Research report

Synaptic contacts between gonadotropin-releasing hormone-containing fibers and neurons in the suprachiasmatic nucleus and perichiasmatic area: an anatomical substrate for feedback regulation?

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Abstract

The suprachiasmatic nucleus (SCN) is critically involved in the generation and entrainment of circadian rhythms in mammalian species. Both the occurrence and the timing of the luteinizing hormone surge on the afternoon of proestrus in the female rodent are critically dependent on the integrity of the SCN. Recently, we demonstrated the presence of a monosynaptic pathway from the SCN to the gonadotropin releasing hormone (GnRH) neurons in the preoptic area. In addition, we found that interaction between the SCN and the GnRH system may be found close to the SCN, since we observed apposition of SCN efferents and GnRH fibers at the ultrastructural level in that region. The aim of the present study was to investigate the presence of synaptic contacts between GnRH fibers and structures in the SCN and surrounding perichiasmatic area (periSCN). At the light microscopical level, the immunoreactivity for GnRH showed a considerable overlap with the immunoreactivity for vasopressin and vasoactive intestinal peptide, two neuropeptides synthesized by SCN neurons. At the ultrastructural level, we demonstrated synaptic input of GnRH-containing axons on immunocytochemically unidentified structures in the SCN/peri-SCN region. The present results clearly demonstrate that the SCN and periSCN are postsynaptic targets of GnRH fibers. It is hypothesized that the GnRH input in the SCN region represents an anatomical substrate for feedback-control between these systems. © 1997 Elsevier Science B.V.

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1. Introduction

The gonadotropin-releasing hormone (GnRH) system in the brain constitutes the final common pathway for the central regulation of pituitary gonadotropin release. GnRH is synthesized by cell bodies widely spread throughout the ventral forebrain, with concentrations of neurons in the diagonal band of Broca, surrounding the organum vasculosum of the lamina terminals and in the preoptic area [2,37]. A vast majority of these neurons send fibers to the median eminence (ME) [32], where GnRH is released into the portal blood circulation. Tracing studies using peripheral injections of Flourogold suggested that most, if not all GnRH neurons are neuroendocrine in nature [49]. The GnRH-containing fibers reach the ME by two main routes, a lateral one which parallels the base of the brain and a periventricular one that parallels the third ventricle [4,15,16,31]. Other routes reach the ME via the area between the two suprachiasmatic nuclei (SCN) at the basis of the third ventricle (median-SCN) and along the ventral surface of the optic chiasm, forming the subchiasmatic pathway [15].

In the female, synthesis and release of GnRH from the ME and consequently preovulatory luteinizing hormone (LH) release from the pituitary, are under control of a positive feedback by gonadal steroids [10,11,22,23,25]. Also, the SCN is essential for normal control of cyclic LH secretion in the female rodent [5,6,14,19,24,28]. Recent

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studies have demonstrated the presence of a monosynaptic projection from the SCN to the GnRH neurons in the preoptic area in the female rat, containing vasoactive intestinal peptide-containing (VIP) as a putative transmitter [40,42,43]. This pathway may constitute an anatomical substrate involved in the circadian regulation of preovulatory LH release.

In addition to its role as a neurohormone, GnRH may act as a neurotransmitter within the brain, since GnRH fibers and GnRH receptors are present within various intra- and extra-hypothalamic areas [2,17]. GnRH immunoreactive fibers have been demonstrated in SCN and surrounding area [38], which most probably are axons or axon collaterals of the median and medial pathways to the median eminence, which account for 15–30% of the total GnRH innervation of the median eminence [20]. Recent tracing studies of SCN efferents have shown GnRH-containing axons in apposition to axon- and dendrite-like structures filled with tracer in this region at the ultrastructural level [39].

The objective of the present study was to investigate the occurrence of synaptic interactions between GnRH-containing fibers and neurons in the SCN and peri-SCN. To this end we evaluated the distribution of immunoreactivity for GnRH and for vasopressin (VP) and VIP, two SCN-derived neuropeptides abundantly present in projections of the SCN to the peri-SCN [47], in serial sections through the entire rostrocaudal extent of the SCN at the light microscopical level. Furthermore, we examined the immunoreactivity for GnRH at the ultrastructural level using single labelling and the possible interaction of VP- and VIP-containing structures with GnRH-containing fibers using double labelling immunocytochemistry.

2. Experimental procedures

2.1. Animals

Adult female Wistar rats obtained from Central Animal Facilities (Utrecht University, The Netherlands) weighing 200–280 g were used for electron- ($n = 18$) and light- ($n = 5$) microscopical immunocytochemical studies. Rats were housed under a regular light dark cycle (lights on from 07.00 to 19.00 h) in a temperature controlled room. Food and water were available ad libitum.

2.2. Tissue processing

Animals were perfused between 10.00 and 13.00 h under pentobarbital anaesthesia (0.1 ml/100 g body weight, intraperitoneal) with 100 to 150 ml 0.9% saline followed by 500 ml fixative. For light microscopy, 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) was

used, followed by overnight postfixation of the brain in the same fixative with 0.1% glutaraldehyde added to it. For electron microscopical studies the animals were perfused with: (A) 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, or (B) 5% glutaraldehyde in 0.1 M citrate buffer, pH 4.0, or (C) 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were dissected and postfixed in the same fixative as used for perfusion for 2 h at room temperature. Postfixation included a short 15 min microwave-step as described previously [9]. Vibratome sections of 40 μ m were collected as free floating sections in Tris buffered saline (TBS; 0.05 M Tris-HCl containing 0.9% NaCl, pH 7.4) and washed overnight.

2.3. Immunocytochemistry

The vibratome sections for single light microscopical immunocytochemistry were pretreated with 1% sodium borohydride in TBS for 20 min [7,26]. Alternating series of serial sections were incubated with antisera raised in rabbit against GnRH (1:2,000; No. PLR 005, Euro-Diagnostica, Apeldoorn, The Netherlands), VIP (1:6,000; No. Viper 29–10'86 [7]), or Arginine-VP⁸ (1:8,000; No. Truus 10-4'86 [7]) respectively diluted in TBS containing 1% bovine serum albumin and 0.5% Triton X-100 (supermix) for 1 h at room temperature followed by 48 h at 4°C. Immunoreactivity was detected with biotinylated Goat-anti Rabbit IgG (H + L) (GaR-bio; 1:500 in supermix, Vector Laboratories, Burlingame, USA) for 1 h and Avidin-Biotin Complex-Elite (ABC; final dilution of both avidin and biotin 1:1,500 in supermix; Vector Laboratories) for 2 h at room temperature. Between incubation steps, sections were thoroughly washed in TBS. The staining was visualized by incubation with 0.05% 3,3'-diaminobenzidine (DAB; Sigma Chemical Company, St. Louis, MO, USA), containing 0.03% H₂O₂ for 8–12 min. Sections were mounted on glycerin-albumin (Gurr, Poole, England) coated slides, dried, dehydrated through graded series of ethanol and xylene and coverslipped.

Preabsorption of the GnRH, VIP and AVP antisera using respectively 10, 6.7 and 5.3 ng/ μ l homologous synthetic peptide coupled to gelatin-coated nitrocellulose [41] completely abolished the immunocytochemical staining.

The sections for pre-embedding electron microscopical immunocytochemistry were treated with 50% ethanol for 40 min to enhance antibody penetration [27], followed by treatment with sodium borohydride as described above. Sections were either single stained for GnRH, or double stained for GnRH and VIP, or for GnRH and VP. Briefly, all sections were incubated with the antiserum raised against GnRH (1:800) diluted in TBS containing 1% BSA and 0.05–0.1% Triton X-100 (ultramix) for 2–4 h at room temperature, followed by 48 h at 4°C. Staining was detected with GaR-bio (1:500 in ultramix; Vector Laborato-

ries) overnight at 4°C and ABC (1:1,500 in ultramix; Vector Laboratories) for 2 h at room temperature and finally visualized by incubation with DAB as described above.

Sections for double labelling were incubated with the polyclonal rabbit antiserum raised against VIP (1:1,000 in ultramix) or against Arginine-VP⁸ (1:2,000 in ultramix) for 3–5 h at room temperature and overnight at 4°C. Subsequently, the DAB reaction product from the GnRH staining was intensified according to the silver-gold deposit method with a few modifications as described previously [8,13]. Following this intensification procedure, sections were incubated with GaR-bio (1:400 in ultramix) overnight at 4°C and ABC (1:1,200 in ultramix) for 2 h at room temperature and finally visualized by incubation with DAB containing 0.02% H₂O₂ for 15–20 min. Sections were postfixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h and the area containing the SCN and peri-SCN was dissected and fixed in 1% osmiumtetroxide in 0.1 M sodium cacodylate buffer for 1 h. Sections were counterstained with 1% uranyl acetate in distilled water for 45 min, dehydrated through graded series of acetone and flat embedded in Durcupan[®] ACM (Fluka AG, Buchs, Switzerland). Plastic embedded sections were examined under a light microscope and small pieces of the ventral and medial part of the SCN or from the peri-SCN containing fibers immunopositive for GnRH, or comparable pieces from alternating sections double stained for GnRH and VP, or for GnRH and VIP were dissected and mounted on plastic blocks. Also, small pieces of the area between the SCN from the sections single stained for GnRH were dissected. Ultrathin, semi-serial sections were cut on a Reichert–Jung microtome, collected on piliform- (Wacker, Munchen, FRG) coated one-hole grids and examined under an electron microscope (Philips CM10).

3. Results

3.1. Light microscopical studies

The light microscopical staining for GnRH, VIP and VP was evaluated in alternating sections through the hypothalamus. In the SCN and surrounding peri-SCN, a clear overlap was found in the distribution of the GnRH fibers and that of immunoreactivity for VP and VIP (Fig. 1). Immunoreactivity for all three antigens was present along the entire rostro-caudal extent of the nucleus. VIP-immunoreactive neurons were localized primarily in the ventral part of the SCN, whereas VP-immunoreactive neurons were found predominantly in the dorsal portion of the nucleus (Fig. 1). In the SCN and peri-SCN, both VP- and VIP-immunoreactivity were observed in numerous thin fibers, showing numerous varicosities. GnRH-containing fibers were found predominantly in the border zone of the

SCN and peri-SCN, relatively few fibers were found entering the nucleus (Fig. 1). Based on the immunocytochemical staining, distinction between the SCN and surrounding peri-SCN is difficult to make. We therefore considered only the GnRH fibers present in the ventral and medial SCN, close to the optic chiasm, to be within SCN borders. GnRH cell bodies were not detected in the SCN itself and rarely in the surrounding peri-SCN. Most GnRH fibers located in the peri-SCN surrounded the rostral and medial part of the SCN, whereas fibers were sparse in the retrochiasmatic area surrounding the caudal part of the SCN (see Fig. 1 and Fig. 2). The GnRH-fibers appeared as long thin fibers and as thicker fibers, which regularly showed varicosities. Some of the thicker fibers appeared as corkscrew-like structures (Fig. 2). Short thick GnRH-containing fibers were also observed in the median-SCN, the small area between the two SCN located under the third ventricle just above the optic chiasm (see Fig. 1 and Fig. 2).

3.2. Electron microscopical studies

At the ultrastructural level, the periSCN was characterized by the presence of large fields of transversal cuts through small axonal and dendritic structures. All three antigens were present in these axonal fields. GnRH-immunoreactivity was demonstrated by varying amounts of DAB precipitate, partially covered by silver-gold particles that varied in size and number. In the double stained material VIP- and VP-immunoreactive were characterized by a variable amount of diffuse DAB-precipitate. The fixatives used for labelling considerably influenced the detection of the three antigens by immunocytochemistry. In general, the number of immunoreactive structures and the staining intensity were drastically reduced in the electron microscopical labelling compared to light microscopical labelling procedures. In the material processed for electron microscopy, most GnRH-immunoreactivity was detected in sections from brains perfused with fixative B and C, while the number of GnRH-immunoreactive fibers as well as the intensity of the staining were considerably lower after perfusion with fixative A. Most VP-immunoreactivity was found in material perfused with fixatives A and C, whereas for VIP labelling only fixative B appeared to be successful. Ultrastructural detail, however, was best preserved with fixative A.

In the peri-SCN as well as in the SCN itself, the GnRH-containing axons very frequently showed interaction with large dendrites, small dendritic spines and cell somata (Fig. 3). Also, direct apposition of GnRH terminals to smaller axon-like structures, sometimes showing some synaptic specialization, was observed (Fig. 4). In the median-SCN, GnRH-containing structures showed no interaction whatsoever with adjacent unlabelled structures (data not shown).

The majority of GnRH-immunopositive axons in the

Fig. 1. Immunocytochemical staining for GnRH, VP and VIP in serial sections through the rostrocaudal extent of the SCN at the light microscopical level. A–D: GnRH staining; E–H: VP staining; and I–L: VIP staining; bar = 100 μ m.

Fig. 1 (continued).

Fig. 3. GnRH-immunoreactive fibers at the ultrastructural level in single stained material perfused with fixative A. (A) and (B) show synaptic contacts with small dendritic spines in the peri-SCN. The inlays in A and B show the site of synaptic interaction at a higher magnification; (C) a synaptic contact with a small dendritic spine within the SCN; and (D) interaction with an SCN neuron; bar = 500 nm; a, axon; d, dendrite; arrowhead, dendritic spine; N, nucleus.

peri-SCN showed synaptic input to unlabelled structures. VIP- and VP-containing axons, however, were regularly found nearby. VP- and VIP-containing cell bodies, dendrites and axons were observed within the SCN, but we

were not able to detect interaction of GnRH-containing terminals with these cell bodies. Occasionally, input of GnRH and VP-containing axons on the same terminal field were observed in the SCN and peri-SCN region (data not

Fig. 2. GnRH-containing fibers in the SCN area in representative sections of the SCN. (A) in the rostral part of the SCN; (B) in the medial part of the SCN; (C) in a more caudal section through the SCN area: note the presence of varicosities in GnRH-immunoreactive fibers (arrowheads), and of thick fibers in the median SCN (large arrows). Also, some fibers appear as cork-screw like structures (small arrows). V: third ventricle; OC: optic chiasm; bar = 100 μ m.

Fig. 4. Interaction of GnRH-containing axons with unidentified axons in the peri-SCN. (A) and (B) represent two consecutive sections through a GnRH-immunoreactive axon and an adjacent unlabelled axon. Both axons show synaptic input to the same unlabelled dendrite (arrows). The membrane between the two axons shows some synaptic specialization and a possible example of an endo- or exocytotic structure (arrowhead); (C) GnRH-immunoreactive axon in the peri-SCN showing apposition to an unlabelled axonal structure; (D) synaptic interaction between a GnRH-immunoreactive and an unlabelled axon-like structure (arrowheads). Panels A and B: fixative A; panel C: fixative B; panel D: fixative C. Panels A–C, bar = 1 μ m; panel D, bar = 500 nm; a, axon; d, dendrite.

shown). Apposition of VIP-containing dendrites with GnRH-containing axons was found more regularly (Fig. 5).

4. Discussion

The present study demonstrates a marked overlap in the light microscopical distribution of GnRH-immunoreactive fibers and that of VP- and VIP-immunoreactivity in neurons and fibers in the SCN and surrounding peri-SCN. The GnRH-containing fibers were predominantly localized in the border zone of the SCN and surrounding peri-SCN and

between the two suprachiasmatic nuclei as has been reported previously [38]. In addition, GnRH-immunoreactive fibers appeared to be more abundant in the peri-SCN surrounding the rostral and medial part of the SCN, than in the retrochiasmatic area surrounding the SCN more caudally. Since numerous varicosities were observed, extensive synaptic interaction was suspected. Indeed, the GnRH-containing fibers regularly formed synaptic junctions with structures in the SCN region at the ultrastructural level. The transmitter content of the neuronal structures receiving input of GnRH fibers, however, remains to be elucidated, since we did not observe synaptic interaction between these fibers and VP- or VIP-immunoreactive

Fig. 5. Illustrates the presence of GnRH- (DAB-SIG) and VIP- (DAB) immunoreactivity in adjacent structures in the peri-SCN: direct apposition, without synaptic specialization of a GnRH-immunoreactive axon and a small VIP-immunoreactive dendrite (fixative B); bar = 1 μ m; d, dendrite.

structures. Previous tracing studies suggest that the neuronal interaction with GnRH fibers in this area indeed involves SCN-structures [39], although the present results do not exclude interaction with neuronal structures of non-SCN origin.

The present observations accord with evidence that, in addition to its role as a neurohormone, GnRH may act as a neurotransmitter in the central nervous system [2,17]. The physiological role of GnRH in this area may be of special interest, since the SCN has been shown to be crucial for the regulation of the oestrous cycle in small rodents like the rat [5,6,14,19,24,28]. The surrounding peri-SCN projects to essentially the same regions as the SCN and has been considered as a modulatory area for SCN output [33,46]. This area is not only an important target area of SCN-efferents but also sends numerous fibers back to the SCN [33,40,45,47]. Knife cuts in the SCN projection to the subparaventricular zone, which includes the peri-SCN and extends towards the paraventricular nucleus, significantly reduce the magnitude of the diurnal LH surge in OVX E-treated female rats [48]. Also, electrical stimulation of neurons in the peri-SCN results in an inhibition of LH release in OVX rats, but elevates LH levels in OVX E-primed females. Stimulation of SCN neurons, however, elevates LH levels in both OVX and OVX E-primed females, the magnitude of the LH release being much higher after E-treatment [1]. Taken together, this indeed suggests a specific role for the peri-SCN region in the regulation of preovulatory LH release.

Recent anatomical evidence suggests the existence of a monosynaptic pathway between the SCN and the GnRH neurons in the preoptic area [40,43]. Thus, functionally, the

presence of synaptic input of GnRH-containing terminals on neurons in the SCN region, could be interpreted as anatomical evidence for a feedback loop from the GnRH system to the SCN. The existence of feedback pathways between the GnRH system and the neuronal systems involved in its regulation is supported by a number of studies. For instance, neurons in the ventromedial hypothalamus (VMH) and the amygdala, two areas that have been implicated in the control of reproduction, send efferent projections to the regions where the GnRH cell bodies are located [30,35]. In turn, the VMH and amygdala are both sparsely innervated by GnRH containing fibers and local injections of GnRH respectively potentiate sexual behavior or affect the electrical activity of neurons projecting to the VMH [3,12,18,29,44,50]. Another example for the presence of a feedback loop between the GnRH system and putative regulatory systems is found within the preoptic area itself. The neuronal activity of GnRH cell bodies is regulated by the negative and positive feedback of gonadal steroids [34,51]. Since GnRH neurons do not contain estrogen receptors [36], it is likely that other, steroid-sensitive, neurons transduce the steroidal signals. In turn, it has been shown that GnRH-immunoreactive fibers terminate on estrogen receptor-containing neurons in the preoptic area of the female guinea-pig [21].

The evidence cited above strongly supports the presence of feedback loops between the GnRH system and regulatory systems within the central nervous system that are involved in steroid feedback and sexual behavior. The functional significance of the presently described input, however, remains to be elucidated. The present study provides morphological data showing that the SCN region can be considered a postsynaptic target of GnRH terminals. We hypothesize that this interaction could provide an anatomical basis by which feedback information from the GnRH system is relayed to the SCN.

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