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Efferent Projections from the Ovarian Steroid Receptor-Containing Area of the Ventrolateral Hypothalamus in Female Guinea Pigs

K. H. N. Ricciardi*, J. C. Turcotte*, G. J. De Vries and J. D. Blaustein
Neuroscience and Behavior Program and Psychology Department, University of Massachusetts, Amherst, MA 01003, USA.

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Abstract

The ventrolateral hypothalamus (VLH) in female guinea pigs includes a subset of neurons which contain estrogen and progestin receptors, and which are implicated in the regulation of female sexual behavior by steroid hormones. However, little is known about where these neurons project, and consequently which other brain areas are involved in sexual behavior in female guinea pigs. The anterograde tracer *Phaseolus vulgaris*-Leucoagglutinin was used to label efferents from the ovarian steroid receptor-containing part of the VLH. To identify the correct placement of the tracer specifically within the group of neurons containing estrogen receptors, medial hypothalamic sections were also immunostained for estrogen receptors. Forebrain areas receiving dense projections from the ventrolateral hypothalamus included the bed nucleus of the stria terminalis, medial preoptic area, anterior hypothalamic area, anterior ventromedial hypothalamus, and caudal ventrolateral hypothalamus. The midbrain central gray was also heavily labeled. Moderate innervation was observed in the forebrain in the basolateral amygdala, medial preoptic nucleus, lateroanterior hypothalamic nucleus, dorsal hypothalamic areas, posterior hypothalamus, zona incerta, and in the midbrain interspersed among the central and lateral tegmental tracts. The major efferent pathways from the VLH appeared to travel rostrally through the mediobasal hypothalamus and preoptic area, and caudally via the medial thalamic nuclei and periventricular fiber system. These findings are similar to those of previous studies tracing the efferents from the ventromedial nucleus in rats and from the lateral hypothalamus in guinea pigs. Many of these areas that receive input from the steroid receptor rich area within the VLH are likely to be involved in the regulation of female sexual behavior.

Within the hypothalamus of guinea pigs, there are numerous estrogen and progestin receptor immunoreactive neurons in the area immediately ventral and lateral to the ventromedial nucleus (VMN) (1–5). The ovarian steroid receptor-rich region within the ventrolateral hypothalamus (VLH) extends rostrally just beyond the level of the ventromedial nucleus, caudally concomitant with the ventromedial nucleus, and in its mid-region, extends dorsally up to the fornix (6). The VLH appears to be crucial to the expression of female sexual behavior, since lesions of this area inhibit lordosis (7). Small estradiol implants in the rostral VLH are sufficient to both induce progestin receptors in this area and to prime guinea pigs to express progesterone-facilitated lordosis (6). The VLH in guinea pigs is probably comparable to the ventrolateral VMN in rats, since both areas contain estrogen and progestin receptors and are involved in hormonal regulation of lordosis. (5, 8, 9). However, the efferent projections of this specific area in guinea pigs have not been determined, leaving it unclear which neuroanatomical areas are likely to be influenced by the ovarian steroid hormone-sensitive neurons of the VLH.

A great deal of work has been done in rats to determine the efferent projections from the VMN (10–15), and more specifically its ventrolateral division (16). In guinea pigs, the lateral hypothalamus, including part of the VLH, appears to have similar, but slightly more lateral projections (17). However, this previous anterograde tracing study in guinea pigs, was based on injection sites that were aimed at the cytoarchitecturally-defined area, rather than at the specific region adjacent to the VMN which contains steroid receptor-containing cells.

In the present study, *Phaseolus vulgaris*-Leucoagglutinin (Pha-L) was chosen as an anterograde tracer primarily because labeled axons and terminals are completely filled with Pha-L. As a result fibers of passage can be easily distinguished from terminal fields (18, 19). Pha-L labeling was combined with immunocytochemistry for estrogen receptors in the VLH to verify that injections encompassed the precise area where numerous ovarian steroid receptor-containing neurons are found. Thus, Pha-L permitted precise labeling of the efferent projections from the ovarian hormone-receptor containing region of the guinea pig VLH, a likely site for modulation of the expression of lordosis by estrogens and progestins.

Results

Eleven guinea pigs had Pha-L deposits which overlapped with the estrogen receptor-rich area within the VLH to varying extents.

*K. H. Nielsen Ricciardi and J. C. Turcotte should be considered co-first authors of this manuscript based upon each of their contributions to the work. *Correspondence to:* Jeffrey D. Blaustein, Psychology Department and Neuroscience and Behavior Program, Tobin Hall—Box 37710, University of Massachusetts, Amherst, MA 01003–7710, USA. E-mail: Blaustein@psych.umass.edu

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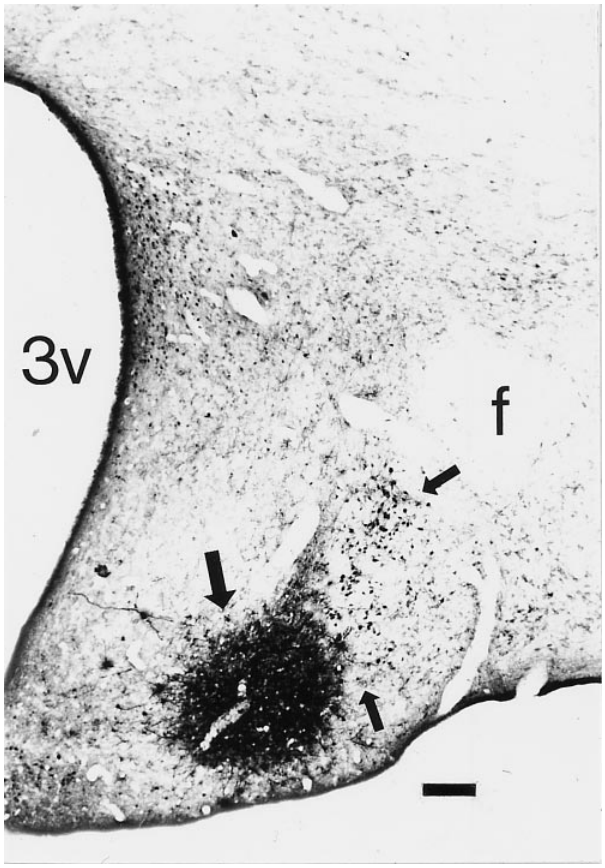

FIG. 2. Photomicrograph showing the Pha-L deposit (large arrow) centered over estrogen receptor-immunoreactive cells (small arrows) in the ventrolateral hypothalamus. Scale bar = 100 μm. For neuroanatomical abbreviations see Fig. 1.

forebrain and the dorsal midbrain. The following areas (from rostral to caudal) received the heaviest projections in terms of the density of putative terminal boutons and varicosities: the bed nucleus of the stria terminalis and medial preoptic area (Figs 3B, C; although in animal 203, the medial preoptic area was only moderately labeled), anterior hypothalamic area (Fig. 3C), lateroanterior hypothalamic nucleus (Fig. 3C), anterior ventromedial hypothalamic nucleus (Fig. 3D), caudal ventrolateral hypothalamus (Fig. 3E), and midbrain central gray (Figs 3G, H).

Moderately dense terminal fields were observed in the medial preoptic nucleus (Fig. 3B; although in animal 203, the medial preoptic area was only heavily labeled), dorsal hypothalamic area, zona incerta, dorsomedial hypothalamus (Fig. 3E), posterior hypothalamus (Fig. 3F), and lateral tegmental tract (Fig. 3G).

Some of the forebrain areas with sparse terminal fields included: the ventrolateral septum and septohypothalamic nucleus, diagonal band of Broca, ventral pallidum (Fig. 3A), the anterior commissure and its posterior part, lateral preoptic area (Fig. 3B), the lateral hypothalamic area, the amygdala at the middle rostrocaudal extent (Fig. 3D), including the basomedial and basolateral cortical amygdaloid areas in and around the central nucleus of the amygdala (Fig. 3D), the paraventricular area of the thalamus, the paraventricular hypothalamic nucleus, the anterior arcuate nucleus, the caudal ventromedial hypothalamic nucleus, the medial lemniscus (Fig. 3E), the parafascicular thalamic nucleus, the area of posterior thalamic nucleus, and the medial mammillary nucleus (Fig. 3F).

In the midbrain, sparse terminal fields were seen in the superior colliculus, retrorubral field (Fig. 3G), medial to the sagulum nucleus, central tegmental tract, and in the general area of the oral part of the pontine reticular nucleus (Fig. 3H).

No evidence of Pha-L labeling was noted in the olfactory bulbs, cerebral cortex, hippocampus, cerebellum or medulla.

Pathways

Areas with fibers of passage with few or no terminals were easily distinguished from areas with terminal fields, where axons branched extensively and had numerous varicosities and/or putative terminal boutons (Fig. 4). The dense terminal fields observed in the bed nucleus of the stria terminalis, preoptic area and anterior hypothalamus (Figs 3B, C) do not seem to originate from a restricted pathway, but rather seem to form one continuous plexus. At the anterior extent of this plexus, fibers appear to ascend through the diagonal band anterior to the anterior commissure toward the bed nucleus of the stria terminalis and septum (Fig. 3A). Some fibers also appear to extend dorsolaterally, toward the region of the bed nucleus of the stria terminalis (Figs 3A, B). Other fibers, some following a medial path and others which may have exited the medial forebrain bundle, form dense terminal fields in the medial preoptic area and anterior hypothalamus (Figs 3B, C). These anterior projections also extend into the amygdala via the substantia innominata and the stria terminalis (Figs 3B, C).

Small but cohesive projections exit the injection site laterally and were observed travelling along the supraoptic commissures into the amygdala extending through the capsule of the central amygdaloid nucleus (Fig. 3D). A few fibers also reach the amygdala by way of diffuse projections through the lateral hypothalamus and into the anterior amygdaloid area (Figs 3C, D).

Other fibers traveling laterally from the injection site seem to take a dorsolateral route into the zona incerta or a lateral route into the medial forebrain bundle (Fig. 3D). Only a few fibers are found in the arcuate nucleus (Figs 3D, E). At the level of the caudal ventromedial nucleus, some fibers form terminals in the region of the ventrolateral nucleus and continue dorsally through the dorsal portion of the ventromedial nucleus and through the dorsomedial nucleus with fibers branching laterally into the zona incerta, medial lemniscus and lateral hypothalamus (Figs 3E and 5A, B). More caudally, a small group of fibers curves laterally around the end of the cerebral peduncle (Fig. 5C) and branches into the ventrolateral tegmentum in the midbrain (Figs 3F and 5D). These ventrolateral fibers seem to enter the midbrain through the region of the peripeduncular nucleus where they form a small number of terminals. This pathway (Figs 5A–D) contains only a few labeled fibers traveling medially toward the midbrain central gray. However, these fibers appear to be joined by a more significant number of fibers entering the lateral tegmental tract via a more dorsal route above the medial geniculate nucleus and by fibers from the region of the posterior hypothalamus entering the midbrain dorsal to the medial lemniscus. These groups of fibers form terminals along the ventral border of the superior colliculus and along a medial trajectory terminating in the lateral midbrain central gray. A few of these fibers seem to continue caudally through the lateral midbrain central gray.

The medial group of fibers leaving the VLH travelling in the

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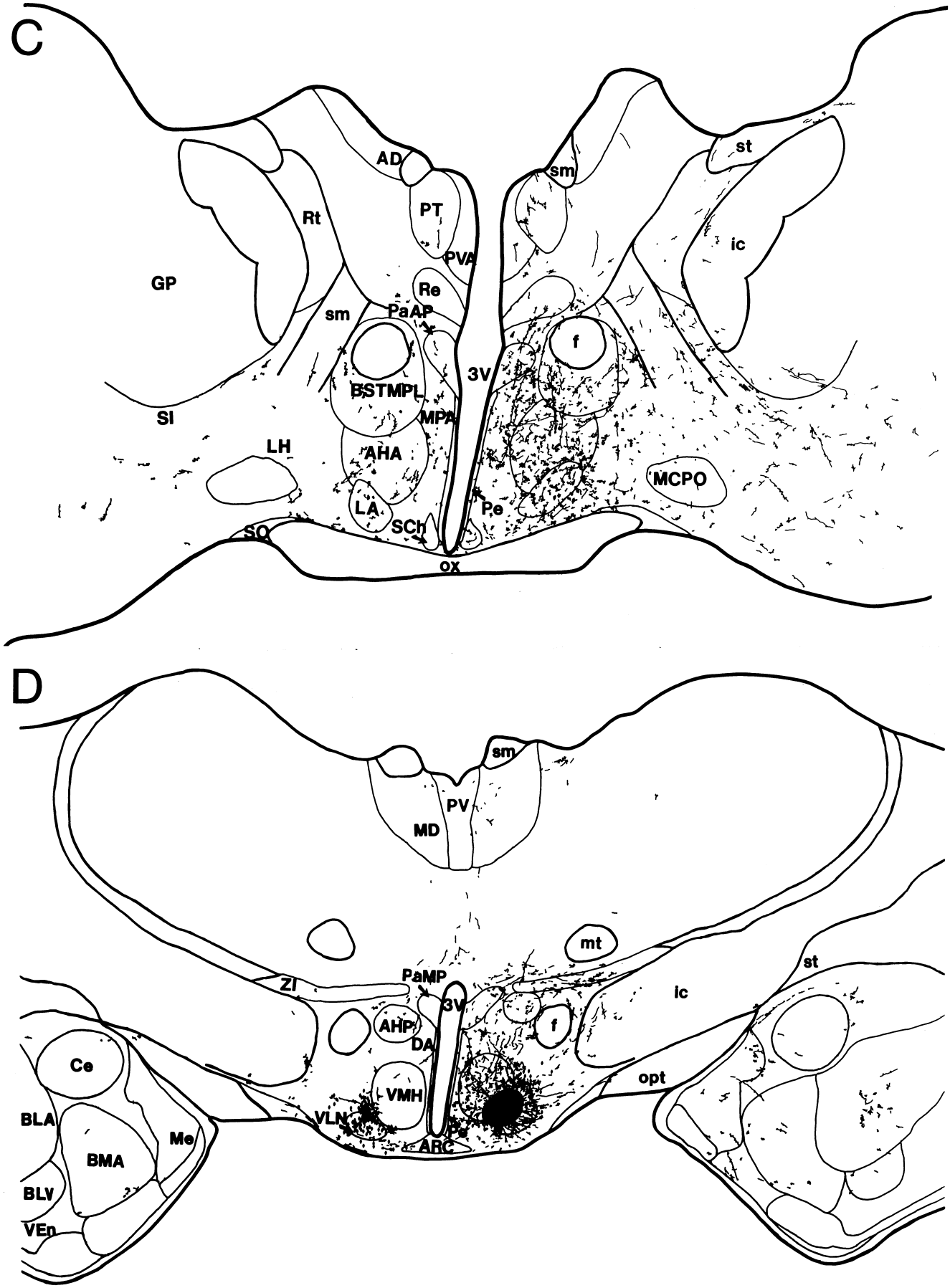

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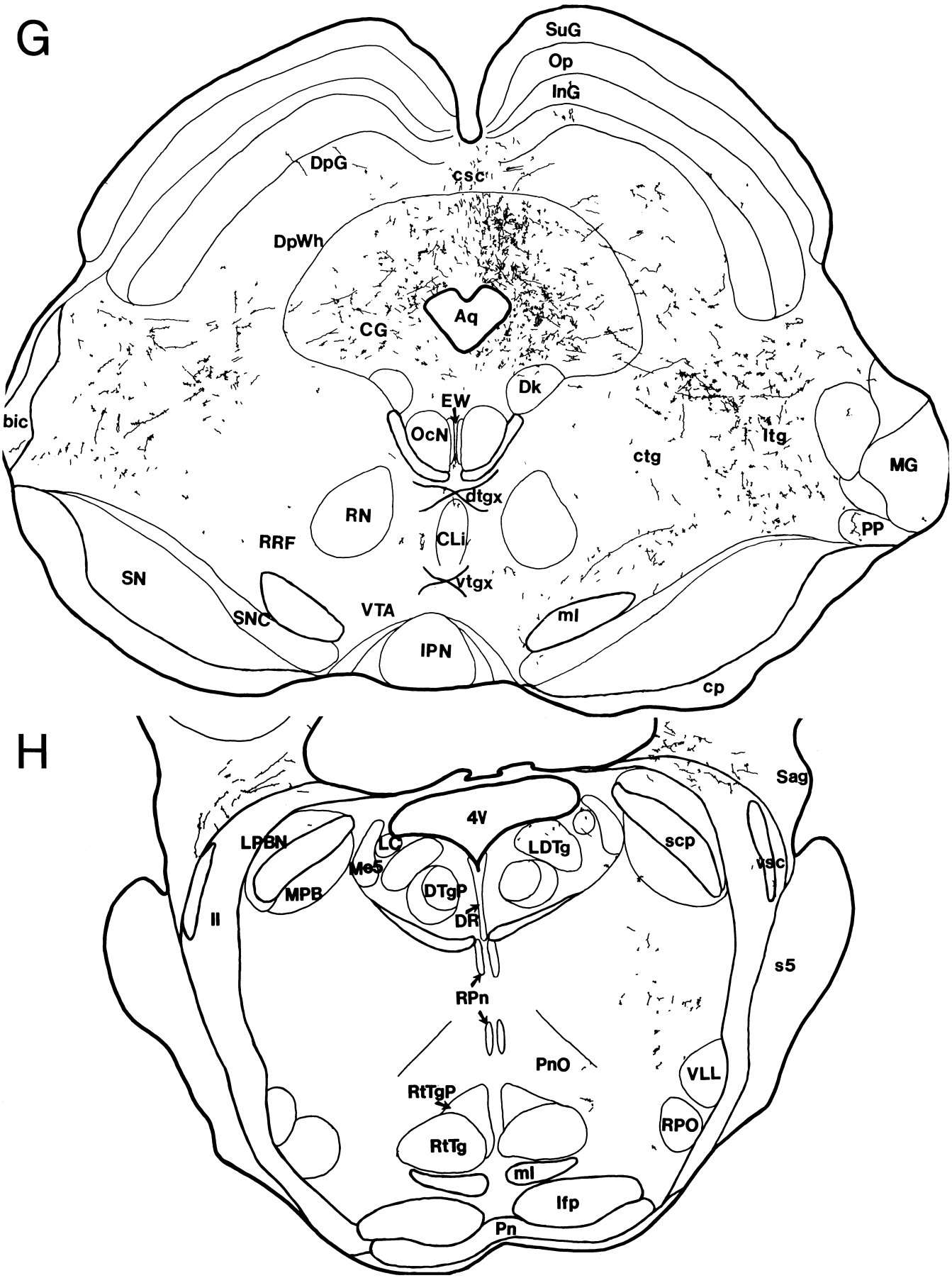

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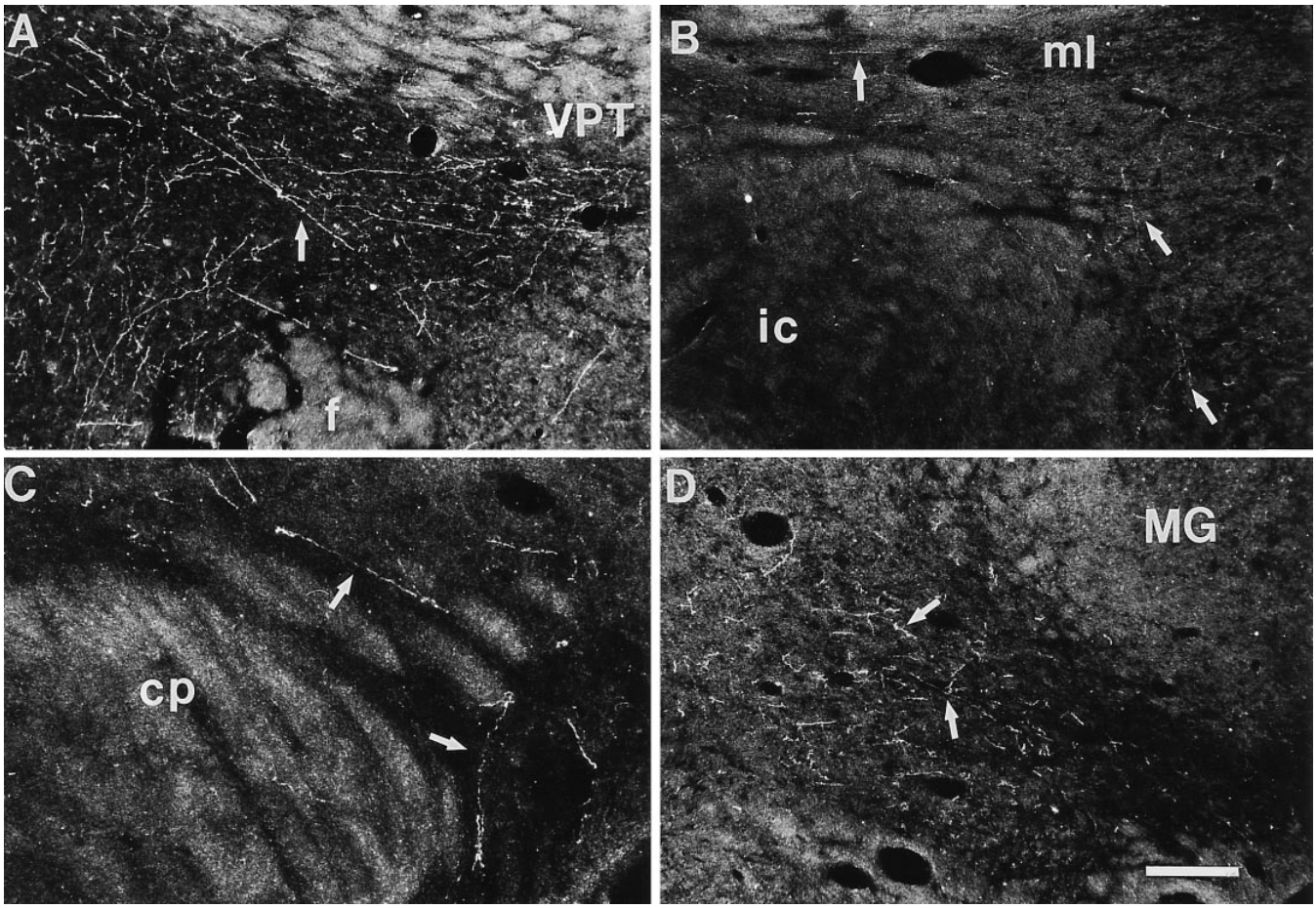

FIG. 5. Dark field photomicrographs from animal 206 of Pha-L labeled fibers in the lateral descending pathway, ipsilateral to the injection site, as they progress from the level of hypothalamus to the midbrain. Fibers appear, A) traveling laterally in the zona incerta, B) around the medial lemniscus, C) lateral to the cerebral peduncle, D) descending into the ventrolateral midbrain. Note that, although the Pha-L labeling in this guinea pig was heavier than in the example depicted in the camera lucida drawings, the labeling in this pathway is sparse. Scale bar 250 = μm in A, B, D, and 100 μm in C. For neuroanatomical abbreviations see Fig. 1. In all figures, dorsal is up, and lateral is right.

This analysis of projections from the VLH in guinea pigs is unique in that Pha-L was deposited into a region of the VLH previously shown to contain a large proportion of retrogradely labeled ovarian hormone receptor-containing cells (22). This is a region of the VLH, ventral to the fornix and lateral to the VMN, that would not be labeled specifically by tracer injections into cytoarchitecturally defined areas such as the ventrolateral region of the VMN or ventrolateral nucleus. However, in this study it was not possible to determine which Pha-L immunoreactive cells also contained immunoreactivity for estrogen receptors due to the intensity of the Pha-L immunostaining. Furthermore, while we can apply Pha-L to an area enriched in estrogen receptor-immunoreactive neurons, we cannot restrict our application only to estrogen receptor-containing cells. Thus, the anterograde labeling in this study also reflects projections from non-steroid receptor-containing cells adjacent to estrogen receptor-containing cells in the VLH.

Most of the VLH projections labeled by Pha-L are very similar to the efferents that have been described from the ventrolateral VMN described in rats (11, 12, 16), an area apparently analogous to the steroid receptor-rich region within the VLH of guinea pigs, or more specifically to the ventrolateral nucleus defined by Bleier (23). The projections discussed in the present paper are similar to those from the ventromedial part of the lateral hypothalamus as identified in guinea pigs (17). Some of the overlap between these findings might be attributed to a few cell bodies outside the VLH picking up the Pha-L, since Pha-L is incorporated primarily by neuronal dendrites. Similarly, it may be the case that in previous studies targeting the VMN or lateral hypothalamus, some neurons between these areas (which might correspond to the guinea pig VLH) may have taken up and transported tracer, since Golgi stains in rats indicate that many neurons in the VMN and lateral hypothalamus (and in between) have long, laterally-extending dendrites (24, 25).

Although the majority of the projections from the estrogen receptor rich area of the guinea pig VLH was similar to the previous studies discussed above, a few inconsistencies between the present results and previous findings were noted. Earlier papers which reported efferents from the ventromedial nucleus of the hypothalamus in rats (11, 12) and from the lateral hypothalamus in guinea pigs (17) described a few projections which we did not observe. For example, all three papers reported substantial projections to the arcuate nucleus and median eminence, while we found few fibers especially in caudal regions. Our results most

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(6), the areas to which the VLH projects are candidates for components of the neural circuit underlying lordosis. All of the areas receiving dense projections from the VLH (including the bed nucleus of the stria terminalis, medial preoptic area, and midbrain central gray), have estrogen and progestin receptors (1, 20, 26, 27), suggesting their participation in physiological and behavioral events that are influenced by ovarian steroid hormones.

Several forebrain areas that receive VLH efferents have been directly implicated in lordosis, including the preoptic area, septum, amygdala, and the zona incerta. Lesions of the medial preoptic area, where we observe abundant inputs from VLH, facilitate lordosis in hormonally-primed male and female guinea pigs (28). Furthermore, implants of estradiol into the preoptic area also facilitate lordosis in female guinea pigs (29), an effect that may be due to inhibition of an inhibitory system. The septum may inhibit lordosis, since septal lesions facilitate lordosis in hormonally-primed rats (30, 31). Although lesions of the entire amygdala in rats have no apparent effect on sexual receptivity (30, 31), anterior corticomedial amygdaloid lesions inhibit lordosis in female rats, and stimulation of this area facilitates lordosis (32). Lesions which spare fibers of passage in the medial zona incerta disrupt female sexual behavior in rats (33), suggesting that axonal terminals in the zona incerta may contribute to the regulation of female sexual behavior.

Estrogen receptor- and progestin receptor-containing neurons in the mediobasal hypothalamus project to the dorsal midbrain, including the central gray (14, 22, 34), another area important to sexual behavior. Dorsal midbrain lesions, which include the central gray, disrupt lordosis in rodents (35–38), as do transections of the connections between the VMN and midbrain (39). However, stimulating the midbrain central gray in estrogen-primed rats has yielded opposing results (40, 41). Although neither estrogens nor progestins are sufficient to induce lordosis when implanted in the dorsal midbrain alone, many neurotransmitters and peptides influence lordosis when infused into the dorsal midbrain (e.g. 42–44). The area where we observe the lateral fiber pathway from the VLH entering the ventrolateral midbrain in guinea pigs may participate in female sexual behavior as well as lactation in rats (45). In conclusion, the midbrain may be an important output of the VLH with respect to female sexual behavior.

The expression of lordosis is only one of many behavioral (46) and physiological changes that accompany sexual receptivity. In addition, the VLH undoubtedly mediates other behaviors, including aggression (47, 48). Consequently, the areas receiving efferents from the VLH probably participate in a variety of functions. As an example, the midbrain central gray, which receives numerous VLH inputs, mediates several functions in addition to lordosis such as: defensive behavior (44, 49, 50); somatosensory perception (51), including the effects of vaginal stimulation during mating (52); analgesia (53–55); and proceptivity (56), including vocalization (57, 58). Thus, the efferent VLH pathways identified in this study are likely to be part of a complex, neural circuit implicated in numerous functions.

Materials and methods

Adult, female, Hartley strain guinea pigs (approximately 350 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA) and maintained on a 14/10 h light/dark cycle, with lights on at 06.00 and food and water freely available. One week after arrival, the animals were ovariectomized through bilateral dorsal incisions. This was esssential, as endogenous estradiol would be expected to decrease estrogen receptor-immunostaining using the H 222 antibody (3). All surgeries were performed while the animals were anesthetized with a combination of sodium pentobarbital (15.1 mg/kg) and chloral hydrate (72.3 mg/kg) administered intraperitoneally. Methoxyflurane (Metofane; Pitman Moore, Inc., Washington, Crossing, NJ, USA; administered via inhalation) was used as a supplemental anesthetic when necessary. Prior to ovariectomy, a combination of droperidol (2%), fentanyl (0.04%), methylparaben (0.18%), and propylparaben (0.02%) in 0.04 ml was injected intramuscularly as a muscle relaxant. All animal procedures were in compliance with guidelines of the National Institutes of Health and were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

One week after ovariectomy, Pha-L was iontophoretically applied to the VLH. A glass micropipette with a tip diameter of 15 μm was filled with a 2.5% solution of the anterograde tracer Pha-L (Vector Laboratories, Burlingame, CA, USA) in 0.1 M phosphate-buffered saline, pH 8.0, and stereotaxically lowered to the VLH (0.6 mm caudal to bregma, 1.9 mm lateral to the midline, 3.1 mm dorsal to the intraaural line, and bregma and lambda level). The tracer was injected iontophoretically by applying a positive current of 5 μA in pulses (7 s on, 7 s off) for 10 min.

Two weeks later, the subjects were deeply anesthetized with an overdose of chloral hydrate and sodium pentobarbital. After clamping the descending aorta and the inferior vena cava, the anticoagulant heparin (5000 units, Sigma Chemical Co., St. Louis, MO, USA) in 1 ml saline was injected directly into the left cardiac ventricle. The animals were first perfused with approximately 75 ml of 0.15 M saline followed by 250–350 ml of fixative (4% paraformaldehyde with either 15% saturated picric acid or 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 at room temperature) at a rate of 25 ml/min. After the brains were removed, they were blocked immediately and stored in 20% sucrose buffer (in 0.1 M sodium phosphate) for at least 12 h at 4 °C before sectioning. Fifty micron frozen transverse sections were cut through the forebrain, midbrain and brainstem. Alternate sections were processed immunocytochemically as follows.

Pha-L immunocytochemistry

The procedure for immunostaining of Pha-L was adopted from De Vries *et al.* (59). After rinsing for 5 min, the free-floating sections were pretreated with 0.1% sodium borohydride, rinsed four times, and incubated in 0.3% Triton X-100, 10% normal rabbit serum, and 1% hydrogen peroxide for 10 min. All rinses and dilutions in this pretreatment were made with 0.5 M tris-buffered saline (TBS), pH 8.6 at room temperature. The sections were then incubated at 4 °C for two days in goat anti-Pha-L (Vector Labs; diluted 1 : 2000 in TBS with 0.3% Triton X-100, 2% normal rabbit serum, pH 8.6 at 4 °C).

After incubation in the primary antibody, sections were brought to room temperature and rinsed three times in TBS containing 3% Triton X-100 and 0.1% gelatin (pH 8.6 for two rinses, switched to pH 7.6 for the third rinse). Unless otherwise noted, this buffer (pH 7.6) was used for the remaining solutions and rinses. The sections were then processed for 45 min in biotinylated rabbit anti-goat serum (1 drop/15 ml buffer; ABC kit, Vector Labs, Burlingame, CA, USA); rinsed twice, followed by a final rinse in TBS, and then incubated 45 min in the avidin DH-biotinylated horseradish peroxidase H complex (1 drop each of reagent A and B per 5 ml buffer; ABC kit). These last two incubations were repeated once and after two final rinses in TBS, the sections were incubated for 15 min in 0.5% diaminobenzidine with 0.003% hydrogen peroxide in TBS, or in nickel-intensified diaminobenzidine (see below).

As a control for crossreactivity of the Pha-L antiserum, the primary antibody and the biotinylated rabbit anti-goat serum were each omitted separately to ensure that they did not crossreact with any endogenous antigens. Pha-L immunostaining was completely eliminated in these procedures.

Estrogen receptor immunocytochemistry

Half of the hypothalamic sections processed for Pha-L immunostaining (above) were incubated with nickel-intensified diaminobenzidine (4 mg/ml nickel ammonium sulfate, 0.15 mg/ml diaminobenzidine, 0.01% hydrogen peroxide, pH 8.0) instead of diaminobenzidine, and those containing

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