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Evidence for a Direct Neuronal Pathway From the Suprachiasmatic Nucleus to the Gonadotropin-Releasing Hormone System: Combined Tracing and Light and Electron Microscopic Immunocytochemical Studies

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ABSTRACT

The timing and occurrence of the preovulatory luteinizing hormone (LH) surge in the female rodent are critically dependent on the integrity of the suprachiasmatic nucleus (SCN). Destruction of the SCN leads to a cessation of the ovarian cycle, whereas implantation of estrogen in ovariectomized rats results in daily LH surges. The anatomical substrate for these effects is not known. Previous studies involving lesions of the SCN have suggested the presence of a direct vasoactive intestinal polypeptide (VIP)-containing pathway to gonadotropin-releasing hormone (GnRH) neurons. To further investigate the direct connection between the SCN and the GnRH system, we have used tract-tracing with the anterograde tracer *Phaseolus vulgaris*-leucoagglutinin (PhaL) in combination with an immunocytochemical staining for GnRH in light and electron microscopic studies. Small, unilateral PhaL deposits, especially when they were placed in the rostral ventrolateral portion of the SCN, revealed a bilateral projection to the preoptic area, where PhaL-immunoreactive fibers were regularly found in close apposition to GnRH neurons. Ultrastructural studies showed synaptic interaction of PhaL-containing fibers with GnRH-immunoreactive (IR) cell bodies, thus demonstrating a direct SCN-GnRH connection. Taken together, these data provide evidence for the existence of a monosynaptic pathway from the SCN to the GnRH system in the hypothalamus of the female rat. We suggest that this pathway may contain at least VIP as a putative transmitter and may play a role in the circadian regulation of the estrous cycle in the female rat. J. Comp. Neurol. 384:569–579, 1997. © 1997 Wiley-Liss, Inc.

Indexing terms: female rat; circadian regulation; estrous cycle; synapse

Gonadotropin-releasing hormone (GnRH), the releasing hormone for the pituitary gonadotropin-luteinizing hormone (LH) and follicle-stimulating hormone, is secreted in a pulsatile way into the portal vasculature at the level of the median eminence. It is well established that, in rodents, only females posses a neurogenic mechanism that triggers the GnRH neuronal system and thereby induces the ovulatory surge of LH on the afternoon of proestrus (Fink et al., 1991). The suprachiasmatic nucleus (SCN) constitutes an essential component for normal control of preovulatory LH secretion in rodents. The SCN is the major component of the biological clock responsible for the

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generation of rhythms and the entrainment of those rhythms to the environmental light-dark cycle. Destruction of the SCN blocks the preovulatory LH surge and induces persistent estrus in intact female rats (Brown-Grant and Raisman, 1977). Even though the LH surge occurs only once every 4-5 days in intact females, there is strong evidence that the actual neural signal is generated daily. For instance, treatment of ovariectomized (OVX) female rats with estrogen (E) results in a daily proestrous-like surge of LH (Legan and Karsch, 1975), which is eliminated by complete lesions of the SCN (Kawakami et al., 1980; Ma et al., 1990).

The anatomical substrate for the circadian regulation of the estrous cycle in the female is not known. Based on the literature showing circadian and 24-hour rhythms in most SCN transmitters (Albers et al., 1992; Inouye et al., 1993), it has been hypothesized that the SCN transmits rhythmic information to target neurons by means of timed release of transmitters from its efferent projections (Kalsbeek and Buijs, 1992). Efferent projections of the SCN have been described by using neuronal transport of tritium-labelled amino acids (Berk and Finkelstein, 1981; Swanson and Cowan, 1975) and of the anterograde tracer *Phaseolus vulgaris*-leucoagglutinin (PhaL; Buijs et al., 1993; Watts et al., 1987). The SCN projects to the rostral forebrain, and both vasoactive intestinal polypeptide (VIP), synthesized in cell bodies in the ventrolateral part of the SCN (Card et al., 1981), and vasopressin (VP), synthesized predominantly in the dorsomedial part of the SCN (Swaab et al., 1975), appear to be present in efferent projections to the preoptic area (Watts and Swanson, 1987), the region that contains the majority of the GnRH-synthesizing neurons in the rat (Wray and Hoffman, 1986). Recently, we demonstrated an input of VIP-containing, but not VP-containing, fibers on GnRH neurons in the preoptic hypothalamus of the female rat at the light microscopic (LM) level (Van der Beek et al., 1993). By using thermic lesions of the SCN, we have shown that well over 50% of the VIP input on hypothalamic GnRH neurons is derived from this nucleus; therefore, it may be involved in the transmission of circadian information from the SCN directly to the GnRH system.

The present study was designed to further investigate the existence of a direct neuronal connection between the SCN and the GnRH system and to establish the monosynaptic nature of this pathway. To this end, unilateral injections of the anterograde tracer PhaL in the SCN were used in combination with immunocytochemistry for GnRH at both the LM level and the electron microscopic (EM) level.

MATERIALS AND METHODS

Animals

A total of 36 mature female Sprague-Dawley rats (obtained from Charles River, Montreal, Quebec; 8-10 weeks of age), weighing between 190 g and 220 g, were used for the LM tracing and immunocytochemical studies (exp I). An additional 10 adult female and male Sprague Dawley rats, weighing between 250 g and 280 g, were used for EM studies with a modified protocol (exp II). All rats were housed under a regular light/dark cycle (12:12 hours light:dark) in a temperature-controlled room. Food and water were available ad libitum.

Iontophoresis of PhaL in the SCN

The animals were anesthetized with Innovarvet (10 mg fluanison and 0.135 mg fentanylcitrate per ml, 0.1 ml/100 g bodyweight i.m.; Janssen Pharmaceuticals, Beerse, Belgium), and unilateral injections of the lectin PhaL (2.0–2.5%; Vector Laboratories, Burlingame, CA) in Tris-buffered saline (TBS; 0.05 M Tris/HCl containing 0.9% NaCl, pH 7.4) were placed stereotactically into the SCN (coordinates: A-P, −0.8 to 1.3 mm; L, 0.2 mm; V, 9.6 mm; according to Paxinos and Watson, 1986). Iontophoretic deposits were made with a glass micropipette (tip diameter 15–30 mm) by using a positive current of 6 mA for 10–15 minutes turned on for 5 seconds and off for another 5 seconds, according to Gerfen and Sawchenko (1984). Following surgery, animals were housed individually and were killed after a survival period of 5–7 days.

Tissue processing

Animals were perfused under pentobarbital anaesthesia (Nembutal; 0.1 ml/100 g body weight, i.p.) with 100–150 ml heparinized saline followed by 250–500 ml 4% paraformaldehyde (PAF) with either 1% acrolein (exp I) or 15% picric acid and 0.2% glutaraldehyde (exp II) in 0.1 M phosphate buffer (PB; exp I, pH 8.6; exp II, pH 7.4) added to it. The brains were removed from the skull and postfixed in PAF/acrolein fixative (exp I), or PAF/picric acid fixative without glutaraldehyde (exp II) for 1-2 hours at room temperature. The postfixation of exp I included a period of 15 minutes in a microwave at low power while chilling on ice and water (Buijs et al., 1993). Coronal sections (40 µm) of the hypothalamus, including the diagonal band of Broca (DBB) and the preoptic area, were cut on a Vibratome and collected as free-floating sections in TBS (exp I) or in PB (exp II) pH 7.4.

LM and EM immunocytochemistry for PhaL and GnRH

Every fifth section containing the SCN was single stained for PhaL to evaluate the size and site of the tracer injection. Approximately half of the sections from animals with tracer deposits in the SCN were processed for LM (exp I) or EM (exp II) double labelling for PhaL and GnRH. A few animals with spots partially or completely outside the SCN were processed for LM double labelling as well.

Sections processed for LM were pretreated with sodium borohydride (5 mg/ml for 10 minutes) and were extensively washed in TBS prior to incubation. Sections were incubated with the antiserum against PhaL (raised in goat; 1:3,000; Vector Laboratories) or, for double staining, were incubated simultaneously with the antiserum against PhaL (1:3,000) and an antiserum against GnRH (raised in rabbit; 1:6,000; no. 20-4; kindly provided by Prof H.J. Th. Goos, Department of Zoology, Utrecht University, The Netherlands) both diluted in TBS containing 1% bovine serum albumin (BSA) and 0.5% Triton X-100 (TBBT). Specificity of the GnRH antiserum was checked on blot and hypothalamic sections by preabsorbing the antiserum with homologous antigen according to procedures described previously (Van der Beek et al., 1992). This resulted in a complete absence of staining. Incubation lasted for 1 hour at room temperature and was followed by incubation overnight at 4°C. Immunoreactivity for PhaL was detected with biotinylated horse-anti-goat (HaG-bio; 1:400 in TBBT; Vector Laboratories) for 1 hour at room

temperature, and avidin-biotin complex-Elite (ABC; final dilution of both avidin and biotin, 1:1,200 in TBBT; Vector Laboratories) for 2 hours at room temperature. Between incubation steps, sections were thoroughly washed in TBS. The immunoreaction was visualized by incubation with 0.05% 3,3′-diaminobenzidine (DAB; Sigma Chemical Company, St. Louis, MO) containing 0.03% H_2O_2 (Merck, Darmstadt, Germany) for 12 minutes, with 0.2% nickel-ammonium sulphate (Merck) dissolved in it for LM double staining. Subsequently, sections for double staining were incubated with biotinylated goat-anti-rabbit (GaR-bio; 1:400, Vector Laboratories) and ABC (1:1,000), as described above. The immunoreaction was visualized by incubation with 0.05% DAB containing 0.03% hydrogen peroxide for 12–20 minutes. Finally, the sections were mounted on glycerin albumin-coated slides (Gurr, Poole, England), dried, dehydrated through graded series of ethanol and xylene, followed by embedding in Depex (Gurr).

Sections for EM double labelling were pretreated with sodium borohydride, as described above. Sections were then incubated with a biotinylated antiserum against PhaL (raised in rabbit; 1:250; Vector Laboratories) diluted in PB containing 1% normal goat serum for 48 hours at 4°C. Subsequently, sections were incubated with ABC (1:500) followed by a DAB reaction, as described above. After several rinses in PB, sections were further processed for GnRH immunocytochemistry with a commercial GnRH antiserum, because pilot experiments with the remaining material from exp I showed that the GnRH antiserum used for our LM staining did not stain any neurons after omission of Triton from the incubation medium. Sections were incubated for 48 hours at 4°C in GnRH antiserum (raised in rabbit; 1:5,000 in PB containing 0.1% sodium azide and 1% normal goat serum; INCSTAR Corporation, Stillwater, MN), followed by 10 nm immunogold-conjugated GaR (1:10 in PB; Polysciences, Warrington, PE) for 2 days at 4°C. Subsequently, sections were postosmicated in 1% osmiumtetraoxide in PB for 30 minutes, dehydrated through graded series of ethanol using 1% uranyl acetate in the 70% ethanol (30 minutes), followed by flat embedding in Araldite between liquid release-coated (Electron Microscopy Sciences, Fort Washington, PA) slides. Embedded sections were examined under a light microscope, and the preoptic area was dissected and mounted on Araldite blocks. Ultrathin sections were cut on a Reichert-Jung microtome, collected on Formvar-coated, single-slot grids, and examined under a Philips CM10 electron microscope.

RESULTS

Localization of iontophoretic injections of PhaL

Of the 36 injections of PhaL made for LM studies, 11 were limited to the SCN. These small injections labelled cell bodies primarily located in the ventral part of the nucleus in six animals (animals 44, 52, 54, 59, 66, and 71; Fig. 1A) and in the dorsal part of the nucleus in two animals (57 and 73; Fig. 1B). Larger PhaL injections with labelled cells in both subdivisions of the SCN were observed in two animals (46 and 47; Fig. 1C). One animal (51) showed a partially bilateral deposit of PhaL that was strictly limited to the ventral part of the SCN just above the optic chiasm (Fig. 1D).

In several animals, PhaL-labelled cell bodies were located within the rostral-ventral part of the SCN as well as just outside the SCN in the ventral preoptic area (animals 40, 42, 65, and 68). One animal showed PhaL-labelled cells in the dorsal part of the SCN as well as in the retrochiasmatic area outside the SCN more caudally (animal 64). Six animals had small PhaL injections located completely outside the SCN, either in the perichiasmatic area surrounding the rostral and medial part of the SCN (animals 48, 60, 62, 63, and 72) or in the ventral periventricular area (animal 74). In four animals (70, 67, 61, and 56) only a very few SCN neurons were filled with the tracer, and almost no transport of the tracer to rostral areas in the brain was detected. Ten animals showed no labelling, probably because the injections were placed too far ventral, e.g., below the optic chiasm or in the cavity of the third ventricle. Material from these animals was not processed for double staining. Sections from the animals with injections placed in the SCN (n = 11) and from a few animals with injections partially in the SCN (animals 40, 42, 64, and 68) or outside the SCN (animals 48, 65, 72, and 74) were processed for LM double staining for PhaL and GnRH.

Efferent projections of the SCN

The description of the efferent projections of the SCN to the area rostral of this nucleus is based on observations in seven animals with small PhaL injections into the SCN (animals 44, 52, 54, 57, 66, 71, and 73). PhaL-IR fibers were detected in a number of previously described targets of the SCN. Scattered fibers were observed in the lateral ventral part of the lateral septal nucleus and in the ventral part of the DBB. Fibers were more numerous in the region of the organum vasculosum of the lamina terminalis (OVLT) and preoptic area (PO), particularly in the periventricular part of the rostral preoptic area (AvPv), and in the anterior hypothalamic area (AHA) just above the optic chiasm (Fig. 2). Dense fiber plexuses were observed in the border zone just outside the SCN, i.e., the perichiasmatic area (peri-SCN; see Fig. 1), the subparaventricular zone (sub-PVN), the PVN nucleus of the thalamus, and the contralateral SCN (see Fig. 1). Projections to the PO and OVLT region, the AHA, as well as the septal area were bilateral but were less dense at the contralateral side than at the ipsilateral side of the brain (see Fig. 2). Most PhaL-IR fibers in the above-described regions were long, thin fibers with widely spaced varicosities. In addition, fibers with more narrowly spaced varicosities, which showed regular branching that occasionally formed pericellular-like structures, were observed in the peri-SCN, the contralateral SCN, the AvPv, the AHA, and the ventral PO (see Fig. 2).

The exact localization of the injection in the SCN, the number of neurons filled with PhaL in the SCN, and the transport of the tracer showed large individual differences. In animals with only a few PhaL-IR cell bodies in the SCN, numerous fibers were observed in the sub-PVN, just under the PVN. PhaL-containing fibers were also numerous in the PO at the ipsilateral side but were less prominent at the contralateral side. Large injections that filled a substantial part of the SCN showed not only intense innervation of the ipsilateral side of the brain but, in addition, showed numerous fibers at the contralateral side in the PO and OVLT region. The efferent projections visualized in animal 51 (see Fig. 1D), the animal with a partially bilateral

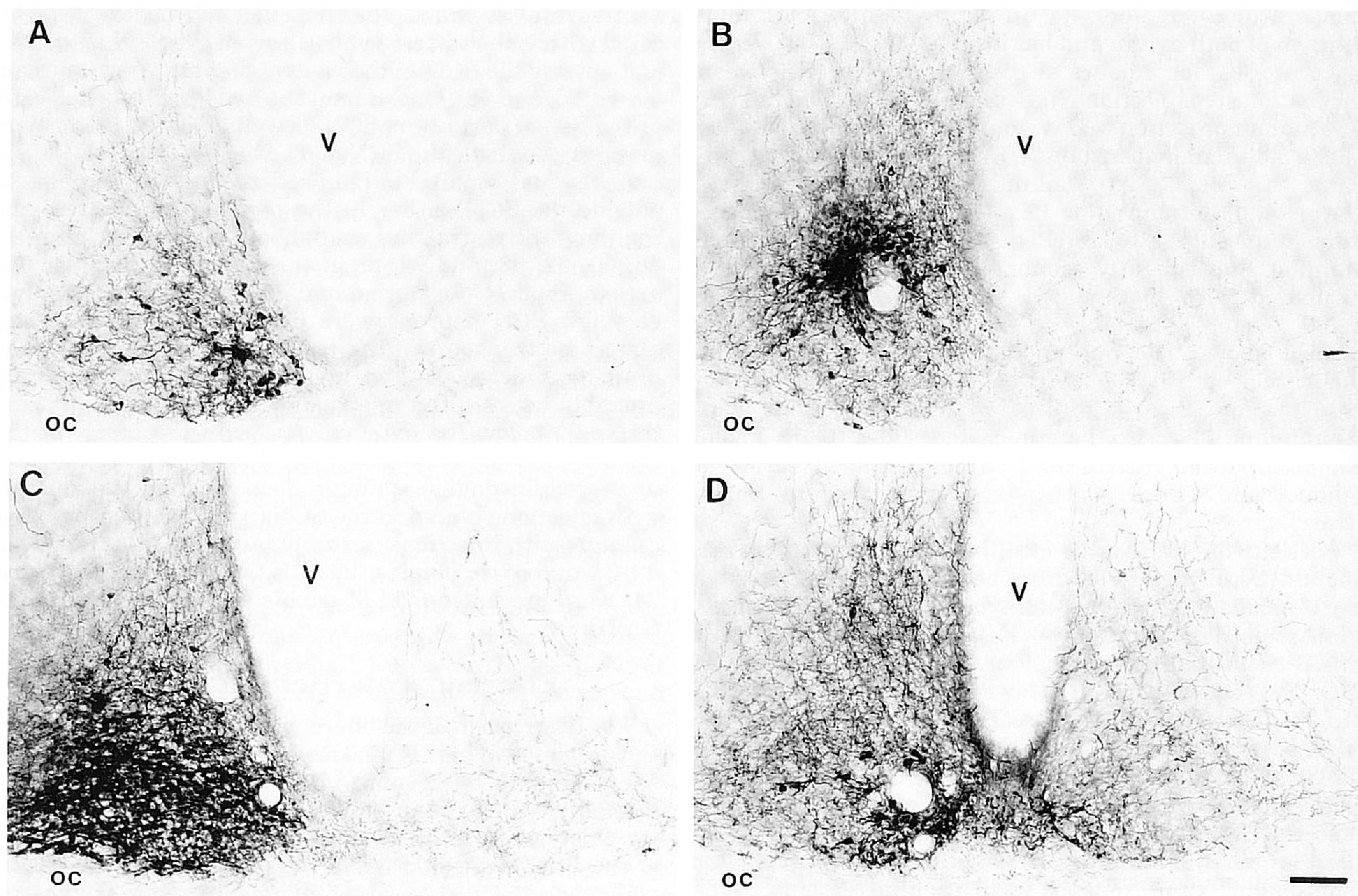

Fig. 1. Representative photographs of *Phaseolus vulgaris*-leucoagglutinin (PhaL) injections in the suprachiasmatic nucleus (SCN) of female rats: An injection that filled neurons located predominantly in the ventral part (**A**; animal 54) or primarily in the dorsal part (**B**; animal 57) of the SCN, a large PhaL deposit that filled neurons in both subdivisions of the SCN (**C**; animal 46), and a partially bilateral injection in the ventral part of the SCN (**D**; animal 51). V, ventricle; oc, optic chiasm. Scale bar = 100 μm.

injection, were much more dense than those observed in animals with unilateral injections. Also, rostrally directed projections in this animal were more evenly distributed through both sides of the brain, especially in the regions of the OVLT and the PO. Finally, in the animals with large injections into the SCN (animals 46 and 47; see Fig. 1C), extensive projections to the ventral septal area, to the area around the OVLT, and to the PO and AHA were visualized.

A number of differences were observed between injections located in the ventral parts and those in the dorsal parts of the SCN. In animals with dorsally placed injections, more fibers were observed in the AvPv and the PO (see Fig. 2). In addition, the projection to the sub-PVN contained more fibers close to the third ventricle in the periventricular nucleus.

In the animals with injections that filled neurons in the SCN as well as a number of neurons outside the SCN, more fibers showing extensive branching were found in the medial PO and AHA. In addition, more fibers were found in the ventral septal nucleus and the ventral part of the DBB. This larger number of fibers was found almost exclusively on the ipsilateral side. This pattern of staining was also observed in the animals with injections placed in the peri-SCN. In addition, these animals showed projections to the ipsilateral SCN, whereas almost no fibers were observed in the contralateral SCN.

Combined tract tracing and immunocytochemistry

GnRH-IR neurons were found scattered throughout the forebrain, with concentrations of neurons in the PO and OVLT region (see Fig. 2). In the animals with tracer injections into the SCN, PhaL-containing fibers were found in close apposition to a number of GnRH neurons at the LM level (Fig. 3). The input was scored as positive when the PhaL-containing fibers showed one or more varicosities in contact with a GnRH-IR perikaryon or with one of its extending dendrites. The PhaL-innervated GnRH neurons were predominantly localized in the OVLT and PO region, in the area adjacent to the third ventricle just below the anterior commissure, and in the rostral part of the AHA close to the optic chiasm. GnRH neurons located above the level of the anterior commissure, in the DBB, and in the more rostral areas showed no PhaL-IR input.

GnRH neurons innervated by PhaL-containing fibers were regularly observed at the ipsilateral side in all animals with injections into the SCN. Occasionally, contacts of PhaL-IR fibers were seen with GnRH neurons located at the contralateral site (see Fig. 2D,H). The PhaL-GnRH contacts were more frequent in the animals with larger injections, and, in the animal with a bilateral deposit (i.e., in animal 51). In this animal, 19.8% of the

GnRH neurons at the aimed ipsilateral side and 11.3% of the GnRH neurons at the aimed contralateral side received an input of PhaL-containing fibers, whereas the percentage at either side varied between 1.3% and 11.0% in the animals with smaller unilateral deposits in the SCN. Contacts were also frequently observed in animals with injections into the peri-SCN, predominantly at the ipsilateral side of the brain. Complete evaluation of the interaction between PhaL and GnRH in animals with PhaL-IR neurons located in the ventral PO (animals 40, 42, 65, and 68) was often not possible, because the intense blue-black staining for PhaL masked the GnRH staining in this area. In these animals, however, interaction of PhaL fibers with GnRH neurons was sparse at the contralateral side of the brain.

In the animals used for EM studies, two out of the total of ten PhaL injections were located within SCN borders. These injections primarily labelled neurons located in the ventrolateral portion of the SCN (see Fig. 1A). At the ultrastructural level, PhaL immunoreactivity was characterized by a moderate-to-dense DAB reaction product. Immunoreactivity for GnRH appeared as small gold particles over the entire cell body. The PhaL-containing axons showed synaptic interaction with GnRH neurons in material selected from the PO and OVLT region in both animals (Fig. 4). PhaL-IR axons also frequently contacted unlabelled dendrites and cell somata and regularly showed synaptic specialization of the membranes (data not shown).

DISCUSSION

The present study describes the extensive rostral projections of the SCN to the PO, the region of the OVLT, and the DBB, areas known to contain the majority of GnRH-synthesizing neurons in the rat brain (Wray and Hoffman, 1986). The synaptic interaction of PhaL-containing fibers with cell bodies and dendrites at the ultrastructural level indicates that these areas represent an important target zone of SCN efferents, as suggested previously (Buijs et al., 1993; Watts et al., 1987). Also, the combined tracing of SCN efferents and the immunocytochemical double staining for PhaL and GnRH demonstrate the existence of a direct neuronal connection between the SCN and the GnRH system. At the LM level, SCN efferents were in close apposition to a subset of GnRH neurons in all animals with PhaL injections restricted to the SCN. Most interaction was observed in the medial PO, just caudal to the PO close to the third ventricle, and in the AHA near the optic chiasm, whereas no LM-innervation was found more rostrally. At the ultrastructural level, we observed synapses between PhaL-containing axons and GnRH-IR cell bodies.

The results of the tracing experiments were strongly dependent on the effective uptake and transport of the tracer by SCN neurons and on the number of SCN neurons filled with the tracer. Density of the innervation patterns in general as well as the number of SCN efferents contacting GnRH neurons showed a consistent relation to the size and localization of the tracer deposit. At the LM level, GnRH neurons received considerably more innervation in the animals with a larger or a bilateral deposit of the tracer. In addition, the contralateral side showed more SCN efferents, and more GnRH neurons received an input of these fibers in the animals with PhaL injections that filled more SCN neurons.

Synaptic input on GnRH neurons is generally accepted to be sparse (Jennes et al., 1985; Witkin and Silverman, 1985), but more input has been found in female rats compared with males (Chen et al., 1990). Ultrastructural studies have suggested even less input on GnRH neurons in the male hamster (Lehman and Silverman, 1988), which may indicate that the degree of innervation of GnRH neurons also shows some differences between species. Recent tracing studies of SCN efferents in OVX female golden hamsters, however, have also revealed close associations of SCN efferents with GnRH neurons at the LM level (De la Iglesia et al., 1995). The results of the present study clearly demonstrate that the SCN can influence at least a part of the GnRH system in the rat through a monosynaptic pathway.

Recently, we demonstrated VIP-containing fibers in apposition to GnRH neurons in the PO and OVLT region and in the AHA, and lesion studies demonstrated that the majority of this VIP-containing input on GnRH neurons originates in the SCN (Van der Beek et al., 1993). The localization and distribution of these VIP-innervated GnRH neurons show a strong similarity to that of the PhaL-innervated GnRH neurons observed in the present study. Most innervated GnRH neurons were localized in the PO and were sparse more rostrally. The GnRH neurons localized in these areas appear to be critically involved in the regulation of the proestrous LH surge, as illustrated by the induction of the protooncogene c-fos in these neurons at the time of the LH surge (Lee et al., 1990). An important role for the VIP input on GnRH neurons, especially during the onset of the afternoon LH surge, has been suggested by our recent observation that, during the afternoon LH surge, c-fos immunoreactivity is preferentially induced in those neurons that receive a VIP input (Van der Beek et al., 1994). Indeed, VIP has been implicated in the regulation of cyclic LH release in studies using injections of this peptide into the ventral forebrain. VIP injections eliminate (Kimura et al., 1987) or significantly decrease the E-induced LH surge in OVX females (Weick and Stobie, 1992, 1995), depending on the site of administration.

The VIP-synthesizing neurons of the SCN are likely candidates for the entrainment of circadian rhythms with the light-dark cycle (Albers et al., 1992; Inouye et al., 1993). The effect of light on VIP production in SCN neurons shows some intriguing similarities to the effect of light on the occurrence and timing of the LH surge. Moreover, these effects of light are comparable to the effects of central application of VIP. Constant light (LL) for a period of one ovarian cycle in intact rats (Watts and Fink, 1981a) as well as VIP injections into the third ventricle of OVX E-treated rats (Weick and Stobie, 1992) considerably reduce the magnitude of the LH surge. Longer periods in LL result in anovulation in intact females (Critchlow, 1963) and eliminate the E-induced LH surge in OVX females (Watts and Fink, 1981b), which is comparable to the effect of VIP injections directly into the medial PO (Kimura et al., 1987). Taken together, we propose that the VIP-synthesizing cell population of the SCN represents a likely candidate for a neuronal signal from the SCN that is involved in the circadian regulation of cyclic LH release in the female. The presence of daily proestrous-like surges of LH in OVX E-treated rats suggests that this signal is expressed daily but only results in an afternoon surge of LH when circulating estrogen levels are elevated (Fink et al., 1991; Legan and Karsch, 1975). In view of this, it is of special interest that hypothalamic VIP mRNA and peptide content are affected by gonadal steroids (Gozes and Bren-

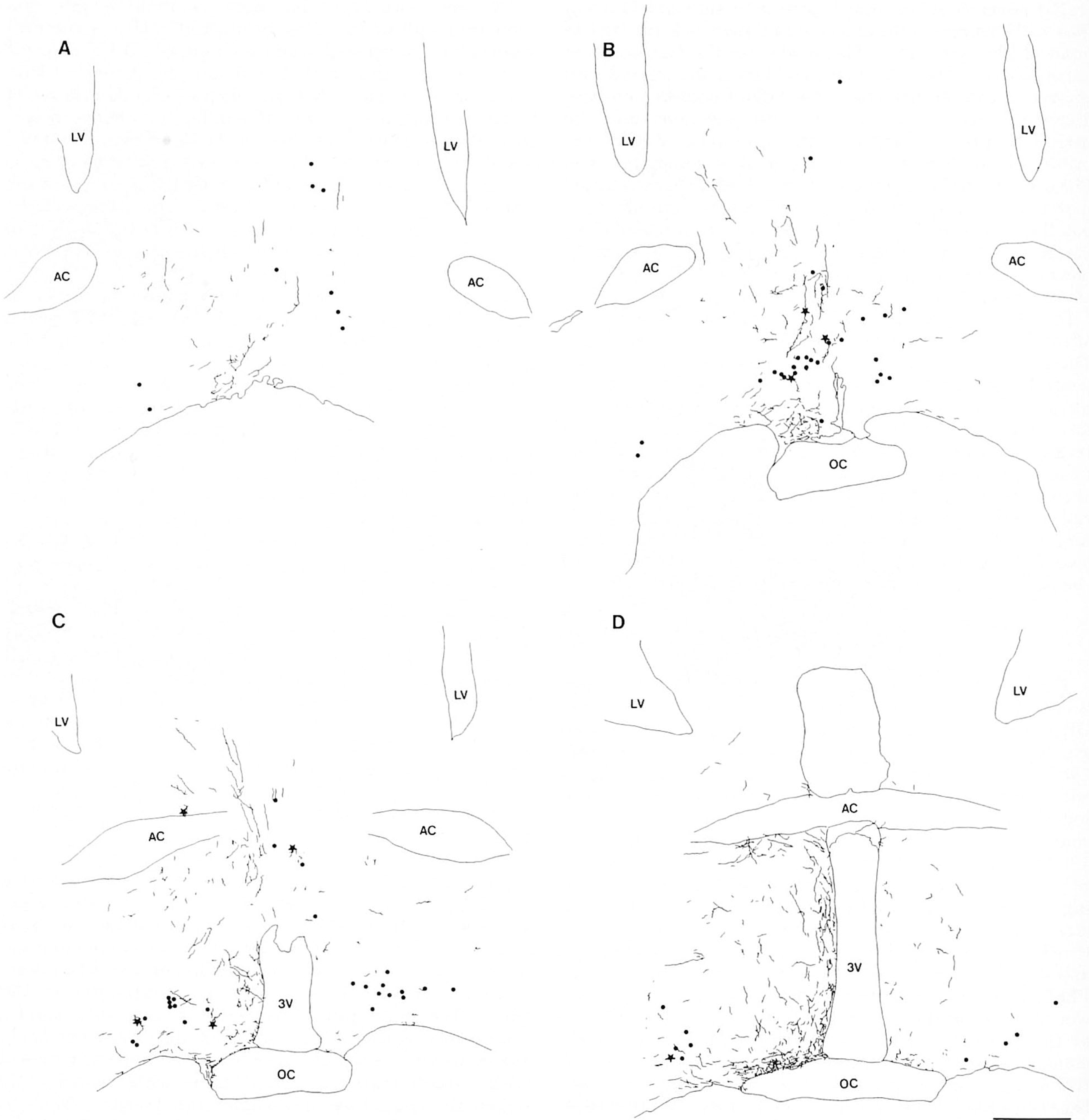

Fig. 2. Camera lucida drawings of the efferent projections of the SCN following small, unilateral, iontophoretic injections of PhaL. The localization and distribution of SCN-efferent projections and of gonadotropin-releasing hormone (GnRH) neurons without (circles) and GnRH neurons with (asterisks) apposition of PhaL containing fibers at the light microscopical (LM) level in representative sections of the diagonal band of Broca (DBB; A,E), the region of the organum vasculosum of the lamina terminalis (OVLT; B,F), in the preoptic area (PO; C,G), and in the anterior hypothalamic area (AHA; D,H). **A–D:** Animal 54, with a small tracer injection into the ventral part of the SCN in which 6.5% of the GnRH neurons at the ipsilateral side of the brain and 1.6% of the neurons at the contralateral side of the brain showed input of SCN fibers. **E–H:** Animal 73, with a small PhaL injection into the dorsal part of the SCN at the contralateral side of the brain in which 3.9% of the neurons at the ipsilateral side of the brain and 14.4% of the GnRH neurons at the contralateral side of the brain showed innervation of SCN efferents at the LM level. LV, lateral ventricle; OC, optic chiasm; 3V, third ventricle; AC, anterior commissure. Scale bars = 500 μm.

neman, 1989; Maletti et al., 1982) and, in addition, that gonadal steroids affect the onset and period length of circadian activity rhythms in the rat (Albers, 1981). In addition to the monosynaptic pathway described in the present study, other multisynaptic pathways may be involved in the circadian regulation of LH release. Such an

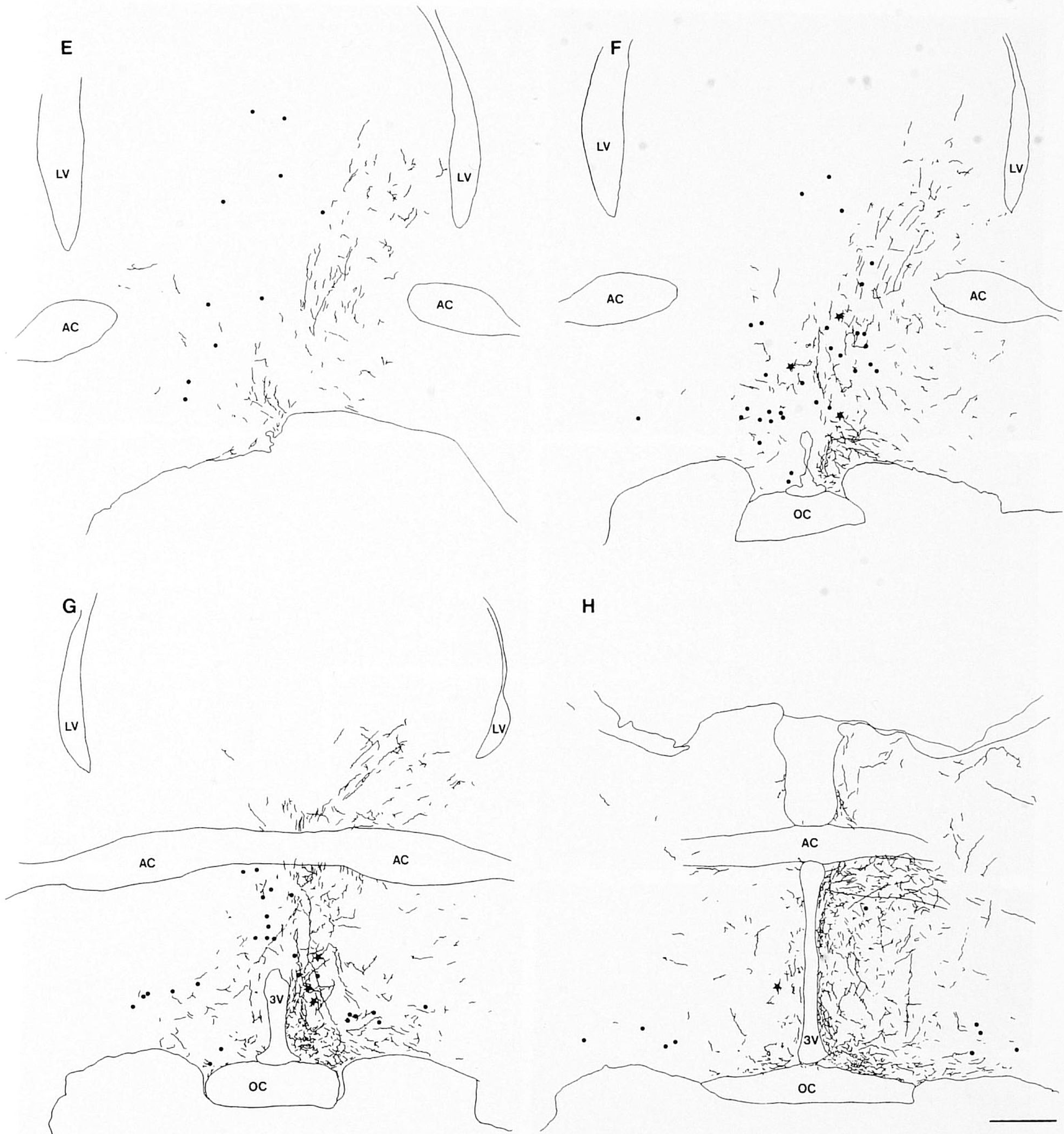

Figure 2 (Continued.)

intermediate role has been proposed for the sub-PVN (Watts et al., 1989; Weick and Stobie, 1995) but could also involve rostral projections of the SCN. The present study confirmed earlier reports showing that the rostral medial preoptic area, i.e., the anteroventral periventricular region, is an important target of SCN efferents. Recently, Watson et al. (1995) demonstrated synaptic input of SCN efferents on neurons containing estrogen receptors in this region. Implantation studies using an antiestrogen have demonstrated that this area is essential for the positive feedback effects of estrogen (Petersen et al., 1989), and lesions of this area block the preovulatory surges of LH and eliminate steroid-induced LH surges in OVX females (Kawakami et al., 1980; Ma et al., 1990; Wiegand and Terasawa, 1982; Wiegand et al., 1980). These data suggest that the timing of preovulatory LH secretion may be regulated at least in part by interaction of SCN projections with steroid-sensitive neurons in the anteroventral periventricular area.

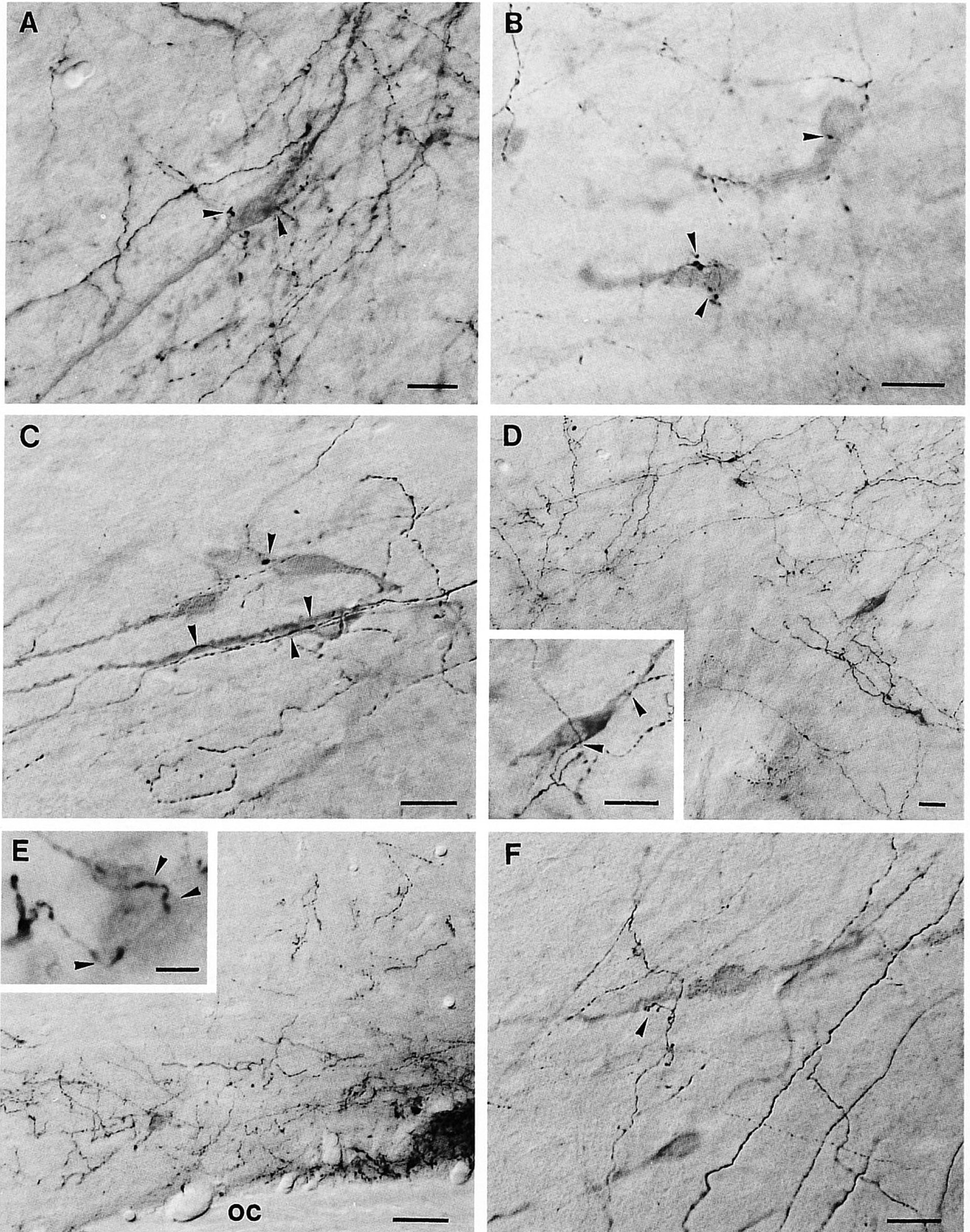

Fig. 3. PhaL input (blue-black; arrowheads) on GnRH neurons (brown) at the LM level in the PO (animal 46; **A**), (B) close to the OVLT (animal 52; **B**), in the PO (animal 51; **C**), rostral from the OVLT (animal 51; **D**; **inset** represents the same neuron at a higher magnification), in the AHA just above the optic chiasm (not completely in focus; animal 54; **E**; **inset** represents the same neuron at a higher magnification), and in the PO (animal 57; **F**). OC, optic chiasm. Scale bars = 20 μm in A–F, 5 μm in inset E.

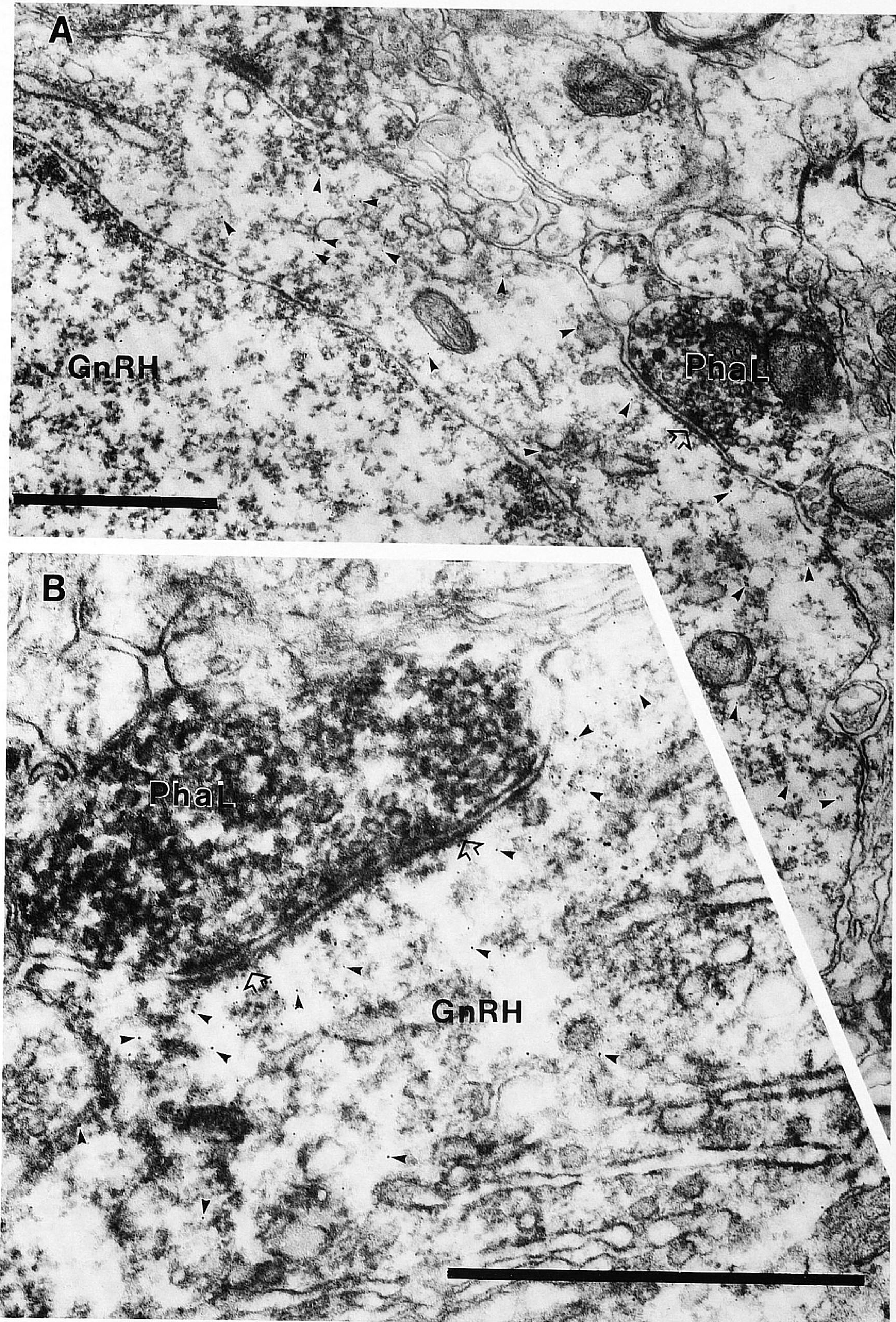

Fig. 4. Interaction of fibers containing PhaL (3,3′-diaminobenzidine; DAB) with GnRH neurons (gold particles; arrowheads) at the ultrastructural level. **A,B:** Synaptic input (open arrows) of PhaL-immunoreactive axons onto GnRH-containing cell bodies in the PO. Scale bars = 1 μm.

The present study clearly indicates the presence of a monosynaptic neuronal connection between the SCN and the GnRH system in the female rat. Tracing studies showed that at least 70% of the GnRH neurons project to the portal vasculature (Merchenthaler et al., 1989; Witkin, 1990), which suggests that most GnRH neurons are neuroendocrine in nature. In view of the role of SCN in the entrainment of rhythms to the environmental light-dark cycle, it is feasible that the presently described monosynaptic pathway constitutes an anatomical substrate that is involved in the circadian regulation of preovulatory LH release in the female rodent.

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