Lesions of the Suprachiasmatic Nucleus Indicate the Presence of a Direct Vasoactive Intestinal Polypeptide-Containing Projection to Gonadotrophin-Releasing Hormone Neurons in the Female Rat

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Abstract

In non-seasonal breeders like the rat, the influence of the suprachiasmatic nucleus (SCN) on reproduction is most clearly expressed in the female. Complete lesions of the SCN induce persistent oestrus (anovulation) in intact female rats, whereas oestrogen implantation in ovariectomized rats results in daily luteinizing hormone surges. Vasoactive intestinal polypeptide (VIP), a peptide synthesized in cell bodies of the SCN, inhibits the increase in pulsatile luteinizing hormone release observed in ovariectomized female rats. In search of the anatomical basis for these observations, the present study employs an immunocytochemical double staining for VIP and gonadotrophin-releasing hormone (GnRH) at the light microscopical level. It was demonstrated that approximately 45% of the GnRH positive neurons in the diagonal band of Broca, the preoptic and anterior hypothalamic area of female rats are innervated by VIP-containing processes. To investigate whether these VIP-containing fibres represent a direct projection of the SCN to the GnRH system, unilateral thamic SCN lesions were made.

Lesions that unilaterally destroyed the majority of the VIP synthesizing cells in the SCN resulted in at least a 50% decrease of the VIP innervation of GnRH cell bodies at the lesioned side compared to the intact side. Lesions not affecting the VIP synthesizing cell population in the SCN did not change the percentage of GnRH neurons innervated by VIP-containing fibres, while partial lesions resulted in intermediate effects.

These results indicate that the majority of the light microscopical VIP-containing input on GnRH neurons in the hypothalamus is derived from the SCN. It is suggested that the reported effects of VIP on luteinizing hormone release may, at least in part, be induced via a direct effect of VIP on GnRH cell bodies. This direct SCN–GnRH pathway provides an anatomical basis for diurnal influences on the regulation of the female reproductive cycle.

Gonadotrophin-releasing hormone (GnRH), the releasing hormone for the pituitary gonadotrophs, is secreted in a pulsatile way into the portal vasculature at the level of the median eminence. Although no steroid receptors have been demonstrated in GnRH cell bodies (1), synthesis and release of GnRH from the hypothalamus in the female rat are under control of a positive feedback of gonadal steroids (see review in 2). The afternoon luteinizing hormone (LH) surge preceding ovulation occurs simultaneously with an activation of GnRH cell bodies in the hypothalamus, and an increased release of GnRH from the median eminence. Activation of GnRH cell bodies by oestrogens is illustrated by the presence of c-fos in these neurons at the time of the LH surge (3, 4), as well as an increased message for pro-GnRH (5).

The regulation of the ovarian reproductive cycle in the female is an example of a hormonal process controlled by the suprachiasmatic nucleus (SCN). The SCN has been shown to be the endogenous circadian pacemaker involved in the generation of

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many hormonal and behavioural rhythms, and the entrainment of those rhythms with the light-dark cycle (6).

 Destruction of the SCN in intact female rats induces persistent oestrus (anovulation) (7), and abolishes progesterone-induced LH surges (8). Daily LH surges, however, occur in the afternoon in oestrogen-implanted ovariectomized (OVX) rats (9), while lesions of the SCN eliminate these daily LH surges (8, 10–12). Moreover, high mean LH levels in plasma of OVX rats are significantly reduced by SCN lesions (12). These observations support the concept of the involvement of the SCN in the regulation of the activity of the GnRH system.

 Efferent projections of the SCN have been studied using autoradiography after injection of radioactive amino-acids (13, 14), and more recently with Phaseolus vulgaris leucoagglutinin (15). Vasovagal intestinal polypeptide (VIP) synthesized by cell bodies in the SCN (16, 17) appears to be present in projections to the preoptic area (PO) (18), the region that contains the largest concentrations of GnRH cell bodies in the rat brain (19). A possible role of VIP in the regulation of GnRH release is indicated by observations that intracerebroventricular infusion of VIP inhibits the increased pulsatile LH release in OVX female rats (20). Infusion of VIP antagonists prevented this effect indicating the involvement of VIP receptors (21).

 To explore the anatomical basis of the reported effects of VIP, we investigated the existence of VIP-containing processes on GnRH-containing cell bodies, using an immunocytochemical double labelling technique. The presence of such contacts at the light microscopic (LM) level prompted us to investigate the putative relationship between this nucleus and the VIPergic input on GnRH neurons by lesioning the SCN.

 Results

 GnRH immunoreactivity (GnRH-IR) was found in cell bodies and fibres in the diagonal band of Broca (DBB), PO and anterior hypothalamic area (AHA) with concentrations of GnRH neurons in the medial PO (MPO) as previously described (19) (see review in 22). VIP-IR processes were found throughout the PO, and the AHA and some fibres in the medial part of the DBB. Only a few VIP-IR cell bodies were visualized within these areas, predominantly located close to the DBB in the medial septal area. Using an LM double labelling procedure, VIP-containing processes (VIP fibres) seemed to be in close apposition to a number of GnRH cell bodies. This innervation at the LM level was observed in every area where GnRH cell bodies were present, with the highest concentration in the PO. Some cell bodies received a dense input (Fig. 1a, 1c) while on others just a few VIP-IR varicosities could be visualized (Fig. 1b). VIP-IR fibres running along GnRH-IR cell bodies and dendrites were also frequently observed (Fig. 1b, 1c). The innervation was scored as positive when the cell body received an input of at least two VIP-IR varicosities on either the perikaryon itself or on one of the dendritic processes. In the double stained sections the total number of GnRH-IR cell bodies and the number of GnRH-IR cell bodies receiving an input of VIP fibres were counted in both hemispheres in each animal separately. In intact control animals 45%±5.4% (SEM) of the GnRH-IR neurons received a VIP innervation. In these animals no differences were found in the percentage of VIP innervation of GnRH-IR cells between the left and right hemisphere (see Fig. 3).

 The unilateral lesions were evaluated in sections containing the SCN single stained for VIP. The damage inflicted upon the SCN was estimated by comparing the VIP-IR at the lesioned side with that of the contralateral control side, and divided into four types: i.e. type I, II, III and bilateral lesions. In type I lesions the proportion of the VIP synthesizing cell population that was destroyed unilaterally was estimated to be 70% or more (n=13; Fig. 2a). The damage to the VIP cell population in the SCN in type II lesions was estimated to be less than 70% (n=7; Fig. 2b). In type III lesions VIP-IR in the SCN was still intact, but either the optic chiasm (animals 12 and 8) or the ventricle wall (animal 28) was damaged (Fig. 2c). Lesions that resulted in bilateral damage to the VIP synthesizing cell population in the SCN were evaluated separately for damage both at the side at which the lesion was aimed and at the contralateral side (bilateral: I/I; animal 2 (Fig. 2d), and I/II; animals 4, 6 and 23), while the fifth group consisted of the control animals without lesions (n=11).

 The lesion itself had no effect on the number of GnRH-IR cell bodies detected in sections of the DBB, PO and AHA when the lesioned sides were compared with the contralateral intact sides (Fig. 3), although immunoreactivity seemed slightly increased at the lesioned side of the brain in some animals. In addition, no significant differences were found in the number of GnRH-IR cell bodies when intact animals were compared with lesioned animals, or between animals in the different experiments. The mean number of GnRH-IR cell bodies visualized in the present study was 480±106 (SEM). The number of GnRH-IR neurons varied considerably between individual animals. In one control animal only 323 GnRH-IR cell bodies were counted (animal 35), while as many as 799 cells were identified in another, unilateral lesioned animal (animal 1).

 The percentage of GnRH-IR neurons that received a VIP fibre input varied between the two experiments (Fig. 3), probably due to a slight increase in staining efficiency. At the intact side in animals in experiment A (experiment A; 2.5 weeks survival after the lesion, animals 1–16) 33%±5.2 of the GnRH-IR neurons received a VIP input in the type I, II and III lesioned animals, while this percentage was 46%±7.9 in experiment B (experiment B; 4 weeks survival after the lesion, animals 17–29). This percentage was reduced to 13%±4.6 (n=8, experiment A), or 24%±6.9 (n=5, experiment B) at the lesioned side in the successful (type I) lesioned animals. A decrease in the percentage of VIP innervated GnRH cell bodies at the lesioned side was also seen in the type II lesioned animals in both experiments, but this decrease was less prominent. In the animal with a complete bilateral lesion (animal 2) VIP innervation of GnRH cell bodies was reduced to 7% on the ipsilateral and 12% on the contralateral side. The percentage and number of GnRH-IR cell bodies that received an innervation of VIP fibres at the lesioned side in the type III lesioned animals were comparable to those at the intact side, and to those found in control animals.

 The effect of lesions on the VIP innervation of GnRH-IR cell bodies was evaluated by comparing the percentage of VIP innervated GnRH cell bodies at the lesioned with that of the intact side, which is represented as the ratio between the percentage innervation at the lesioned versus the intact side in each individual animal (Fig. 4). Lesions that effectively damaged a major part of the SCN estimated at 70% or more (type I) diminished the number of GnRH neurons that received a VIP neuronal input, which was reflected in a decreasing ratio. Type I lesions resulted
Fig. 1. Light microscopical double labelling for VIP (blue-black) and GnRH (brown) in the preoptic area of intact female rats. (A) Dense VIPergic innervation of a GnRH-containing cell body. (B and C) VIP-containing fibres running along GnRH-IR cell bodies and dendritic processes; (D) input of one VIP-IR structure on the perikaryon. (×1,625).

In a decrease of this ratio to at least 50% of that found in control animals both in experiment A and B. In the type III lesioned animals the ratio was not different from that found in intact control animals, while the animals with a type II lesion showed intermediate results. The longer survival time in experiment B did not result in a different decrease in the percentage of VIP
innervation at the lesioned side compared to the contralateral intact side.

Discussion

For the purpose of the present experiments we decided to make unilateral lesions which would provide us with a control side within each animal. Thus, we circumvented effects of differences in the number of GnRH cell bodies between animals on the outcome of the study. Moreover, it allowed us to evaluate the effect of the lesion not only on the VIP input but also on the GnRH neurons themselves. A disadvantage of this approach is that it may also result in a conservative impression of the percentage of neurons receiving a VIP input of SCN origin, since projections of the SCN are never strictly unilateral (15).

In spite of this obvious disadvantage the percentage of GnRH-IR neurons innervated by VIP fibres was significantly decreased at the lesioned side as compared to the intact side following successful unilateral lesioning of the VIP synthesizing cell population in the SCN (type I). The results of the first experiment (A) showed that a part of the VIP innervation of GnRH cell bodies was still present at the lesioned side after successful lesions of the SCN (type I) following a survival time of 2.5 weeks. To be certain of a complete outflow and degeneration of the VIP-containing fibres we decided to increase the survival time to 4 weeks in the second experiment (B). In this experiment the total number of VIP innervated GnRH cell bodies appeared to be slightly higher in each animal irrespective of the lesion results, probably due to a more intense VIP staining. Despite these differences, experiment B confirmed the results obtained in experiment A. Again a marked decrease in the number of VIP innervated GnRH cell bodies was found after successful lesioning. In accord with results of experiment A, the results of experiment B showed that still a number of GnRH neurons were receiving a VIP input. This observation is in agreement with earlier studies that showed a complete outflow of immunoreactive vasopressin from degenerating fibres of the SCN in most animals after a survival time of 2 weeks (23). It is not likely that in 50 μm thick vibratome sections many remaining VIP synthesizing neurons in the lesioned SCN have been missed. Thus, incomplete lesions cannot fully
Fig. 3. Bars represent the number of GnRH-IR cell bodies innervated by VIP-containing fibres (shaded area) as a percentage of the total number of GnRH immunopositive cell bodies both for the ipsilateral lesioned and the contralateral intact side separately. (A) Type I, (B) type II, (C) type III, (D) bilateral lesions, and (E) in the left and right hemisphere of intact animals.
Fig. 4. The ratio between the percentage of VIP innervated GnRH-IR cell bodies at the lesioned versus the intact side in each individual animal: control animals (□); type III lesions (■); type II lesions (■); type I lesions (■). Animals are presented (left to right) in the same order as in Fig. 3 (top to bottom); bilateral lesions are not represented.

explain the remaining VIPergic innervation both in the unilateral and the bilateral lesioned animals. The remaining VIPergic innervation of GnRH cell bodies in animals with a type I lesion at the lesioned side, however, could be a consequence of contralateral innervation. That the SCN may indeed have strong contralateral projections is illustrated by the fact that in the bilaterally lesioned animal (animal 2) the number of GnRH neurons receiving a VIP input was much more diminished. Yet, the remaining VIP input on a very small part of the GnRH cell population, if not from undetected VIP cells in the SCN or contralateral innervation, might be derived from elsewhere, for instance from the amygdala (16).

The total number of GnRH neurons that were visualized in the present study showed large fluctuations between individual animals, as has been reported previously (24). In addition, immunoreactivity for GnRH in some animals with a type I or II lesion seemed slightly increased at the lesioned side of the brain. This might be a consequence of damage at the level of the SCN to GnRH axons projecting to the median eminence, resulting in obstruction and accumulation of the product in the cell bodies. The number of GnRH-IR cell bodies that were detected, however, were comparable between lesioned and intact animals, and between the ipsilateral lesioned, and contralateral intact side within each animal. Earlier studies also reported that bilateral lesions of the SCN, which destroyed a major part of the VIP synthesizing neurons in the SCN, had neither an effect on the number of GnRH neurons, nor on the GnRH levels in the hypothalamus measured in an RIA, although pro-GnRH levels were significantly reduced (12).

One may argue that VIP fibres just accidentally contact GnRH neurons. Intense LM innervation patterns that were regularly observed and were absent in the bilaterally lesioned animal contradict such assumption. In addition when we investigated putative contacts between GnRH and vasopressin, another SCN transmitter which is also present abundantly in the MPO, we were unable to find evidence for such interaction. These results indicate that it is likely that a subpopulation of SCN neurons consisting of at least VIP-producing cells is able to influence a considerable part of the GnRH system in a direct way. This direct projection of VIP-producing cells in the SCN to the GnRH-containing system provides an anatomical basis for previously obtained results with VIP injections in the ventral forebrain on LH secretion. Not only infusion of VIP into the third ventricle (20), but also injection of a single dose of VIP directly into the MPO rapidly decreased mean LH levels and LH pulse frequency in OVX rats (25). Moreover, the predictable diurnal LH surge in oestrogen-treated OVX rats was eliminated in 4 of 6 animals when VIP was injected directly into the MPO (26). In addition to this direct pathway of the SCN to the GnRH system, indirect routes may also be involved. As indicated by studies with the Phaseolus vulgaris leucoagglutinin tracer the main terminal field of SCN efferents is the subparaventricular zone, which projects to essentially the same regions as the SCN (15). Deafferentation of this projection by knife cuts between the SCN and the subparaventricular zone resulted in a marked decrease of the size of the diurnal LH surge in oestrogen-treated OVX rats (27). Moreover, the paraventricular nucleus has been considered as a medulary nucleus in the effect of VIP on LH release (28).

The VIP synthesizing neurons of the SCN are considered likely candidates for the synchronization of rhythms with the light-dark cycle (29). Both immunoreactivity and mRNA for VIP in these neurons display a distinct 24 h rhythmicity with a peak value during the dark period (30–32), and the activity of the VIP-producing neurons is selectively altered by constant light (33). Timing and onset of the LH surge during pro-oestrus in intact rats is also strongly associated with the light-dark cycle. Constant light applied during a short period (one ovarian cycle) delays the onset and reduces the magnitude of the pro-oestrous LH surge (34), while a longer period of constant light results in anovulation (persistent oestrus) in intact rats (35), and in a blockade of the diurnal LH surges in OVX oestrogen-primed rats (36). These data tie in with our present observations suggesting an important role for SCN derived VIP in the direct control of the GnRH system.

The present results indicate that a considerable part of the VIP-containing input on the GnRH system at the LM level is derived from the SCN. Several electron microscopical studies have focused on the synaptology of GnRH neurons in the rat (37–39). The density of innervation of these cells is relatively low, although more synaptic input has been found in the female (39). Therefore, further double labelling electron microscopical studies will be necessary to reveal the presence of synaptic connections between VIP-containing fibres and GnRH cell bodies. In conclusion, the present data suggest the presence of a monosynaptic VIP-containing pathway of the SCN to the GnRH system which provides an anatomical explanation for the observed diurnal influences on the regulation of the reproductive cycle in the female rat.

Materials and Methods

Animals

Adult female Wistar rats (10 weeks of age, U; WU, home bred) weighing between 190 and 230 g were housed under a regular light-dark cycle (lights on from 0700 to 1900 h) in a temperature controlled room. Food and water were available ad libitum.
LM innervation of GnRH neurons by VIP-containing SCN efferents

Experimental design
The animals (n = 28) were lesioned at the age of 12 weeks and sacrificed after a survival time of 2.5 weeks (experiment A, n = 15, animals 1–16) or 4 weeks (experiment B, n = 12, animals 17–28). In addition, a group of control animals was sacrificed together with the animals in experiment B (n = 3, animals 29–31), and separately in experiment C (n = 8, animals 32–39).

Lesion procedure
The rats were anaesthetized with Hypnorm* (10 mg fluanisone and 0.135 mg fentanyl citrate per ml, 0.1 ml/100 g body wt, intraperitoneally; Janssen Pharmaceutica, Beerse, Belgium), and unilateral lesions of the SCN were stereotaxically made with a thermic electrode insulated with Epoxylitite except at the 0.5 mm tip (TCZ, diameter 0.3 mm; Radiodics Inc., Burlingame, CA, USA). Coordinates for the lesion were determined in pilot experiments using tannic acid + 5; from bregma, rostral + 1.4, lateral + 0.2 and ventral − 9.5 from skull. Lesions were produced by applying a variable current producing a constant temperature of 80 °C for 60 s (Grass DC, Heat-Lesion Generator RGF-4A).

Tissue processing
The tissues were perfused under pentobarbital anaesthesia (1 mg/kg body wt, intraperitoneally) with 50 to 70 ml 0.9% saline followed by 300 ml paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed from the skull and coronal slices containing the DDB, PO, area AHA and SCN were dissected, and immersed overnight in the same fixative containing 0.05% glutaraldehyde, at 4 °C on a rocking table. Subsequently, the slices were embedded in gelatin and postfixed overnight as described previously (40). Vibratome sections of 50 μm were made on a BioRad vibratome and collected as free-floating sections in 0.05 M Tris-HCl containing 0.9% NaCl (TBS, pH 7.4).

Immunocytochemistry
Sections were pretreated with sodium borohydride (5 mg/ml, 5 to 10 min; Merck, Darmstadt, Germany) prior to incubation (41, 42). Free-floating serial sections of the DDB, PO and AHA were double stained for VIP and GnRH. Sections containing the SCN were single stained for VIP to evaluate the damage made by the lesion to the VIP synthesizing cell population in the SCN.

Briefly, sections were stained with an antiserum against VIP (Viper, p29–1086; final dilution 1:4,000(42)) diluted in TBS containing 1% bovine serum albumin and 0.5% Triton X-100 (TBBT, pH 7.4) for 1 h at room temperature, followed by 48 to 72 h at 4 °C. Staining was detected with biotinylated goat anti-rabbit IgG (H + L) (Gn-Rab, 1: 500 in TBBT; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature, and avidin-biotin complex elite (ABC, final dilution of both avidin and biotin 1:1,500 in TBBT; Vector Laboratories) for 2 h at room temperature. The immunoreaction was visualized with 0.05% 3,3'-diaminobenzidine (DAB; Sigma Chemical Company, St. Louis, MO, USA) containing 0.2% nickel ammonium sulphate (Merck) and 0.03% H2O2 (Merck), for 12 min. Between incubation steps, sections were thoroughly washed in TBS. The sections for double labelling were washed in graded series of methanol and finally in 0.3% H2O2 (Merck) in TBS to inhibit the ABC peroxidase activity from the first sequence (43), followed by extensive washing in TBS. Subsequently, sections were incubated with a polyclonal rabbit antibody directed against GnRH ([pL461, final dilution 1:2,000; Eurodiagnostics, Apeldoorn, The Netherlands) for 1 h at room temperature followed by 24 to 48 h at 4 °C, and detected with GcR-bio and ABC as described above. The reaction was visualized with DAB containing 0.03% H2O2, for 20 min. Finally, the sections were mounted on glycerin-albumin (Gurr, Poole, England) coated slides, dried, dehydrated through graded series of ethanol and xylene followed by embedding in Depex* (Gurr). Specificity of the immunocytochemical staining was checked by preabsorbing the antisera with their homologous antigen. This resulted in a complete absence of immunocytochemical staining throughout the hypothalamus.

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