Abstract #3:

Female reproduction requires the precise temporal organization between estradiol and the master circadian clock located in the anterior hypothalamus. The phasic estradiol release functions to activate the phasic GnRH release mode that leads to a preovulatory surge of LH and thus ovulation. The central biological clock, in the SCN, might play a role in the activation of the GnRH network, probably through VIP acting on the intrinsic clock-controlled genes expressed in the GnRH neurons. The mechanism of this structure and function relationship is initiated by the release of estradiol from the ovaries. Then, estradiol acts on the SCN which causes VIPergic neurons in the SCN to project monosynaptically to the VIP receptor (VPAC₂) that is located on GnRH neurons. Consequently, GnRH is released into the hypophyseal portal system which stimulates the LH surge. The importance of maintaining a light-dark cycle is critical for the proper functioning of the SCN. In a constant light and estradiol environment, two LH surges will occur in a 24 hour period, each surge in antiphase oscillation between the bilateral SCN. Thus, the importance of the SCN in stimulating the LH surge through the release of GnRH via estradiol and VIP is critical in the female reproductive system.
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.

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Indexing terms: female rat; circadian regulation; estrous cycle; synapse
generated 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 proestrus-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., 1981), 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 fentanyl citrate per ml, 0.1 ml/100 g bodyweight i.m.; Janssen Pharmaceuticals, Beersel, 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/HC1 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 (TB1T). 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 TB1T; Vector Laboratories) for 1 hour at room temperature. The immunoreactivity for GnRH was detected with biotinylated sheep-anti-goat (HAS-bio; 1:400 in TB1T; Vector Laboratories) for 1 hour at room temperature. Double immunoreactivity was detected with a 1:400 dilution of biotinylated horse-anti-sheep (HAS-bio; 1:400 in TB1T; Vector Laboratories) for 1 hour at room temperature. The immunoreactivity for GnRH was detected with a 1:400 dilution of biotinylated horse-anti-sheep (HAS-bio; 1:400 in TB1T; Vector Laboratories) for 1 hour at room temperature.
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$_2$O$_2$ (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, PA) 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, 57, 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 perichiasmatic 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

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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, and in the rostral part of the AHA, 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 (Bujs 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-
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

![Diagram](image-url)

**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.
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. It 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.

ACKNOWLEDGMENTS

Part of this work was carried out during a research visit of Eline Van der Beek to the Neuroscience Department of the Loeb Research Institute (Ottawa Civic Hospital, Ottawa, Canada), which was supported by the C.G. van ’t Hooft Foundation and the Dutch Foundation for Medical Sciences.

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MONOSYNAPTIC SCN-GnRH PATHWAY
Suppression of Vasoactive Intestinal Polypeptide in the Suprachiasmatic Nucleus Leads to Aging-Like Alterations in cAMP Rhythms and Activation of Gonadotropin-Releasing Hormone Neurons

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Input from the suprachiasmatic nucleus (SCN) to gonadotropin-releasing hormone (GnRH) neurons is critical to the occurrence of regular cyclic GnRH secretion. It is thought that an essential neuropeptide in the SCN that communicates this cyclic information to GnRH neurons is vasoactive intestinal polypeptide (VIP) and that it may act through cAMP. We tested the hypothesis that (1) aging involves a blunting of cAMP diurnal rhythmicity in the SCN; (2) administration of antisense oligonucleotides (anti-oligos) against VIP, which produces an aging-like pattern in VIP, would lead to an aging-like suppression of cAMP; and (3) this in turn would lead to inhibition of the steroid-induced activation of GnRH neurons. We measured cAMP concentrations in the SCN and rostral preoptic nucleus throughout the day in young and middle-aged rats that were ovariectomized (OVX) or OVX and treated with estradiol.

Our results show that cAMP concentrations exhibit a diurnal rhythm in young rats, and that this rhythm is totally abolished by the time rats are middle age. Administration of antisense oligonucleotides against VIP or random oligos suppresses VIP concentrations and abolishes the cAMP rhythm, leading to significantly reduced activation of GnRH neurons. Together, these findings strongly suggest that the SCN conveys diurnal information to GnRH neurons by driving VIP-dependent cAMP rhythms. In addition, aging involves deterioration in this VIP-driven rhythmicity, which impacts the ability of steroids to induce GnRH neuronal activation.

Key words: GnRH; aging; medial preoptic area; vasoactive intestinal polypeptide; cAMP; reproduction

Introduction
An intricate and complex interplay of hypothalamic and peripheral endocrine events controls the cyclic synthesis and secretion of gonadotropin-releasing hormone (GnRH), which in turn, leads to cyclic secretion of luteinizing hormone (LH) from the anterior pituitary. The synchronization of these events is tightly coupled to environmental cues, such as the light/dark cycle, and circulating ovarian hormones, such as estradiol (Wise, 1999). As rats approach middle age, age-related deterioration in hypothalamic function is a crucial element that contributes to reproductive decline (Wise et al., 1997, 1999; Wise, 1999, 2000; Wise and Smith, 2001). We found that the ability of the suprachiasmatic nucleus (SCN), the circadian pacemaker of the brain, to drive an array of diurnal neurochemical events diminishes with age, and this may explain the dampening and desynchronization of multiple neurotransmitter rhythms that lead to the age-related improper timing of GnRH–LH surges (Lloyd et al., 1994; Krajnak et al., 2001; Le et al., 2001). Assessment of activation of GnRH neurons, by measuring Fos within the nuclei of GnRH neurons (Lee et al., 1990), reveals that although the concentration of GnRH does not change, the extent of activation is markedly attenuated during middle age (Lloyd et al., 1994; Rubin et al., 1997; Krajnak et al., 2001; Legan and Tsai, 2003). Therefore, decreased activity of GnRH neurons on proestrus and a decrease in the magnitude and a delay in the timing of the preovulatory LH surge characterize this functional decline in reproduction.

Several lines of evidence suggest that a diurnal pattern of vasoactive intestinal polypeptide (VIP) gene and protein expression, originating from the SCN, is an essential component of the constellation of stimulatory signals leading to the proper timing of preovulatory GnRH (Harney et al., 1996; Horvath et al., 1998; van der Beek et al., 1999) and that changes in the dynamics of this neuropeptide may contribute to age-related alterations in the pattern of GnRH secretion (Krajnak et al., 2001; Le et al., 2001). First, VIP neurons in the SCN directly innervate GnRH neurons in the rostral preoptic nucleus (rMPN) (van der Beek et al., 1999; Horvath et al., 1998). Second, GnRH neurons express the VIP/PACAP receptor subtype 2 (VPAC2) receptor subtype (Smith et al., 2000), which is coupled to the stimulatory G-protein (Gs) signal transduction pathway that leads to an accumulation of cAMP (McCulloch et al., 2002). Interestingly, Chappell et al. (2000) reported that cAMP levels in the anteroventral periven-
tricular nucleus (AVPV) exhibit a diurnal rhythm that is markedly analogous to the rhythm of VIP. Third, aging affects the rhythm of VIP in the SCN (Krajinak et al., 1998a), and suppression of this rhythm in young rats leads to delayed and attenuated LH surges that are strikingly similar to those observed in middle-aged (MA) female rats (Harney et al., 1996; van der Beek et al., 1999).

The goal of this study was to determine whether the diurnal rhythm of cAMP in the SCN and rMPN changes with age, and whether attenuation of VIP leads to a disruption of the cAMP diurnal rhythm and concomitant suppression of steroid-induced activation of GnRH neurons.

Materials and Methods

Animals. Young (2–3 months of age) and middle-aged (9–12 months of age) female Sprague Dawley rats (Zivic-Miller, Penelope, PA) were maintained on a 14/10 hr light/dark cycle (lights on at 0400 h) with food and water available *ad libitum*. Estrous cyclicity was monitored by daily vaginal lavage for at least 3 weeks before use. Only young rats that exhibited at least two consecutive 4 d estrous cycles and middle-aged rats that exhibited estrous cycles of regular or irregular length (5–8 d) were used. All rats were ovariectomized (OVX; day 0) and implanted subcutaneously with a SILASTIC capsule (0900 h on day 7; Konigsberg Instruments, Pasadena CA) containing either sesame oil or 17β estradiol (Sigma, St. Louis, MO) dissolved in sesame oil (180 μg/ml; young, 30 mm capsule; middle-aged, 40 mm capsule; 0.062 × 0.125 inches, inner × outer diameter). This estradiol treatment paradigm produces estradiol in the SCN. At the same time, rats were im-

Oligonucleotide treatment. Oligonucleotides were synthesized with a Sonic Disemembrator (Fisher Scientific, Houston, TX) in ice-cold 5% trichloroacetic acid (Sigma). Homogenates were centrifuged at 600 × g, and supernatants were removed, placed in microcentrifuge tubes, lyophilized in a Speed-Vac evaporator (Savant Instruments, Farmington, NY), and reconstituted in assay buffer and measured for cAMP (Assay Designs, Ann Arbor, MI). Pellets were resuspended in buffer solution from the cAMP kit. Protein content (10 μl of reconstituted homogenates) was determined using the Bradford assay (Bio-Rad, Hercules, CA). Data were expressed as picomoles of cAMP per milligram of protein.

Immunocytochemistry. Coronal brain sections (40 μm) were cut in a cryostat (Microm, Kalamazoo, MI) starting at the level of the medial diagonal band of Broca (bregma, −0.20 mm) (Paxinos and Watson, 1997) through the accumbens nucleus (bregma, −3.80 mm). Each brain was collected in a series of six sets with every sixth section represented in a group and stored in cryoprotectant until processed for ICC. Antibodies against GnRH (LR-1; Benoit, Montreal, Canada) and Frog (Santa Cruz Biotechnology, Santa Cruz, CA) (recognizes Fos and Fra-related antigens) were used to determine activation of GnRH neurons. On day 1, sections from one series that contained every sixth section were rinsed in 0.1 M Tris-buffered saline (TBS) and blocked with 10% normal horse serum (NHS) plus 0.4% Triton X-100 (NHS-X) for 1 hr at room temperature. Next, sections were incubated in goat anti-Fos (1:25,000) in 2% NHS-X overnight at 4°C. On day 2, sections were rinsed in TBS, incubated in biotinylated anti-goat IgG diluted 1:500 in 2% NHS-X for 1 hr at room temperature, followed by incubation in avidin–biotin complex (ABC) (Vectastain kit; Vector Laboratories, Burlingame, CA) in TBS plus 0.4% Triton X-100 for 1 hr at room temperature. Finally, sections were incubated in 0.025% diaminobenzidine (DAB; Sigma) with 0.02% ammonium nickel sulfate (Fisher Scientific) and 0.1 μl/ml of 30% hydrogen peroxide in TBS for 10 min at room temperature. After the DAB reaction, sections were rinsed in TBS and then incubated in rabbit anti-GnRH (1:100,000) in 2% NHS-X overnight at 4°C. On day 3, sections were rinsed in TBS, incubated in biotinylated anti-rabbit IgG diluted 1:500 in 2% NHS-X for 1 hr at room temperature, followed by incubation in avidin–biotin complex (ABC) (Vectastain kit; Vector Laboratories) in TBS plus 0.4% Triton X-100 for 1 hr at room temperature. Finally, sections were incubated in 0.025% DAB (Sigma) with 0.1 μl/ml of 30% hydrogen peroxide in TBS for 10 min at room temperature. Each step was followed by rinses in TBS [three rinses in TBS (5 min each)]. Sections were mounted on slides, and coverslips were applied using Permount (Fisher Scientific).

**V**IP radioimmunoassay total protein in **SCN**. In antisense-treated animals, the frozen SCN tissue punches were divided into two groups for each animal. One group was used in the cAMP ELISA. The other group was used to determine the concentration of VIP in the SCN tissue punches. Tissue punches were extracted as described above. The pellets were assayed for VIP using a kit (Peninsula Laboratories, Belmont, CA). Total protein in the SCN punches was measured using the DC Protein Assay kit (Bio-Rad). Data were expressed as picograms of VIP per microgram of protein.

**Statistical analysis.** cAMP levels in the rMPN, SCN, and cerebral cortex were analyzed by Student’s *t* test. VIP concentrations in the SCN were measured by two-way ANOVA followed by Bonferroni’s *post hoc* test for effects of treatment × time interaction. One-way ANOVA followed by Newman–Keuls *post hoc* tests were performed on cAMP concentrations in antisense and random oligo-treated animals in the rMPN, SCN, and cerebral cortex to test for effects of time. Two-way ANOVA followed by Bonferroni’s *post hoc* test were used to determine the percentage of GnRH expressing compared with all GnRH neurons in antisense and random sequence oligo-treated rats for effects, treatment, time, and treatment × time interactions. All statistics were performed using Prism 4.0 software (Graphpad, San Diego, CA).
Results

cAMP

SCN
In young OVX and OVX rats treated with estradiol (OVX-E₂), cAMP levels (picomoles per milligram of protein) in the SCN exhibited a diurnal rhythm (Fig. 1A). Regardless of steroidal milieu, cAMP levels were the lowest at 0300 and 0800 h (Fig. 1A). By 1200 h, cAMP levels rose and remained high through 1400 h. By 1600 h, cAMP levels returned to baseline and were not significantly different from 0300 and 0800 h. In middle-aged OVX and OVX-E₂-treated rats, cAMP levels did not exhibit a diurnal rhythm (Fig. 1B).

rMPN
In young rats, cAMP levels (picomoles per milligram of protein) exhibited diurnal rhythms that were similar regardless of steroidal milieu (Fig. 1C). In OVX rats, cAMP levels were the lowest at 0300 and 0800 h (Fig. 1C). By 1200 h, cAMP levels rose and peaked at 1400 and 1600 h. By 1800 h, cAMP levels returned to baseline and were not significantly different from 0300 and 0800 h. In OVX-E₂-treated young rats (Fig. 1C), cAMP levels were low at 0300 h, 0800 h, and 1200 h, peaked at 1400 and 1600 h (p < 0.05), and returned to baseline at 0600 h. In middle-aged rats, cAMP levels did not exhibit a diurnal rhythm in either OVX or OVX-E₂-treated rats (Fig. 1D).

Cerebral cortex
cAMP (picomoles per milligram of protein) did not exhibit a diurnal rhythm in the cortex, and neither E₂ nor age altered the pattern or level of cAMP (Fig. 1E, F).

Effects of antisense oligo treatment on VIP concentrations in the SCN

VIP levels (picograms per microgram of protein) in the SCN in random sequence oligo-treated rats displayed a diurnal rhythm (Fig. 2). VIP concentrations were low at 0300 h (43 hr after injection), significantly greater at 1400 h (53 hr after injection; p < 0.05), and decreased by 1800 h (57 hr after injection). Treatment with antisense oligo against VIP blocked the VIP rhythm (Fig. 2).

Effects of antisense oligo treatment on cAMP concentrations in the SCN, rMPN, and cerebral cortex
cAMP levels in the SCN and rMPN of control random sequence oligo-treated rats displayed a diurnal rhythm similar to cAMP levels in Figure 1 (Fig. 3A). In the SCN, cAMP levels in control random sequence oligo-treated rats were lower at 0300 h (Fig. 3A) and increased at 1400 h (p < 0.001). By 1800 h, cAMP levels were significantly decreased compared with 1400 h (p < 0.001) but were still more than at 0300 h (p < 0.05). Antisense oligo treatment suppressed cAMP levels and abolished the rhythm (Fig. 3A). In the rMPN, cAMP levels were significantly low at 0300 h and increased at 1400 h (p < 0.001) and returned to baseline (Fig. 3B). Antisense oligo treatment significantly
Our study clearly establishes four important points. First, in young rats, cAMP levels exhibit a robust diurnal rhythm in the SCN and rMPN, two brain regions that play crucial roles in the occurrence of cyclic GnRH release. Second, this rhythm in both brain regions disappears by the time rats are middle-aged, a time when cyclic GnRH release and reproductive cycles become irregular. Third, VIP from the SCN appears to drive this rhythm, because suppression of the VIP rhythm abolishes the cAMP rhythm in both brain regions. Fourth, the VIP-driven cAMP rhythm plays a critical role in activating GnRH neurons, because blockade of the VIP rhythm suppresses this activation. Thus, we have established, for the first time, a functional relationship between VIP, cAMP, and GnRH neurons, a pathway that appears to be important in reproductive cyclicity.

The SCN of the hypothalamus is the major circadian pacemaker of the brain and consists of the following: (1) biological oscillators that are endogenous and maintain a self-sustained circadian periodicity in the absence of environmental time cues; (2) input pathways that convey environmental information, especially light cues, that entrain circadian oscillations to local time; and (3) output pathways that drive overt circadian rhythms, such as the rhythms of locomotor activity and a variety of endocrine rhythms (Goldman, 1999). Ablation of the SCN disrupts virtually all rhythms and leads to the disappearance of cyclic reproductive function in female rodents (Bethea and Neill, 1980).

Our results clearly establish that cAMP rhythms exist in the SCN and rMPN, and that aging involves a waning of these cAMP rhythms in middle-aged rats. These brain regions work in concert to regulate the cyclic synthesis and secretion of GnRH and to induce reproductive behaviors (Wise and Smith, 2001). Our findings confirm and extend the previous study by Chappell et al. (2000) who showed that cAMP levels display a diurnal rhythm in the AVPV. It is particularly intriguing that the disappearance of the cAMP rhythms occur at the same age as previously reported alterations in VIP rhythms in the SCN and in the activation of GnRH neurons, which is required for LH surges to occur (Lloyd et al., 1994; Krajnak et al., 1998a, 2001; Le et al., 2001; Legan and Tsai, 2003). Therefore, we explored whether these three neurochemicals (VIP, cAMP, and GnRH) are functionally linked.

We asked whether VIP drives the cAMP rhythm. VIP is a logical candidate for several reasons. First, VIP is a major neuropeptide in the SCN and receives retinal projections directly from the retinohypothalamic tract (Stephan et al., 1981; Tanaka et al., 1993) and indirectly from the retinorecipient intergeniculate leaflet via the geniculohypothalamic tract (Tanaka et al., 1993). Second, VIP mRNA and protein exhibit diurnal rhythmicity and are entrained to the light/dark cycle (Shinohara et al., 1993; Krajnak et al., 1998b). Third, VIP binding to the VPAC2 receptor activates the Gs pathway leading to the accumulation of cAMP (McCulloch et al., 2002). Fourth, we have shown that the rhythm in VIP mRNA in the SCN disappears during middle age analyzed a group of estradiol-treated middle-aged rats. The percentage of GnRH-expressing Fos-IR was identical to antisense-treated rats and did not show the characteristic afternoon activation that we observed in young rats in either the OVLT (Fig. 5A) or the rMPN (Fig. 5B).

Discussion

Effects of antisense oligo treatment on GnRH activation in the OVLT and rMPN compared with MA rats

The percentage of GnRH expressing Fos immunoreactivity (IR) in the OVLT (Fig. 4A) and rMPN (Fig. 4B) in control random sequence oligo-treated rats was low at 0300 h and rose at 1400 h (p < 0.01) and 1800 h (p < 0.001). In previous studies, we have established that the LH surge occurs between 1400 and 1800 h. Thus, the expression of Fos within GnRH neurons is an index of activation of these neurons and stimulation of the LH surge. In rats treated with antisense oligos to VIP, the percentage of GnRH neurons expressing Fos-IR in the OVLT and rMPN did not rise at 1400 or 1800 h (p < 0.001) (Fig. 4A, B). We were interested to determine whether antisense treatment mimicked the level of GnRH activation in middle-aged rats. Therefore, in parallel, we analyzed a group of estradiol-treated middle-aged rats. The percentage of GnRH-expressing Fos-IR was identical to antisense-treated rats and did not show the characteristic afternoon activation that we observed in young rats in either the OVLT (Fig. 5A) or the rMPN (Fig. 5B).

Figure 3. A, cAMP levels (picomoles per milligram of protein) in the SCN of rats treated with random sequence oligos exhibit a diurnal rhythm, whereas treatment with antisense oligos disrupts the cAMP diurnal rhythm. B, cAMP levels (picomoles per milligram of protein) in the rMPN of rats treated with random sequence oligos exhibit a diurnal rhythm, whereas treatment with antisense oligos disrupts the cAMP diurnal rhythm. C, cAMP levels in the cortex do not exhibit diurnal rhythms, regardless of treatment. Symbols indicate levels of significance between time points (*p < 0.05; **p < 0.01; ***p < 0.001) and between treatments (##p < 0.01; ###p < 0.001).

Figure 4. The percentage of GnRH neurons expressing Fos-IR in the OVLT (A) and the rMPN (B) of rats treated with random sequence oligos throughout the day exhibited a diurnal rhythm. Treatment with antisense oligos in the SCN disrupted the diurnal rhythm in the OVLT and rMPN. Symbols indicate levels of significance between time points (*p < 0.05; **p < 0.01; ***p < 0.001) and between treatments (##p < 0.01; ###p < 0.001).

Figure 5. GnRH neurons expressing Fos-IR in the OVLT (A) and in the rMPN (B) in middle-aged rats did not exhibit a diurnal rhythm and were not different from the antisense oligo-treated rats.

decreased cAMP levels in the rMPN at all time points and abolished the rhythm (Fig. 3B). Antisense treatment did not significantly affect cAMP levels in the cerebral cortex (Fig. 3C).
(Krajnak et al., 1998a), at the same age we observed the disappearance in cAMP rhythms in the current study. VIP dynamics are sexually dimorphic and play essential roles in cyclic reproductive function in females. The number of VIP boutons that terminate on GnRH neurons and the percentage of GnRH neurons contacted by VIP axons are greater in female rats than in male rats (Horvath et al., 1998). Krajnak et al. (1998b) showed that the rhythm of VIP mRNA in the SCN of females is 12 hr out of phase with that of male rats. In females, peak VIP mRNA occurs during the early afternoon of the light phase, whereas in males, peak VIP mRNA and protein occur in the middle of the dark phase. This sexually dimorphic pattern of VIP activity gave rise to the speculation that VIP neurons are critical in conveying circadian information to GnRH neurons and may indicate the involvement of VIP in the sex-specific regulation of GnRH release in rats.

To test whether VIP drives the cAMP rhythm, we administered antisense oligos to VIP into the peri-SCN region of young rats. This treatment had three effects: (1) it suppressed the concentrations of VIP in the SCN, replicating the effects that we observed previously (Harney et al., 1996); (2) it abolished the rhythm of VIP peptide expression; thus, both the concentration and absence of rhythmicity mimicked the profile normally observed in MA rats (Krajnak et al., 1998a); and (3) GnRH neurons that are closely apposed to VIP fibers preferentially express Fos, an index of activity (Krajnak et al., 2001). We show that injections of VIP antisense oligos into the SCN decreased the level of estradiol-induced Fos activation in GnRH neurons in the OVLT and rMPN in the afternoon at a time when we observed elevated GnRH activation in young rats. This antisense-induced suppression of GnRH neuronal activation is similar to what we observed in middle-aged rats and suggests that although VIP innervation of GnRH neurons does not change with age (Krajnak et al., 2001), the SCN itself may not be functioning properly to activate GnRH neurons. Our findings add to the increasing evidence: (1) VIP fibers from the SCN innervate GnRH neurons (van der Beek et al., 1997; Horvath et al., 1998); (2) GnRH neurons express the VPAC2 receptors (Smith et al., 2000); and (3) GnRH neurons that are closely apposed to VIP fibers preferentially express Fos, an index of activity (Krajnak et al., 2001). We show that injections of VIP antisense oligos into the SCN decreased the level of estradiol-induced Fos activation in GnRH neurons in the OVLT and rMPN in the afternoon at a time when we observed elevated GnRH activation in young rats. This antisense-induced suppression of GnRH neuronal activation is similar to what we observed in middle-aged rats and suggests that although VIP innervation of GnRH neurons does not change with age (Krajnak et al., 1998a), the SCN itself may not be functioning properly to activate GnRH neurons.

Our results clearly demonstrate that an aging-like pattern of VIP in the SCN leads to an aging-like profile in cAMP rhythms in the SCN and may lead to an aging-like profile in cAMP rhythms in the rMPN, which in turn may lead to an aging-like suppression of GnRH neuronal activation. Our findings add to the increasing body of evidence that aging results in fundamental changes in the SCN because of the following: (1) the size and number of different neuronal populations change with age (Roozendaal et al., 1987; Chee et al., 1988); (2) the amplitude of electrical activity rhythms in slice and dispersed cultures becomes dampened (Satinoff et al., 1993; Ruby et al., 1998; Aujard et al., 2001); and (3) the free-running period and the light-induced production of the clock genes Per1 and Per2 change with age (Yamazaki et al., 2002; Asai et al., 2001). These changes in the pacemaker itself or the coupling to its outputs may cause temporal desynchronization of rhythms that are critical for the precise timing of GnRH secretion and ultimately LH secretion ( Wise et al., 1996, 1997; Krajnak et al., 1998a).

The AVPV, OVLT, and rMPN play critical and interactive roles regulating cyclic GnRH–LH surges. The AVPV serves as a nodal relay station where information on steroid milieu is communicated to GnRH neurons (Simerly et al., 1990; Simerly, 1998; Simonian et al., 1999). We show that the SCN sends circadian signals directly to the OVLT and rMPN areas of the brain that contain a subpopulation of GnRH neurons that are dedicated to regulating the cyclic timing of GnRH–LH surges. Both the steroid and circadian information converging on GnRH neurons are necessary but not sufficient by themselves to induce GnRH–LH surges. We demonstrate how age leads to deterioration in the circadian signal that is relayed to GnRH neurons and how this circadian signal is important in the timing of the GnRH–LH surges. Our microdissected punches contained the AVPV, rMPN, and preoptic area (POA). Thus, it is possible that inputs from the POA could affect GnRH activation. However, because we reduced cAMP and the level of activation of GnRH neurons with the VIP antisense oligo treatment, we conclude that VIP from the SCN is necessary for the timing of the LH surge but not sufficient to induce a LH surge.

In summary, we have established a functional relationship between VIP in the SCN and GnRH neurons in the rMPN that is mediated through the cAMP pathway. These data suggest that VIP is a key messenger from the SCN that communicates time-of-day information to GnRH neurons. Thus, maintenance of the normal rhythm of VIP is of great importance to the synchronization of the SCN with GnRH neurons. Additionally, we have uncovered that aging affects this pathway and may be one component that contributes to the gradual deterioration of reproductive cyclicity in aging females. Subtle changes in the ability of the biological clock to drive reproductive rhythms in middle-aged rats may underlie the transition to acyclicity and decline in reproductive function. Thus, these findings demonstrate the crucial role the SCN plays in coordinating the timing of GnRH–LH surges and reproduction.

References

Krajnak K, Kashon ML, Rosewell KL, Wise PM (1998a) Aging alters the...


A surge of GnRH release signals the LH surge that triggers ovulation. The GnRH surge is dependent on a switch in estradiol feedback from negative to positive and, in rodents, a daily neural signal, likely from the suprachiasmatic nuclei. Vasoactive intestinal polypeptide (VIP) may be involved in suprachiasmatic nuclei-GnRH neuron communication. Here we assessed the effects of acute VIP (5 min treatment) on GnRH neuron function using targeted extracellular recordings of firing activity of GnRH neurons in brain slices. We examined the effect of VIP on firing rate at different times of day using an established ovariectomized, estradiol-treated (OVX+E) mouse model that exhibits daily LH surges timed to the late afternoon. Cells from OVX animals (no estradiol) did not respond to VIP, regardless of time of day. With estradiol, the effect of VIP on GnRH neurons was dependent on the time of recording. During negative feedback, OVX+E cells did not respond. VIP increased firing in cells recorded during surge onset, but this excitatory response was reduced at surge peak. Acute treatment of OVX+E cells during surge peak with a VIP receptor antagonist decreased GnRH neuron firing. This suggests endogenous VIP may both increase GnRH neuron firing during the surge and occlude response to exogenous VIP. These data provide functional evidence for VIP effects on GnRH neurons and indicate that both estradiol and time of day gate the GnRH neuron response to this peptide. VIP may provide an excitatory signal from the circadian clock that helps time the GnRH surge. (Endocrinology 149: 3130–3136, 2008)
Materials and Methods

Animals

Adult female mice (2–4 months old) in which green fluorescent protein is expressed under the control of the GnRH promoter (21) were used for all experiments. Animals were housed on a 14-h light, 10-h dark cycle with lights off at 1630 h Eastern Standard Time and had ad libitum access to food (Harlan 2916 chow; Harlan, Indianapolis, IN) and water. Mice were bilaterally ovariectomized under isoflurane anesthesia (Burns Veterinary Supply, Westbury, NY) and treated with a sc SILASTIC brand capsule (Dow-Corning Co, Midland, MI) containing 0.625 μg estradiol suspended in sesame oil (OVX containing (in m

Two to four days after surgery, coronal or sagittal brain slices (300 μm) were prepared with slight modifications (4, 22) of previous descriptions performed during negative feedback (1100–1400 h), surge onset (1400–1600 h), and surge peak (1600–1900 h). During negative feedback, five of six cells (83%) did not respond (Fig. 1, A and B). In contrast, VIP increased firing in nearly half of OVX + E cells recorded at surge onset (46%, n = 6 of 13) (Figs. 1, A and C, and 2); the remaining cells did not respond. In responding cells, the percentage increase in firing was 673 ± 318% (P < 0.05 vs. pre-VIP levels) (Fig. 2). Baseline mean firing rate during surge onset was not significantly different from mean firing rate during negative feedback, largely because seven of the 13 cells showed very low levels of firing (0–0.01 Hz), likely due to variability in the time of transition from negative feedback to surge onset. Basal firing rate, however, did not affect the rate of response to VIP during surge onset because it was not significantly different between responders and nonresponders (responders, 0.18 ± 0.1 Hz vs. nonresponders, 0.07 ± 0.04 Hz, P > 0.25). Of note, this rate of response corresponds well with the percentage of GnRH neurons in the rat that express receptors for VIP (13). During the surge peak, the rate of response to VIP was reduced because only four of 14 cells (27%) showed a change in firing; furthermore, the direction of response was not consistent with two cells increasing and two cells decreasing firing rate (Figs. 1, A and D, and 2). VIP can thus excite GnRH neurons from OVX + E mice, but this effect appears limited to specific times of day.

The orientation of brain slice preparation may affect the response to drug application because different populations of synaptic afferents are maintained in different orientations. For example, γ-aminobutyric acid (GABA) transmission to GnRH neurons during surge peak is affected by slice orientation (26), and other forms of neurotransmission may be altered as well. To examine whether the response to VIP during surge peak is dependent on maintenance of specific
afferent connections, the effect of VIP on firing of GnRH neurons in sagittal slices from OVX/E mice was tested. Sagittal slices are of interest because they can contain both the SCN and GnRH neurons and thus may preserve endogenous SCN-GnRH pathways. They also can contain the AVPV, a known target of VIP that subsequently projects to GnRH neurons and is thus poised to convey transsynaptic signals. Similar to the data in coronal slices during surge peak, eight of 15 cells (53%) did not respond to VIP. Of the remaining cells, five (33%) increased firing and two (13%) decreased firing. Although this indicates no net response across the population of cells tested (Fig. 2), the change in firing in those cells that showed an increase was 159 ± 44%, indicating a substantial subpopulation of GnRH neurons in sagittal slices can respond to VIP with an increase in firing rate during surge peak.

**Estradiol is required for a response to VIP**

Estradiol is required for surge generation (4–6). We thus hypothesized that estradiol is required for a response to VIP. To test this, the effects of VIP on cells from OVX mice that were not treated with estradiol were examined. The vast majority of OVX cells (n = 9 of 10) did not respond to VIP application either during times corresponding to negative feedback or surge onset in OVX+E-treated animals (negative feedback, control 0.04 ± 0.02 Hz vs. VIP 0.01 ± 0.01 Hz, n = 5 cells, P > 0.25; surge onset, control 0.06 ± 0.05 Hz vs. VIP 0.06 ± 0.06 Hz, n = 5 cells, P > 0.72) (Fig. 3). Thus, estradiol is required for a GnRH neuron response to VIP, just as it is for the expression of diurnal changes in GnRH neuron firing activity (4).

**Endogenous VIP can excite GnRH neurons during the GnRH/LH surge**

The reduced rate of response to VIP during surge peak (when OVX+E cells show an elevated level of firing activity) could be at least partially due the presence of VIP in the slice from an endogenous VIP input, in which case exogenously applied VIP would likely have no effect. To examine whether GnRH neurons receive an excitatory endogenous VIP input during the surge peak, the effect of a VIP receptor antagonist (100 nM) on GnRH neuron firing rate was tested in OVX+E cells in sagittal slices. VIP antagonist application reversibly decreased firing in four of nine cells (44%) (Fig. 4). This percentage is similar to that which was excited by VIP application around the time of surge onset, and the percentage of cells expressing VIP receptors (13). In the cells that responded, the average decrease was 67 ± 15% (P < 0.05). Basal firing rate did not affect the rate of response to VIP receptor antagonism as firing rate was not significantly different between responders and nonresponders (responders, 0.99 ± 0.31 Hz vs. nonresponders, 0.88 ± 0.22 Hz, P > 0.73). In coronal slices, which do not contain SCN cell bodies, the response to VIP antagonist was weak. Although a similar percentage of cells did not respond to antagonist as in sagittal slices (58%, eight of 14), there was not a consistent direction of change in cells that did change firing at the time of drug application. Specifically, three increased firing and three de-
increased firing, averaging out to no net response across the population of cells tested (P > 0.5; Fig. 4). Thus, the response to VIP antagonism appears dependent on the maintenance of connections from the SCN within the brain slice preparation. These data suggest endogenous VIP may play a role in increasing GnRH neuron firing during the surge in a substantial subpopulation of GnRH neurons.

**Discussion**

In some species, input from the circadian timing system appears to be critical in the regulation of reproduction, particularly in the neural control of the ovulatory process. The precise nature of this input is poorly understood. Previous studies examining the effect of VIP or VIP antagonism in rats have been inconclusive, with some indicating an inhibitory effect and others indicating an excitatory effect (17–19). Because measurement of GnRH levels in mice are problematic because of low peptide levels, we used electrophysiological techniques to assess the effects of VIP on the activity of GnRH neurons in brain slices. Here we present evidence supporting a role for VIP in driving increased firing of a substantial subpopulation of GnRH neurons during the GnRH surge. The effects of VIP on GnRH neurons are dependent on both estradiol feedback state and time of day, two elements that are essential in surge regulation, at least in rodent species (4–6). Thus, VIP is a candidate as a component of the daily neuronal signal that is required for surge generation.

Based on these results, we postulate that both estradiol- and time-of-day-dependent gates must be open to allow for VIP excitation of GnRH neurons (Fig. 5). Without estradiol, VIP is blocked from exerting an effect on the activity of GnRH neurons (Fig. 5A). Estradiol opens this gate, but a second, time-of-day-dependent gate is closed during negative feedback (Fig. 5B), preventing VIP action on GnRH neurons. The time-of-day-dependent gate opens near the time of surge onset, allowing for effects of exogenously applied VIP (Fig. 5C). This gate remains open during the surge peak, but at this time, GnRH neurons are already receiving an endogenous
VIP input, so the response to exogenously applied VIP is at least partially occluded (Fig. 5D).

The SCN are a strong candidate source of a daily signal required for surge generation. Lesions of the SCN eliminate both the LH surge and ovulation (9). SCN grafts capable of restoring locomotor rhythmicity do not restore LH surges in OVX+E hamsters (27), indicating that a neural, rather than humoral, SCN output may be required for LH surge generation. Furthermore, anatomical studies have indicated the presence of ipsilateral, direct SCN-GnRH neuron connections (10, 11, 28, 29). VIP is synthesized in the ventrolateral portion of the SCN, which receives retinal information about external light conditions both directly (30, 31) and indirectly (31), and VIP fibers are found in the vicinity of GnRH neurons (11, 12, 32–34). VIP is therefore a prime candidate for transmitting information about the external light-dark cycle to GnRH and other neurons.

Generation of the daily signal (or signals) may be independent of steroid hormones (35), but estradiol is required to couple circadian input to the GnRH system (4–6); integration of estradiol feedback and circadian signals is thus crucial for proper surge regulation. The α-isofrom of the estradiol receptor (ER) is required for surge generation (36, 37). Although ERα expression has been found in the human SCN, with higher expression in females (38), it has not been detected in the rodent SCN (39). The SCN receives projections from cells expressing ERα (40), however, providing a pathway for indirect actions of estradiol on the SCN. The AVPV appears to be a site of convergence of circadian and estradiol signals because these cells express ERα, receive inputs from the SCN, and project directly to GnRH neurons (15, 16, 41, 42). The increased percentage of GnRH neurons excited by VIP during surge peak in sagittal slices, compared with coronal slices, in the present study may indicate VIP acts through afferent pathways that are maintained in a sagittal, but not a coronal, slice preparation, such as connections from the AVPV and SCN. VIP receptor expression in the preoptic area appears to be largely confined to GnRH neurons and the AVPV (13, 14), suggesting that VIP action within the preoptic area is primarily involved in the control of reproduction. The requirement for both estradiol treatment and a particular time of day for a GnRH neuron response to VIP is consistent with a possible role for this neuropeptide in transmitting successful pre- or post-SCN convergence of estradiol feedback and circadian signals to GnRH neurons.

Alterations in GnRH neuron responsiveness to VIP provide a potential point of integration of circadian and estradiol signals directly at the level of the GnRH neuron. The percentage of GnRH neurons in the rat immunoreactive for VIP2 receptor protein does not appear to change during the day of proestrus (13). Other changes, such as alterations in receptor expression or binding and activation properties, may alter the responsiveness of GnRH neurons to VIP. Future work is needed to examine whether these mechanisms play a role and display an estradiol and/or circadian dependence. Estradiol increases GnRH neuron electrical excitability (43, 44), which may increase the response of GnRH neurons to depolarizing input such as VIP, providing a possible level of integration of these signals directly at the GnRH neuron.

The data presented here add to the growing body of evidence for widespread effects of VIP on neural function through changes in both neurotransmission and intrinsic properties of target cells. VIP can activate a hyperpolarization-activated cation current in thalamocortical neurons (45), reduce the amplitude of the Ca2+-dependent K+ current mediating the slow afterhyperpolarization current in hippocampal pyramidal neurons (46), and inhibit Ca2+ currents in sympathetic neurons of the rat pelvic ganglia (47). In cultures of hippocampal neurons, VIP increases GABA transmission (48). VIP also stimulates brain-derived neurotrophic factor mRNA transcription and c-fos expression in primary cultures of mouse cortical neurons (49, 50). With regard to GnRH neurons, VIP may act directly on these cells through the VIP2 receptor (13) and/or indirectly through other neurons (14) or glial elements (51). VIP may also act within the SCN to modulate GnRH-targeting outputs, such as through changes in intra-SCN GABA transmission (52). In the present studies, synaptic transmission within the brain slice network was kept intact to minimize disruption to the input pathways through which VIP may act on GnRH neurons; thus, both intrinsic and synaptic effects may contribute to the observed action of VIP.

The function of GnRH neurons can be regulated both by neuromodulators and fast synaptic transmission, which in GnRH neurons is mediated by GABA and glutamate acting through ionotropic receptors (22, 53). GABAreceptor activation can excite adult GnRH neurons (54–56), although inhibitory actions have also been suggested (57, 58). Recent work has indicated a role for GABAergic mediation of estradiol feedback in surge regulation. Specifically, estradiol

**Fig. 5.** Working model of VIP effects on GnRH neuron firing activity, in which estradiol and time of day both gate the GnRH neuron response to VIP. A, An estradiol-dependent gate blocks exogenous VIP from exerting an excitatory effect on OVX cells, regardless of time of day. B, OVX+E treatment opens the estradiol-dependent gate, but a time-dependent gate is closed during negative feedback. C, Near surge onset; both the estradiol gate and the time-dependent gate are open, and VIP is able to exert an excitatory effect on GnRH neurons. D, Both gates remain open during surge peak, but GnRH neurons are already receiving excitatory input from endogenous VIP, so the response to exogenously applied VIP is occluded.
decreases GABA transmission to GnRH neurons during negative feedback but increases it during positive feedback (26). Further, the SCN appears to be a potential source of increased GABA transmission because disrupting inputs from the SCN to GnRH neurons in a slice preparation lowers the frequency of GABA transmission during positive feedback (26). VIP colocalizes with GABA in the SCN, and it is possible that increased GABA transmission during the surge may reflect increased co-release of GABA and VIP. Preliminary data suggest similar diurnal changes in glutamate transmission to GnRH neurons in OVX+E mice, with low glutamate transmission during negative feedback and increased transmission during positive feedback (59).

The importance of fast synaptic transmission in surge generation is demonstrated by the disruptive effects of blocking ionotropic GABA and glutamate receptors on estradiol negative and positive feedback effects on GnRH neurons. This treatment reverses some, but not all, diurnal changes in GnRH neuron firing activity (60). If the GnRH surge were dependent solely on fast synaptic transmission, the reversal should be complete. This suggests that in addition to fast synaptic transmission, neuromodulators [such as VIP, vasoressin (61–63), or kisspeptin (64)] likely also play a significant role in regulating GnRH neurons in different estradiol feedback states. Given the importance of the surge for ovulation and reproductive success, multiple signals may regulate diurnal changes in GnRH neuron firing activity. Only a portion of the GnRH surge itself is required to generate an LH surge, providing a further safety net. This redundancy likely accounts for fertility in VIP and VIP receptor knockout mice (23).

In summary, VIP can excite GnRH neurons, and this effect is dependent on both estradiol feedback and time of day. Alterations in VIP release, along with other neuropeptides and fast synaptic transmission, are thus poised to play a role in regulating the diurnal and estradiol-dependent changes in GnRH neuron firing that appear to underlie surge generation.

Acknowledgments

We thank Debra Fisher and Xu-Zhi Xu for expert technical assistance and Justyna Pielecka-Fortuna, Alison Roland, Pei-San Tsai, and Heidi Walsh for helpful editorial comments.

Received August 9, 2007. Accepted February 28, 2008.

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This work was supported by National Institute of Child Health and Human Development/National Institutes of Health Grant R01 HD41469 and National Institute of Neurological Disorders and Stroke National Research Service Award F31 NS53253 (to C.A.C.).

Author Statement: C.A.C. and S.M.M. have nothing to disclose.

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