Implication of novel neurotransmitter systems in the regulation of gonadotropin-releasing hormone neurons

Ph.D. Thesis

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The adaptive regulation of the hypothalamo-pituitary-gonadal (HPG) axis is crucial in the successful reproduction among the vertebrates. The central unit of the axis is formed by the gonadotropin-releasing hormone (GnRH)-producing neurons that release their neurohormone content into the portal circulation of the hypophysis in a pulsatile manner. The episodic hormone release from the GnRH axon terminals results in a rhythmic discharge of the two gonadotropins, luteinizing hormone and follicle stimulating hormone, by the gonadotroph cells of the anterior pituitary. The gonadotrop hormones reach the gonads via the systemic circulation and stimulate their steroid hormone production and gametogenesis. The gonadal steroids, in turn, exert negative (in females and males) and positive (only in females) feedback actions on the central component of the axis.

In rats, the central regulation of the HPG axis is carried out by about 1000-1600 GnRH-producing neurons that are located in the preoptic area (POA) of the hypothalamus. The humoral and neuronal signals that converge on these neurons modify their functional properties that results in an altered pattern of GnRH release. Among the humoral factors that modify the function of GnRH neurons, the gonadal steroids such as estrogen, progesterone and androgens are of primary importance. With respect to the afferent neuronal regulation of GnRH neurons, electron microscopic studies have established synaptic connections between the GnRH neurons and at least nine different neurotransmitter systems. Among the classical neurotransmitters, GABAergic, glutamatergic, catecholaminergic and serotonergic pathways establish synapses with the GnRH-immunoreactive elements. The range of neuropeptide-containing afferents that innervate GnRH neurons is also wide: the presence of neuropeptide Y (NPY), opioid peptides, corticotropin-releasing hormone, substance P, kisspeptin and GnRH itself have been revealed in presynaptic structures to GnRH neurons. The neurochemical property of GnRH nerve cells, similarly to other hypothalamic neurons, is fairly complex. In addition to the GnRH decapeptide, the expression of galanin, cholecystokinin, neurotensin, delta sleep-inducing peptide and insulin-like growth factor-1 have been reported so far. Moreover, in young, migrating GnRH neurons the transient occurrence of GABA has also been observed.

A large body of evidence exists to indicate the involvement of acetylcholine (Ach) in the regulation of reproductive events, including male and female sexual behavior, and gonadotropin secretion. While the site(s) and mechanism(s) of the cholinergic actions upon the reproductive axis are not clear, they appear to be exerted, at least in part, at the

hypothalamic level. In studies under specific aim 1 of this thesis, we postulated the direct innervation of GnRH neurons by cholinergic pathways. We examined the neuronal communication between central cholinergic pathways and GnRH neurons using light and electron microscopic immunocytochemistry.

NPY exerts profound and diversified effects on the reproductive axis; however, the origins of the NPY afferents to GnRH neurons have been in obscurity. In studies under specific aim 2, we addressed the origin of NPYergic afferents to GnRH neurons. We selected topographical markers, which are co-expressed with NPY exclusively in distinct brain regions. Then, we used the coexpression of these markers in NPY fibers as an indication of their sources of origin. Since our studies have determined the hypothalamic arcuate nucleus (Arc) as a major source of NPY-immunoreactive neuronal contacts on GnRH neurons, under specific aim 3, we investigated the synaptic communication between NPY fibers of Arc origin (using agouti-related peptide (AGRP) as a neurochemical marker for NPY neurons of the Arc) and GnRH-immunoreactive neurons.

The recent discovery of the three vesicular glutamate transporters (Vglut 1-3), which selectively accumulate glutamate into secretory vesicles has enabled the histochemical identification of glutamatergic neurons and fibers in the central nervous system. The abundance of glutamatergic neurons expressing Vglut2 mRNA in the hypothalamus and the high density of Vglut2-immunoreactive axon terminals and ionotropic glutamate receptors in the external zone of the median eminence (ME) raised the possibility that GnRH neurons secrete glutamate as an autocrine/paracrine modulator of their neurohormone output. Therefore, in studies under specific aim 4, we examined the possibility whether mature GnRH neurons in the POA possess glutamatergic characteristics.

Specific aims

Studies of this thesis were aimed at identifying of novel neurotransmitter systems in the neuronal regulation of GnRH neurons

1. **We addressed the direct innervation of GnRH neurons by cholinergic afferent pathways in male rats.**

2. **We determined the sources of origin of NPY-containing afferents to GnRH neurons in male GnRH-GFP transgenic mice.**
   a. Using light microscopic immunocytochemistry, we compared the frequency of NPY- and AGRP-immunoreactive axonal contacts on GnRH neurons in neonatally monosodium glutamate-treated mice (with lesioned Arc) with that of the untreated controls. In this way, we could estimate the ratio of NPY afferents originating in the Arc.
   b. We carried out a series of triple-label fluorescent studies in GnRH-GFP transgenic mice using brain region-specific topographical markers to identify the relative contribution of NPY neurons located in the Arc and in the brainstem catecholaminergic nuclei, respectively, to the NPYergic innervation of GnRH neurons. We used the noradrenaline-synthesizing enzyme, dopamine-β-hydroxylase (DBH), as a topographic marker of NPY fibers with brainstem origin. AGRP was used as a marker of NPY fibers originating from the Arc. The ratios of NPY-immunoreactive neuronal contacts on the surface of GnRH-GFP neurons that contained DBH or AGRP were determined.

3. **We performed ultrastructural studies to reveal the synaptic communication between NPY/AGRP containing fibers and GnRH neurons.**

4. **We addressed the putative glutamatergic phenotype of GnRH neurons.**
   a. We examined the expression of Vglut2 mRNA in GnRH mRNA expressing neurons with double-label *in situ* hybridization histochemistry.
   b. We addressed the presence of Vglut2-immunoreactivity in the axon terminals of GnRH neurons with the aid of confocal laser scanning microscopy.
   c. We determined the subcellular localization of Vglut2 protein in the hypophysiotropic axon terminals of median eminence with immuno-electron microscopy.

Materials and methods

1. Experimental animals

All of the experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee at the Institute of Experimental Medicine.

Mice

To study the sources of NPY afferents to GnRH neurons, experiments were carried out in a transgenic mouse strain, in which a portion of the mouse GnRH promoter directs the selective expression of GFP to the majority of GnRH neurons. Adult (8 wk old) male GnRH-GFP transgenic mice (n= 16) were bred and housed at the Institute of Experimental Medicine under conditions of 12 h light-12 h dark schedule (lights on at 07:00 h, lights off at 19:00 h), in a temperature (22 ± 2 °C) and humidity (60 ± 10%) controlled environment, with free access to laboratory food (Sniff Spezialdiaeten GmbH, Soest, Germany) and tap water.

GnRH-GFP mice treated neonatally with monosodium glutamate

To eliminate NPY fibers arising from the Arc, the chemical lesion of this region was performed by monosodium glutamate (MSG) treatment of four neonatal mice. Briefly, the neonatal animals were injected *s.c*. with increasing volumes of an 8% MSG solution dissolved in water: 4 mg/g body weight (BW) MSG solution on postnatal days 1 and 3; followed by 8 mg/g BW MSG solution on postnatal days 5, 7, and 9. The treated animals and four age-matched untreated mice were allowed to reach postnatal wk 8 and then killed by transcardiac perfusion. Brain tissues from the two groups were processed in parallel for comparative histological studies of the Arc and POA.

Rats

Adult male Wistar rats (N=6; 260–280 g BW) were purchased either from the local breeding colony of the Medical Gene Technology Unit of the Institute of Experimental Medicine or from Charles River Hungary Ltd. (Isaszeg, Hungary). The animals were kept under a 12 h light-12 h dark schedule (lights on at 07:00 h, lights off at 19:00 h), in a temperature (22 ± 2 °C) and humidity (60 ± 10%) controlled environment with free access to laboratory rat food and tap water.

2. Single- and double-labeling immuno-peroxidase histochemistry for light microscopic studies (Aims 1, 2a)

The deeply anesthetized animals were perfused via the ascending aorta with a fixative solution, which contained 4% paraformaldehyde in phosphate buffered saline (PBS). Coronal sections were cut on Leica freezing microtome, processed for immunocytochemistry and incubated in dilutions of specific primary antisera. Antigens were reacted with biotin-conjugated species-specific antibodies (Jackson ImmunoResearch Laboratories) followed by ABC Elite solution (Vector Laboratories). The color peroxidase reaction was visualized by an ammonium nickel (II) sulfate – diaminobenzidine (DAB) containing developer. Finally, the ammonium nickel (II) sulfate – DAB reaction product was enhanced by the silver-gold intensification (SGI) method.

In the case of double-labeling experiments, after the SGI step the sections were incubated with the second primary antibody, which was detected by a second immuno-peroxidase reaction and with the application of a DAB containing developer. In this way, the black signal of the SGI-ammonium nickel (II) sulfate – DAB could be clearly distinguished from the brown DAB precipitate at the light microscopic level.

3. Double-labeling immuno-peroxidase histochemistry for electron microscopic studies (Aim 1)

The deeply anesthetized animals were perfused via the ascending aorta with a fixative solution containing 2% paraformaldehyde and 4% acrolein in PBS. Coronal sections were cut on Vibratome and processed for the double immuno-peroxidase method according to the protocol described in section 2, with two modifications. (1) The sections were pretreated with 0.5% sodium borohydride dissolved in PBS in order to eliminate the residual aldehydes of the fixative. (2) Sections intended for electron microscopy were immersed into ascending concentrations of sucrose in PBS and permeabilized by three repeated freeze-thaw cycles on liquid nitrogen, instead of the use of Triton X-100. The combined use of SGI-Ammonium nickel (II) sulfate – DAB and DAB precipitates was also optimal for electron microscopic examinations since the SGI-ammonium nickel (II) sulfate – DAB precipitate was apparently more electron dense than DAB, making the distinction of the two labels easy at the ultrastructural level. The immunostained sections were osmicated, contrasted with uranyl-acetate and flat-embedded in TAAB 812 Epoxy resin (TAAB Laboratory Equipments Ltd.). Ultrathin sections were cut with Leica Ultracut UCT ultramicrotome, collected onto Formvar-coated single-slot grids, contrasted with a stabilized solution of lead citrate, and studied with a transmission electron microscope. Digital images were captured with a cooled CCD camera.

4. Double-labeling immunofluorescent histochemistry (Aims 2b, 4b)

Coronal sections of paraformaldehyde-perfused brains were cut on Leica freezing microtome, processed for immunocytochemistry and incubated in dilutions of specific primary antisera. The antigen-primary antibody reaction was detected by species-specific Cy3 fluorochrome conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) or by a peroxidase-mediated biotin tyramide amplification method followed by avidin-conjugated AMCA (Vector Laboratories) or FITC (Jackson ImmunoResearch Laboratories) fluorochromes. The immunofluorescent sections were mounted on microscope slides, coverslipped (Vectashield, Vector Laboratories) and analyzed with an Axiophot (Zeiss) epifluorescent microscope (Aim 2b) or Bio-Rad Radiance 2000 confocal microscope (Aim 4b).

5. Pre-embedding immuno-gold labeling (Aims 3, 4c)

In the case of study 3, the pre-embedding immuno-gold labeling was combined with immuno-peroxidase method in order to reveal two antigens. Coronal sections of acrolein-paraformaldehyde perfused brains were cut on a Vibratome, processed for immunocytochemistry and incubated in a dilution of anti-GnRH primary antibodies raised in rabbit. The primary antibodies were detected by anti-rabbit IgG conjugated with ultra small gold particles (1:100). The gold particles were silver intensified according to the instructions provided with the Aurion R-Gent SE-LM silver enhancing kit (AURION ImmunoGold Reagents & Accessories). The silver intensification was followed by the incubation in rabbit anti-AGRP antibodies, followed by biotin-conjugated secondary antibodies. The immunoreaction was detected by an immuno-peroxidase method that yielded DAB precipitate as it is described in section 2. Finally, the double-labeled sections were flat-embedded in TAAB 812 Epoxy resin (TAAB Laboratory Equipments Ltd) sectioned on a Leica Ultracut UCT ultra microtome and examined with a transmission electron microscope.

For electron microscopic localization of Vglut2 in the ME (Aim 4c), the adult male rats were anesthetized with pentobarbital and killed by decapitation. The brains were removed rapidly, the basal hypothalami dissected out and fixed for 24 h by immersion into a freshly made fixative solution containing 4% paraformaldehyde, 0.3% glutaraldehyde and 15% (V/V) saturated picric acid in PBS. The tissues were fixed further for 3 days in the same fixative, with the omission of glutaraldehyde. For the immuno-gold labeling, we used rabbit anti-Vglut2 primary antibodies (Synaptic Systems). The immunolabeling and embedding processes of these sections followed the above-described protocol.

6. Dual-label in situ hybridization histochemistry (ISHH)(Aim 4a)

Four adult rats were decapitated, and their brains were snap-frozen on powdered dry ice. Twelve-micrometer thick coronal sections through the POA were cut with a cryostat and collected serially on gelatin-coated microscope slides. For dual-label ISHH detection of the GnRH and Vglut2 mRNAs, the sections were hybridized with digoxigenin labeled GnRH antisense and ^{35}S-UTP-labeled Vglut2 antisense riboprobes. The digoxigenin probes were detected by peroxidase-conjugated anti-digoxigenin antibody (Roche), which was followed by a biotin tyramide amplified peroxidase reaction, which resulted in a brown DAB end-product. Then the slides were dipped into Kodak autoradiographic emulsion (Kodak Co.; NTB-3) in order to detect the autoradiographic signal of ^{35}S-labeled Vglut2 probes. Finally, the sections were coverslipped and examined with an Axiophot (Zeiss) microscope equipped with a digital camera, under bright field and dark field illumination.

7. Statistics

The summarized data from the light microscopic double-labelings were analyzed with one-way ANOVA ($p<0.05$ was considered significant). All data are presented as mean ± SEM.

Methods used for the different experiments of the thesis are summarized in Table 1.

<table>
<tr><th>Aims</th><th>Method</th><th>Antibodies</th></tr>
<tr><td>(1) Cholinergic innervation of GnRH neurons</td><td>Double-labeling immuno-peroxidase histochemistry at light and electron microscopic levels</td><td>Anti-ChAT (Chemicon, AB144P; 1:1000); anti-VAChT (Sigma, V5387; 1:10,000); anti-GnRH (LR-1; a gift from Dr. R.A. Benoit 1:10,000)</td></tr>
<tr><td rowspan="2">(2) Possible sites of origin of NPY afferents to GnRH neurons</td><td>(2a) Light microscopic single- and double-labeling immuno-peroxidase histochemistry</td><td rowspan="3">Anti-NPY (a gift from István Merchenthaler, 1:100,000); anti-AGRP (Phoenix Pharmaceuticals, 1:8000); anti-DBH (1:8000); anti-GnRH (LR-1)</td></tr>
<tr><td>(2b) Immunofluorescent histochemistry</td></tr>
<tr><td>(3) Innervation of GnRH neurons by AGRP/NPY afferents</td><td>Combined immuno-peroxidase and immuno-gold histochemistry at electron microscopic level</td></tr>
<tr><td rowspan="3">(4) Glutamatergic phenotype of GnRH neurons</td><td>(4a) Double-label in situ hybridization histochemistry</td><td></td></tr>
<tr><td>(4b) Fluorescent double-labeling immunocytochemistry</td><td rowspan="2">Anti-Vglut2 (Chemicon, AB 5907; SYnaptic SYstems AB 135103 1:1000); anti-GnRH (LR-1)</td></tr>
<tr><td>(4c) Electron microscopic immuno-gold labeling</td></tr>
</table>

Results

Identification of cholinergic afferents to GnRH neurons of the rat

The cell bodies of GnRH neurons were scattered in the POA and the same region contained a fine network of varicose cholinergic fibers that were immunoreactive for choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT). Immunostaining with the VAChT antiserum resulted in a somewhat higher fiber density compared to that obtained with the ChAT antiserum. High-power analysis of dual-labeled sections established that the cholinergic axons were frequently apposed to GnRH-immunoreactive neuronal elements. Cholinergic axons contacted the cell bodies as well as the dendrites of GnRH neurons.

At the ultrastructural level, some of the cholinergic axonal profiles established asymmetric synapses with unlabeled dendrites. Gonadotropin-releasing hormone neurons were often contacted by the cholinergic fibers on their somata as well as dendrites. While the appositions were devoid of glial intercalations, state-of-the-art synaptic specializations occurred rarely.

Revealing the origin of NPY-containing afferents to GnRH neurons in male mice

Comparison of cresyl-violet-stained sections from MSG-treated mice *vs.* untreated controls showed a marked reduction in the size of the Arc. Results of single-labeling immunocytochemical experiments showed that the density of NPY-immunoreactive fibers was largely reduced in the POA of Arc-lesioned animals, while the AGRP-immunoreactivity was almost completely absent from this region due to the MSG treatment. This observation indicated that a major, albeit not the sole, source of NPY fibers to the POA is the Arc.

The analysis of dual-labeled sections from intact mice demonstrated numerous contacts between NPY- and AGRP-immunoreactive axons and the perikarya and proximal dendrites of GnRH neurons. The frequency of NPY-containing axonal appositions to individual GnRH cells was significantly higher than the frequency at which AGRP-immunoreactive neuronal contacts occurred on GnRH neurons. The number of AGRP-immunopositive contacts was 56.5 ± 9.8% of that of all NPY-immunoreactive contacts. Furthermore, significantly less

NPY-containing neuronal contacts were visible in neonatally MSG-treated animals *vs.* controls. The lesion of the Arc caused a 63.7 ± 5.0% loss of NPY-immunopositive juxtapositions. Altogether, the two different methodological approaches revealed that about 56.5–63.7% of NPY axons to GnRH cells arise from NPY neurons of the Arc. In our triple fluorescent experiments, the high-power analysis of the fluorescent specimen established that 49.1 ± 7.3% of NPY-containing neuronal contacts also contained AGRP. Moreover, the high-power analysis of dual-immunostained axons established the presence of DBH in 25.4 ± 3.3% of NPY-containing fibers in contact with GnRH neurons.

Electron microscopic detection of AGRP in synaptic afferents to GnRH neurons

At the ultrastructural level, AGRP-immunoreactive fibers contained electron-dense DAB deposits, and they were frequently apposed to GnRH neurons accumulating highly electron-dense silver-gold particles. Synapses of symmetric morphology were observed between AGRP axons and GnRH neurons as well as non-GnRH structures.

Morphological evidence for the glutamatergic phenotype of GnRH neurons in adult male rat

Development of emulsion autoradiographs exposed for 2 wk resulted in strong hybridization signal for Vglut2 mRNA in the POA. Virtually all (99.5 ± 0.2%) of the total 438 GnRH neurons analyzed also contained Vglut2 hybridization signal, usually at moderate levels. Confirmative results obtained with a distinct Vglut2 probe and the lack of Vglut2 signal using the sense strand of Vglut2 RNA transcript provided support for hybridization specificity.

Both Vglut2- and GnRH-immunoreactive fibers formed dense plexus in the organum vasculosum laminae terminalis (OVLT) and the external zone of the ME. High power confocal images demonstrated extensive terminal co-expression of Vglut2 with GnRH immunoreactivities in both circumventricular organs. Vglut2-immunoreactive axons were eliminated from the ME when using primary antibodies preabsorbed with 10 μM immunization antigen. In addition, simultaneous use of two different primary antisera against Vglut2 labeled identical axons throughout the hypothalamus, in further support of labeling specificity.

Pre-embedding colloidal gold labeling for Vglut2 identified numerous glutamatergic axon terminals in the external layer of the ME. Many of these axons established direct contact with the external limiting membrane of portal vessels, indicating that they represented neurosecretory terminals. The labeled structures contained small clear as well as dense-core vesicles at a highly variable ratio. The immunocytochemical labeling clearly tended to occur at subcellular domains occupied primarily by small clear vesicles.

Discussion

Morphological evidence for direct cholinergic afferents to GnRH neurons

We present neuromorphological evidence for a direct cholinergic afferent input to the GnRH neuronal system of the rat. These results reveal a previously unexpected neurotransmitter system, which directly regulates GnRH neurons. Our results indicate that the cholinergic axons often form direct contacts but rarely establish classical synapses with the GnRH neurons. This is highly reminiscent to the previous morphological findings by several other investigators. The observations that out of cholinergic axon varicosities, only 7 % in the hippocampus (CA1, stratum radiatum), 10 % in the neostriatum and 14 % in the parietal cortex were engaged in synapses may indicate nonsynaptic mechanisms whereby cholinergic axons influence their target neurons, including GnRH cells. Therefore, we suggest a dominantly nonsynaptic signaling mechanism in the cholinergic – GnRH interaction.

Origin of the NPY afferents to GnRH neurons

In the first set of our experiments, the possible origins of NPY-containing afferents to GnRH cells were investigated. Our results indicate that NPY neurons of the Arc give rise to 49–64% of the NPY-immunoreactive axonal contacts on the somata and proximal dendrites of GnRH-GFP neurons (depending on the calculation approach we used). The use of AGRP-immunoreactivity as a topographic marker for NPY axons of Arc origin was introduced to our studies based on previous evidence that AGRP and NPY neurons of the Arc are essentially identical. Results of our triple-label fluorescence studies indicate that NPY/AGRP neurons of the Arc give rise to 49% of NPY axons that form contacts with the somata and proximal dendrites of GnRH neurons. A similar ratio for NPY axons of Arc origin (56%) was calculated based on the results of our light microscopic double-labeling experiment from the

comparison of AGRP-immunoreactive *vs.* all NPY-immunoreactive contacts on GnRH neurons in intact animals. A somewhat heavier innervation appears to originate from the Arc (64%) if the lost fraction of NPY contacts in MSG-treated animals is considered. This intense NPYergic innervation from the Arc appears to have a critical role in the mediation of the metabolic cues to the reproductive axis, including leptin and ghrelin signaling from the periphery.

We have revealed that an additional 25% of NPY juxtapositions are related to adrenergic/noradrenergic cell groups of the brain stem, using DBH as topographic marker for these NPY fibers. The adrenergic/noradrenergic input to GnRH cells is consistent with a large body of evidence in the literature indicating the important role of adrenergic stimuli in the regulation of the ovarian cycle and the steroid-induced gonadotropin surge. It is also worth noting that a large subset of DBH-immunopositive axons in contact with GnRH cells was devoid of NPY-immunoreactivity, in concert with the finding that NPY is expressed differentially among distinct noradrenergic/adrenergic cell groups. The most likely sources of NPY innervation are the C1–3 adrenergic and the A1 noradrenergic cell groups where high percentages of neurons were found to contain NPY.

Agouti-related peptide as a novel neurotransmitter in the regulation of the GnRH neurons

In order to analyze the nature of the putative neuronal communication between AGRP-immunoreactive axons and GnRH neurons, we carried out electron microscopic studies and demonstrated that AGRP-immunoreactive axons establish synapses with GnRH neurons.

From a functional viewpoint, it is important to note that AGRP-immunopositive axons formed only symmetric-type synapses with GnRH as well as non-GnRH neurons of the POA. This observation is in concert with the previous findings of symmetric synapses between NPY axons and GnRH neurons of the POA. Furthermore, this synaptic morphology also characterizes inhibitory synapses. Since AGRP administration can suppress or stimulate the HPG axis depending on the experimental paradigm, future research will need to address any difference of chronic *vs.* acute AGRP effects on gonadotropin secretion, as well as the potential sexual steroid dependence of AGRP actions.

Demonstration of Vglut2 mRNA expression and Vglut2-immunoreactivity in GnRH neurons

Our ISHH finding that virtually all GnRH neurons expressed Vglut2 mRNA in the adult male rat strongly suggests that the glutamatergic phenotype is a critically important feature of the GnRH neuronal system. Moreover, we have verified the presence of Vglut2-immunoreactivity in the GnRH axon terminals of the ME and OVLT. Our electron microscopic observations have demonstrated the presence of Vglut2 in the hypophysiotropic nerve terminals and the close association of Vglut2-immunoreactivity with small clear vesicles. The observable ultrastructural distribution of Vglut2 above small clear, and not dense core, vesicles agrees with the presumed site of location of this classical neurotransmitter in neurons. This location is also in agreement with the association of Vglut1 and Vglut2 immunoreactivities with small clear vesicles in excitatory synapses in other brain areas. Considering the occurrence of Vglut2 in GnRH neurons, it is tempting to speculate that intrinsic L-glutamate fulfills autocrine/paracrine regulatory functions that may contribute to the generation of the pulsatile patterns of GnRH neurohormone output. Nevertheless, this hypothesis would require experimental support.

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