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

Ph.D. Dissertation

Gergely F. Túri

Laboratory of Endocrine Neurobiology
Institute of Experimental Medicine, Hungarian Academy of Sciences

Semmelweis University
János Szentágothai Ph.D. School of Neuroscience

Supervisor: Zsolt Liposits Ph.D., D.Sc.

Chairman of committee: Béla Halász Ph.D., D.Sc.

Opponents: József Kiss Ph.D., D.Sc.
Zoltán Rékási Ph.D.

Budapest
2007
# Table of contents

1. List of abbreviations ........................................................................................................5  
2. Introduction ..................................................................................................................6  
   2.1. Regulation of reproduction in mammals .................................................................6  
   2.2. The anatomy of the GnRH neuronal system in rodents ............................................8  
   2.2.1. The development, distribution and structural properties of GnRH neurons in adult rats .............................................................................................................8  
   2.2.2. The efferent pathways and target areas of the GnRH neurons ..............................10  
   2.2.3. The afferent regulation of GnRH neurons ..............................................................14  
   2.2.3.1. Innervation of GnRH neurons by afferents containing classical neurotransmitters .....................................................................................................................14  
   2.2.3.2. Innervation of GnRH neurons by neuropeptide-containing afferents ............17  
   2.2.4. Colocalized neurotransmitters in GnRH neurons .................................................19  
   2.3. Proposed novel neurotransmitter systems in the regulation of GnRH neurons ......22  
   2.3.1. Involvement of acetylcholine (ACh) in the regulation of the reproduction ..........22  
   2.3.2. The role of the NPY in the regulation of reproduction ........................................24  
   2.3.3. Glutamate as a proposed autocrine/paracrine regulator synthesized by GnRH neurons .........................................................................................................................26  
3. Specific aims ..................................................................................................................27  
4. Materials and methods .................................................................................................28  
   4.1. Direct innervation of GnRH neurons by cholinergic afferent pathways in male rats .......................................................................................................................28  
   4.1.1. Double-labeling immunocytochemical detection of cholinergic fibers and GnRH neurons at the light microscopic level ...........................................................................28  
   4.1.2. Double-labeling immunocytochemical detection of cholinergic fibers and GnRH neurons at the electron microscopic level .........................................................30  
   4.2. Determining of the origin of NPY-containing afferents to GnRH neurons in male GnRH-GFP transgenic mice .................................................................31  
   4.2.1. Experimental animals ..........................................................................................31  
   4.2.2. Single-labeling immunocytochemical detection of NPY- and AGRP-containing fibers in the POA of neonatally MSG-treated mice vs. untreated controls ..........................................................32  
   4.2.3. Double-labeling immunocytochemical detection of NPY- and AGRP-containing fibers in contact with GnRH neurons of neonatally MSG-treated mice vs. untreated controls ..........................................................33  
   4.2.4. Immunofluorescent-labeling of AGRP/NPY and DBH/NPY contacts with GnRH-GFP neurons in intact mice......................................................................................34  
   4.3. Electron microscopic analysis of AGRP afferents to GnRH neurons in intact male, GnRH-GFP mice .........................................................................................36  
   4.4. Investigation of the putative glutamatergic phenotype of GnRH neurons ...37  
   4.4.1. Experimental animals ..........................................................................................37  
   4.4.2. Detection of GnRH and Vglut2 mRNAs by dual-label in situ hybridization histochemistry (ISHH) .................................................................................................37
4.4.3. Dual-immunofluorescent labeling for confocal laser scanning microscopic analysis of Vglut2- and GnRH-immunoreactive axons in the OVLT and ME ................................................................. 40
4.4.4. Pre-embedding immuno-gold-labeling of Vglut2 in the hypophysiotropic nerve terminals of the ME ........................................................................................................ 41

5. Results ................................................................................................................................ 43

5.1. Identification of cholinergic afferents to GnRH neurons of the rat .............................. 43
  5.1.1. Light microscopic evidence for cholinergic neuronal contacts on GnRH neurons .................................................................................................................. 43
  5.1.2. Electron microscopic evidence for cholinergic afferents to GnRH neurons .................................................................................................................. 45

5.2. Revealing the origin of NPY-containing afferents to GnRH neurons in male mice ................................................................. 47
  5.2.1. Comparative studies of NPY- and AGRP-containing fibers in contact with GnRH neurons of neonatally MSG-treated mice vs. untreated controls .... 47
  5.2.2. Triple-label fluorescent detection of AGRP/NPY and DBH/NPY fibers forming neuronal contacts with GnRH-GFP neurons .............................................. 50

5.3. Electron microscopic detection of AGRP in synapt ic afferents to GnRH neurons ........................................................................................................................ 53

5.4. Morphological evidence for the glutamatergic phenotype of GnRH neurons in adult male rat ........................................................................................................ 55
  5.4.1. Results of in situ hybridization experiments .................................................................................................................. 55
  5.4.2. Results of dual-label immunofluorescent studies ........................................................................................................ 56
  5.4.3. Electron microscopic localization of Vglut2 in hypophysiotropic axon terminals in the ME ...................................................................................................... 58

6. Discussion ................................................................................................................................ 59

6.1. Proposed nonsynaptic role and receptorial mechanisms of ACh on GnRH neurons and the possible sites of origin of the direct cholinergic afferents ........................................................................................................ 59
  6.1.1. Ach as a proposed nonsynaptic modulator of GnRH neurons ................................................................. 59
  6.1.2. Receptorial mechanisms of ACh actions on GnRH neurons ........................................................................................................ 60
  6.1.3. Possible sites of origin of the cholinergic innervation to GnRH neurons ........................................................................................................ 61

6.2. The functional roles of the NPYergic afferents of Arc and brainstem in the regulation of GnRH neurons and the receptorial mechanisms of NPY action on GnRH neurons ........................................................................................................ 62
  6.2.1. The Arc and the brainstem as two major sources of origin of NPY fibers innervating GnRH neurons ........................................................................................................ 62
  6.2.2. Receptorial mechanisms of NPY actions on GnRH neurons ........................................................................................................ 67
  6.2.3. Methodological considerations .................................................................................................................. 68

6.3. Agouti-related peptide as a novel neurotransmitter in the regulation of the GnRH neurons ........................................................................................................ 68

6.4. Demonstration of glutamatergic phenotype of the GnRH neurons ............................. 69
  6.4.1. Central effects of glutamate on the regulation of reproduction .................................................................................................................. 70
  6.4.2. Glutamatergic phenotype of the GnRH neurons ........................................................................................................ 70
  6.4.3. Subcellular localization of Vglut2 in the axon terminals of the ME ........................................................................................................ 72
  6.4.4. Methodological considerations .................................................................................................................. 73

7. Conclusions ................................................................................................................................ 75

8. Summary .................................................................................................................................. 77
1. List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>ac</td>
<td>Anterior commissure</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AGRP</td>
<td>Agouti-related protein</td>
</tr>
<tr>
<td>AMCA</td>
<td>Aminomethylcoumarin acetate</td>
</tr>
<tr>
<td>Arc</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>Cy3</td>
<td>Cyanine dye 3</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DBH</td>
<td>Dopamine-β-hydroxylase</td>
</tr>
<tr>
<td>DSIP</td>
<td>Delta sleep-inducing peptide</td>
</tr>
<tr>
<td>DV</td>
<td>Dense core vesicle</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GLU</td>
<td>Glutamate</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>HDB</td>
<td>Horizontal limb of the diagonal band of Broca</td>
</tr>
<tr>
<td>HPG</td>
<td>Hypothalamo-pituitary-gonadal axis</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>INF</td>
<td>Infundibular stalk</td>
</tr>
<tr>
<td>ISHH</td>
<td>In situ hybridization histochemistry</td>
</tr>
<tr>
<td>KA-2</td>
<td>Kainate-2 ionotropic glutamate receptor subunit</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>ME</td>
<td>Median eminence</td>
</tr>
<tr>
<td>MPN</td>
<td>Median preoptic nucleus</td>
</tr>
<tr>
<td>MPOA</td>
<td>Medial preoptic area</td>
</tr>
<tr>
<td>MSG</td>
<td>Monosodium glutamate</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR-1</td>
<td>N-methyl-D-aspartate glutamate receptor subunit</td>
</tr>
<tr>
<td>NT</td>
<td>Neurotensin</td>
</tr>
<tr>
<td>OVLT</td>
<td>Organum vasculosum laminae terminalis</td>
</tr>
<tr>
<td>ox</td>
<td>Optic chiasm</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCS</td>
<td>Pericapillary space</td>
</tr>
<tr>
<td>POA</td>
<td>Preoptic area</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard saline citrate</td>
</tr>
<tr>
<td>SV</td>
<td>Small clear vesicle</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>VACHT</td>
<td>Vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>Vglut1-3</td>
<td>Types 1-3 of the vesicular glutamate transporters</td>
</tr>
<tr>
<td>αMSH</td>
<td>α-melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>β-END</td>
<td>β-endorphin</td>
</tr>
</tbody>
</table>
2. Introduction

2.1. Regulation of reproduction in mammals

The adaptive regulation of the hypothalamo-pituitary-gonadal (HPG) axis is crucial in the successful reproduction among the vertebrates. The central unit of this axis is formed by the gonadotropin-releasing hormone (GnRH)-producing neurons which 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 (LH) and follicle stimulating hormone (FSH), 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 components of the axis. (Figure 1.)

The gonadal steroids are crucial hormonal factors in the regulation of the HPG axis. Gonadectomy results in increased levels of LH/FSH in the systemic circulation and elevates the level of GnRH in the hypophyseal portal circulation. This confirms the tonic inhibitory role of the gonadal steroids on the hypothalmo-pituitary unit in intact animals. The negative feedback effect is a common regulatory mechanism in both genders. After puberty, the steroid hormone genesis is continuous in males. High levels of androgens produced by the Leydig cells of the testicles exert a constant inhibitory action on the hypothalamus and pituitary either via acting on the androgen receptors or following its aromatization to estradiol, via acting on estrogen receptors (1). In contrast, in females the gonadal functions show a cyclic activity. In the early phases of ovarian cycle, the relatively low level of estrogen exerts negative feedback effects on the hypothalamus and hypophysis. This way estrogen constrains its own follicular synthesis and constrains the GnRH neurons as well to function in a pulse mode, i.e. to release their hormone content at regular time intervals. As the follicles mature, they show a dramatic increase in their secretion of estrogen and a modest increase in their output of progesterone. These changes in the circulating blood levels of ovarian steroids switch the function of GnRH neurons to surge mode, which results in a rapid increase of
hypothalamic GnRH (2) and consequential pituitary gonadotropin secretion. This phenomenon is referred to as positive feedback and it is a prerequisite for ovulation (3).

In addition to the feedback effects of the steroid hormones on the HPG axis, many external and internal factors influence GnRH neuronal functions. These include environmental toxins, stressful stimuli, malnutrition or the overfed status of the animal. These factors modify the spontaneous electrical (4) and secretory activities (5, 6) of GnRH neurons either via direct actions or via the regulation of neuronal inputs from various neurotransmitter systems, which impinge on GnRH nerve cells. The focus of this thesis is on the neuronal regulation and neurochemical properties of GnRH neurons, therefore, following the anatomical characterization of the hypophysiotropic GnRH neurons, their efferent pathways and main target areas, the thesis will discuss the chemical nature and the possible sources of origin of the regulatory neurotransmitters within specific neuronal afferents to GnRH neurons. Finally, the co-expressed neurotransmitters by GnRH neurons will be reviewed briefly.

Figure 1. The schematic drawing depicts the components of the HPG axis. The hypophysiotropic GnRH is released by the hypothalamus and it liberates the gonadotropins from the hypophysis. LH and FSH stimulate gonadal functions. Gonadal steroids, in turn, regulate the functions of the HPG axis by negative (in males and females) and positive (only in females) feedback mechanisms. (FSH: follicle stimulating hormone, GnRH: gonadotropin-releasing hormone, LH: luteinizing hormone)
2.2. The anatomy of the GnRH neuronal system in rodents

2.2.1. The development, distribution and structural properties of GnRH neurons in adult rats

In rats, the central regulation of the reproductive axis is carried out by about 1000-1600 GnRH neurons (7, 8). These neurons ontogenetically originate outside the brain in the olfactory placodes (9) and migrate to their final location during the embryonic life. In addition to these data from mice, results of other experiments performed in chicks (10), rhesus macaques (11), rats (12) and humans (13) indicate that the olfactory origin of GnRH neurons is a general phenomenon of vertebrate development.

While GnRH neurons in the two genders show functional differences, their number is equal in the two sexes. Although the hypophysiotropic GnRH neurons serve the same function, i.e. the stimulation of gonadotropin release from the anterior pituitary, they are scattered in different anatomical areas. From a topographic viewpoint, GnRH immunoreactive perikarya can be divided into several subpopulations with the help of immunocytochemical approaches (7). One group of GnRH neurons can be observed in the medial septal nucleus, nucleus of the vertical and horizontal limbs of the diagonal band and in the vicinity of the organum vasculosum laminae terminalis (OVLT). Another group can be detected in the medial preoptic area with the majority of the GnRH cells situated in the medial preoptic nucleus (MPN). Fewer GnRH neurons are found in the periventricular portion of the preoptic nucleus and in the lateral preoptic area (7). All of these anatomical regions are located rostral to and above the optic chiasm. Therefore, for the sake of simplicity, in this thesis we will commonly refer to these sites as the preoptic area (POA).

In addition to hypophysiotropic GnRH neurons in the POA, the detailed light microscopic mapping of GnRH-immunoreactive perikarya has revealed sparse neurons in other brain regions as well (7); the function of these non-hypophysiotropic GnRH neurons needs clarification.
Figure 2. (A) Distribution of GnRH containing neuronal elements in the rat brain. The gray dots in the schematic sagittal drawing (A) correspond to GnRH synthesizing perikarya. The hypophysiotropic GnRH neurons are located in the POA (delineated with a dashed line) and project to the OVLT (yellow area above the optic chiasm) and to the ME. (B) Coronal section of a rat brain trough the POA-OVLT region. The immunostained section illustrates the typical distribution of the hypophysiotropic GnRH neurons (arrows) and fibers in this area (Scale bar = 50 µm) (III: third ventricle; ac: anterior commissure; ME: median eminence; OVLT: organum vasculosum lamiae terminalis; ox: optic chiasm; POA: preoptic area)

The spreading of immunocytochemical methods and highly specific antibodies against the GnRH decapeptide allowed to characterize the light (7) and electron microscopic features of GnRH neurons (8, 14, 15). It is worth of note that the immunocytochemical visualization of GnRH-immunoreactivity limits the microscopic analysis to the cell body, proximal dendrites, and nerve terminals of GnRH cells, which contain the neuropeptide abundantly. In contrast, the fluorescent visualization and subsequent confocal microscopic analysis of biocytin-filled GnRH neurons have revealed an extensive dendritic arborization (16), which could not be detected reliably by labeling for the GnRH decapeptide itself.

In most species, the majority of GnRH neurons are oval or fusiform in shape, with a maximum diameter of 10 to 20 µm. The fine structural properties of these neurons do not differ from the general neuronal attributes, except that some of the GnRH nerve cells are ciliated (8, 15). Most of the GnRH neurons show either uni- or bipolar shapes. The dendritic processes of GnRH cells originate from one or both poles of the cell body.
The dendrite with the greatest circumference extending from the GnRH soma is designated as the primary dendrite.

With the aid of validated and highly specific antibodies against GnRH decapetide, GnRH neurons can also be divided into two basic morphologic categories: those, which possess a smooth contour, and those, which have a more ragged surface (8). Somewhat contrasting this categorization, Campbell and colleagues examined 45 biocytin-filled cells and observed that all of them exhibited some somal and dendritic spines with a density up to 1 spine/µm plasma membrane (16). They found that the majority of the spines were of the simple sessile type, but thin pedunculated and mushroom-like spines were observed as well.

2.2.2. The efferent pathways and target areas of the GnRH neurons

The GnRH axons follow the septo-preoptico-infundibular tract that emerges from the GnRH neurons located in the medial septum and the POA (7). The GnRH fibers run in ventral, caudal and somewhat lateral direction and converge into several fascicles. The unpaired median fascicle is formed at the rostral level of the optic chiasm. One bundle of this fascicle is localized immediately beneath the ependymal lining of the floor of the third ventricle, while the other is widely spread out in a superficial position along the ventral surface of the optic chiasm. Lateral to the suprachiasmatic nucleus, the majority of the GnRH fibers forming the septo-preoptico-infundibular tract gather into two fascicles. The medial fascicle projects to the retrochiasmatic area, arcuate nucleus (Arc) and inframammillary region, while the lateral fascicle terminates in the median eminence (ME) (17).

Many fibers originating from the medial septal and preoptic regions pass through the dorsomedial hypothalamus making up a periventricular subependymal GnRH network that accounts for most of the extrahypothalamic projections. The scattered, extrahypothalamic GnRH fibers innervate the following areas: mammillary complex, raphe nuclei, medial nucleus of the amygdala, medial habenular nuclei, and ventral tegmental area. In addition to these nuclei, the GnRH neurons compose a dense axonal network around the aqueductus cerebri in the mesencephalic central gray (7). Moreover, GnRH neurons that are located in the medial septum, the nucleus of diagonal band, the
olfactory tubercle and along the medial surface of the hemispheres innervate the medial layers of the olfactory bulb (7). This implies that the GnRH system may be involved in the transmission or modulation of olfactory stimuli, many of which are closely related to reproductive functions and behavior (18).

Nevertheless, the major efferent targets of the GnRH neurons are two circumventricular organs: the OVLT and the median eminence (ME) (8, 19).

The OVLT is located at the rostral tip of the third ventricle (7, 8, 20) and receives its innervation from the GnRH cell bodies of the adjacent MPN (21). The OVLT extends dorsally approximately 1 mm from the optic chiasm and the roof of the optic recess and it is made up of ependymal cells, neurons and glial cells. The ependymal cells have tight junctions near their apical surfaces, which prevent the free flow of the cerebrospinal fluid from the ventricle to the brain parenchyma. Similarly to other circumventricular organs, the OVLT also contains a rich vascular network with specialized fenestrated capillaries (20). The highly vascularized outer layer of the OVLT together with the pia mater composes the external zone, which receives its blood supply from the preoptic artery. The internal zone contains a complex neuropil with neurons, fibers, glial elements, capillary loops and perivascular spaces (22). The OVLT is innervated by axons containing several neuropeptides and neurotransmitters, including GnRH, thyrotropin-releasing hormone, somatostatin, dopamine, norepinephrine, serotonin, acetylcholine, oxytocin and vasopressin (20). Although the function of the OVLT is not entirely understood, the presence of estrogen receptors implies its important role in estrogen feedback to the brain (23). The OVLT also responds to osmotic challenges (24), which also suggests its important function in osmotic regulation.

The ME is a rostro-caudally elongated vascular organ on the ventral surface of the hypothalamus and it is innervated by GnRH axons via the septo-preoptico-infundibular pathway. In the rat, its extension is over 2.5 mm from the most rostral to the most caudal part (25). The ME receives an extremely rich blood supply from the superior hypophyseal artery, which forms a dense, mosaic-like external plexus, and runs along the ventral surface of the ME. The internal plexus arises from the external plexus and forms several small, twisted capillary loops spanning between the external and internal zones of the ME. On the surface or in the close vicinity of this fenestrated capillary
network terminate the nerve endings of the hypothalamic homeostatic hormone producing neurons. The capillaries are re-collected to the long portal veins that run on the surface of the infundibular stalk, the direct continuation of the caudal end of the ME. The long portal veins then supply for the portal circulation of the pituitary. The ME can be divided three zones, starting from the bottom of the third ventricle: the ependymal layer, the internal zone and the external zone (for a review see :(26)).

The main cell type of the ependymal layer is the ependymal cell, which has microvilli on its apical surface. Adjacent ependymal cells overlying the ME are interconnected by tight junctions, which limit passive exchange of materials between the ventricle and the extracellular space of the ME. The other typical cell type of the ependymal layer is the tanycyte, which has a bipolar shape. Its soma is located in the ependymal layer, and its basal, column-like process reaches the basal part of the ME.

The internal zone is subdivided into a hypependymal layer and a fiber layer and in addition to the supportive cells (subependymal cells and pituicytes) it is composed of axons that originate from the Arc, magnocellular cells of supraoptic and paraventricular nuclei, and aminergic neurons of the brainstem.

The external zone lies ventral to the internal zone and may be subdivided into a reticular layer and a palisade layer. The reticular layer contains ependymal and glial processes and axons of aminergic and parvicellular peptidergic systems. The palisade layer lies at the surface of the ME. Here one can find nerve terminals of the hypophysiotropic parvicellular systems, pituicytes, ependymal end-feet and capillaries.

The GnRH fibers preferentially localize in the lateral part of the ME (Figure 3.). The majority of the GnRH fibers comes from the main septo-preoptico-infundibular GnRH tracts, but the oral and caudal parts of the ME are also innervated by fibers originate in the periventricular GnRH network (7). Within the nerve terminals, the GnRH decapeptide is packed into large granular (also called dense core) vesicles (25). Although GnRH is released into the portal capillary system, GnRH terminals in direct contact with the pericapillary space can rarely be observed, except for the day of proestrus (25). The rest of the time tanycyte processes separate the GnRH nerve endings from the capillaries (25).
Figure 3. GnRH immunostained photomicrographs demonstrate the main termination fields of the GnRH axons. (A) The OVLT is located in the rostral tip of the third ventricle (III.) above the optic chiasm. The GnRH axons (brown fibers) originating from the medial preoptic nucleus densely innervate this area. (B) The coronal section illustrates the topographical distribution of GnRH axons in the ME. GnRH fibers originating from the POA preferentially localize in the lateral parts of the ME. (C) Parasagittal section through the ME and its direct caudal continuation, the infundibular stalk (INF). The photomicrograph demonstrates the rostro-caudal distribution of GnRH fibers. (III.: third ventricle; INF: infundibular stalk; ME: median eminence; OVLT: organum vasculosum laminae terminalis; Scale bars: 50 µm)

Besides the hypophysiotropic axons, neurotransmitter-containing fibers also terminate in the areas of the OVLT and ME and release their peptide content in the blood stream. For example in the ME, axons containing acetylcholine (27), gamma-aminobutyric acid (GABA) (28), galanin (29), substance P, neurotensin (30, 31), cholecystokinin (31), δ-sleep inducing peptide (32) and catecholamines (33) have been identified. While the role of these transmitters in the ME is not fully understood, the expression of their receptors on the glial and neuronal elements supports the view that these neurotransmitters can exert local paracrine effects. Moreover, their high
concentration in the hypothalamic portal circulation suggests that they may influence pituitary functions as well (29, 30).

2.2.3. The afferent regulation of GnRH neurons

Although double immuno-labeling techniques identified various neurotransmitter and neuropeptide containing synapses along the surface of GnRH neuronal perikarya and dendrites, GnRH nerve cells receive a relatively sparse synaptic input. While the interneurons in the hippocampus display 2000 – 16,000 synaptic connections (34, 35), the ultra structural analysis revealed only few dozen synapses on the cell bodies and dendrites of GnRH neurons (15, 36). This apparent paucity of synaptic inputs may partly reflect the previously mentioned technical limits to detect immunocytochemically the dendritic spines and filopodia of GnRH neurons where excitatory terminals may be present at higher densities (37). It is also worth noting that the GnRH neurons of female rats exhibit about a 2-fold greater proportion of plasma membrane occupied by synapses than do those in males (36). Such a trend cannot be revealed at the level of the dendrites (36). In addition, Campbell at al. failed to detect any dimorphism in the spine number at any neuronal segment of biocytin filled GnRH neurons (16).

2.2.3.1. Innervation of GnRH neurons by afferents containing classical neurotransmitters

*Catecholamines.* The effects of catecholamines on GnRH neurons is not regarded as a conventional inhibitory or excitatory drive (for review see: (38, 39)). Instead, catecholamines rather play a neuromodulator role that enables interactions within the network depending upon its local concentrations (38). The primary substrate of catecholamine biosynthesis is tyrosine. Among the four enzymes that are involved in the biosynthetic pathway of catecholamines, the following ones are generally used as marker enzymes of catecholaminergic neurons. Tyrosine hydroxylase (TH), which produces dopamine from tyrosine, dopamine-β-hydroxylase (DBH), which converts dopamine to noradrenaline and finally, phenylethanolamine- N-methyltransferase, which can metabolize further noradrenaline to adrenaline. The ultrastructural analysis of
the direct interactions between DBH-immunoreactive axons and GnRH dendrites has demonstrated synaptic connection between noradrenergic fibers and GnRH neurons in mice, although the number of the synaptic specializations was low (40). In rats, Léránth and colleagues have also reported that a small number of axons immunopositive for TH establishes synaptic contacts with GnRH somata and dendrites. The synapses belonged to the symmetric (Gray II type) category (41). Since the biosynthetic pathways of dopamine, noradrenaline and adrenaline synthesis commonly require TH, the presence of TH-immunoreactivity in the catecholaminergic synapses of GnRH neurons (41) does not show unequivocally the chemical nature and origin of the fibers. The cell bodies of noradrenaline and adrenaline synthesizing neurons (forming the bulk of the catecholaminergic nuclei A1–A7 and C1-C3, respectively) make up bilateral cell clusters in the pons/medulla region of the brainstem, therefore these areas can be considered as the only sources of the noradrenergic/adrenergic innervation to GnRH neurons.

**Excitatory amino acids.** Glutamate and aspartate are important excitatory neurotransmitters within the neuroendocrine hypothalamus (42) and exert powerful actions on the neurohormone output of the GnRH network (43). Although a number of immunoreactive ionotropic and metabotropic glutamate receptor subunits and their encoding mRNAs could be identified in the hypothalamus and also in GnRH neurons (44-46), the distribution of glutamatergic fibers and cell bodies in the CNS was unknown for a long time due to the lack of appropriate glutamatergic neuronal markers. The detection of the recently discovered vesicular glutamate transporters 1 and 2 (Vglut1, 2) provides a novel tool for neuromorphological studies to reveal glutamatergic neuronal structures. These transporters show highly specific and saturable ATP-dependent transport of glutamate into synaptic vesicles *in vitro* (47) and show subtype-specific anatomical distributions (48). Recently, Kiss et al. have described the direct glutamatergic innervations of GnRH neurons (49). According to their report, specifically the Vglut2-immunoreactive terminals often formed asymmetric synapses with GnRH dendritic shafts but hardly ever with the somata. It is difficult to estimate the putative sources of the glutamatergic innervation of GnRH neurons because of the ubiquity of glutamatergic cell bodies in the CNS. Vglut1 and Vglut2 positive neurons form two distinct subsets of glutamatergic neurons, with complementary patterns of
distribution (50). The lack of Vglut1-immunoreactive synapses on the surface of GnRH neurons (49) suggests that the Vglut1 expressing glutamatergic nerves cells do not contribute to the glutamatergic innervation of GnRH neurons. Moreover, as it has been revealed by $[^3]H$D-aspartate injection into the medial preoptic area, several telencephalic (lateral septum, bed nucleus of the stria terminalis and amygdala) and diencephalic (medial preoptic area, hypothalamic paraventricular, suprachiasmatic, ventromedial, arcuate, ventral premammillary, supramammillary and thalamic paraventricular nuclei) areas may give rise to the glutamatergic innervation of the POA, and possibly, of GnRH neurons (51).

**GABA.** The main inhibitory neurotransmitter in the CNS as well as in the hypothalamus is GABA (52). Léranth et al. demonstrated glutamic acid decarboxylase positive axons forming synaptic contacts with GnRH positive neurons (53). All of these synapses belonged to the symmetric type, which is a common feature of GABAergic synapses (54). GnRH neurons express GABAA receptors (55) and results of in vivo microinfusion experiments indicate that GABA inputs within the vicinity of the GnRH cell bodies exert a powerful inhibitory influence on the secretory activity of GnRH neurons in several species (56). Nevertheless, the role of GABAA receptor signaling on the electrophysiological properties of postnatal GnRH neurons is somewhat controversial in the literature. Moenter and colleagues have shown that endogenous synaptic activation of GABAA receptors can be excitatory to mature GnRH neurons due to their unusual intracellular ionic milieu (57). In contrast, other investigators found evidence that GABA exerts inhibitory effects on adult GnRH neurons (58). This controversy has been clarified somewhat by the recent observation that depolarizing effects of GABA upon adult GnRH neurons only occur when all glutamatergic receptor signaling is blocked within the brain slice preparation (57). These findings indicate the importance of the balance between GABA and glutamate neurotransmission in determining the firing activity of GnRH neurons. The major GABAergic innervation of GnRH neurons is supposed to arise from the local GABAergic interneurons (38).

**Serotonin (5-HT).** Serotonin is synthesized by neurons located in the raphe nuclei and it plays an important role in the generation of LH surge (59). Anatomical studies have shown that about 5% of nerve terminals apposed to GnRH neurons are serotonergic (60). Recently, Campbell and co-workers have revealed the median and
dorsal raphe nuclei as the sources of serotonergic innervation to a subset of GnRH neurons that are located in the rostral POA (61). Nevertheless, the pharmacological studies have provided somewhat conflicting results about the physiological role of serotonin in the control of LH secretion (59) and some controversy also exists between the results obtained by dual in situ hybridization and with single-cell microarray experiments. The former method has failed to detect the mRNA for 5-HT1A, 5-HT1C and 5-HT2 receptors (62), while the microarray has revealed the presence of 5-HT1A, 5-HT3A and 5-HT4 receptors in a subset of GnRH neurons (63).

2.2.3.2. Innervation of GnRH neurons by neuropeptide-containing afferents

Corticotropin-releasing hormone (CRH). It is well known that prolonged or repetitive stress inhibits reproductive functions (64). Several neurotransmitters and hypothalamic nuclei that control reproduction, also take part in the regulation of stress responses (64). As the paraventricular nuclei of the hypothalamus are activated in all stressful situations, CRH is one of the candidates that might account for the reduced GnRH secretion during stress (for a review see: (64)). Its effect on GnRH neurons can be exerted directly, given that CRH-immunoreactive axon terminals form synaptic connections with GnRH dendrites (65). Although there are no experimental data concerning the origins of CRH innervation to GnRH neurons, the possible sources can be speculated by the comparison of the distribution of CRH neurons (66) and the results of retrograde tract tracing studies (18, 67). The putative sources of the CRH innervation to GnRH neurons are the followings: paraventricular nucleus of the hypothalamus, central amygdala, bed nucleus of stria terminalis, medial and lateral preoptic area, lateral hypothalamus, central grey, locus coeruleus, parabrachial complex and the area of the A1 noradrenergic cell group.

GnRH. The light microscopic observations that GnRH neurons often form cell groups containing two or three neurons led some investigators to examine the nature of these anatomical associations. They found appositions between GnRH cell bodies in female rodents and rhesus macaques; the number of the appositions did not correlate with the stage of the female ovarian cycle (15). The electron microscopic examination of GnRH-GnRH contacts did not reveal classical types of intercellular communication
or membrane specializations, including gap junctions. Nevertheless, in some cases cytoplasmic bridges were found between GnRH neurons where the cytoplasms of different neurons were apparently confluent (15). Moreover, several investigators have observed GnRH processes forming synaptic contacts with other GnRH neurons (15, 68, 69). A putative functional significance of GnRH-GnRH connections may be in the synchronization of neurohormone release from the network.

**Kisspeptin.** There is a growing number of reports on the role of kisspeptin in the regulation of reproduction. Intracerebroventricular administration of kisspeptin, a recently isolated product of the *kiss-1* gene elicits rapid and profound elevation in serum gonadotropin levels due to the increased activation of the secretory activity of GnRH neurons (70, 71). This fast effect of kisspeptin is apparently exerted directly on GnRH neurons because the potent receptor of this peptide (G protein coupled receptor-54) occurs in the majority of GnRH neurons (70, 71). While the hypothalamus of the female mouse exhibits more kisspeptin positive neurons and a denser plexus of kisspeptin positive fibers than it is observable in males (72), the kisspeptin positive fibers establish direct contacts with GnRH neurons in both genders, as revealed by dual-label immunofluorescence (72). The possible sources of kisspeptin positive fibers to GnRH neurons are the anteroventral periventricular nucleus and the Arc (73).

**Neuropeptide Y (NPY).** Axons immunoreactive for NPY establish synapses with GnRH neurons (74). The physiological significance of NPY in the regulation of the HPG axis and the possible sources of NPYergic innervation to GnRH neurons will be discussed in 2.3.2.

**Opioid peptides, β-endorphin (β-END).** Substantial literature has been built up around the role of β-END in the regulation of the gonadal axis (38). β-END results from the enzymatic cleavage of the precursor pro-opiomelanocortin (POMC) protein. The intracellular processing of POMC also gives rise to other peptide hormones including α-melanocyte-stimulating hormone (αMSH) and adrenocorticotropic hormone (ACTH). Neurons synthesizing β-END have been found in the Arc (75) and in the caudal region of the nucleus of the solitary tract of the brainstem (76, 77), although only the β-END synthesizing neurons of the Arc have been implicated in the regulation of GnRH functions (38, 78). Léránth and colleagues investigated the connection between the opioid system and GnRH neurons at the ultrastructural level. They used ACTH as a
marker peptide for opioid fibers and found that ACTH-immunopositive axons establish synaptic connections mainly with the GnRH-immunoreactive dendrites in female rats (78).

**Substance P (SP).** SP belongs to the peptide family of tachykinins which act as excitatory neurotransmitters in the CNS (79). Neurons containing SP are widely distributed throughout the brain (79). Some physiological experiments show the profound effect of SP on the HPG axis; however, these data are controversial. While intracerebroventricular administration of SP stimulates the release of LH, it has no effect on FSH liberation (80). In contrast, SP decreases the elevation of LH as well as FSH levels when injected subcutaneously to cycling female rats on the day of proestrus (81). The direct effect of SP on GnRH neurons is supported by the presence of synapses between SP terminals and GnRH neurons (82). The synapses belonged to the asymmetric (Gray I type) category (82). As revealed from the use of retrograde tracer injections into the POA, the possible sources of SP afferents to GnRH neurons may include the Arc and the ventrolateral areas of the hypothalamus (82).

2.2.4. Colocalized neurotransmitters in GnRH neurons

*Cholecystokinin (CCK), neurotensin (NT).* Only one paper (31) reported the presence of CCK and NT in GnRH neurons. Among substances localized within GnRH neurons, galanin (38), CCK and NT (31) all exhibit pronounced sexual dimorphism and steroid hormone dependence. In colchicin treated female adult rats quantitative multiple immunolabeling experiments revealed the presence of CCK in more than half (54.5%) and NT in one-third (29.4%) of GnRH neurons. The colocalization of both neuropeptides is unambiguous in the GnRH fibers of the ME without colchicin treatment. In contrast with females, in male rats NT was undetectable in GnRH cells and only few (1.1%) GnRH neurons contained CCK. Interestingly, ovariectomy of adult animals resulted in a complete disappearance of both neurotransmitters from GnRH neurons, but CCK and NT expression could be restored to the normal level when applying an estrogen regimen. According to Ciofi’s anatomical characterization, the “multipeptidergic” GnRH neurons are concentrated immediately around the OVLT and
the optic chiasm but the functional significance of this distribution pattern remains obscure.

**GABA.** In addition to existing proof for the GABAergic innervation of GnRH neurons (53), Tobet and colleagues have demonstrated that GABA is transiently present in a large populations of the migrating GnRH neurons during the embryonic development of rats and mice (83). Data exist to indicate that GABA is an essential regulator for the migration of GnRH neurons. The involvement of GABA<sub>A</sub> receptor in this process is well established. The migration rate of GnRH neurons out of the olfactory pit is inhibited in the embryos by the subcutaneous administration of the GABA<sub>A</sub> receptor agonist muscimol to pregnant mice (84). Moreover, in the same paradigm, the antagonist bicuculline leads to a disorganized distribution of GnRH cells in the forebrain and a concomitant dissociation of GnRH cells from fibers of guidance (84). The understanding of the role of GABA during development is further complicated by the presence of GABA<sub>B</sub> receptors, as well, along the GnRH migratory route (85). The developmental role of GABA appears to include inhibiting the rate of GnRH neuronal migration out of the olfactory pit, controlling the association of GnRH neurons to fibers of guidance, and regulating GnRH fiber extension toward the ME (85).

**Galanin.** The highest galanin concentrations in the brain are observed in the hypothalamus, particularly in the ME (86). Galanin producing neurons that project to the ME, as do classical hypophysiotropic neurons, release their galanin content into the portal circulation. Beyond these neurohormonal properties, galanin peptide and mRNA are expressed by a subpopulation of GnRH neurons in a sexual dimorphic manner (29, 38). In female rats with normal gonadal cycle, galanin mRNA expression is the highest on the afternoon of proestrus (87), suggesting the profound effect of estrogen on the expression of this gene. Indeed, ovariectomy results in a decrease of galanin mRNA expression, while estrogen replacement restores the transcript to intact levels. The positive feedback effect of estrogen stimulates GnRH and galanin mRNA levels sequentially at the time of the LH surge (38), although the physiological significance of this differential regulation needs further clarification.

**Insulin-like growth factor-1 (IGF-1).** The neurotrophic factor IGF-I is thought to play a role in the onset of reproductive ability at puberty and the control of reproductive function throughout adult life (88). It is believed that these effects are mediated at least
in part by the activation of GnRH neurons by IGF-I, but the interactions of IGF-I with
GnRH neurons in vivo are unknown. Recently, Miller and Gore have shown that more
than two-thirds (78%) (89) of GnRH neurons co-express IGF-1 protein and the majority
of them also possess the receptor for this neurotrophic factor (90). In the GnRH nerve
terminals at the level of ME, only a weak if any IGF-1- immunoreactivity can be
observed (89). The in vivo detected increase in IGF-1 gene expression between different
stages of puberty (89) and the decreasing expression of this substance during the
reproductive senescence (89) suggest that the presence of IGF-1 in GnRH neurons may
indeed play an important role in the regulation of gonadal maturity.

**Δ-sleep-inducing-peptide (DSIP).** DSIP is presented in different areas of the brain,
including the hypothalamus and has been shown to induce slow-wave (delta) sleep in
various species. The early physiological experiments suggested that DSIP stimulates LH
release at the level of the hypothalamus. Intracerebroventricular injection of DSIP to
ovariectomized and estrogen- and progesterone-treated rats elicits rapid LH release (91).
Moreover, its in vitro administration to hypothalamic ME fragments results in increased
GnRH release (92). In contrast, DSIP has no effect on either basal or GnRH-induced in
vitro LH release when it is applied to hemi pituitaries of ovarian steroid-primed rats
(91). One laboratory have demonstrated the colocalization of DSIP in GnRH neurons
and in GnRH fibers at the level of the ME in several species including cat (93), rat (32),
rabbit (94) and human (95). Interestingly, the consequences of the reported co-
localization phenomenon have not been studied further or published.
Figure 4. The schematic representation of a GnRH neuron illustrates synaptic inputs and co-expressed neurotransmitters. (5-HT: serotonin; β-END: β-endorphin; CCK: cholecystokinin; CRH: corticotropin-releasing hormone; DSIP: delta sleep-inducing peptide; GABA: gamma-aminobutyric acid, GLU: glutamate, GnRH: gonadotropin-releasing hormone, IGF-1: Insulin-like growth factor-1, NPY: neuropeptide Y; NT: neurotensin; SP: substance P; TH: tyrosine hydroxylase)

2.3. Proposed novel neurotransmitter systems in the regulation of GnRH neurons

2.3.1. Involvement of acetylcholine (ACh) in the regulation of the reproduction

The markers of the central cholinergic systems are choline acetyltransferase (ChAT) which is involved in the synthesis of Ach, and vesicular acetylcholine transporter (VACht) which accumulates Ach into secretory vesicles (96). The visualization of ChAT-positive cell bodies by immunocytochemistry or by in situ hybridization defines five major cholinergic neuronal groups in the CNS. These include: (i) the efferent cranial nerve nuclei and motoneurons of the spinal cord; (ii) the parabrachial complex; (iii) the brainstem reticular complex; (iv) the neostriatal complex; and the (v) cholinergic neurons of the basal forebrain (97, 98). Beyond these principal cholinergic
cell groups ChAT-immunoreactivity has been localized within neurons of the medial habenula, hippocampus, neocortex (97, 98) and of the Arc (99). Retrograde (100), as well as anterograde tract tracing (18, 67) studies have revealed the connection between some of the above-delineated cholinergic areas and the POA, suggesting the contribution of cholinergic neurons to the regulation of GnRH neurons. Indeed, a large body of evidence exists to indicate the involvement of ACh in the regulation of reproductive events, including male and female sexual behavior (101-106) and gonadotropin secretion (107-109). Atropine, the muscarinic acetylcholine receptor antagonist blocks both spontaneous and reflex ovulation (107). In the spontaneously ovulating species rat, atropine implants in the anterior lateral hypothalamus disturb the ovarian cycles and prolong the diestrous phase (108). 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. Simonovic and colleagues examined the ACh-stimulated FSH release from halved adenohypophyses of male rats and found enhanced FSH release only if the adenohypophyses were co-incubated with hypothalamic fragments (109). On the other hand, nanomolar concentrations of ACh could markedly stimulate GnRH release from dissected mediobasal hypothalamic fragments in vitro and this effect could be abolished by the nicotinic ACh receptor antagonist hexamethonium (110). The presence of both muscarinic and nicotinic receptors have been reported on at least a subpopulation of GnRH neurons (63) which suggests a direct effect of ACh on GnRH neurons; however the anatomical proof for the direct cholinergic innervation is missing from the literature.

In the light of evidence for central cholinergic actions on the reproductive axis, and considering the presence of muscarinic and nicotinic ACh receptors in GnRH neurons, in studies under specific aim 1 of this thesis we postulated the direct innervation of GnRH neurons by cholinergic pathways. Male rats were used as experimental animals, and immunocytochemical experiments were performed at the light and electron microscopic levels to investigate the putative cholinergic innervation of GnRH neurons.
2.3.2. The role of the NPY in the regulation of reproduction

Neuropeptide Y has a bimodal effect on the reproductive axis. Depending on the mode of its administration, further, the gender, the age and/or the hormonal status of the experimental animal and the time course of the treatment, NPY can exert both suppressive and excitatory effects on the HPG axis. Chronic increase in NPY tone inhibits gonadotropin release (111), delays sexual maturation (112) and suppresses estrous cyclicity (113). In contrast, the direction of acute NPY effects is markedly influenced by the sexual steroid status of the experimental animals. Likewise, in castrated rats, rabbits, and monkeys, central administration of NPY decreases gonadotropin secretion (114-119), whereas, in intact or gonadectomized and steroid-primed rodents, NPY increases serum gonadotropin levels (111, 114, 117, 119-121).

Neuropeptide Y plays important roles in the regulation of reproduction via acting at multiple levels of the HPG axis (122). The majority of work indicates that NPY mainly acts centrally to modulate gonadotropin secretion (111-116, 118-121, 123-127) and the targets of the central NPY action are directly the GnRH neurons of the POA (74, 128-131). However, evidence also exists to support the direct stimulation of pituitary gonadotropins by NPY (123, 132-134). This multilevel effect of NPY may be due to the different origins of NPYergic fibers to GnRH neurons. In the rodent brain, NPY-immunoreactive perikarya or fibers were found in most brain areas except the cerebellum (135). The highest number of NPY-synthesizing neurons can be found in the hypothalamic Arc, moreover a high number of NPY nerve cells can be detected in the cerebral cortex, dorsal hypothalamic area, parvicellular part of the ventrolateral geniculate nucleus, locus coeruleus and the nucleus of the solitary tract (135). The possible sites of origin of NPY fibers may be diverse and in order to clarify the cause of the multiple effect of NPY on HPG axis it would be useful to reveal the relative contribution of the various sources of NPYergic fibers to GnRH neurons. The determination of possible sources of NPY origin by retrograde tract-tracing studies is largely complicated by the scattered distribution of GnRH neurons in a relatively large area. Thus, as an alternative strategy, in studies under specific aim 2, we have selected topographical markers, which are only co-expressed with NPY in distinct brain regions. Then we used the co-expression of these markers in NPY fibers as an indication of their
sources of origin and for the estimation of the relative contribution of the putative sources of the NPYergic fibers to GnRH neurons. In our experiments, we used male GnRH-GFP mice in which a portion of the mouse GnRH promoter directs the selective expression of GFP to the majority of GnRH neurons (136). We injected four neonatal animals with a solution of monosodium glutamate (MSG) that caused an extensive ablation of the neuronal population of the Arc (137-140). Using dual-labeling light microscopic immunocytochemistry, we compared the frequency of NPY- and agouti-related protein (AGRP)-immunoreactive (see below) axonal contacts on GnRH neurons in neonatally MSG-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. Moreover, we carried out a series of triple-label fluorescent studies in intact male GnRH-GFP transgenic mice using brain region-specific topographical markers. This way, we tried to identify the relative importance of NPY neurons located in the Arc and those corresponding to catecholaminergic nuclei of the brainstem, two possible sources of NPY afferents to GnRH neurons. AGRP was used as a marker of NPY fibers originating from the Arc. We used the noradrenaline-synthesizing enzyme DBH, as a topographic marker of NPY fibers with brainstem origin. The ratios of NPY-immunoreactive neuronal contacts on the surface of GnRH-GFP neurons that contained DBH or AGRP were determined.

Neuropeptide Y may be one of the essential messenger molecules that serve as a communication bridge between the neural processes that regulate reproduction and those that maintain energy homeostasis (141). Chronic central increase in NPY tone has been implicated in hypogonadism that accompanies malnutrition (111, 113). Moreover, the significant inhibition of the gonadotrophic axis can be observed in genetically obese mice (142). The Arc is involved in the integration of the satiety signals of the periphery (143) and contains a high number of NPY neurons (135), that co-express AGRP (137, 144). AGRP also takes part in the regulation of energy metabolism as an orexigenic signal (143) and beyond this function, its central impact on the HPG axis is suggested as well (145).

Since NPY afferents with Arc origin contain AGRP, we carried out dual-label immuno-electron microscopic studies to reveal the synaptic communication between
NPY fibers of Arc origin (using AGRP as a neurochemical marker for NPY neurons of the Arc) and GnRH-immunoreactive neurons.

2.3.3. Glutamate as a proposed autocrine/paracrine regulator synthesized by GnRH neurons

The recent discovery of the three vesicular glutamate transporters which selectively accumulate glutamate into secretory vesicles has enabled the histochemical identification of glutamatergic fibers in the central and in the peripheral nervous systems. The abundance of glutamatergic neurons expressing Vglut2 mRNA in the hypothalamus (146, 147) and the high density of Vglut2-immunoreactive axon terminals (146) and ionotropic glutamate receptors (148, 149) in the external zone of the ME raised the possibility that peptidergic neuroendocrine cells regulating anterior pituitary functions may secrete glutamate as an autocrine/paracrine modulator of their neurohormone output. Corroborating this notion, beside the dense core vesicle (DV)-containing (peptidergic and catecholaminergic) and electron lucent vesicle containing (GABAergic, glutamatergic and cholinergic) nerve terminals, “mixed” phenotyped nerve endings could also be detected by electron microscopy at the level of the ME (26). Moreover, the POA of postnatal rodents is also populated by high numbers of glutamatergic neurons containing Vglut2 mRNA (146, 147).

In studies under specific aim 4, we examined the possibility whether mature GnRH neurons in the POA possess glutamatergic characteristics. We examined the expression of Vglut2 mRNA in GnRH mRNA-expressing neurons with double-label in situ hybridization histochemistry. In addition, we addressed the presence of Vglut2-immunoreactivity in the axon terminals of GnRH neurons with the aid of confocal laser scanning microscopy. Finally, we determined the subcellular localization of Vglut2 protein in the hypophysiotropic axon terminals of ME with pre-embedding immunoelectron microscopy.
3. Specific aims

Studies in this thesis were aimed at the identification of novel neurotransmitter systems in the neuronal regulation of the GnRH system.

1. We addressed the direct innervation of GnRH neurons by cholinergic afferent pathways in male rats at light and electron microscopic levels.

2. We addressed the sources of origin of NPY-containing afferents to GnRH neurons in male GnRH-GFP transgenic mice.
   a. Using neonatally monosodium glutamate treated (with lesioned Arc) and intact mice we estimated the ratio of NPY afferents originating in the Arc.
   b. Using intact mice we carried out a series of triple immuno-fluorescent studies in GnRH-GFP transgenic mice by brain region-specific topographical markers to identify the relative importance of NPY neurons located in the Arc and those corresponding to catecholaminergic nuclei of the brainstem, two putative sources of NPY afferents to GnRH neurons. AGRP was used as a marker of NPY fibers originating from the Arc. We used the noradrenaline-synthesizing enzyme DBH, as a topographic marker of NPY fibers of brainstem origin.

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 ME with immuno-electron microscopy.
4. Materials and methods

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.

4.1. Direct innervation of GnRH neurons by cholinergic afferent pathways in male rats

4.1.1. Double-labeling immunocytochemical detection of cholinergic fibers and GnRH neurons at the light microscopic level

*Animals.* Adult male Wistar rats [N=3; 260–280 g body weight (BW)] were purchased from the local breeding colony of the Medical Gene Technology Unit of the Institute of Experimental Medicine. They 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 (Sniff Spezialdiaeten GmbH, Soest, Germany) and tap water.

*Tissue preparation.* The animals were deeply anesthetized with sodium pentobarbital (45 mg/kg BW, *i.p.*) and perfused via the ascending aorta first, with 20 ml of 0.1 M phosphate buffered saline (pH 7.4) (PBS) and then with 100 ml fixative solution containing 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS.

Brains were rapidly removed and immersed into 30% sucrose in PBS overnight and snap frozen on powdered dry ice. Then 30-μm thick, free-floating coronal sections were prepared from the POA region with a freezing microtome (Leica Microsystems, Wetzlar, Germany).

The free-floating sections were collected in anti-freeze solution (30 V/V % ethylene glycol, 25 V/V % glycerol in 0.05 M phosphate buffer) and stored at minus 20 °C until use.

*Immunocytochemistry.* The sections were rinsed in PBS, incubated in 10% thioglycolic acid for 30 min to suppress tissue argyophilia (150) and then pretreated
with a solution of 0.5% H$_2$O$_2$ and 0.2% Triton X-100 in PBS for 30 min. Non-specific binding of the antibodies was reduced using a 2% normal horse serum blocker in PBS for 10 min. All primary and secondary antibodies were diluted with 2% normal horse serum in PBS or Tris buffered saline (TBS; 0.1 M Tris-HCl with 0.9% NaCl; pH 7.8), which also contained 0.1% sodium azide as a preservative. To detect cholinergic neuronal elements, the sections were incubated in polyclonal anti-ChAT antiserum raised in a goat (AB144P; 1:1000; Chemicon, Temecula, CA) for 72h at 4 °C. The primary antibodies were reacted with donkey, biotin-SP-anti-goat IgG (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h. Alternatively, in a second set of sections the cholinergic system was detected using a rabbit antiserum against VACHT (V5387; 1:10,000; Sigma), followed by biotin-SP-anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories). The biotinylated secondary antibodies were reacted with the ABC Elite working solution (Vector Laboratories, Burlingame, CA; 1:1000 dilutions of solutions A and B in 50 mM TBS; pH 7.6) for 1 h. Then the sections were rinsed in TBS and the color reaction visualized with a developer containing 0.05% diaminobenzidine (DAB), 0.15% ammonium nickel (II) sulfate and 0.005% H$_2$O$_2$ in TBS. Finally, the reaction product was silver intensified according to Liposits et al. (150) to obtain a final black reaction product. Briefly, the sections were rinsed for several times in 2% sodium acetate and then placed into a silver developer. The developer consisted of equivalent amount of 5% sodium carbonate and silver development solution (0.15% formalin-solution, 0.2% ammonium nitrate, 0.2% silver nitrate, 1% tungstosilicic acid, dissolved in distilled water). The progress of the intensification reaction was monitored under the light microscope and terminated in 0.5% acetic acid solution when the color of the originally purple nickel-DAB precipitate turned black. After several rinses in 2% sodium acetate, the sections were dipped into cold 0.05% of gold-chloride (2x5 min) to stabilize the black silver- ammonium nickel (II) sulfate -DAB precipitate and then into 3% sodium thiosulfate (2x5 min) solutions to remove the residual metallic ions.

After the detection of cholinergic neuronal elements, the sections were transferred into rabbit LR-1 anti-GnRH antibodies (1:10,000 a gift from Dr. R.A. Benoit, Montreal, Canada) for 2 days at 4 °C. Immunoreactivity for GnRH was visualized with the ABC method, with the application of biotin-SP-anti-rabbit secondary antibody (1:1000;
Jackson ImmunoResearch Laboratories, West Grove, PA) and a developer consisting of 0.025% DAB and 0.0036% H$_2$O$_2$ in TBS which yielded a brown reaction product.

Beyond the use of well-characterized primary antibodies in this study against the cholinergic system (99, 151), the specificity of labeling was further indicated by the absence of immunocytochemical signal if the primary or the secondary antibodies were omitted from the staining procedure.

The dual-immunostained sections were mounted on microscope slides, dehydrated in ethanol, coverslipped with DePeX mounting medium (Fluka Chemie, Buchs, Switzerland) and studied with an Axiophot microscope (Zeiss, Göttingen, Germany) equipped with an RT Spot digital camera (Diagnostic Instruments, Sterling Heights, MI).

4.1.2. Double-labeling immunocytochemical detection of cholinergic fibers and GnRH neurons at the electron microscopic level

**Animals.** Adult male Wistar rats (N=3; 260–280 g BW) were purchased from the local breeding colony of the Medical Genetec Technology Unit of the Institute of Experimental Medicine. 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 (Sniff Spezialdiaeten GmbH, Soest, Germany) and tap water.

**Tissue preparation.** The deeply anesthetized animals were perfused via the ascending aorta with 20 ml of 0.1 M PBS followed by 50 ml mixture of 2% freshly depolymerized paraformaldehyde (Sigma Chemical Co., St. Louis, MO) and 4% acrolein (Sigma Chemical Co., St. Louis, MO) solution in 0.1 M PBS. The brains were postfixed in 4% paraformaldehyde for 24 h. Preoptic sections were prepared from the tissue blocks at 50 µm with a Vibratome (Technical Products International, St. Louis, Mo., USA).

**Immunocytochemistry.** Vibratome sections were pretreated with 0.5% sodium borohydride in PBS for 30 min to eliminate residual aldehydes. Then they were infiltrated with sucrose for cryoprotection (15% for 1 h and then, 30% overnight) and permeabilized by three repeated freeze-thaw cycles on liquid nitrogen. The immunocytochemical dual-labeling procedures were carried out as described for light
microscopy. The dual-labeled sections were treated with 1% osmium tetroxide in 0.1 M PBS for 30 min and dehydrated in an ascending series of ethanol. A 30-min contrasting step using 1% uranyl acetate in 70% ethanol was inserted in this procedure and the fully dehydrated sections were immersed in propylene oxide. Finally, the sections were infiltrated with TAAB 812 Embedding resin (TAAB Laboratory Equipments Ltd., Aldermaston, Berks, UK) and flat embedded on liquid release agent (Electron Microscopy Sciences) coated slides. The coverslipped sections were polymerized at 60 °C for 24 h. Ultra thin (50-60 nm) sections were cut with Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany), collected onto Formvar-coated single-slot grids and contrasted with a stabilized solution of lead citrate. Digital images were captured with a cooled CCD camera.

The electron microscopic digital images were processed with the Adobe Photoshop 7.0 software.

4.2. Determining of the origin of NPY-containing afferents to GnRH neurons in male GnRH-GFP transgenic mice

4.2.1. Experimental animals

To examine the origin of NPY afferents to GnRH neurons, studies 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 (136). 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 day-12 h night 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 MSG treatment of four neonatal mice. The neonatal animals were injected s.c. with increasing volumes of 8% MSG solution (dissolved in water), using a treatment paradigm adapted from Légrádi et al. (139): 4 mg/g body weight MSG solution on
postnatal days 1 and 3; followed by 8 mg/g body weight 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.

Tissue preparation. The animals were deeply anesthetized with sodium pentobarbital (45 mg/kg BW, i.p.) and perfused via the ascending aorta first, with 20 ml of 0.1 M PBS and then with 100 ml fixative solution containing 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS.

Brains were rapidly removed, cryoprotected in 30% sucrose in PBS overnight and snap frozen on powdered dry ice. Then 30-μm thick, free-floating coronal sections were prepared from the POA and Arc regions with a freezing microtome (Leica Microsystems, Wetzlar, Germany).

The free-floating sections were collected in anti-freeze solution (30 V/V % ethylene glycol, 25 V/V % glycerol in 0.05 M phosphate buffer) and stored at minus 20 °C until use.

4.2.2. Single-labeling immunocytochemical detection of NPY- and AGRP-containing fibers in the POA of neonatally MSG-treated mice vs. untreated controls

Coronal sections through the Arc were obtained from neonatally MSG-treated mice as well as untreated controls and stained with cresyl violet to verify the chemical lesion in the former group. Sections containing the POA were used for comparative immunocytochemical studies of NPY- and AGRP-immunoreactivities in these two groups, as outlined below. Sections were rinsed in PBS and pretreated with 10% thioglycolic acid (Sigma) for 30 min to suppress tissue argyophilia (150) and with 0.5% H2O2 in PBS for 15 min to eliminate endogenous peroxidase activity. Nonspecific antibody binding sites were blocked with 2% normal horse serum then tissues permeabilized in 0.4% Triton X-100 (Sigma) in PBS for 20 min. Every other preoptic section was transferred into a 1:100,000 dilution of a polyclonal sheep antiserum to NPY (FJL no. 14/3A; diluted with Triton X-100-free blocking reagent), which was generously provided by Dr. István Merchenthaler (Wyeth Research, Collegeville, PA),
and applied to the sections for 48 h at 4 °C. The remaining sections were incubated in a 1:8000 dilution of a polyclonal rabbit antiserum to human AGRP (H-003-053; Phoenix Pharmaceuticals, Inc., Mountain View, CA). Immunoreactivities were detected after tissue incubations in species-specific biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:1000), then in ABC-Elite reagent (Vector Laboratories, Burlingame, CA; 1:1000 dilutions of solutions A and B in TBS), for 1 h each. The peroxidase developer contained 10 mg DAB, 30 mg ammonium nickel (II) sulfate, and 0.002% H₂O₂ in 24 ml TBS. Then silver intensification of the peroxidase reaction product was carried out as described in section 4.1.1.

4.2.3. Double-labeling immunocytochemical detection of NPY- and AGRP-containing fibers in contact with GnRH neurons of neonatally MSG-treated mice vs. untreated controls

The majority of single-labeled sections immunostained for AGRP or NPY were processed further for the immunocytochemical identification of GnRH neurons, using sequential incubations in rabbit polyclonal antibodies to GnRH (LR-1; 1:25,000; gift from Dr. R. Benoit, Montreal, Canada) for 48 h, biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories; 1:1000) for 1 h, and then ABC Elite working solution for 1 h. The developer solution contained 10 mg DAB and 0.001% H₂O₂ in 50 ml TBS. The immunostained sections were mounted on microscope slides, dehydrated with ethanol, coverslipped with DPX mounting medium (Fluka Chemie, Buchs, Switzerland), and studied with an Axiophot microscope (Zeiss, Göttingen, Germany) equipped with an RT Spot digital camera (Diagnostic Instrument, Sterling Heights, MI).

Quantitative microscopic analysis of dual-labeled sections. Sections dual-immunostained for NPY and GnRH from MSG-treated and untreated control animals, as well as sections dual-labeled for AGRP and GnRH from control animals were mounted on microscope slides in a random sequence. The sections were individually labeled with a code, and in each, the number of axonal contacts along the outlines of GnRH neurons was determined. The analysis was carried out using a x100 objective lens with immersion oil by an investigator who was blind to the experimental procedures and the sequence of section mounting. Studies of sections in random
sequence ensured the homogenous analysis of groups to compare. A case was considered as a “contact” based only on highly stringent criteria that were applied consistently. The axon and GnRH neuron had to occur in the same focal plane without the presence of an intervening gap, and instances of partial overlap were excluded from the counting. Two different approaches were used in parallel to estimate the ratio of NPY afferents of Arc origin to GnRH neurons. First, the average number of AGRP-immunoreactive neuronal appositions to GnRH cells was calculated in intact animals (after the analysis of 561 GnRH neurons at high power) and compared with the mean of NPY-immunoreactive contacts on individual GnRH cells (with 546 GnRH neurons analyzed). Second, the average number of NPY-containing juxtapositions to single GnRH cells was calculated for neonatally MSG-treated mice (analysis included 408 GnRH neurons) and related to the number determined for the untreated controls. The summarized data from the light microscopic double-labelings were analyzed with one-way ANOVA (p<0.05 was considered significant) and the results of the analysis were expressed as mean ± SEM.

4.2.4. Immunofluorescent-labeling of AGRP/NPY and DBH/NPY contacts with GnRH-GFP neurons in intact mice

To determine the relative contributions of the Arc and brainstem to the innervation of GnRH neurons, a triple-fluorescence strategy was used, enabling the detection of NPY in axonal contacts to GnRH neurons simultaneously with the immunofluorescent visualization of a site-of-origin-specific topographic marker. The presence of AGRP in NPY axons showed their origin in the Arc, whereas the identification of the adrenergic/noradrenergic biosynthetic enzyme DBH in NPY fibers served as a selective marker for NPY axons arising from catecholamine cell groups of the brainstem. Although DBH is present exclusively in adrenergic/noradrenergic cells of the brainstem, the fine distinction among various catecholamine cell groups that express NPY differentially (152, 153) was not possible via the use of this marker. First, a cocktail of two primary antibodies (sheep anti-NPY, 1:12,000 and rabbit anti-AGRP, 1:4000; sheep anti-NPY, 1:12,000, and rabbit anti-DBH, 1:8000) (154) was applied to the sections for 48 h at 4 °C. This was followed by sequential incubations in a mixture of secondary
antibodies, donkey biotin-conjugated anti-rabbit IgG (1:1,000; Jackson ImmunoResearch Laboratories), and antisheep-Cy3 (1:250; Jackson ImmunoResearch Laboratories), and then in the ABC-Elite working solution (Vector Laboratories, Burlingame, CA; 1:1000 dilutions of solutions A and B in 50 mM TBS; pH 7.6) for 1 h each. The detection of AGRP and DBH fibers was completed by treating sections with biotin tyramide working solution (described below) for 30 min and finally with avidin-conjugated AMCA fluorochrome (1:200; Vector Laboratories).

To prepare the biotin tyramide solution, a stock solution was first synthesized according to instruction by Adams (155). This stock was stored in frozen aliquots and diluted at 1:1000 in TBS/0.005% H2O2 immediately before use.

The GFP expression in GnRH neurons unequivocally revealed the cell bodies and primary dendrites of the GnRH nerve cells, thus we did not have to apply a third immunolabeling against the GnRH decapeptide.

Following steps of the dual-immunofluorescent labeling procedure, sections were mounted on microscope slides and coverslipped with Vectashield mounting medium (Vector Laboratories). Fibers immunoreactive for NPY appeared in red, AGRP-, or DBH-containing axons appeared in blue, and GnRH-GFP neurons appeared in green using the following epifluorescent filter sets:

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Excitation filter (nm)</th>
<th>Band pass filter (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3</td>
<td>540–590</td>
<td>595</td>
<td>600–660</td>
</tr>
<tr>
<td>AMCA</td>
<td>320–400</td>
<td>400</td>
<td>430–490</td>
</tr>
<tr>
<td>GFP</td>
<td>460–500</td>
<td>505</td>
<td>510–560</td>
</tr>
</tbody>
</table>

Quantitative microscopic analysis of the fluorescent specimen. To determine the percent ratios of dual-labeled NPY fibers in each set of experiment, individual NPY juxtapositions were analyzed at high power and categorized as being either positive or negative for the topographic markers, AGRP or DBH. Each study included the analysis of more than 200 NPY-containing neuronal contacts with GnRH neuronal cell bodies and proximal dendrites and percent data were expressed as mean from four animals ±SEM. The fluorescent specimen was analyzed using an x100 objective lens with
immersion oil. First, NPY contacts apposed to GnRH profiles were identified using the same stringent criteria as for bright-field microscopy. Then in each contact identified, we determined whether the NPY axon contained AGRP (or DBH) by switching between the fluorescent filter sets.

4.3. Electron microscopic analysis of AGRP afferents to GnRH neurons in intact male, GnRH-GFP mice

*Animals.* For electron microscopic experiments we used male mice from the same GnRH-GFP mouse strain (N=3) as for the light microscopic experiments (section 4.2.1.).

*Tissue preparation.* The deeply anesthetized animals were perfused via the ascending aorta with 20 ml of PBS followed by 50 ml mixture of 2% freshly depolymerized paraformaldehyde (Sigma Chemical Co., St. Louis, MO) and 4% acrolein (Sigma) solution in PBS. The brains were postfixed in 4% paraformaldehyde for 24 h. Preoptic sections were prepared from the tissue blocks at 50 µm with a Vibratome (Technical Products International, St. Louis, Mo., USA).

*Immunocytochemistry.* A combination of a preembedding immuno-peroxidase histochemistry and immuno-gold reaction was used for the ultrastructural analysis of neuronal contacts between AGRP-immunoreactive axons and GnRH cells of the POA. Sections were treated with 0.5% sodium borohydride in PBS for 30 min to eliminate residual aldehydes, infiltrated with sucrose for cryoprotection (15% for 1 h and then, 30% overnight), and permeabilized by three repeated freeze-thaw cycles on liquid nitrogen. The immunocytochemical detection of AGRP used sequential incubations in primary antibodies to AGRP (Phoenix; 1:2000) for 4 days, biotinylated anti-rabbit antibodies (Jackson ImmunoResearch Laboratories; 1: 1000) for 2 h, and ABC working solution (Vector Laboratories, Burlingame, CA; 1:1000 dilutions of solutions A and B in 50 mM Tris buffer; pH 7.6) for 1 h. Triton X-100 was omitted from all solutions and immunoreactivity to AGRP was visualized with a DAB in the peroxidase developer. The detection of AGRP axons was followed by a 4-day incubation (4 °C) of sections in rabbit polyclonal antibodies to GnRH (LR-1; 1:25,000), a 30-min blocking step using 0.1% cold-water fish gelatin (Electron Microscopy Sciences, Fort Washington, PA) and 1% BSA (fraction V; Sigma) in PBS, and then, a 1-h incubation in goat anti-rabbit IgG
conjugated with ultra small colloidal gold (Electron Microscopy Sciences; diluted at 1:100 with the blocking reagent). The sections were rinsed briefly with the same blocking reagent then with PBS and treated for 10 min with 1.25% glutaraldehyde in PBS, and rinsed in 0.2 M sodium citrate (pH 7.5). The silver intensification of gold particles was carried out according to instructions provided with the IntenSE kit (Amersham, Arlington Heights, IL). The dual-labeled sections were osmicated (1% osmium tetroxide in 0.1 M PB; 30 min) and dehydrated in serial dilutions of ethanol. A 30-min contrasting step using 1% uranyl acetate in 70% ethanol was inserted in this procedure and the fully dehydrated sections were finally infiltrated with propylene oxide and flat embedded in Durcupan ACM epoxy resin (Fluka, Ronkonkoma, NY) on liquid release agent (Electron Microscopy Sciences) coated microscope slides at 56 °C. Ultrathin section (50–60 nm) were cut from the resin blocks with an Ultracut UCT Ultramicrotome (Leica Microsystems AG, Wetzlar, Germany), collected onto Formvar-coated single-slot grids, and examined with a Jeol-100C transmission electron microscope (JEOL, Tokyo, Japan).

4.4. Investigation of the putative glutamatergic phenotype of GnRH neurons

4.4.1. Experimental animals

Adult male Wistar rats (N=11; 260–280 g BW) were purchased 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 (Sniff Spezialdiaeten GmbH, Soest, Germany) and tap water.

4.4.2. Detection of GnRH and Vglut2 mRNAs by dual-label in situ hybridization histochemistry (ISHH)

*Tissue preparation for in situ hybridization.* 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 CM 3050 S Cryostat (Leica, Deerfield, IL) and collected serially on gelatin-coated microscope slides.

**ISHH.** For dual-label ISHH detection of the GnRH and Vglut2 mRNAs, every sixth section was processed for the following prehybridization steps. Sections were fixed for 30 min with 4% solution of freshly depolymerized paraformaldehyde in PBS. After several step of washing in 2x standard saline citrate solution (SSC; 1x SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) the sections were placed into 0.25% acetic anhydride/0.9% NaCl/0.1 M triethanolamine (pH 8.0; Sigma) solution for 10 min. Following a brief rinse in 2x SSC, sections were dehydrated in a serial dilution of ethanol (2 min each); delipidated in chloroform (5 min) then partially rehydrated in 100% followed by 96% ethanol (2 min each). The slides were finally air-dried and processed further for the hybridization procedure.

For the dual-label ISHH, we used a digoxigenin-labeled GnRH probe (transcribed from a 330-bp long cDNA template; a gift from Dr. J. P. Adelman, Vollume Institute, Oregon Health Science University, Portland, OR) and one of two non-overlapping 35S-UTP-labeled Vglut2 probes (Vglut2-879 or Vglut2-734).

The dried slides were placed into plastic boxes in which the air was humified using caps of 50 ml centrifuge tubes filled with distilled water. For hybridization, we dissolved both the 35S-labeled and the digoxigenin-labeled probes in a SSC-buffered hybridization solution, which consisted of 50% formamide (Fisher BP228-100), 10% dextran sulfate (Sigma D8906), 1x Denhardt’s solution, 0.5 mg/ml yeast tRNA (Roche Diagnostics, St. Louis, MO), 500μg/ml heparin sodium salt (1760 USP units, Sigma H3391) and 1 M dithiothreitol (Sigma D9779). The hybridization solution was pipetted onto each slides and covered with glass coverslips. According to a recently introduced novel approach to enhance hybridization sensitivity (156), we applied unusually high radioisotopic probe (80,000 cpm/μl) and dithiothreitol (1 M) concentrations in the hybridization solution and extended the time of hybridization from 16 to 40 h.

After the hybridization process, the slides were subjected to post hybridization treatments. The coverslips were floated off the slides in 1x SSC solution and the excess of hybridization solution was rinsed off in several changes of 1x SSC solution. Then the slides were loaded into metal racks and the following incubation steps were carried out under agitation.Slides were rinsed in 1x SSC solution twice for 20 min, which was
followed by 1 hour incubation in a 50 μg/ml RNase A solution (Roche Diagnostics, St. Louis, MO) prepared with RNase buffer (500 mM NaCl/10 mM Tris-HCl/1 mM EDTA, pH 7.8, 37 °C). The RNase A-treated sections were rinsed in 1x SSC several times, followed by an incubation in 0.1x SSC buffer at 60 °C for 1 hour. Finally, the slides were rinsed in 2x SSC buffer and placed into PBS before the detection of the digoxigenin-labeled GnRH probe.

The digoxigenin-labeled cRNA probe to GnRH mRNA was detected by immunocytochemistry as follows. The slides were pretreated with a solution that contained 0.2% Triton-X 100 and 0.5% H₂O₂ in PBS in order to permeabilize the tissue and to eliminate the endogenous peroxidase. After extensive rinsing in PBS, the slides were dipped into a blocker solution (Roche Diagnostics, St. Louis, MO) for 30 min. The sections were incubated overnight with anti-digoxigenin antibodies conjugated to horseradish peroxidase (1:200; anti-digoxigenin-POD, Roche, St. Louis, MO). After several rinses in PBS, the sections were incubated overnight with anti-digoxigenin antibodies conjugated to horseradish peroxidase (1:200; anti-digoxigenin-POD, Roche, St. Louis, MO). After several rinses in PBS, the biotin tyramide signal amplification procedure was applied to the sections to amplify the non-isotopic hybridization signal for GnRH mRNA. This included a 1-hour incubation in a 1:1000 dilution of biotin tyramide stock solution (155) in TBS/0.005% H₂O₂, followed by a 1 hour incubation in ABC Elite working solution (Vector Laboratories, Burlingame, CA; 1:1000 dilutions of solutions A and B in 50 mM Tris buffer; pH 7.6). The final peroxidase reaction was visualized with DAB chromogen in the peroxidase developer (0.025% DAB and 0.0036% H₂O₂ in TBS). Subsequently, the ³⁵S-labeled cRNA probes to Vglut2 mRNA were detected on autoradiographic emulsion (NTB-3; Eastman Kodak Co., Rochester, NY) after a 2-wk exposure. Finally, the sections were coverslipped and examined with an Axioptot microscope (Zeiss, Göttingen, Germany) equipped with an RT Spot digital camera (Diagnostic Instruments, Sterling Heights, MI), under bright field and dark field illumination.

Hybridization controls. The ³⁵S-labeled Vglut2-879 probe corresponded to bases 522-1400 of Vglut2 mRNA (GenBank accession no. NM_053427). As a positive control for hybridization specificity, a second series of dual-label ISHH experiment was carried out with a distinct Vglut2-734 probe that was complementary to bases 1704–2437 (The template was a gift from Dr. J. P. Herman, University of Cincinnati Medical Center, Cincinnati, OH). Negative control experiments were performed with the combined use of the GnRH probe and the sense strand Vglut2 RNA transcripts.
4.4.3. Dual-immunofluorescent labeling for confocal laser scanning microscopic analysis of Vglut2- and GnRH-immunoreactive axons in the OVLT and ME

**Tissue preparation.** The animals (N=4) were deeply anesthetized with sodium pentobarbital (35 mg/kg BW, *i.p.*) and perfused via the ascending aorta, with 150 ml mixture of 2% freshly depolymerized paraformaldehyde (Sigma Chemical Co., St. Louis, MO) and 4% acrolein (Sigma) in PBS.

**Section preparation and storage.** Brains were rapidly removed, immersed into 30% sucrose in PBS overnight and snap frozen on powdered dry ice. Then 20-μm thick, free-floating coronal sections were prepared from the regions of interest (POA, ME and Arc) with a Leica CM 3050 S cryostat (Meyer Instruments, Houston, TX).

The free-floating sections were collected in anti-freeze solution (30 V/V % ethylene glycol, 25 V/V % glycerol in 0.05 M phosphate buffer) and stored at minus 20 °C until use.

**Double-labeling immunocytochemistry.** The sections were rinsed in TBS, treated with 0.5% sodium borohydride (Sigma-Aldrich Corp.; 30 min) and 0.5% H2O2 (15 min), and finally treated with a mixture of 0.2% Triton X-100 (Sigma-Aldrich Corp.) and 2% normal horse serum in TBS (30 min). After these pretreatments, the sections were incubated (72 h; 4 °C) in anti-Vglut2 primary antibodies raised in a guinea pig (AB 5907; Chemicon International, Temecula, CA; 1:1000). Steps to enhance the fluorescent signal included sequential incubations with biotinylated anti-guinea pig IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:1000; 2 h), ABC Elite solution (Vector Laboratories, Burlingame, CA; 1:1000 dilutions of solutions A and B in 50 mM TBS; pH 7.6; 1 h), biotin tyramide working solution prepared and used as described previously (section 4.2.4.), and streptavidin-conjugated FITC fluorochrome (Jackson ImmunoResearch Laboratories; 1:200; 12 h). Immunoreactivity for GnRH was detected with rabbit LR-1 primary antibodies (1:30,000; gift from Dr. R. Benoit, Montreal, Canada). This antiserum was applied to the sections for 48 h (4 °C) and then reacted with Cy3- conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories; 1:200; 12 h). The colocalization of Vglut2 and GnRH immunoreactivities was examined with a
Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK) using the following parameters:

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Laser excitation line (nm)</th>
<th>Dicroic mirror/ Emission filter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC</td>
<td>488</td>
<td>560 /500–530</td>
</tr>
<tr>
<td>Cy3</td>
<td>543</td>
<td>565–625 /565–625</td>
</tr>
</tbody>
</table>

To distinguish axonal colocalizations from cases of overlap, individual optical slices of minimal thickness (<0.7 µm) were obtained using a x60 objective lens (with immersion oil) and an optimized pinhole size.

Control experiments. Parallel control experiments used Vglut2 antibodies preabsorbed with 10 µM immunization antigen (AG209, Chemicon International). As a positive control for the specificity of Vglut2 immunostaining, dual-immunofluorescent experiments were carried out with the combined use of guinea pig anti-Vglut2 primary antibodies (AB 5907, Chemicon) and rabbit anti-Vglut2 primary antibodies (1:5000; AB 135103; SYnaptic SYstems, Göttingen, Germany). The primary antibodies were detected with anti-guinea pig-FITC (Jackson ImmunoResearch Laboratories; 1:200) and anti-rabbit-Cy3 (Jackson ImmunoResearch Laboratories; 1:200) conjugates, respectively, and were analyzed by confocal microscopy.

4.4.4. Pre-embedding immuno-gold-labeling of Vglut2 in the hypophysiotropic nerve terminals of the ME

Tissue preparation. For electron microscopic localization of Vglut2 in hypophysiotropic axon terminals in the ME immersion fixation was used. Three adult male rats were anesthetized with pentobarbital (35 mg/kg BW, i.p.) and killed by decapitation. The hypothalami were removed rapidly 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 0.1 M PBS (pH 7.4). The tissues were fixed further for 3 days in the same fixative, with the omission of glutaraldehyde. The basal hypothalami were sliced at 50 µm with a Vibratome (Technical Products International, St. Louis, MO, USA).
Immunogold-labeling. Pre-embedding immunoelectron microscopy was used for the ultrastructural analysis of Vglut2-immunoreactivity in the ME. The sections were first treated with 0.5% sodium borohydride in 0.1 M PBS for 20 min to eliminate residual aldehydes and then infiltrated with increasing concentrations of sucrose in 0.1 M PBS for cryoprotection (7.5%, 15% and 30%; 20 min for each). Subsequently the tissues were permeabilized by three repeated freeze-thaw cycles on liquid nitrogen. For the immunocytochemical detection of Vglut2, the sections were incubated for four days in a 1:1000 dilution of the rabbit primary antibodies against Vglut2 (AB 135103; SYnaptic SYstems). These antibodies performed better than the guinea-pig anti-Vglut2 antibodies following the use of glutaraldehyde in the tissue fixative. The primary antibody incubation was followed by a 30-min blocking step using 0.1% cold-water fish gelatin (Electron Microscopy Sciences, Fort Washington, PA, USA) and 1% BSA (fraction V; Sigma) in 0.1 M PBS, and then, an overnight incubation in goat anti-rabbit IgG conjugated with ultra small gold particles (Electron Microscopy Sciences; diluted at 1:100 with the blocking reagent). The sections were rinsed briefly with the same blocking reagent, followed by PBS. Then they were treated with 2% glutaraldehyde in PBS for 10 min and rinsed in distilled water (3x5 min). The silver-intensification of gold particles was carried out according to the instructions provided with the Aurion R-Gent SE-LM silver enhancing kit (AURION ImmunoGold Reagents & Accessories, Wageningen, The Netherlands). Hypothalamic sections were osmicated (0.5% osmium tetroxide in 0.1 M PBS, 20 min) and dehydrated in serial dilutions of ethanol. A 30-min contrasting step using 1% uranyl acetate in 70% ethanol was inserted in this procedure and the fully dehydrated sections were finally infiltrated with propylene oxide and flat embedded at 60 °C in TAAB 812 Embedding resin (TAAB Laboratory Equipments Ltd., Aldermaston, Berks, UK) on microscope slides that were pre-coated with liquid release agent (Electron Microscopy Sciences). Ultra thin (50–60 nm) sections were cut from the resin blocks with a Leica Ultracut UCT Ultramicrotome (Leica Microsystems AG, Wetzlar, Germany), collected onto Formvar-coated single-slot grids and examined with a Hitachi 7100 (Yokohama, Japan) transmission electron microscope.
5. Results

5.1. Identification of cholinergic afferents to GnRH neurons of the rat

5.1.1. Light microscopic evidence for cholinergic neuronal contacts on GnRH neurons

The cell bodies of GnRH neurons, immunostained with brown DAB, were scattered around the OVLT and in the MPOA (Figs. 5A, B), and their regional distribution followed the characteristic patterns described earlier by others (7). The same regions also contained a fine network of varicose cholinergic fibers that were immunoreactive for ChAT (Fig. 5A) and VACHT (Fig. 5B) and contained gray-to-black silver-intensified ammonium nickel (II) sulfate-DAB chromogen. Immunostaining with the VACHT antiserum resulted in a somewhat higher fiber density compared to that obtained with the ChAT antiserum, corroborating previous observations with the same antisera by others (99). High-power analysis of dual-labeled sections revealed that the cholinergic axons were apposed to GnRH-immunoreactive neuronal elements. Cholinergic axons contacted the cell bodies (Fig. 5C) as well as the dendrites (Figs. 5D-F) of GnRH neurons.
Figure 5. Cholinergic afferents to gonadotropin-releasing hormone (GnRH) producing neurons in the male rat. A, B: The cell bodies of GnRH neurons (arrowheads), immunostained with brown diaminobenzidine chromogen, are scattered within the organum vasculosum laminae terminalis (OVLT) and the medial preoptic area (MPOA). GnRH-immunoreactive fibers abundantly innervate the OVLT. Cholinergic neuronal cell bodies (gray-to-black immunostaining with silver-intensified ammonium nickel (II) sulfate-diaminobenzidine), immunoreactive for choline acetyltransferase (ChAT; A) and vesicular acetylcholine transporter (VACHT; B) occur in high numbers in the horizontal limb of the diagonal band of Broca (HDB). C, D: Thin and varicose cholinergic axons (arrows) immunoreactive for ChAT show an overlapping distribution with that of GnRH neurons and form appositions (arrowheads) to GnRH-immunoreactive cell bodies and dendrites. E, F: the cholinergic afferent contacts (arrowheads) on GnRH neurons are also detectable using VACHT as the cholinergic marker. Scale bars: A, B: 100 µm; C, D: 15 µm E, F: 20 µm.
5.1.2. Electron microscopic evidence for cholinergic afferents to GnRH neurons

At the ultrastructural level, the cholinergic axons were labeled with highly electron dense silver-gold-intensified ammonium nickel (II) sulfate – DAB precipitate. Some of the cholinergic axonal profiles established asymmetric synapses with unlabeled dendrites (Fig. 6A). Gonadotropin-releasing hormone neurons, labeled with electron dense DAB, were contacted by the cholinergic fibers on their somata as well as dendrites (Figs. 6B-D). While the appositions were devoid of glial intercalations (Figs. 6C, D), state-of-the-art synaptic specializations occurred rarely (Figs. 6E, F).
5.2. Revealing the origin of NPY-containing afferents to GnRH neurons in male mice

5.2.1. Comparative studies of NPY- and AGRP-containing fibers in contact with GnRH neurons of neonatally MSG-treated mice vs. untreated controls

Comparison of cresyl-violet-stained sections from MSG-treated mice (Fig. 7B) vs. untreated controls (Fig. 7A) showed a marked reduction in the size of the Arc, whereas neurons of the ventromedial nuclei remained intact.

Results of immunocytochemical experiments showed that the density of NPY-immunoreactive fibers was largely reduced in the POA of Arc-lesioned animals (Fig. 7D), in comparison with untreated controls (Fig. 7C). This observation indicated that a major, albeit not the sole, source of NPY fibers to the POA is the Arc. Axons containing AGRP-immunoreactivity (Fig. 7E) formed a somewhat less dense plexus in the POA than did NPY fibers (Fig. 7C) in control animals. As opposed to NPY immunostaining (Fig. 7D), AGRP-immunoreactivity was almost completely absent from the POA (Fig.
7F) in MSG-treated animals, corroborating previous data that the Arc is the only brain region in which this neuropeptide is synthesized (137).

The analysis of dual-labeled sections from intact mice demonstrated numerous contacts between NPY-immunoreactive axons and the perikarya and proximal dendrites of GnRH neurons (Fig. 8A). The frequency of NPY-containing axonal appositions to individual GnRH cells was significantly higher by ANOVA (P < 0.05) than the frequency at which AGRP-immunoreactive neuronal contacts occurred on GnRH neurons (Fig. 8B). The number of AGRP-immunopositive contacts was 56.5 ± 9.8% of that of NPY immunoreactive contacts. Furthermore, significantly less (P < 0.05) NPY-containing neuronal contacts were visible in neonatally MSG-treated animals (Fig. 8C vs. 8A) vs. controls. The lesion of the Arc caused a 63.7 ± 5.0% loss of NPY-immunopositive juxtapositions. Altogether, the two different methodological approaches suggested that about 56.5–63.7% of NPY axons to GnRH cells arise from NPY neurons of the Arc.
Figure 7. Effects of neonatal monosodium glutamate (MSG) treatment on the morphology of the Arc and innervation pattern of the medial POA (MPOA) by NPY and AGRP fibers. A, Cresyl violet-stained section shows the normal structure of the Arc. B, Neonatal MSG treatment of mice results in a marked shrinkage and cell paucity of this region (dashed line) due to a major neuronal cell loss. Note that the lesion is region specific, and neurons outside the Arc escape the effects of treatment. C and D, Innervation of the MPOA by NPY-immunoreactive axons is largely reduced in the Arc-lesioned animal (D) vs. the control (C). The source of the NPY network that survives the treatment is likely outside the Arc. E and F, Neuronal fibers immunoreactive to AGRP densely innervate the MPOA in untreated animals (E). Because the Arc is the only brain region synthesizing AGRP, immunoreactive axons almost completely disappear after neonatal lesioning of the Arc by MSG treatment (F). Note that the loss of NPY fibers in (D) and AGRP fibers in (F) reflects necrosis of neurons that synthesize both AGRP and NPY in the Arc. Scale bar, 100 µm (A–F).
Figure 8. Innervation of GnRH neurons by NPY and AGRP fibers. Effects of neonatal monosodium glutamate (MSG) treatment. (A), Axons immunoreactive to NPY form juxtapositions (arrowheads) with the cell bodies and proximal dendrites of GnRH neurons. (B), Neuronal contacts (arrowheads) between AGRP-immunoreactive axons and GnRH cells are less frequent. (C), GnRH neurons of neonatally MSG-treated mice are contacted (arrowheads) by significantly less NPY axons than GnRH cells of untreated controls (A). Scale bar, 10 µm (A–C).

5.2.2. Triple-label fluorescent detection of AGRP/NPY and DBH/NPY fibers forming neuronal contacts with GnRH-GFP neurons

The distributions of NPY- (Fig. 9A) and AGRP-containing (Fig. 9B) fibers largely overlapped (Fig. 9C) in the POA, and the heaviest immunolabeling for both was observed in the ventral aspect of this region. Axons double labeled for NPY and AGRP often formed serial contacts with the cell bodies and proximal dendrites (Fig. 9 D–F) of GnRH-GFP neurons that exhibited bright green fluorescence. High-power analysis of the fluorescent specimen established that 49.1 ± 7.3% of NPY-containing neuronal
contacts also contained AGRP (Figs. 9 D–F). In addition, nearly all AGRP fibers were immunoreactive to NPY.

Similar to AGRP-immunoreactive axons, fibers containing DBH (Fig. 9H) showed overlapping distribution (Fig. 9I) with that of NPY axons in the POA (Fig. 9G). High-power analysis of immunostained axons established the presence of DBH in 25.4 ± 3.3% of NPY-containing fibers in contact with GnRH neurons (Fig. 9 J–L). Other types of fibers observed in the POA contained NPY without DBH or DBH without NPY (Figs. 9 J–L), and they were apposed to GnRH neurons.
Figure 9. Immunofluorescent identification of AGRP and DBH in NPY afferents to GnRH neurons. (A–C), Both NPY (A; red Cy-3 fluorochrome) and AGRP-immunoreactive (B; blue AMCA fluorochrome) fibers form dense plexus in the medial POA (MPOA). The purple-to-white color that dominates over red in the merged figure (C) indicates that many NPY-immunoreactive axons co-contain AGRP. Note the bright green fluorescence in GnRH neurons (A–C) due to the presence of the GnRH-GFP transgene product. D–F, High-power photomicrographs reveal an NPY axon (D) that also contains AGRP (E). Arrows indicate the same points of contact between a dual-labeled NPY/AGRP axon and a GnRH neuron in unmerged (D, E) and merged (F) digital images. G–I, The distribution of NPY-immunoreactive axons (G; red Cy-3 fluorochrome) also overlaps with that of DBH-containing fibers (H; blue AMCA fluorochrome) in the MPOA. Note that this match (purple-to-white color in the merged panel; I) is of lower degree than in case of NPY- and AGRP-immunoreactive fibers (C). J–L, A noradrenergic/adrenergic axon that contains NPY (J) as well as DBH (K) establishes contact (arrows) with the soma of a GnRH neurons. The merged image in L clearly shows that many NPY axons (red color) are devoid of DBH, and vice versa, DBH-immunoreactive axons (blue color) often remain immunonegative for NPY. The scale bar for A-C and G-I is presented in I (100 µm). Sale bar for D–F and J–L is presented in L (10 µm).
5.3. 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 (Fig. 10 A and B). Synapses of symmetric morphology were observed between AGRP axons and GnRH neurons as well as non-GnRH structures (Fig. 10B). The absence of metal particles over AGRP fibers indicated that antibody cross-reaction did not arise from the species identity of the two primary antibodies.
Figure 10. Electron microscopic evidence for synaptic communication between AGRP-containing axons and GnRH neurons. A and B, an AGRP-immunoreactive axon terminal (at) labeled by electron-dense DAB deposits is closely apposed to the perikaryon of a GnRH-immunoreactive neuron (GnRH), which contains highly electron-dense silver-intensified gold particles (arrows). High-power image in B reveals axosomatic synaptic communication between the labeled structures. Arrowheads point to the postsynaptic density of a symmetrical synapse. Also note the presence of a symmetric synapse between the same AGRP-immunoreactive terminal and an unlabeled dendrite (d). Scale bars, 0.8 μm in A and 0.5 μm in B.
5.4. Morphological evidence for the glutamatergic phenotype of GnRH neurons in adult male rat

5.4.1. Results of *in situ* hybridization experiments

Development of emulsion autoradiographs exposed for 2 wk resulted in strong hybridization signal for Vglut2 mRNA in the POA (Fig. 11A). Heavy accumulation of silver grains was observed frequently above glutamatergic cells in the vicinity of GnRH neurons (Fig. 11 A1, A2, and B). Virtually all (99.5 ± 0.2%) of the total 438 GnRH neurons analyzed (Fig. 11 A1–A3) also contained Vglut2 hybridization signal, usually at moderate levels. Confirmative results obtained with a distinct Vglut2 probe (Fig. 11B) and the lack of Vglut2 signal using the sense strand Vglut2–879 RNA transcript (Fig. 11C) provided support for hybridization specificity.

Figure 11. Dual-label ISHH experiments reveal high expression levels of Vglut2 mRNA (autoradiographic grain clusters in (A) in regions also populated by GnRH neurons (brown histochemical staining in (A1–A3), including the medial preoptic area (MPOA) and the median preoptic nucleus (MPN). High power insets (A1–A3) illustrate moderate levels of Vglut2 mRNA in GnRH neurons (arrows) from frames in (A) after use of the Vglut2–879 probe. Demonstration of Vglut2 mRNA-containing GnRH neurons with a distinct antisense probe (Vglut2–734; B) and lack of such dual-labeled GnRH neurons after use of the sense strand Vglut2–879 RNA transcript (C) serve as controls for hybridization specificity.
5.4.2. Results of dual-label immunofluorescent studies

Both glutamatergic and GnRH-containing fibers formed dense plexus in the OVLT (Fig. 12A) and the external zone of the ME (Fig. 12C). High power confocal images demonstrated extensive terminal co-expression of Vglut2 with GnRH immunoreactivities in both circumventricular organs (Fig. 12B, and D). Although most dual-labeled axons occurred laterally in the ME, a dense Vglut2-immunoreactive plexus was also observed medially (Fig. 12C). Vglut2-immunoreactive axons (Fig. 12E) were eliminated (Figs. 12F) from the ME when using primary antibodies preabsorbed with 10 μM immunization antigen. In addition, simultaneous use of the two different primary antisera against Vglut2 labeled identical axons throughout the hypothalamus, in further support of labeling specificity (Fig. 12 G–I).
Figure 12. Dual-immunofluorescent images (A and C) illustrate the overlapping distribution of fibers immunoreactive for Vglut2 (green fluorochrome) and GnRH (red) in the OVLT (A) and the ME (C). Although sites of overlap (yellow) occur mostly in the lateral part of the ME, the dense Vglut2-immunoreactive plexus is also present medially (C). Arrows in high power confocal images (B and D) point to dual-labeled axon varicosities (yellow) immunoreactive for both GnRH and Vglut2. The specificity of Vglut2 immunostaining in the ME (E) is indicated by the lack of labeled axons after adding the immunization antigen (10 µM) to the working dilution of AB 5907 (F). In addition, dual-immunofluorescent labeling of hypothalamic axons (arrows in confocal images G, H, and merged panel I) using two distinct primary antibodies for Vglut2 further supports the authenticity of Vglut2-immunoreactivity. Scale bars, 200 µm in A, 30 µm in D and F, and 10 µm in the other panels.
5.4.3. Electron microscopic localization of Vglut2 in hypophysiotropic axon terminals in the ME

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 (Fig. 13 A, B), indicating that they represented neurosecretory terminals. The labeled structures contained small clear as well as DV at a highly variable ratio. The immunocytochemical labeling clearly tended to occur at subcellular domains occupied primarily by small clear vesicles (Fig. 13A, B).

Figure 13. Electron microscopic localization of Vglut2-immunoreactivity in the external zone of the ME. (A) Three neuroendocrine axon terminals (AT1-3) freely communicate with the pericapillary space (PCS) of portal blood vessels. Arrowheads delineate the basal lamina. AT1 and AT2 contain both dense core vesicles (DV; arrows) and small clear vesicles (SV), whereas AT3 comprises SVs only. The silver-intensified gold particles identify the Vglut2 content of the terminals. Note that the immunocytochemical label is preferentially associated with subcellular sites densely populated by SVs, indicating that Vglut2 is contained in SVs. (B) A glutamatergic axon terminal (AT) terminates on the external limiting membrane (arrowheads) of a portal capillary. Arrowheads delineate the basal lamina. Note the presence of DVs (arrows) as well as SVs in the same terminal profile and the association of the Vglut2 immune signal with SVs. Scale bars=0.5 µm.
6. Discussion

6.1. Proposed nonsynaptic role and receptorial mechanisms of ACh on GnRH neurons and the possible sites of origin of the direct cholinergic afferents

6.1.1. Ach as a proposed nonsynaptic modulator of 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 unknown neurotransmitter system, which directly regulates GnRH neurons and suggest a dominantly nonsynaptic signaling mechanism in this interaction.

Our results indicate that the cholinergic axons often formed direct contacts but rarely established 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) (157), 10 % in the neostriatum (158) and 14 % in the parietal cortex (159) were engaged in synapses may indicate nonsynaptic mechanisms (160) whereby cholinergic axons influence their target neurons, including GnRH cells. The main properties of nonsynaptic communication include the long lasting and tonic action (s, min, h), axonal varicosities without synaptic contacts, the high affinity receptors and the high concentration of neurotransmitters in the extracellular space (160). The nonsynaptic localization of cholinergic heteroreceptors allows the modulation of GABAergic, glutamatergic, noradrenergic and serotonergic neurotransmission by ACh in the hippocampus (160, 161). These neurotransmitter systems also innervate the GnRH neurons (see in section 2.2.3.1.), therefore, the proposed nonsynaptic cholinergic interactions on GnRH neurons may involve the modulation of the above-mentioned neurotransmitter-containing neuronal afferents of the GnRH nerve cells. Further neuropharmacological experiments and the anatomical localization of cholinergic receptors on GnRH nerve cells and their surrounding neural elements will be needed to clarify this hypothesis.

Our anatomical observations showed that GnRH neurons receive cholinergic innervation on their cell bodies as well as dendrites. Campbell and colleagues who used
biocytin-filled GnRH neurons of mice detected a surprisingly rich dendritic arborization, which was unknown previously (16). While the number of spines showed a trend to decrease with increasing distance from the cell body, they remained detectable even on the most distal elements of the primary dendrite (16). Considering that cholinergic axons preferentially form contacts and synapses with dendritic branches and spines in other brain regions (157, 159, 162, 163), our study likely left a significant proportion of cholinergic contacts undetected at the distal dendrites and spines of GnRH neurons, which might remain unlabeled for GnRH.

Finally worth of note, that the external zone of the ME exhibits a high density of VACHT- and ChAT-immunoreactivities (99, 164). This observation suggests the possible paracrine regulatory role of ACh at the level of the GnRH nerve terminals as well.

6.1.2. Receptorial mechanisms of ACh actions on GnRH neurons

To estimate the exact functional consequences of ACh release on GnRH neurons the determination of the receptor subtypes and their site of locations (i.e. non-, pre- or postsynaptic) would be crucial. The receptorial mechanisms of cholinergic actions on GnRH neurons appear to be multiple. Krsmanovic and coworkers studied perifused hypothalamic primary cell cultures and immortalized GnRH producing GT 1-7 cells and concluded a dual receptorial effect of ACh on GnRH neurons. While the cholinergic activation of M2 muscarinic receptors reduced basal GnRH release via inhibiting G protein-coupled intracellular cAMP elevation, the activation of M1 receptors led to stimulation of phosphoinositide hydrolysis, with the result of a rapid and transient increase in GnRH neurosecretion (165). In addition, ACh also increased GnRH secretion via causing increased Na\(^+\) and Ca\(^{2+}\) influx due to nicotinic receptor activation (165). The Ca\(^{2+}\) delivery from intracellular Ca\(^{2+}\) stores also forms an important component of cholinergic signaling as shown by Morales and coworkers. The ACh-induced [Ca\(^{2+}\)], elevation from the endoplasmic reticulum of GT 1-7 cells could be blocked by atropine. Moreover, the cholinergic stimulation of [Ca\(^{2+}\)], delivery showed a strong modulation by estrogen and was reduced rapidly by 50-60% in the presence of 10 nM 17\(\beta\)-estradiol. The fast onset of this effect and the similar action of a membrane
impermeant estradiol conjugate implicated a membrane associated estrogen receptor in this modulatory action (166). Since the M1 muscarinic and several nicotinic receptors (Chrnβ 1-2, Chrnbg) are represented in a subpopulation of GnRH neurons \textit{in vivo} (63), the direct cholinergic afferents on GnRH neurons revealed by us may stimulate (109, 110) the functional properties of the GnRH neurons via the above-described second messenger systems. In contrast, GnRH neurons \textit{in vivo} appear to lack the M2 receptors, which would suggest the presynaptic role of this cholinergic receptor, although further pharmacological and/or anatomical experiments would need to clarify this question.

6.1.3. Possible sites of origin of the cholinergic innervation to GnRH neurons

Cholinergic neurons of the basal forebrain (167) may represent one possible source for the cholinergic innervation of GnRH neurons. Retrograde tact tracing studies in rat (67) and mouse (18) revealed retrogradely labeled nerve cells in the following parts of the basal forebrain: nucleus of the horizontal and vertical limbs of the diagonal band (18, 67) and medial septal nucleus (18). Although the chemotype of retrogradely labeled neurons has not been determined. The main projection field of the cholinergic neurons of the basal forebrain are neocortical areas (167), however the cholinergic neurons in the septum and diagonal band indeed possess extensive local collaterals (168) from which they are capable of releasing ACh locally (for a review see: (168)). This observation suggests that ChAT- and VACHT-immunoreactive fibers in the POA revealed by us may arise from particular parts of basal forebrain.

The parabrachial complex also contains a major cholinergic cell population (98). The idea that these cells may contribute to the cholinergic innervation of the GnRH system gains support from the observation that a large number of neurons in the central and a somewhat lower number of cells in the dorsal and superior lateral parabrachial nuclei could be retrogradely labeled from the POA (67, 100).

Both of the basal forebrain and the parabrachial complex belong to the ascending arousal system (169) that contributes to the generalized arousal state of the organism. Sexual arousal, which is a subcategory of the former (170), is a state of the animal that is requisite of the activation for those complex cognitive and physiologic processes that result in sexual behavior (106, 170). Cholinergic activation of the POA is involved in
the normal pattern of the sexual behavior (104, 105). Although for the activation effect of ACh, the presence of the gonadal steroids appears to be essential components (101). These observations emphasize the importance of both the adaptive functioning of arousal systems and the appropriate level of the gonadal steroids in the governing of the reproductive behavior. Thus, the basal forebrain and/or the parabrachial nuclei as a proposed source of cholinergic afferents to the GnRH neurons may provide a novel relation between the neural processes of sexual behavior and its accompanying hormonal adaptations.

Aside from tract tracing studies (18, 67, 129), several pieces of indirect evidence indicate that the Arc as one primary site of origin of the cholinergic afferents, to the GnRH neurons. About a third (37%) of the POMC neurons in the Arc was found to contain the cholinergic marker VAChT (99). The POMC neurons establish synaptic contacts with GnRH neurons (78) and the proposed source of the opioid innervation to GnRH neurons is the Arc (56, 78). The presence of cholinergic/αMSH positive fibers in the POA (99) also raises the possibility that the Arc is a potential source of cholinergic innervation to GnRH neurons. The POMC neurons in the Arc are an important neuron population in the regulation of food intake and in energy homeostasis (143), which would indicate the role of POMC/cholinergic neurons as a possible linkage between the regulation of body weight and the HPG axis. Nevertheless, the functional role of the possible co-release of ACh and one of the POMC derived peptides on the GnRH neurons would need further clarifications, because the GnRH neurons do not express either the melanocortin receptors (receptors of αMSH and ACTH) or the opioid receptors (receptors of β-END) (171) in rats.

6.2. The functional roles of the NPYergic afferents of Arc and brainstem in the regulation of GnRH neurons and the receptorial mechanisms of NPY action on GnRH neurons

6.2.1. The Arc and the brainstem as two major sources of origin of NPY fibers innervating 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 neurons (depending on the calculation approach we used), and an additional 25% of juxtapositions originate in adrenergic/noradrenergic cell groups of the brainstem (Figure 14.).

Figure 14. The scheme illustrates the relative contribution of the hypothalamic Arc and the catecholaminergic nuclei of the brainstem to the NPY innervation of GnRH neurons. Our results indicate that NPY/AGRP neurons of the Arc give rise to 49–64% of the NPY-immunoreactive axonal contacts on the somata and proximal dendrites of GnRH neurons (depending on the calculation approach we used), and an additional 25% of juxtapositions originate in adrenergic/noradrenergic cell groups of the brainstem. (Arc: arcuate nucleus; A1, C1, C2, C2: catecholaminergic nuclei of the brainstem; POA: preoptic area)

These quantitative data supplement results of a previous report by Simonian et al. (131), who described retrograde labeling of NPY neurons in both the Arc and ventrolateral medulla after tracer injection around GnRH neurons. 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 (137). Results of our triple-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 nerve cells. 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.

The intense NPYergic innervation, which arises from the Arc is involved in the mediation of the nutritional imbalance and metabolic disturbances of the organism toward the reproductive axis (141), since the Arc is one among the main target areas of the metabolic signals from the periphery (143, 172). The classic example in this context is the adipocyte-derived hormone leptin, a satiety factor that not only reduces food intake and energy consumption but also influences the HPG axis (173). The obese leptin deficient ob/ob mouse is known to harbor serious reproductive effects including variable degrees of hypogonadotropic hypogonadism and infertility (142). Most of the effects of leptin in the control of food intake are exerted within the brain, predominantly in the hypothalamus, where leptin is known to modulate both orexigenic (mostly those co-expressing NPY and AGRP) and anorexigenic neurons (mostly those co-expressing POMC and cocaine- and amphetamine-regulated transcript) (174). A subpopulation of NPY neurons express the long form of leptin receptors (175, 176) and exhibit a pronounced inhibition in their electrophysiological activities in response to leptin administration (177). Leptin concentration falls during starvation, which accompanied by the increased firing rate of the NPY-synthesizing cells (177), a concomitant increase of NPY mRNA in the Arc (176) and increased NPY peptide release (178). There is strong evidence to indicate that the hyperfunction of NPY neurons in the ob/ob mouse inhibits the reproduction axis: leptin replacement therapy to these mice restores normal LH secretion and fertility (142) with a concomitant suppression of hypothalamic NPY gene expression. Moreover, crossbreeding of ob/ob mice with NPY-knockout animals results in a significant improvement of the reproductive phenotype (179). Given that a subpopulation of NPY neurons express GAD-65 a biosynthetic enzyme of GABA (180), the inhibition of HPG axis in fasting and leptin deficient animals might be due to the inhibitory effect of increased electrophysiological and/or secretory activity of the leptin sensitive NPY/GABA neurons on the GnRH cells. Further support for the inhibitory influence of NPY afferents is the observed symmetric morphology (i.e. inhibitory) of NPY synapses on GnRH neurons (74).
In addition to AGRP/NPY contacts of Arc origin, our analysis found DBH immunofluorescence within 25% of NPY immunoreactive axonal contacts on GnRH neurons. This observation indicates that noradrenergic/adrenergic pathways directly regulate GnRH neurons and is also in accordance with previous results by others (131, 152, 181) showing noradrenergic cell groups projecting to the immediate vicinity of GnRH neurons in rats and mice (61). The present work did not address the ultrastructural characteristics of DBH-immunoreactive juxtapositions to GnRH neurons. One previous study (40) suggested that noradrenergic neurons could communicate with GnRH cells through classical synaptic mechanisms, whereas other investigators (41) debated the abundance of such synapses. Potential difficulties to reveal synaptic specializations between adrenergic/noradrenergic terminals and GnRH neurons may indicate the involvement of nonsynaptic routes in the catecholamine-GnRH communication. Although this concept will require formal support by the immunoelectron microscopic analysis of DBH contacts on GnRH cells, there is little doubt that the proposed nonsynaptic mechanisms often play a role in noradrenergic neurotransmission (160). For example, only a small fraction of DBH-immunoreactive juxtapositions establishes classical synapses with neurons of the cerebral cortex and the subcellular distribution of α2A adrenoceptors is not restricted to the postsynaptic membranes (182). 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 (131, 153, 183). Likewise, high percentages of C1–3 adrenergic neurons and A1 noradrenergic neurons were found to contain NPY, whereas A2 and A6 noradrenergic neurons often lacked any NPY immunostaining (131, 153). The adrenergic/noradrenergic input to GnRH cells is consistent with a large body of evidence in the literature indicating the important role of noradrenergic stimuli in the regulation of the ovarian cycle and the steroid-induced gonadotropin surge (38, 39). A prominent increase can be observed in the turnover rate of noradrenaline within the vicinity of the GnRH nerve bodies and terminals before and at the time of the steroid induced or proestrus LH surge. The increased noradrenaline concentration is assigned to the estrogen receptor positive A2 neurons, which were found to express the immediate early gene Fos on the morning of proestrus. Moreover, the noradrenaline concentration
in the POA also exhibits a circadian periodicity, which is probably maintained by A1 neurons exhibiting a diurnal pattern of Fos expression. Thus, it is possible that the A1 input is liable for the circadian rhythm of noradrenaline in the POA, while the A2 neurons provide a steroid dependent component to the noradrenaline release. (for a review see: (39))

Finally, while it is likely that at least some of the AGRP/NPY input that reaches GnRH neurons from the Arc is GABAergic (180, 184), the recent demonstration of Vglut2 in C1 adrenergic and other catecholamine neurons of the brainstem (185), which also synthesize NPY (153), raises the possibility that the excitatory neurotransmitter glutamate is co-released with NPY and epinephrine/norepinephrine from afferents to GnRH neurons.

Although the Arc and brainstem can be viewed as the most important sources of central NPY, neuronal perikarya synthesizing NPY in other brain areas that are interconnected with the POA may also contribute to the NPYergic innervation of GnRH nerve cells. The putative sources can be speculated by the comparison of the distribution of NPY neurons (135) and the results of retrograde tract tracing studies (18, 67). The possible brain regions are the followings: dorsomedial hypothalamic nucleus, bed nucleus of the stria terminalis, anterior horn of the anterior commissure, lateral preoptic area, dorsal hypothalamic area and the mesencephalic central gray (18, 67). Among these areas, the neurons in the dorsomedial hypothalamic nucleus, bed nucleus of the stria terminalis and lateral preoptic area establish synaptic connection with GnRH neurons in the mouse (18). The calculated percentage of these alternative afferents can be up to 20–26% of NPY fibers form the Arc if identified by their AGRP content but somewhat lower (11%) if the percent loss of NPY afferents in MSG-treated animals is considered. Several explanations for this discrepancy may exist. It is possible that MSG treatment altered other NPY systems, in addition to induce the lesion of Arc neurons. Alternatively, the ratio of NPY afferents reaching GnRH neurons from the Arc may be somewhat higher than we estimated based on their AGRP content. A subset of NPY neurons in the Arc (5%) seem to be devoid of AGRP (137), and these cells may contribute to the innervation of GnRH neurons. Finally, we cannot rule out the possibility that the immunocytochemical assay conditions were suboptimal to reveal
low levels of DBH or AGRP in subsets of NPY axons, despite the high detection sensitivity provided by the tyramide signal amplification technique (155).

Finally, we would note that, the NPYergic innervation of GnRH neurons is known in human brain as well (186). It would be interesting to find out whether the sources of this NPY innervation are similar to those we revealed in the present studies in mice.

6.2.2. Receptorial mechanisms of NPY actions on GnRH neurons

Neuropeptide Y receptors are signed with a Y character and belong to the family of the seven-transmembrane domain, G protein-coupled receptors, which act primarily via inhibiting adenylate cyclase (187). In the CNS, six types of Y receptors have been characterized up to now, and except for Y6, all appear to play role in the regulation of reproduction. Pharmacological studies using selective ligands to distinct Y receptor subtypes for intracerebroventricular acute injections into castrated rats (118) and chronic infusions to intact male rats and mice (111) provided evidence that the Y5 receptor plays a crucial role in the inhibitory control of gonadotropin release by NPY. Given that more than half (55%) of GnRH neuronal perikarya bear this receptor subtype (128), the Y5 receptor-mediated reproductive actions of NPY may be exerted, at least partly, on GnRH cells. In pentobarbital-blocked, proestrus rats, i.v. pulses of Y1 receptor antagonist blocked the endogenous LH surges and prevented the amplification of the GnRH-induced LH surges by NPY, implicating Y1 receptor activation in the stimulatory control of LH secretion (188). Morphological evidence supports the presence of the Y1 receptor subtype in axon terminals of GnRH neurons in the median eminence as well as in NPY fibers that innervate GnRH neurons from the Arc (129). It is reasonable to speculate that Y1 receptors presynaptic to GnRH neurons may modulate the synaptic release of NPY, AGRP, or a putative classic neurotransmitter of these neurons, GABA (184). Accordingly, NPY agonism on presynaptic Y1 and Y2 receptors has been shown to inhibit glutamate (189) GABA (190) and norepinephrine (191) release. Beyond the Y1 and the Y2 receptor subtypes, a recently published paper (192) has provided evidence for the role of Y4 in the regulation of the HPG axis. The authors compared GnRH mRNA levels in Y2 and Y4 knock out and Y2/Y4 double-knock out animals. They found significantly higher GnRH mRNA in the Y4- and
double-knock out vs. Y2-knock out or wild type mice. In addition, after 24h fasting GnRH mRNA decreased in Y2-knock out mice similarly to wild type animals, while the measured GnRH mRNA levels in Y4 and double-knock out animals did not show any decline. The lack of Y4 receptor expression in the POA (193) appears to suggest that the Y4 receptor does not act directly on GnRH neurons.

6.2.3. Methodological considerations

The AGRP and DBH contents of NPY afferents to GnRH neurons were analyzed in GnRH-GFP transgenic mice (136) in which green fluorescence is detectable in the vast majority of GnRH neurons, without a significant ectopic expression of the transgene (136). This approach alleviated the need of using three primary antibodies, all of which should be generated in different species to avoid cross-reactions. A second technical consideration is that the innervation pattern we observed reflects only the situation on the somata and proximal dendrites of GnRH neurons. Because distal dendrites of GnRH neurons may not contain GFP fluorescence, our studies do not allow us to conclude about their putative regulation by NPY afferents.

6.3. 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 axons and GnRH neurons, we carried out electron microscopic studies and demonstrated that AGRP-immunoreactive axons establish symmetric synapses with GnRH neurons.

From a functional viewpoint, it is important to note that AGRP-immunoreactive 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 (74). Furthermore, this synaptic morphology also characterizes GABAergic synapses (54). Therefore, the identification of the biosynthetic enzyme of GABA, GAD-65, in 30% of NPY neurons in the Arc (180) along with the light and electron microscopic demonstration of GABA-immunoreactivity in subsets of NPY axons in the POA (184) highly indicate that at least
some AGRP/NPY terminals that innervate GnRH neurons may co-contain GABA. Although this hypothesis awaits confirmation, recent evidence supports the physiological importance of a GABA/NPY interplay in the regulation of gonadotropin secretion (184).

Little is currently known about the role of AGRP, an endogenous antagonist of melanocortin 3 and 4 receptors in the regulation of the reproductive axis. It is likely that AGRP primarily stimulates gonadotropin secretion via acting at hypothalamic sites. Increased gonadotropin secretion was observed 40 min after intracerebroventricular injection of AGRP to male rats, whereas AGRP was unable to alter either basal- or GnRH-stimulated gonadotropin secretion from dispersed pituitary cells (194). Furthermore, AGRP also stimulated GnRH release from mediobasal hypothalamic explants in vitro, and this effect was prevented by the presence of αMSH in the medium (194), indicating that AGRP partially acts via antagonizing melanocortin receptors in the Arc-ME region. Somewhat contrasting the finding that in vivo chronic infusion of AGRP to ovariectomized female rhesus monkey suppresses pulsatile LH release (115, 145). The above-mentioned observations concerning the GABAergic phenotype of a subpopulation of the NPY neurons (180, 184) emphasize the possible inhibitory role of AGRP. The symmetric, i.e. inhibitory properties of the synapses between AGRP- and GnRH-immunoreactive profiles revealed by us also confirm the hypothesis that at least a subpopulation of AGRP neurons transmits inhibitory effects toward the GnRH neurons. We can conclude that the effect of AGRP may depend on the gender and/or the hormonal status of the animal or whether or not the innervating AGRP/NPY neurons possess GABAergic chemotype. Identification of the type and cellular location of NPY and AGRP receptors will provide a better insight into mechanism whereby NPY/AGRP neurons modify GnRH neuronal functions. Future research will also 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, features well-characterized for the regulation of the reproductive axis by NPY.

6.4. Demonstration of glutamatergic phenotype of the GnRH neurons
We have provided several pieces of anatomical evidence for the novel glutamatergic phenotype of the GnRH neurons. Our ISHH experiments have shown that nearly all GnRH neurons (~99%) in male rats express the glutamatergic marker Vglut2 mRNA. The confocal microscopic observations have confirmed the presence of Vglut2 protein in the GnRH axon terminals of the OVLT and the ME. Finally, our preembedding electron microscopic examinations have verified the presence of Vglut2 in the axon terminals of the ME and established the association of Vglut2-immunoreactivity with small clear vesicles.

6.4.1. Central effects of glutamate on the regulation of reproduction

Glutamate is a critically important neurotransmitter in the regulation of the GnRH neuronal system. Intravenous N-methyl-D,L-aspartate infusion can induce precocious puberty in immature rats (195) and activation of ionotropic glutamate receptors plays a crucial role in both pulse (5) and surge (196) modes of GnRH secretion. Administration of glutamate can enhance circulating LH levels and this effect is exerted centrally, since exposure of pituitary slices to glutamate or glutamate injection directly into the hypophysis do not alter LH release (44). Indeed, glutamate can regulate GnRH neurons at the level of GnRH cell bodies and dendrites, which receive Vglut2-immunoreactive synapses (49, 146) and exhibit immunoreactivity for ionotropic glutamate receptors (45, 46). Moreover, compelling evidence indicates that an additional major site of action for glutamate is the ME. GnRH terminals in the ME are apposed to glutamatergic axons (146, 149) and express immunoreactivity for the kainite-2 (KA2) and N-methyl-D-aspartate-1 ionotropic glutamate receptor subunits (NMDAR-1) (149). Further, glutamate and agonists of ionotropic glutamate receptors can induce Ca\(^{2+}\)-dependent release of GnRH from superfused ME fragments (148).

6.4.2. Glutamatergic phenotype of the GnRH neurons

Our ISHH finding that virtually all GnRH neurons expressed Vglut2 mRNA in the adult male rat strongly suggests that the glutamatergic chemotype is a critically important feature of the GnRH neuronal system. It is important to note that the ME also
contained a large number of Vglut2-immunoreactive terminals that were devoid of immunostaining for GnRH. A likely source of some of these fibers is the parvicellular part of the hypothalamic paraventricular nucleus, where Vglut2-expressing neurons occur in high numbers (146, 147). Indeed, according to our subsequent studies, the parvicellular hypophysiotropic neurons including thyrotropin-releasing hormone, CRH (197) and somatostatin-synthesizing neurons (198) express both the mRNA and the transporter protein of Vglut2.

A large subset of GnRH neurons in rats, mice, and humans exhibit GABA-immunoreactivity (83, 85) during fetal migration from the olfactory placode to the forebrain (9). Tobet and co-workers (83) found no GABA-immunoreactivity in GnRH neurons that migrated further caudal to the olfactory bulbs, suggesting that GnRH neurons may switch from the GABAergic to the glutamatergic phenotype perinatally. Nevertheless, the image about the GABA/glutamate switch in the GnRH neurons during the prenatal migration may be more shaded. Interestingly, Vglut2-immunoreactive cell groups also occur in the olfactory placode at E11.5 (199), around the time when the first juvenile GnRH neurons can be detected there and begin their migration toward the POA (200). Moreover, Honma and coworkers were able to reveal GnRH-associated protein immunoreactivity in a few migrating Vglut2-immunoreactive nerve cells between E14.5 - E16.5 (199). Considering that the peak in the number of GABA positive neurons in the peripheral olfactory sites (i.e. vomeronasal organ and around the vomeronasal and olfactory nerve) is around E15 (83), the juvenile GnRH neurons or at least a subpopulation of them may use both GABA and glutamate signaling. The question whether or not the adult GnRH neurons also have such a double GABA/glutamatergic phenotype remains opens. We showed that virtually all GnRH neuron in adult male rats possessed Vglut2 mRNA. This finding does not exclude the possibility that the GABA synthetic enzymes and the vesicular GABA transporter may continue to be expressed together with Vglut2 in a subset of adult GnRH neurons. In this context, it is interesting to note that Ottem et al. observed neurons in the anteroventral periventricular nucleus that possess such a GABA/glutamate double phenotype (201). Similarly, prenatal GnRH neurons may also express a dual GABA/glutamate phenotype.

The putative existence of autocrine/paracrine glutamatergic mechanisms in the terminal regulation of GnRH neurosecretion receives substantial support from i) our
present observation that GnRH terminals co-contain Vglut2; ii) the identification of immunoreactivities for the KA-2 and the NMDAR-1 ionotropic glutamate receptor subunits on GnRH-immunoreactive terminals (148, 149, 202); and iii) the ability of ionotropic glutamate receptor agonists to elicit GnRH release from the mediobasal hypothalamus (44). Glutamate receptors may also be present on glial and vascular elements of the ME and posterior pituitary to influence their functions, possibly including morphological plasticity. Tanycytes lining the ventral wall of the third ventricle and astrocytes of the ME were, indeed, found to contain mRNAs and immunoreactivity for kainate receptors (203-205) and to also express the activity marker c-Fos immunoreactivity in response to stimulation by kainate (204).

6.4.3. Subcellular localization of Vglut2 in the axon terminals of the ME

Our electron microscopic observations have demonstrated the presence of Vglut2 in the hypophysiotropic nerve terminals of the ME and the close association of Vglut2-immunoreactivity to the small clear vesicles.

It is worth noting that neuroendocrine axons were particularly rich in small clear vesicles, close to their termination around the pericapillary space in both the ME and the posterior pituitary. Inversely, DVs were often decreased in number as the neuroendocrine axons approached the basal lamina. Previous studies have revealed a similar change in the vesicular composition of CRH-containing axons in the ME in that DVs dominated the preterminals, whereas small clear vesicles became more characteristic to terminals reaching the pericapillary space (206). Glutamate-immunoreactive small clear vesicles also had a tendency to accumulate at the terminal segment of magnocellular axons in the posterior pituitary (207), which is in line with our recent finding of Vglut2-immunoreactive small clear vesicles in magnocellular terminals (208). To some extent, the differential distribution of the two types of vesicles is also reminiscent to their relationship at chemical synapses of various species. The presynaptic specialization contains primarily small clear vesicles, whereas dense core granules do not tend to occur close to the synaptic specializations (209, 210). Although the astrocytes in vitro possess all types of Vgluts (211) we could not observed significant in situ Vglut2-immunolabeling above the glial elements of the ME with our
pre-embedding electron microscopic labeling. In contrast, 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 (47, 212, 213) in other brain areas.

To elucidate the role of glutamate co-secretion in neuroendocrine regulation, it is
of prime importance to first find and characterize glutamate receptors in the ME and
hypothalamus. Theoretically, such receptors may be located on the neuroendocrine
terminals to inhibit or facilitate peptide neurosecretion. In a paper published recently
(202), Yin and coworkers compared the distribution of the ionotropic NMDAR-1
subunit with GnRH-immunoreactivity by a sensitive double-labeling postembedding
electron microscopy in the axon terminals of the ME. Their analysis showed that
NMDAR-1 subunits frequently colocalized with GnRH decapetide even within the
same DV, while the plasma membrane exhibited low immunoreactivity for this receptor.
The authors concluded that since the sparse occurrence of synaptic connections in the
ME, the liberating glutamate from the nerve terminals might triggers the release of other
dense core granules, which are located either in the neighboring nerve terminals or even
within the same nerve ending.

From the occurrence of Vglut2 in functionally and neurochemically diverse
neurosecretory endings, it is tempting to speculate that intrinsic L-glutamate fulfills
similar regulatory functions in several neuroendocrine systems and may contribute to
the generation of the pulsatile patterns of neurohormone output. Nevertheless, this
hypothesis would require experimental support. The molecular mechanisms by which
 glutamate endogenous to GnRH neurons regulates reproduction require future
clarification.

6.4.4. Methodological considerations

The reason why previous confocal and electron microscopic studies (49, 146, 148,
149) failed to reveal the glutamatergic phenotype of the GnRH neuronal system is not
clear. In view of the moderate Vglut2 mRNA and protein levels we found in GnRH
cells, the use of amplification methods could be essential for our colocalization studies
to succeed. Furthermore, although Vglut2 mRNA was present in virtually all GnRH neurons, Vglut2-immunoreactivity often remained undetectable in GnRH terminals. This discrepancy may be attributable to a general limitation of the immunocytochemical detection method due to the low amount of Vglut2 protein. Another possibility is the heterogeneity of GnRH axon varicosities, in that some may mostly contain DVs with GnRH and only few small clear vesicles with Vglut2.
7. Conclusions

1. We have provided anatomical proof for the direct innervation of hypophysiotropic GnRH neurons by the cholinergic neurotransmitter system in male rats. We have suggested that this neuronal communication takes place mostly via nonsynaptic mechanisms.

2. We have determined that in male mice 49-64% of the NPY innervation of GnRH neurons arises from the Arc of the hypothalamus, while 25% of NPY contacts originate from the catecholaminergic nuclei of the brainstem.

3. We have revealed synaptic contacts between AGRP-immunoreactive terminals, which originate from the Arc and the hypophysiotropic GnRH neurons in male mice. The symmetric morphology we observed is typical of inhibitory synapses.

4. We have provided evidence for the presence of Vglut2 mRNA and protein in the hypophysiotropic GnRH neurons of male rats. Moreover, we have verified the presence of Vglut2 in the axon terminals of the ME and the close association of Vglut2-immunoreactivity to small clear vesicles. These data suggest a novel glutamatergic phenotype of GnRH neurons and can serve as the basis of a novel autocrine/paracrine regulation of neurohormone release.
Figure 15. The schematic drawing represents the known synaptic inputs to GnRH neurons (orange color). The terminals indicated by orange color correspond to the novel transmitter systems that directly innervate GnRH neurons and were revealed by our studies in this thesis. Inside the GnRH neuron can be found neurotransmitters/neuromodulators co-expressed in GnRH cells. The presence of Vglut2 in GnRH neurons, which was revealed in our studies, suggests the previously unknown glutamatergic phenotype of GnRH neurons. (5-HT: serotonin; β-END: β-endorphin; ACh: acetylcholine; AGRP: agouti-related peptide; DSIP: delta sleep-inducing peptide; CCK: cholecystokinin; CRH: corticotropin-releasing hormone; DBH: dopamine-β-hydroxylase; GABA: gamma-aminobutyric acid; GnRH: gonadotropin-releasing hormone; GLU: glutamate; NT: neuropeptide Y; SP: substance P; TH: tyrosine hydroxylase; Vglut2: vesicular glutamate transporter-2)
8. Summary

The hypothalamo-pituitary-gonadal (HPG) axis regulates the reproductive functions. The central unit of the axis is the gonadotropin-releasing hormone (GnRH)-producing neuron population located in the preoptic area (POA). The neurosecretory activity of GnRH neurons is under the influence of hormonal factors and several neurotransmitter systems. The aim of this dissertation is the identification of novel neurotransmitter systems in the neuronal regulation of the GnRH neurons. (1) Acetylcholine (ACh) is able to modulate the activity of GnRH neurosecretion and sexual behavior. We have provided neuroanatomical evidence for the direct innervation of GnRH neurons by cholinergic axons in male rats. Our results indicate that the cholinergic axons often formed direct contacts but rarely established classical synapses with the GnRH neurons. Therefore, similarly to other brain areas, we propose nonsynaptic mechanisms whereby ACh modulates the functions of GnRH neurons in the POA. (2) Despite the profound central effect of neuropeptide Y (NPY) on the HPG axis, the sources of NPY afferent to GnRH neurons were unidentified. Therefore, we addressed the sources of origin of NPY-containing afferents to GnRH neurons in male GnRH-GFP transgenic mice. Our results indicate that NPY/AGRP neurons of the Arc gave rise to 49–64% of the NPY-immunoreactive axonal contacts on the somata and proximal dendrites of GnRH neurons (depending on the calculation approach we used), and an additional 25% of juxtapositions originated in adrenergic/noradrenergic cell groups of the brainstem. (3) Moreover, we have revealed with electron microscopy that the AGRP-immunoreactive terminals establish symmetric synapses with GnRH neurons. (4) The neurotransmitter glutamate is packed into synaptic vesicles by the three recently discovered vesicular glutamate transporters (Vglut1-3). Using the Vglut2 as a marker of glutamatergic neuronal phenotype, we have provided conclusive evidence for a marked glutamatergic phenotype of GnRH neurons in the adult male rat by demonstrating Vglut2 mRNA expression in the perikarya and Vglut2-immunoreactivity in the axons of these cells. Using immuno-electron microscopy, we have demonstrated that the Vglut2-immunoreactivity is localized to small clear vesicles in the neurosecretory endings of the ME. The physiological significance of endogenous glutamate in the regulation of GnRH secretion requires clarification.
9. Összefoglalás

A szaporodást és az azzal összefüggő élettani folyamatokat a hypothalamo-
hypophyseo-gonadális tengely szabályozza. Rágszálókban a tengely centrális
szabályozó elemei a preoptikus area területén elhelyezkedő gonadotropin-releasing
hormon (GnRH)-t termelő idegsejtek, melyek neuroszekréciós működését humorális
faktorok és neurotransmitter rendszerek együttesen modulálják. A doktori értekezés
témája a GnRH idegsejtek neurális szabályozásban részt vevő új neurotransmitterek
azonosítása volt. (1) Az acetilkolin (ACh) in vitro már nanomoláris koncentrációban is
képes serkenteni a GnRH neuroszekréciót. Fény- és elektronmikroszkópos
vizsgálatainkkel kimutattuk, hogy hím patkányokban a kolinerg axonok közvetlenül
beidegzik a GnRH neuronokat, azonban a klasszikus morfológiájú szinapszisok a
kolinerg idegnyúlványok valamint a GnRH idegsejtek és dendritjeik között ritkák voltak.
Megfigyeléseink alapján azt feltételeztük, hogy - hasonlóan a központi idegrendszer
egyéb területeihez -, az ACh a hypothalamusban is főként nem-szinaptikus módon hat
az idegsejtek, így a GnRH neuronok működésére. (2) A centrálisan adott neuropeptid Y
(NPY) reprodukció szabályozására gyakorolt élettani hatásai jól ismertek. A NPY
tartalmú idegnyúlványok beidegzik a GnRH idegsejteket, azonban az afferensek pontos
eredete nem ismert. A GnRH idegsejtek NPY afferenseinek kvantitatív analízisével
megállapítottuk, hogy hím egerekben az NPY beidegzsés két fő forrása a hypothalamus
acuatus (Arc) idegmagja (~49-64%), valamint az agytörzsi katekolaminerg idegmagok
(~25%). (3) További immun-elektronmikroszkópos vizsgálatainkkal bebizonyítottuk,
 hogy az Arc eredetű NPY-t és agouti-releated peptidet egyaránt tartalmazó
idegnyúlványok szimmetrikus szinapszisokat létesítenek a GnRH neuronokkal. (4) A
glutamátot a központi idegrendszer területén a vezikuláris glutamát transzporterek 3
típusa (Vglut1-3) csomagolja szinaptikus vezíkdalakba. Igazoltuk, hogy a hím patkányok
GnRH idegsejtjeinek döntő többsége (~99%) glutamáterg kemotípusú is, mivel a GnRH
sejtestekben Vglut2 mRNS in situ hibridizációs jelet, az axonokban pedig Vglut2-
immunreaktivitást tudtunk kimutatni. Vizsgálatainkkal megállapítottuk, hogy a Vglut2-
immunreaktivitás ultrastruktúrális szinten a neuroszekretoros idegvégződésekből
található szinaptikus vezíkdalkhoz kötődik. A GnRH neuronhálózat újonnan leírt
glutamáterg kemotípusának funkcionális jelentőségére egyelőre nincsen magyarázat.
10. Literature cited


34 Gulyas AI, Megias M, Emri Z, Freund TF (1999) Total number and ratio of excitatory and inhibitory synapses converging onto single interneurons of different types in the CA1 area of the rat hippocampus. J Neurosci, 19:10082-10097


Kiss J, Halasz B (1985) Demonstration of serotonergic axons terminating on luteinizing hormone-releasing hormone neurons in the preoptic area of the rat using a combination of immunocytochemistry and high resolution autoradiography. Neuroscience, 14:69-78

Campbell RE, Herbison AE (2007) Definition of brainstem afferents to gonadotropin-releasing hormone (GnRH) neurons in the mouse using conditional viral tract tracing. Endocrinology,


Todman MG, Han SK, Herbison AE (2005) Profiling neurotransmitter receptor expression in mouse gonadotropin-releasing hormone neurons using green


65 MacLusky NJ, Naftolin F, Leranth C (1988) Immunocytochemical evidence for direct synaptic connections between corticotrophin-releasing factor (CRF) and gonadotrophin-releasing hormone (GnRH)-containing neurons in the preoptic area of the rat. Brain Res, 439:391-395


Everett JW (1964) Central Neural Control of Reproductive Functions of the Adenohypophysis. Physiol Rev, 44:373-431


Raposinho PD, Pierroz DD, Broqua P, White RB, Pedrazzini T, Aubert ML (2001) Chronic administration of neuropeptide Y into the lateral ventricle of C57BL/6J male mice produces an obesity syndrome including hyperphagia, hyperleptinemia, insulin resistance, and hypogonadism. Mol Cell Endocrinol, 185:195-204.


Lendvai B, Vizi ES (in press) Nonsynaptic Chemical Transmission through Nicotinic Acetylcholine Receptors. Physiological Reviews,


Zaborszky L, Duque A (2003) Sleep-wake mechanisms and basal forebrain circuitry. Front Biosci, 8:d1146-1169

171 Sannella MI, Petersen SL (1997) Dual label in situ hybridization studies provide evidence that luteinizing hormone-releasing hormone neurons do not synthesize messenger ribonucleic acid for mu, kappa, or delta opiate receptors. Endocrinology, 138:1667-1672
177 Takahashi KA, Cone RD (2005) Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons. Endocrinology, 146:1043-1047


205 Kawakami S (2000) Glial and neuronal localization of ionotropic glutamate receptor subunit-immunoreactivities in the median eminence of female rats: GluR2/3 and GluR6/7 colocalize with vimentin, not with glial fibrillary acidic protein (GFAP). Brain Res, 858:198-204


11. Bibliography

11.1. List of publications related to the subject of the thesis

1. **Turi GF**, Liposits Z, Hrabovszky E.  
Cholinergic afferents to gonadotropin-releasing hormone neurons of the rat  
Neurochemistry International 2007 Sep 8; [Epub ahead of print]

2. Hrabovszky E, Deli L, **Turi GF**, Kallo I, Liposits Z.  
Glutamatergic innervation of the hypothalamic median eminence and posterior pituitary  
of the rat  

3. Hrabovszky E, **Turi GF**, Kallo I, Liposits Z.  
Expression of vesicular glutamate transporter-2 in gonadotropin-releasing hormone  
eurons of the adult male rat  
Endocrinology 2004 Sep; 145(9):4018-21.

Origin of neuropeptide Y-containing afferents to gonadotropin-releasing hormone  
eurons in male mice  

11.2. List of other publications

Expression of vesicular glutamate transporter-2 in gonadotrope and thyrotrope cells  
of the rat pituitary. Regulation by estrogen and thyroid hormone status  

6. Hrabovszky E, Csapo AK, Kallo I, Wilheim T, **Turi GF**, Liposits Z.
Localization and osmotic regulation of vesicular glutamate transporter-2 in magnocellular neurons of the rat hypothalamus

7. Hrabovszky E, Turi GF, Liposits Z. Presence of vesicular glutamate transporter-2 in hypophysiotropic somatostatin but not growth hormone-releasing hormone neurons of the male rat


12. Acknowledgements

First, I would like to thank my supervisor Professor Zsolt Liposits for giving me the opportunity to work in his research group in a very stimulating atmosphere. I also would like to thank Dr. Erik Hrabovszky who was my tutor during the undergraduate and Ph.D. years and helped my Ph.D. work with valuable ideas and practical advices.

Further, I am grateful to all of my colleagues in the Laboratory of Endocrine Neurobiology, Bekó Norbertné Hajni, Dr. Imre Farkas, Dr. Csaba Fekete, Dr. Balázs Gereben and Dr. Imre Kalló. I could always turn to them with my questions and problems and they helped my work with invaluable advices.

I would like to express my heartfelt gratitude for my closest colleagues Levente Deli, Tamás Füzesi, Vivien Hársfalvi, Edit Juhász, Andrea Kádár, Judit Menyhért, Ágnes Simon, Dr. Patrícia Varjú, Barbara Vida, Gábor Wittmann and Dr. Anikó Zeöld for the joyful atmosphere in the laboratory and the great hours that we spent together.

Finally, I will be grateful forever to my beloved parents and my sister for their encouragement and support during my studies and my life.