Localization of the VIP$_2$ Receptor Protein on GnRH Neurons in the Female Rat

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Abstract

In mammals, the timing and occurrence of the preovulatory LH surge critically depends on the proper functioning of the suprachiasmatic nucleus (SCN). Recent evidence suggests that vasoactive intestinal polypeptide (VIP) conveys time of day information from the SCN to GnRH neurons. However, it is not completely clear whether this action is exerted directly at the level of the GnRH neuron. To determine if GnRH neurons are direct targets for VIP, triple-label immunofluorescence was utilized to simultaneously localize GnRH, VIP and VIP$_2$ receptor protein. The present results demonstrate that about 40% of all GnRH neurons analyzed contain VIP$_2$ receptor immunoreactivity and that VIP-containing processes were seen in close apposition to a significant number of VIP$_2$ receptor-positive GnRH neurons. GnRH neurons that exhibit immunoreactivity for the VIP$_2$ receptor are located predominantly in the OVLT region and the rostral preoptic area. In the median eminence, where the majority of GnRH neurons terminate, VIP$_2$ receptor immunoreactivity was absent. In summary, these findings indicate that VIP can communicate directly with GnRH neurons.

The preovulatory surge of luteinizing hormone (LH) is initiated by a dramatic increase in the secretion of GnRH into the hypophyseal portal vasculature (1, 2). In addition, estradiol drives the hypersecretion of GnRH by coupling a daily neuronal signal originating from the suprachiasmatic nucleus (SCN) to the neurotransmitters and neuropeptides that regulate GnRH secretion. Experimental evidence using a wide variety of methods demonstrates that signals from the SCN are critical to the occurrence of the LH surge and govern its timing. First, ablation of this nucleus blocks the LH surge in intact animals (3). Second, in ovariectomized, estradiol-primed rats, daily afternoon surges of LH occur at a specific time relative to the light/dark cycle (4). Third, pentobarbital blocks the neural signals required for the proestrous LH surge and leads to a surge that occurs at the expected time 24 hours later (5).

The exact mechanisms by which the SCN transmits circadian information to the GnRH neuronal system are not completely understood. However, recent evidence suggests that vasoactive intestinal polypeptide (VIP), synthesized in the ventrolateral aspect of the SCN (6), is a critical neurochemical messenger that relays time-of-day information from the SCN to GnRH neurons. First, VIP-containing fibers are found in close apposition to GnRH cell bodies and dendrites, and lesion studies have shown that the majority of this input to GnRH neurons originates in the SCN (7). Second, a substantial proportion of the GnRH neurons that receive VIP input express cFos during the afternoon of proestrus (8). Lastly, infusion of VIP antisense oligonucleotides directly into the SCN (9) or central administration of antiserum to VIP (10) delays and attenuates the steroid-induced LH surge. Taken together these data suggest that VIP plays a critical role in the regulation of the surge of LH.

VIP exerts its effects by binding to and activating two G protein-coupled receptors (VIP$_1$ and VIP$_2$) with very different amino acid sequences (11). Only the VIP$_2$ receptor mRNA is expressed in the OVLT/rostral preoptic area (12), a region containing a subpopulation of GnRH neurons that regulates the LH surge (13). The present study was designed to test the hypothesis that VIP may communicate directly with GnRH neurons. We have used specific antibodies to the VIP$_2$ receptor to investigate whether GnRH neurons possess VIP$_2$ receptor protein.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (Zivic-Miller, Allison Park, PA) were maintained on a 14-h light, 10-h dark schedule (lights on at 0400 h) and provided food and water ad libitum. Animals that exhibited at least two consecutive 4-day estrous cycles were anesthetized with ketamine-acepromazine maleate (50 and 5 mg/kg BW, respectively) at 0900 or 1600 h on proestrus (n = 4 animals/time point). The animals were then transcardially perfused with 350 ml of a modified Zamboni’s fixative (4% paraformaldehyde + 7.5% saturated picric acid in 0.1 M Phosphate Buffer, pH 7.4). Brains were rapidly removed and post-fixed in the above fixative overnight. Coronal vibratome sections (40 μm) were collected in four series from the medial septum to the suprachiasmatic nucleus and stored in cryopreservant at −20°C until processed for immunocytochemistry.

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Antibodies

Mouse monoclonal VIP<sub>2</sub> receptor antibody, which was raised against amino acids 105-122 of the N-terminus region of the human VIP<sub>2</sub> receptor, was purchased from Antibody Solutions (Palo Alto, CA). The VIP antisera (DiaSorin, Stillwater, MN) was raised in rabbits against porcine VIP. Guinea pig GnRH antisera (GP8-4) was raised in our laboratory and has been characterized previously. An affinity-purified rabbit VIP<sub>2</sub> receptor antibody (R6192-3) was also raised in our laboratory against a portion of the third intracellular loop (amino acid residues 312-323) of the rat VIP<sub>2</sub> receptor.

Immunocytochemistry

Free-floating brain sections were washed in Tris-HCl buffer (0.05 M; pH 7.6) and then incubated in blocking buffer (10% normal horse serum, 0.2% Triton X-100 and 0.1% sodium azide in the above Tris buffer) for 1 h. For dual-label immunofluorescence, sections were then incubated overnight in a mixture of either mouse monoclonal or rabbit anti-VIP<sub>2</sub> receptor and guinea pig anti-GnRH in blocking buffer at final dilutions of 1:500 or 1:1,000 and 1:750, respectively. For triple-label immunofluorescence, rabbit anti-VIP (1:1000) was added to the guinea pig anti-GnRH and mouse anti-VIP<sub>2</sub> receptor mixture. After incubation overnight in the primary antisera, sections were rinsed thoroughly in Tris buffer and incubated in a mixture of cross-absorbed and affinity-purified secondary antibodies labeled with different fluorescent markers (Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. For dual-labeling, donkey anti-rabbit or anti-mouse antibody coupled to Texas Red (1:100) was coincubated with FITC-coupled donkey anti-guinea pig antibody (1:50). For triple-labeling, a Cy 5-labeled anti-rabbit antibody (1:100) was added to the mixture. Sections were then rinsed thoroughly in Tris buffer, mounted on glass slides, air-dried and coverslipped with ‘ProLong’ antifade mounting medium (Molecular Probes, Inc., Eugene, OR) prior to analysis. Dual-labeled sections were analyzed with the aid of a Nikon E 400 microscope equipped with the appropriate excitation-barrier filter combinations. Images of the triple-labeled sections were acquired by confocal microscopy (Leica, Germany) and processed using Adobe Photoshop 5.5 software.

Controls included omission of the primary antibody and tests for possible cross-reactivities of the second antibodies. In addition, to verify that both VIP<sub>2</sub> receptor antibodies recognize the same protein, we performed another control experiment: incubation of sections with both antibodies against the VIP<sub>2</sub> receptor.

Results

We observed GnRH-immunoreactive neurons throughout the forebrain as previously described (14). The highest density of neurons was located in the region surrounding the organum vasculosum of the lamina terminalis (OVLT), and the rostral preoptic area. Absorption of the GnRH antiserum with 10μg synthetic GnRH/ml antiserum resulted in the absence of specific staining (data not shown).

Immunohistochemistry for the VIP<sub>2</sub> receptor reveals that in the region stretching from the medial septum-diagonal band of Broca through the medial preoptic area, numerous VIP<sub>2</sub> receptor-immunoreactive neurons were present. In contrast, in the median eminence, where the majority of GnRH neurons terminate, immunoreactivity for VIP<sub>2</sub> receptor was absent. VIP<sub>2</sub> receptor antibodies raised in mice (Figure 1A) or rabbit (Figure 1B) reveal VIP<sub>2</sub> receptor-expressing cells in the medial septum and overlay of these sections demonstrates that both antibodies identify the same population of cells. Furthermore, preincubation of the rabbit VIP<sub>2</sub> receptor antibody with the immunizing peptide resulted in the complete loss of specific staining (Figure 1D).

Figure 1. Immunohistochemical controls. Immunostaining is shown at the level of the medial septum by using antibodies to the VIP<sub>2</sub> receptor raised in mouse (A) or rabbit (B) in the same section. Note that cells in the overlap of A and B. Panel C shows a complete overlap of VIP<sub>2</sub> receptor-containing cells. Immunostaining was prevented after preabsorption of the rabbit antibody with the immunizing peptide (D).

Dual-label immunofluorescence for GnRH and VIP<sub>2</sub> receptor protein demonstrates that about 40% of all GnRH neurons exhibited immunoreactivity for VIP<sub>2</sub> receptor. The percentage of double-labeled cells did not change on proestrus at a time when the LH surge occurs (1600h) compared to when LH concentrations are basal (0900h). VIP<sub>2</sub> receptor-containing GnRH neurons were located predominantly in the OVLT region and in the rostral preoptic area. The distribution of GnRH neurons from a
representative animal expressing or not expressing the VIP₂ receptor protein is shown in Figure 3.

Triple-labeling for GnRH, VIP and VIP₂ receptor protein shows that VIP-containing terminals are in close proximity to a significant number of GnRH neurons that also expressed VIP₂ receptor protein. An example of a GnRH neuron containing VIP₂ receptor protein that is in close apposition to VIP terminals is shown in Figure 2, A-D.

![Figure 2](https://academic.oup.com/endo/article-abstract/141/11/4317/2988114)

**Figure 2.** Confocal micrograph showing a GnRH neuron (A) at the level of the rostral preoptic area that expresses VIP₂ receptor protein (B) and is in close apposition to VIP (C). D is an overlay of A-C.

**Discussion**

Several lines of evidence indicate that VIP may be a critical component of the daily neural signal originating from the SCN that is necessary for the induction of the LH surge (see Introduction). The present results provide the first evidence that GnRH neurons express the VIP₂ receptor protein and therefore, demonstrate that VIP neurons can communicate directly with a subpopulation of GnRH neurons in the forebrain. Interestingly, both the percentage as well as the distribution of GnRH neurons that express VIP₂ receptor immunoreactivity found in the present study are consistent with data reported previously in the literature. Van der Beek and colleagues (7) have reported that about 45% of all GnRH neurons examined received VIP innervation. These results complement those of Olcese et al. (15) who showed that GT1-7 cells, immortalized neurons which secrete GnRH, express the mRNA for the VIP₂ receptor.

Our study establishes that a large proportion of GnRH neurons (approximately 40%) express VIP₂ receptor, suggesting that VIP neurons may be a significant modulator of GnRH secretion. Since VIP neurons originate in the portion of the SCN that receives retinal input, our findings further suggest that VIP neurons directly communicate time-of-day information to GnRH neurons and may influence the timing of the LH surge. They suggest that changes in the pattern of VIP expression, such as those observed during aging, may impact the pattern of GnRH secretion on proestrus. We found that the percentage of GnRH neurons that co-express VIP₂ receptor does not change from the morning to the afternoon of proestrus; however, these methods do not allow us to monitor whether the density of VIP₂ receptors change during the day. Previous studies have shown that GnRH neurons that are closely apposed to VIPergic terminals express fo during proestrus afternoon. Together, these findings suggest that changes in the level of neuropeptide expression and/or release probably account for the ability of VIP to influence the activation of GnRH neurons.

In conclusion, the present investigation demonstrates that a significant proportion of GnRH neurons express the VIP₂ receptor protein. This finding provides further evidence for the possibility of a direct VIP-containing pathway from the SCN to GnRH neurons.

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**References**

Figure 3. Anatomical distribution of GnRH neurons that express (+) or do not express (o) the VIP$_3$ receptor protein. Diagrams of coronal sections were taken from five hypothalamic levels (approximately 200 µm apart) where the majority of GnRH neurons are located: (A) the level of the vertical limb of the diagonal band of Broca; (B) OVLT; (C) anteroventral periventricular nucleus (AVPV); (D) rostral medial preoptic nucleus and (E) caudal medial preoptic nucleus. Images are based upon the atlas of Konig and Klippel (16), with modifications.


