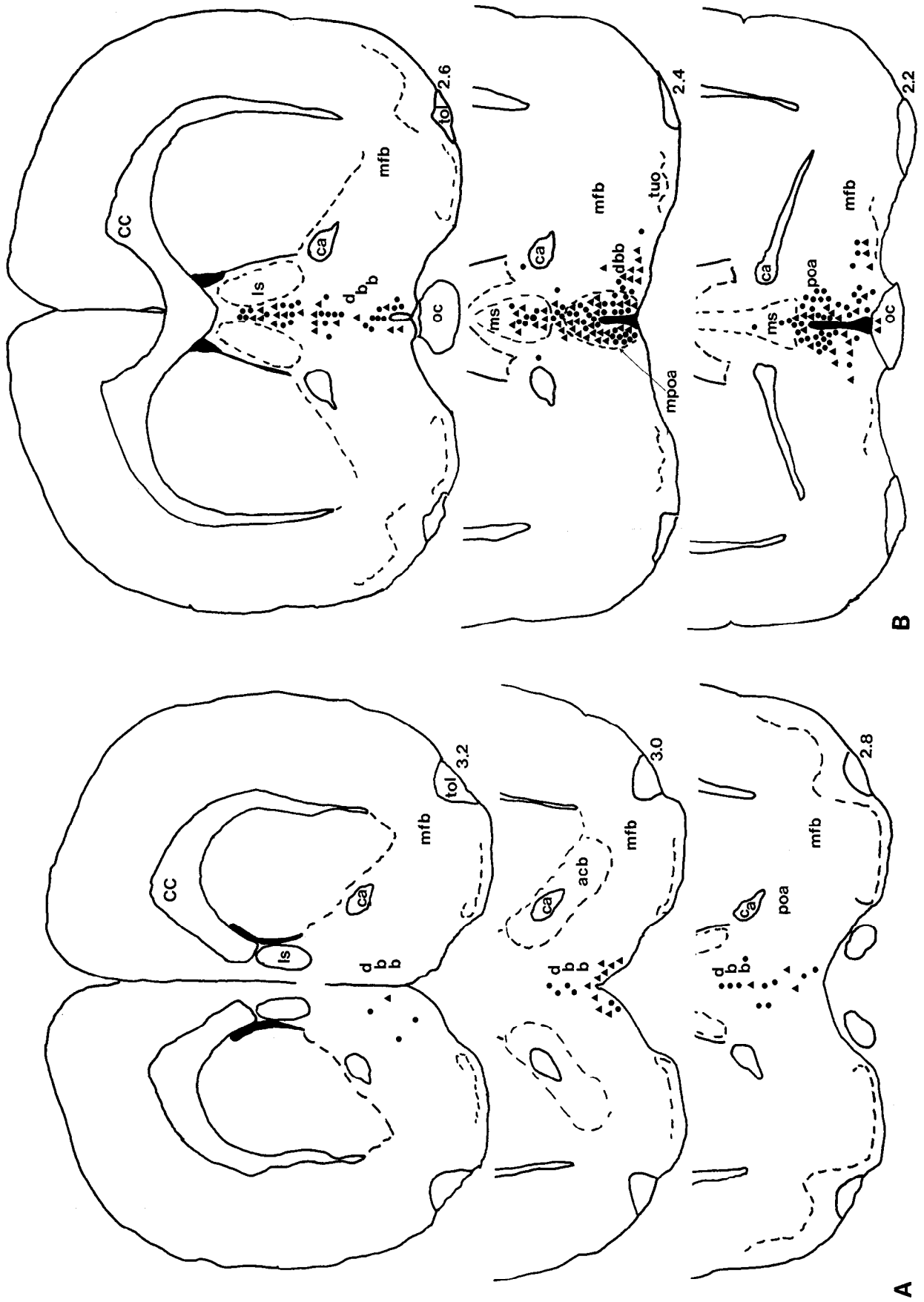
Table 1. Number of LHRH neurons containing WGA after injections of the lectin into brain tissue, cerebrospinal fluid, or vascular system

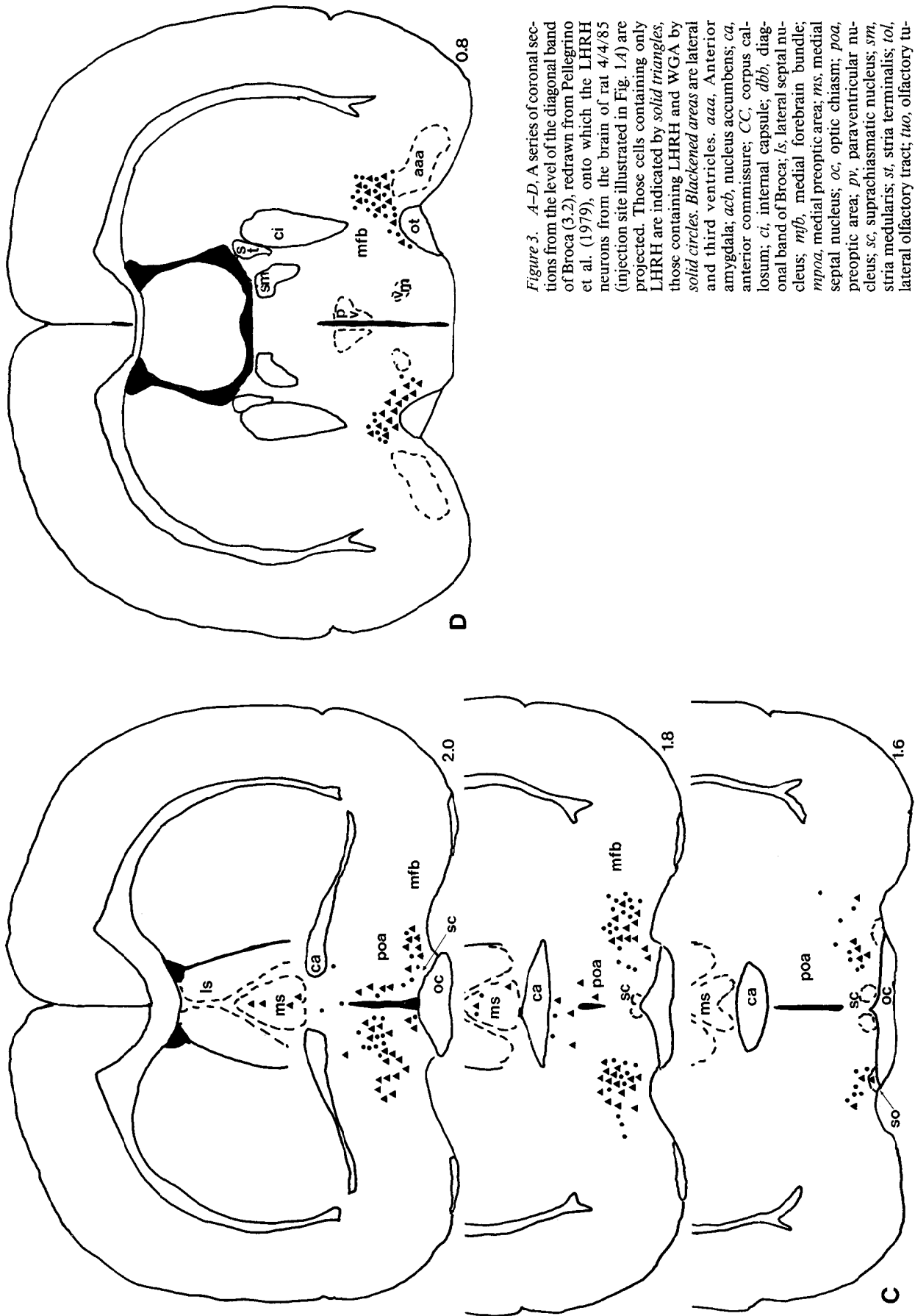
Injection site	LHRH neurons containing WGA	LHRH only
Median eminence	124	125
Median eminence	101	70
Median eminence	165	193
Median eminence	62	77
Median eminence	230	201
Arcuate nucleus	7	300
Arcuate nucleus	30	230
Arcuate nucleus	24	391
Lateral ventricle	1	260
Jugular vein	0	247

not organized into distinct clusters of readily identifiable cell groups, nor were they distinguishable, using morphological criteria, from LHRH neurons that were not double-labeled. Two questions need to be addressed: (1) Do LHRH neurons in all of these regions contribute significantly to the control of gonadotropin secretion? (2) What is the role of LHRH neurons that are not doubly labeled?

It has been well established in the rodent that electrical or electrochemical stimulation of the preoptic/anterior hypothalamic area results in LHRH release and an elevation in circulating gonadotropins that, in females, can induce ovulation (Everett, 1964; Kubo et al., 1975; Fink and Jamieson, 1976; Eskay et al., 1977). Experiments with bilateral deafferentations (Blake and Sawyer, 1974) or lesions (Brown-Grant et al., 1977; Wiegand et al., 1980) strongly suggest that preoptic nuclei contribute significantly to the neurogenic signal for gonadotropin secretion. There remains the question, Are all of the LHRH neurons that send axons to the median eminence essential for control of gonadotropin secretion? For example, in the guinea pig, placement of lesions in the septal region (which in this species did not reduce median eminence LHRH) or in the preoptic area (destroying a considerable percentage of LHRH axons terminating in the median eminence) did not interfere with tonic or cyclic gonadotropin release, while destruction of the medial basal hypothalamus and the LHRH cells therein disrupted both patterns of gonadotropin release (Krey and Silverman, 1978, 1981). This question as to the role of various LHRH neurons has recently been addressed in the rat by Koves and Molnar (1986). These authors found that interruption of LHRH pathways from septal regions decreased median eminence LHRH by 30%, but did not interfere with ovulation. Similarly, unilateral cuts at the level of the retrochiasmatic area, which decreased LHRH in the median eminence by 50%, did not interfere with the occurrence of ovulation. Only bilateral cuts at the retrochiasmatic or suprachiasmatic levels, which resulted in a 70–90% depletion of LHRH at the neurosecretory terminus, also resulted in subjects with persistent estrus and polyfollicular ovaries. These knife-cut studies substantiate our findings that septal LHRH neurons do project to the median eminence, but suggest that these particular immunoreactive cells are not essential for cyclic gonadotropin secretion.

It is possible that we have underestimated the percentage of median eminence-projecting cells. The sources of error could be (1) application of insufficient amounts of tracer, (2) lack of





sensitivity of the immunocytochemical detection of the WGA, or (3) insufficient uptake by the LHRH terminals in the median eminence. As is evidenced by our injection sites, we covered the entire median eminence region with several separate injections of WGA so that availability of tracer is unlikely to have been a problem. The sensitivity of the method was assessed in preliminary experiments in which WGA conjugated to HRP was applied to the median eminence in similar quantities, and cells were localized using the sensitive tetramethylbenzidine (TMB) procedure of Mesulam (1976). The number of retrogradely filled cells found in the septal/preoptic area was similar with both methods. These latter findings suggest that the immunocytochemical detection of WGA is at least as sensitive as the TMB histochemical detection of WGA-HRP. The final possible source of error, insufficient uptake and transport from the terminal, is the most possible. Given the surgical procedure used to expose the median eminence, survival times were limited. Nonetheless, our results are in very good agreement with those of Jennes and Stumpf (1986), in which tracer was available to nerve terminals via the circulation for an extensive period of time. It seems possible to conclude, therefore, that some LHRH neurons that were not retrogradely labeled have projections to other regions of the CNS (see below). We cannot, however, completely rule out the possibility that there is a greater percentage that project to the median eminence.

There was considerable variability from animal to animal in absolute numbers of LHRH-containing cells that were immunofluorescent, although the percentage that contained WGA was quite consistent. The most likely source of variability in LHRH neuronal numbers was the quality of fixation of the tissue. The stress of the surgery necessary to expose the injection site may have led to a state of shock, resulting in cardiovascular insufficiencies and to less than perfect perfusions. Brains required differing times of fixation by immersion for sectioning to be carried out, and longer exposure to the fixative may well have reduced the LHRH immunoreactivity.

There does not seem to have been any quenching of the immunofluorescence by the presence of the diaminobenzidine (DAB) reaction product used for the detection of the WGA immunoreactivity. We have recently checked the problem of quenching by carrying out an identical double-label procedure to localize neurophysin and either oxytocin or vasopressin, which are present within the same precursor protein and therefore within the same secretory granule. The presence of DAB for the visualization of the neurophysin did not block the fluorescent signal for the nonapeptide (A. Hou-Yu, A. J. Silverman, and D. D. Kelly, unpublished observations).

Even within the population of LHRH-containing cells that innervated the median eminence, there was considerable morphological heterogeneity. Double-labeled LHRH neurons spanned the entire continuum from smooth-surfaced to encrusted with somatic spines. It has been suggested (Jennes and Stumpf, 1986) that the LHRH cells with spines receive a denser innervation and are, therefore, presumably regulated by different mechanisms than those of smooth contoured cells. It would also seem obvious that LHRH cells in various regions (e.g., septal versus preoptic) would have differing synaptic inputs. How the different populations of LHRH neurons that project to the median eminence coordinate their activity is not known.

It is well established that gonadotropin secretion occurs in a pulsatile manner (Dierschke et al., 1970). It is supposed that

pulses of LHRH are released from the median eminence to drive the anterior pituitary. Bursts of multiunit electrical activity, which correspond 1 for 1 with a pulse of LH (Wilson et al., 1984) can be recorded from medial basal hypothalamic regions rich in LHRH axons (Silverman et al., 1986) in the rhesus monkey. This suggests that there does exist a coordinated bursting mechanism within the CNS (the "pulse generator") that drives pituitary secretion. Whether the scattered LHRH neuronal system, as described here, accounts for the electrical activity is not known.

If 50% of the LHRH neurons, as shown in Figure 2, project to the median eminence, what of the other 50%? It has been suggested by many authors (cf. Eiden and Brownstein, 1981) that LHRH can be a neurotransmitter and/or a neuromodulatory substance within the CNS, and presumably this second population would form the anatomical substrate for these effects. It is likely that some of the single-labeled LHRH cells project to extrahypothalamic sites. Numerous studies have shown that LHRH fibers from the preoptic area form the dense innervation of the OVLT, while other LHRH fibers leave the preoptic area and bed nucleus of the stria terminalis to project via the stria terminalis to the medial amygdala (cf. Krey and Silverman, 1978; King et al., 1982; Witkin et al., 1982; Merchenthaler et al., 1984). LHRH axons from lateral hypothalamic cells enter the amygdala via a ventral pathway (Leonardelli and Poulain, 1977). Additional LHRH fibers of septal and/or preoptic origin course into the epithalamus and then enter the fasciculus retroflexus to project into the midbrain (Witkin et al., 1982; Merchenthaler et al., 1984). The exact number of cells that contribute to these and other LHRH pathways in the CNS will have to be analyzed with a double-label procedure similar to that described here. It is possible that certain LHRH neurons will be found to project to both the median eminence and to other central sites, as has been proposed for some of the tubero-infundibular neurons located in the mediobasal hypothalamus (Renaud, 1981).

Ultrastructural studies have suggested that some LHRH neurons form local circuit connections with nonimmunoreactive neurons, as well as with LHRH elements of the diagonal band and preoptic areas (guinea pig: Silverman, 1984; rat: Leraneth et al., 1985; Witkin and Silverman, 1985). It is, of course, not known if these synaptic interactions are formed by a group of LHRH neurons separate from those that innervate the median eminence. In this context it is interesting to note that estradiol, which represents one of the major modulators of LHRH release, is not concentrated in LHRH neurons but in other neurons of the preoptic area (Shivers et al., 1983b). It is possible that some of the local circuits (synapses made by LHRH axons within the preoptic area) represent a feedback between estradiol-concentrating neurons and LHRH neurosecretory cells.

In summary, LHRH neurons that form the final pathway for the regulation of gonadotropin secretion are widely distributed in the rat CNS. The contributions made by each separate group to the regulation of gonadotropin secretion are unequal (Koves and Molnar, 1986). The mechanisms by which all of these separate groups are coordinated are still to be determined.

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