

Elucidation of the central regulation of the  
hypophysiotropic corticotropin-releasing hormone-  
and thyrotropin-releasing hormone-synthesizing  
neurons in the rat

Ph.D. Thesis

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## Introduction

The paraventricular nucleus of the hypothalamus (PVN) contains corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) synthesizing neurons that play critical role in the maintenance of energy homeostasis. The hypophysiotropic CRH neurons are the central regulators of the hypothalamic-pituitary-adrenocortical (HPA) axis, while the hypophysiotropic TRH neurons, which are located in the periventricular and parvocellular subdivisions of the PVN in rats, govern the hypothalamic-pituitary-thyroid (HPT) axis. Hypophysiotropic CRH and TRH neurons integrate a wide variety of humoral and neural signals and serve as final common pathways in the regulation of the HPA and HPT axes, respectively.

Neuropeptide Y (NPY), a highly potent orexigenic factor, is one of the most important regulators of the hypophysiotropic CRH neurons. The main sources of the NPY innervation in the PVN are the brainstem catecholaminergic neurons and the arcuate nucleus. The brainstem catecholaminergic neurons are known to mediate the response to several stressors, such as inflammation and glucoprivation. The catecholaminergic NPY innervation originate from the adrenergic C1-3 and the noradrenergic A1, A2 and A6 regions. The adrenergic neurons contain phenylethanolamine-N-methyltransferase (PNMT) and dopamine- $\beta$ -hydroxylase (DBH) enzymes, which are essential for adrenaline synthesis, while the noradrenergic neurons contain only DBH, which produces noradrenaline, without the presence of PNMT, therefore the presence or lack of these enzymes can be used to identify NPY axons originating from the noradrenergic or adrenergic cell groups. The arcuate nucleus is the primary central target of the energy homeostasis related peripheral hormones. This nucleus has crucial role in the regulation of food consumption and metabolism. One of the feeding related neuronal groups of the arcuate is the orexigenic NPY neuronal population that also synthesizes agouti-related protein (AGRP). As AGRP is synthesized exclusively in this neuronal group, this peptide can be used as a maker of NPY-containing axons originating from the arcuate nucleus. Using selective arcuate nucleus lesioning and multiple-labeling immunofluorescence, we determined the contribution of the arcuate nucleus and the brainstem catecholaminergic neurons in the NPY innervation of the hypophysiotropic CRH neurons.

However, the role of NPY in the regulation of the hypophysiotropic CRH neurons is controversial. Acute icv. administration of NPY results a marked increase in the CRH mRNA levels in the PVN, while in contrast, during fasting, when the NPY is stimulated in the arcuate nucleus, the hypophysiotropic CRH neurons are inhibited. To elucidate the role of NPY during fasting, we performed chronic icv. injection of NPY and examined its effect on the CRH mRNA levels in the PVN.

The brainstem catecholaminergic neurons are also known as regulators of the hypophysiotropic TRH neurons. The brainstem catecholaminergic neurons are essential for the mediation of cold stress to the TRH neurons in the PVN. However, the relative contribution of the adrenergic and noradrenergic neurons to this ascending pathway is unknown. To examine this contribution, we performed multiple labeling immunocytochemistry, using antibodies against DBH, PNMT and TRH.

Non-hypophysiotropic TRH neurons located in the anterior parvocellular subdivision of the PVN (aPVN) are suggested to play important function in the regulation of energy homeostasis, since they receive dense innervation from feeding related neurons in the arcuate nucleus. To determine how these TRH neurons are integrated within the brain, the major projection fields of this cell group were studied by anterograde and retrograde tract-tracing methods.

## **Specific aims**

1. Determine the relative contribution of the arcuate nucleus and the brainstem catecholaminergic neurons in the NPY innervation of the hypophysiotropic CRH neurons
2. Examine the effect of chronic NPY administration on the CRH gene expression of the hypophysiotropic neurons
3. Elucidate the relative contribution of the brainstem adrenergic and noradrenergic neurons in the catecholaminergic innervation of the hypophysiotropic TRH neurons
4. Reveal the efferent projections of the TRH neurons located in the aPVN

## Materials and Methods

### Animals

All experiments were carried out on adult male Wistar (TOXI-COOP KKT, Budapest, Hungary) and Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 260-400 g, housed under standard environmental conditions (light between 0600 and 1800 h, temperature  $22 \pm 1$  C, rat chow and water *ad libitum*). All experimental protocols were reviewed and approved by the Animal Welfare Committee at the Institute of Experimental Medicine of the Hungarian Academy of Sciences and Tufts Medical Center.

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

**Table 1.** Summary of main methods used in the different experiments of the thesis

Study	Method
1. NPY-immunoreactive (IR) innervation of CRH-IR neurons in the PVN	Multiple-labeling immunofluorescence, monosodium glutamate treatment
2. Effect of chronic NPY administration on the CRH mRNA level in the PVN	Central peptide administration Quantitative isotopic <i>in situ</i> hybridization
3. Catecholaminergic innervation of hypophysiotropic TRH neurons	Triple-labeling immunofluorescence
4. Efferent projections of the TRH neurons in the aPVN	Anterograde and retrograde neuronal tract-tracing, double-labeling immunofluorescence

### Monosodium glutamate treatment (study 1.)

To ablate the NPY neurons of the arcuate nucleus, a chemical lesion of this region was performed by monosodium glutamate (MSG) treatment. Six neonatal Wistar rats were injected subcutan with MSG solution (dissolved in distilled water): on postnatal days 2 and 4 at a dose of 2 mg/g body weight, and on postnatal days 6, 8, and 10, at a dose of 4 mg/g body weight. Control animals were treated with the same volume of saline.

### **Multiple-labeling immunocytochemistry (studies 1., 3.)**

Low dose (40µg), icv colchicine treatment was used to visualize the CRH and TRH perykaria, and 20 hours later the animals were transcardially perfused with paraformaldehyde containing fixative. Sections from the hypothalamus were cut using a Leica freezing microtome, processed for immunocytochemistry and incubated in dilutions of specific primary antisera. Quadruple-labeling immunofluorescence was performed using antibodies against CRH, NPY, DBH and PNMT to examine the relative contribution of the brainstem adrenergic and noradrenergic neurons to the NPY innervation of the hypophysiotropic CRH neurons (study 1.). For immunofluorescent labeling AMCA, CY<sup>5</sup>, CY<sup>3</sup> and FITC conjugated antibodies (Jackson Immunoresearch) were used, respectively. To elucidate the relative contribution of the arcuate nucleus, triple-labeling immunocytochemistry was performed on the hypothalamic sections of MSG-treated rats using antibodies against CRH, NPY and DBH (fluorochromes: AMCA, CY<sup>5</sup> and CY<sup>3</sup>, respectively). To verify the results of the MSG treatment, additional triple-labeling immunocytochemistry was performed, using antibodies against CRH, NPY and AGRP (fluorochromes: AMCA, CY<sup>5</sup> and CY<sup>3</sup>, respectively). In study 3. antibodies against TRH, DBH and PNMT were used (fluorochromes: CY<sup>5</sup>, CY<sup>3</sup> and FITC respectively). Immunofluorescent specimens were analyzed with a Zeiss epifluorescent microscope or Bio-Rad Radiance 2000 confocal microscope.

### **Quantitative isotopic *in situ* hybridization (study 2.)**

Animals were icv. injected with artificial cerebrospinal fluid (group 1, n = 8), or 10 µg/24 h NPY (Peninsula) (group 2, n = 7) in artificial cerebral spinal fluid for 3 d at a rate of 1 µl/h using osmotic minipumps. Then the rats were perfused transcardially with paraformaldehyde. Sections of the hypothalamus were cut on a cryostat (Leica), mounted on Superfrost Plus glass slides. Every fourth section of the PVN was hybridized with a 976-bp single-stranded [<sup>35</sup>S]UTP-labeled cRNA probe for CRH. Slides were dipped into Kodak NTB2 autoradiography emulsion (Eastman Kodak, Rochester, NY), and the autoradiograms were developed after 2 wk of exposure at 4 °C. Hybridized sections were examined under darkfield illumination and analyzed by quantitative densitometry on a Macintosh G3 computer using Scion Image software.

#### **Anterograde and retrograde tract-tracing experiment (study 4.)**

The anterograde tracer, Phaseolus vulgaris leucoagglutinin (PHAL, Vector Laboratories), was injected by iontophoresis into the region of aPVN of 17 animals. Through a burr hole in the skull, a glass micropipette (20  $\mu\text{m}$  outer tip diameter) filled with 2.5% PHAL in phosphate buffer was lowered into the brain at stereotaxic coordinates corresponding to the aPVN based on the atlas of Paxinos and Watson (6.0  $\mu\text{amps}$  for 11-15 min, pulsed at 7 second intervals). Rats were allowed to survive for 9-14 days and then perfused transcardially.

The retrograde tracer cholera toxin  $\beta$  subunit (CTB; List Biological Laboratories) was injected into specific brain regions where the majority of PHAL/pro-TRH-containing, double-labeled axons were found in the anterograde tract-tracing experiment (0.5% CTB, 6.0  $\mu\text{amps}$  for 11-15 min, pulsed at 7 second intervals). After a 6-10 day transport time, animals were injected with 60  $\mu\text{g}$  colchicine into the lateral cerebral ventricle to enhance the immunocytochemical detection of TRH in cell bodies. After 20 hours of survival, the animals were perfused.

Single-labeling immunohistochemistry for PHAL was performed to evaluate the injection sites, using rabbit PHAL antiserum (Vector Laboratories, visualization: nickel-diaminobenzidine), while double-labeling immunocytochemistry was performed to examine the projections of aPVN TRH using antibodies against PHAL and proTRH (fluorochromes: FITC and CY<sup>3</sup>, respectively).

The location of CTB injection sites and the distribution of CTB-containing TRH neurons in the aPVN and perifornical area were studied in double-immunolabeled sections, using antibodies against CTB and TRH (fluorochromes: FITC and CY<sup>3</sup>, respectively).

#### **Statistics**

All data are presented as mean  $\pm$  SEM. Densitometric data of CRH hybridization signals, and the results of the regional heterogeneity were compared by Student's t-test.

## Results

### **Origin of the NPY-IR innervation of the CRH neurons in the PVN of rats**

#### *Involvement of the brainstem noradrenergic and adrenergic cell groups in the NPY-immunoreactive innervation of CRH neurons*

NPY-, DBH- and PNMT-IR axons densely innervated the parvocellular subdivisions of the PVN. However, the distribution of the three fiber networks showed regional differences. NPY-IR axons and slightly less intensely DBH-IR axons inundated the ventral parvocellular subdivision, whereas the PNMT-IR axons were rare in this location. Furthermore, NPY-IR axons more densely innervated the periventricular parvocellular subdivision and the medial part of the medial parvocellular subdivision than PNMT-IR or DBH-IR fibers.

The area of the medial parvocellular subdivision, where the majority of the hypophysiotropic CRH neurons are located, was heavily innervated by all three afferent systems. As expected, the vast majority of PNMT-IR axons were also labeled for DBH. The majority of adrenergic (PNMT-IR) and a subpopulation of noradrenergic (DBH-IR but not PNMT-IR) axons also showed NPY-immunoreactivity. In the area of hypophysiotropic CRH neurons, the density of catecholaminergic NPY-IR fibers was much higher than the density of single-labeled NPY-IR axons. However, in the ventral and most caudal parts of the medial parvocellular subdivision, single-labeled NPY-IR fibers exceeded the density of catecholaminergic NPY-IR fibers.

NPY/PNMT-IR axon varicosities were found in juxtaposition to the vast majority of CRH neurons ( $94.2 \pm 1.1\%$ ). An average of  $5.5 \pm 0.4$  NPY/PNMT boutons per CRH cell was observed. Noradrenergic NPY/DBH-IR boutons were also found juxtaposed to the  $82.8 \pm 6.2\%$  of CRH neurons with an average of  $3.5 \pm 0.8$  NPY/DBH boutons per cell.

Of all NPY-containing axon varicosities located on the surface of CRH neurons,  $41.2 \pm 5.6\%$  contained both PNMT- and DBH-immunoreactivity, whereas  $22.2 \pm 3.0\%$  were only DBH-IR. An additional  $36.6 \pm 3.1\%$  of NPY-IR axon varicosities were only single-labeled, indicating that these fibers do not originate from catecholaminergic sources. However, the single-labeled NPY-IR fibers were found in juxtaposition to  $89.0 \pm 5.3\%$  of CRH neurons. These single-labeled NPY-IR axon varicosities were unevenly distributed and more frequently contacted the most posterior CRH neurons, and the

CRH neurons located laterally and in the ventral part of the medial parvocellular subdivision. Conversely,  $86.5 \pm 5.6\%$  of PNMT-IR boutons and  $47.8 \pm 12.0\%$  of DBH-IR, PNMT-immunonegative boutons on the surface of CRH neurons contained NPY.

With respect to the catecholaminergic boutons on the surface of CRH neurons,  $48.5 \pm 6.2\%$  contained DBH but not PNMT, suggesting that these varicosities produce noradrenaline, whereas  $51.6 \pm 6.2\%$  contained both DBH and PNMT, indicating their adrenergic phenotype.

#### *Effect of arcuate nucleus ablation on the NPY-IR innervation of CRH neurons*

Immunostaining against NPY in MSG-treated animals demonstrated that the vast majority of NPY-IR cells in the arcuate nucleus were ablated. Only a small number of NPY-IR neurons remained intact in the most caudal part of the arcuate nucleus.

Catecholaminergic axons more densely inundated the medial parvocellular subdivision, and fewer catecholaminergic/NPY-IR fibers were found in the ventral and caudal parts of this subdivision. In the ventral part of the medial parvocellular subdivision where the density of the single-labeled NPY axons was the heaviest in intact rats, only sparse remaining fibers were found in MSG-treated animals. In triple-labeled preparations, the density of single-labeled NPY axons dramatically decreased throughout the PVN. Only  $2.3 \pm 0.3$  single-labeled NPY boutons were found juxtaposed to CRH neurons, and these boutons comprised only  $8.2 \pm 2.3\%$  of the total number of NPY varicosities. The vast majority ( $91.8 \pm 2.3\%$ ) of the NPY boutons on the surface of the CRH neurons also contained DBH-immunoreactivity.

#### *Relative involvement of the arcuate nucleus in the NPY-IR innervation of CRH neurons*

AGRP/NPY-IR fibers arising from the arcuate nucleus densely innervated the parvocellular subdivisions of the PVN. The distribution of the AGRP/NPY fibers was similar to the distribution of single-labeled NPY-IR fibers in the quadruple-labeling studies: the dorsal part of the medial parvocellular subdivision of the PVN was less intensely innervated, whereas the ventral part of the medial parvocellular subdivision was covered by a dense network of AGRP-IR axons. Quantitative analysis of the regional heterogeneity of the AGRP innervation showed that in the caudal region, significantly more AGRP boutons were juxtaposed to CRH neurons than in the other regions of the parvocellular division. The relative contribution of AGRP/NPY-IR

varicosities to the innervation of the CRH neurons was significantly higher in the midventral region than in the middorsal region but significantly lower than in the caudal region.

Double-labeled NPY/AGRP-IR axon varicosities were found in juxtaposition to the vast majority of CRH neurons ( $94.3 \pm 0.7\%$ ). An average of  $7.1 \pm 0.3$  NPY/AGRP boutons per CRH cell was observed. Of all NPY-containing axon varicosities on the surface of CRH neurons,  $33.7 \pm 1.9\%$  contained AGRP-immunoreactivity.

### **Effects of chronic NPY administration on the CRH gene expression in rats**

NPY-treated animals consumed significantly more food (control vs. NPY,  $64.2 \pm 1.8$  vs.  $119.07 \pm 5.6$  g) and gained considerably more weight than controls (control vs. NPY,  $14.8 \pm 1.2$  vs.  $31.00 \pm 5.3\%$ ) during the 3 days of infusion.

In control animals, neurons containing CRH mRNA were readily visualized by *in situ* hybridization histochemistry, symmetrically distributed in the medial parvocellular subdivision of the PVN on both sides of the third ventricle. A 3 day central infusion of NPY resulted in a uniform decrease in the hybridization signal over the CRH neurons throughout the anterior-posterior extent of the PVN. By image analysis, the mean of integrated density values of CRH mRNA in the PVN of NPY-treated animals was approximately 30% of that of the control animals (control vs. NPY,  $77.1 \pm 15.9$  vs.  $23.9 \pm 2.7$  integrated density units).

### **Catecholaminergic innervation of the TRH neurons in the PVN of rats**

Both single-labeled DBH-IR and DBH/PNMT-IR axon varicosities were found in juxtaposition to the vast majority of proTRH-IR neurons ( $97.8 \pm 0.7\%$  and  $100\%$ , respectively). An average of  $7.4 \pm 1.0$  single-labeled DBH-IR boutons per TRH cell was observed while  $11.8 \pm 0.6$  PNMT-IR boutons were juxtaposed to TRH neurons. The relative contribution of noradrenergic axons to the catecholaminergic innervation of the hypophysiotropic TRH neurons was  $36.5 \pm 1.2\%$ . Adrenergic axons formed the remaining two thirds ( $63.5 \pm 1.2\%$ ) of the catecholaminergic innervation of TRH neurons. The results of the quantitative analysis showed that there were significantly more PNMT-IR varicosities juxtaposed to the TRH neurons in the lateral part of the

medial parvocellular subdivision of the PVN compared to the medial part of this subdivision.

### **Efferent projections of the TRH neurons located in the aPVN**

#### *Localization of PHAL injection sites*

To map the projection fields of the TRH neurons residing in the aPVN, PHAL was injected into this subnucleus of the PVN. In four animals, the core of the injection site covered the aPVN. In two of the four brains, cases 116 and 117, the PHAL injection site was centered halfway between the fornix and the third ventricle, corresponding to the lateral border of the aPVN. In these cases, the large cores of PHAL injections substantially overlapped with the location of TRH neurons in both the aPVN and the perifornical area. In case 124, PHAL was injected adjacent to the third ventricle, resulting in an injection site almost entirely confined to the aPVN and the anterior part of the periventricular parvocellular subdivision of the PVN. In case 125, the PHAL injection site covered the ventral part of the aPVN and the adjacent area.

#### *Distribution of double-labeled PHAL/proTRH-IR fibers projecting from the aPVN/perifornical area (cases 116 and 117)*

The distribution of PHAL/proTRH fibers was very similar in both cases. PHAL/proTRH fibers were found primarily on the ipsilateral side, but some scattered double-labeled fibers were also observed on the contralateral side in every major projection area. The contralateral projection was most profound in the lateral septal nucleus.

#### *Hypothalamus and preoptic region*

The density of PHAL/proTRH-IR fibers was moderate in the preoptic region and anterior hypothalamus. Double-labeled fibers were distributed broadly in these areas, extending to the medial preoptic nucleus, anterodorsal preoptic nucleus, strial part of the preoptic area, periventricular nucleus, anteroventral periventricular nucleus, posterior part of the medial preoptic area, and most anterior portions of the anterior hypothalamic area. Laterally, the dorsal part of the lateral hypothalamic area also contained PHAL/proTRH fibers.

More caudally in the hypothalamus, a high density of double-labeled axons was found throughout the retrochiasmatic area and the rostrocaudal extent of the arcuate nucleus. Most double-labeled fibers were distributed in the dorsomedial part of the arcuate nucleus, whereas a moderate density of fibers was observed in the lateral part of the nucleus, with only scattered fibers in its ventromedial part. In the dorsomedial part, the double-labeled fibers were oriented mainly rostrocaudally and frequently established large varicosities.

Large number of PHAL/proTRH-IR axons was found in the ventromedial nucleus. The double-labeled fibers were distributed primarily in the dorsomedial and medial parts of the nucleus. Several fibers ran parallel to the coronal plane and established several varicosities. Many double-labeled axons were also seen immediately rostral to the ventromedial nucleus, in the medial part of the subparaventricular zone. Several varicose PHAL/proTRH-IR axons were present between the arcuate and the ventromedial nuclei and between the borders of the ventromedial and dorsomedial nuclei.

Double-labeled fibers were also observed in the ventromedial part of the dorsomedial nucleus, rostral to the level of the compact part. More caudally, some double-labeled fibers were present in the compact and the ventral parts of the dorsomedial nucleus.

Several PHAL/proTRH-IR fibers were distributed in a broad region ventral to the fornix. This region included the tuber cinereum area, the medial tuberal nucleus, and the ventral premammillary nucleus. PHAL/proTRH-IR fibers were also found close to the ventral surface of the hypothalamus.

Beside these major hypothalamic areas, scattered PHAL/proTRH fibers were seen in several other hypothalamic nuclei, namely, the lateroanterior hypothalamic nucleus, suprachiasmatic nucleus, supraoptic nucleus, paraventricular nucleus, posterior part of the lateral hypothalamic area, posterior hypothalamic area, ventral tuberomammillary nucleus, and supramammillary nucleus.

#### *Thalamus and epithalamus*

A moderate density of PHAL/proTRH-IR fibers was found in the paraventricular nucleus of the thalamus, throughout its rostrocaudal extent. These fibers mainly ran

parallel to the coronal plane. Scattered PHAL/proTRH-IR axons were present in the nucleus reuniens, mediodorsal nucleus, and lateral habenular nucleus.

*Substantia innominata, amygdala, and hippocampus*

Some varicose double-labeled fibers and mediolaterally running, thin PHAL/proTRH axons that established a few boutons were observed in the substantia innominata. Varicose PHAL/proTRH-IR fibers were present in the medial and capsular parts of the central amygdaloid nucleus, especially between anteroposterior levels 1.7 and 2.2 mm caudal to the Bregma.

A high density of varicose PHAL/proTRH-IR axons was found in the medial amygdaloid nucleus, primarily in the posterodorsal and posteroventral subnuclei. These fibers were highly varicose, and many long fibers could be seen running parallel to the coronal plane. A moderate density of double-labeled fibers was also present in the intraamygdaloid division of the bed nucleus of the stria terminalis. More caudally, several PHAL/proTRH-IR axons were observed in a dense network of proTRH-IR fibers located in the amygdalohippocampal transition area. These PHAL/proTRH axons ran parallel to the coronal plane and established several medium-size to large varicosities. Several double-labeled axons were found in a discrete group of varicose proTRH-IR fibers medial to the amygdalohippocampal transition area near the dentate gyrus. PHAL/proTRH fibers were also present diffusely in the ventral hippocampus. Scattered PHAL/proTRH fibers were present in several areas, including the anterior cortical amygdaloid nucleus, posteromedial and posterolateral cortical amygdaloid nuclei, basomedial amygdaloid nucleus, piriform cortex, ventromedial part of the lateral amygdaloid nucleus, and fimbria of the hippocampus.

*Bed nucleus of the stria terminalis and septum*

PHAL/proTRH fibers were found in several divisions of the bed nucleus of the stria terminalis. Many of these fibers could be followed for a long distance, indicating the coronal orientation of the fibers. A high density of double-labeled fibers was present throughout the medial division. Among the nuclei of the lateral division, only the ventral and the posterior part contained significant numbers of double-labeled fibers. In the stria terminalis, passing PHAL/proTRH fibers establishing some boutons were

observed. In the lateral septal nucleus, proTRH-IR fibers established an extremely dense fiber network, frequently forming basket-like structures around the surface of perikarya and completely ensheathed dendrites. Varicose PHAL/proTRH-IR axons were densely distributed in the ventral part of the lateral septal nucleus, some of them forming basket-like shapes. The intermediate part of the lateral septal nucleus contained a moderate number of double-labeled fibers; in its medial part, double-labeled axons frequently ran dorsally, establishing a few en passant boutons. The dorsal part of the lateral septal nucleus contained only scattered double-labeled fibers in its most anterior portions.

#### *Other forebrain regions*

Scattered double-labeled fibers were found in the ventral pallidum, medial septal nucleus, nucleus of the horizontal limb of the diagonal band, nucleus of the vertical limb of the diagonal band, major island of Calleja, posterior part of the anterior olfactory nucleus, and the area adjacent dorsomedially to the dorsal endopiriform nucleus.

#### *Midbrain and hindbrain*

Some scattered PHAL/proTRH-IR fibers could be observed in the periaqueductal gray, lateral and ventrolateral periaqueductal gray, ventral tegmental area, and central part of the lateral parabrachial nucleus.

#### *Cases 124 and 125*

Despite the apparently limited spread of the tracer outside the aPVN, the distribution pattern of PHAL/proTRH axons in case 124 was very similar to that in cases 116 and 117, with only slight differences in the density of double-labeled fibers in some regions. In case 125, the general distribution of double-labeled axons showed similarities to the pattern seen in the brains described above. However, generally, fewer PHAL/proTRH axons were observed in all regions, and only sparse double-labeled axons were present in the medial amygdaloid nucleus, dorsomedial nucleus, and substantia innominata. No PHAL/proTRH fibers were observed near the dentate gyrus or in the ventral hippocampus.

*Distribution of retrogradely labeled TRH-IR cell bodies in the aPVN and perifornical area*

To differentiate between the projection sites of TRH neurons residing in the aPVN and perifornical area, the retrograde tracer CTB was injected into brain regions where a high or moderate density of the PHAL/proTRH-IR fibers was observed in the anterograde tracing experiment. CTB/TRH-IR neurons in the aPVN and perifornical area were found primarily ipsilateral to the injection sites, with fewer double-labeled neurons on the contralateral side. The retrogradely labeled TRH neurons in the aPVN and perifornical region were counted in each brain with a successful CTB injection. Moderate to high numbers of CTB-containing TRH neurons in the aPVN were found after CTB injections into the arcuate nucleus, dorsomedial nucleus, medial preoptic area, caudal tuber cinereum area, ventral premammillary nucleus, anterior and posterior parts of the medial division of the Bed nucleus of stria terminalis, paraventricular nucleus of the thalamus, central amygdaloid nucleus, and ventral part of the lateral septal nucleus. Only a few CTB/TRH-IR neurons, fewer than 10/animal, were found in the aPVN after CTB injections into the ventromedial nucleus, medial amygdaloid nucleus, and amygdalohippocampal area. Moderate to high numbers of CTB-containing TRH neurons were present in the perifornical area after CTB injections into the ventral part of the lateral septal nucleus, ventromedial nucleus, dorsomedial nucleus, medial preoptic region, medial amygdaloid nucleus, amygdalohippocampal area, ventral premammillary nucleus, and posterior part of the medial division of the Bed nucleus of stria terminalis. The greatest number of retrogradely labeled perifornical TRH neurons was observed after CTB injection into the ventral part of the lateral septal nucleus. Only rare (fewer than 10/animal) perifornical TRH neurons contained CTB when injections were made into the arcuate nucleus, paraventricular nucleus of the thalamus, central amygdaloid nucleus, caudal part of the tuber cinereum area, and anterior part of the medial division of the Bed nucleus of stria terminalis.

In all of the CTB-injected brains, very few TRH neurons in hypophysiotropic parts of the PVN contained CTB. In the medial and periventricular parvocellular subdivisions, 10 or more CTB-IR TRH neurons/animal were found in only two cases: after CTB injection into the medial preoptic region (10 neurons) and the dorsomedial nucleus (12 neurons).

## Conclusions

1. We conclude that three major cell populations give rise to the NPY-immunoreactive innervation of hypophysiotropic CRH neurons: adrenergic and noradrenergic NPY-IR neurons of the brainstem, and NPY/AGRP neurons of the arcuate nucleus. Approximately, two thirds of the NPY innervation originates from the brainstem catecholaminergic neurons, while the remaining one third arises from the arcuate nucleus.
2. In spite of the known acute stimulatory effect of NPY on CRH neurons, we have revealed that the chronic administration of NPY decreases CRH mRNA expression in the PVN. Thus, we propose that NPY pathways ascending from the brainstem have an acute activating effect on CRH neurons in response to glucoprivation, infection, and inflammation, whereas neurons located in the arcuate nucleus exert a chronic inhibitory effect on CRH gene expression in association with fasting. The concept that NPY could exert both inhibitory and stimulatory effects on CRH neurons raises the possibility that NPY may exert different effects on the HPA axis depending upon the origin of the NPY input and nature of the specific physiological stimuli.
3. We have elucidated that, the adrenergic and noradrenergic neurons of the brainstem contribute unequally to the catecholaminergic innervation of hypophysiotropic TRH neurons, similarly to the innervation of the hypophysiotropic CRH neurons. We suggest that adrenergic and noradrenergic neurons may each mediate the effects of different physiological conditions on the HPT axis through direct effects on hypophysiotropic TRH neurons.
4. We have described the projection fields of aPVN TRH neurons. Our results raise the possibility that this non-hypophysiotropic TRH cell population might influence energy homeostasis, thermoregulation, prolactin synthesis, and possibly sexual function. In contrast, perifornical TRH neurons may be involved primarily in regulation of limbic functions and, therefore, may be functionally different from aPVN TRH neurons. Partially overlapping projections of the TRH neurons from both regions, and in particular the heavy innervation to the ventromedial nucleus, potentially implicate at least a subpopulation of perifornical TRH neurons in the control of food intake. These

studies provide important initial data from which physiological studies can be designed to better understand the potential role of these cell groups.

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## **List of publications underlying the thesis**

1. T. Füzesi, G. Wittmann, Z. Liposits, R.M. Lechan, C. Fekete  
Contribution of noradrenergic and adrenergic cell groups of the brainstem and agouti-related protein-synthesizing neurons of the arcuate nucleus to neuropeptide-y innervation of corticotropin-releasing hormone neurons in hypothalamic paraventricular nucleus of the rat.  
Endocrinology. 2007 Nov;148(11):5442-50.
2. G. Wittmann, T. Füzesi., P.S. Singru, Z. Liposits., R.M. Lechan, C. Fekete  
Efferent projections of thyrotropin-releasing hormone-synthesizing neurons residing in the anterior parvocellular subdivision of the hypothalamic paraventricular nucleus  
Journal of Comparative Neurology 2009 Jul 20;515(3):313-30
3. T. Füzesi; G. Wittmann, R.M. Lechan, Z. Liposits, C. Fekete  
Noradrenergic Innervation of Hypophysiotropic Thyrotropin-Releasing Hormone-Synthesizing Neurons in Rats  
Brain Research, 2009 Oct 19;1294:38-44. Epub 2009 Aug 6.

## **List of publications related to the subject of the thesis**

4. T. Füzesi, E. Sánchez, G. Wittmann, P.S. Singru, C. Fekete, R.M. Lechan  
Regulation of cocaine-and amphetamine-regulated transcript-(CART) synthesizing neurons of the hypothalamic paraventricular nucleus by endotoxin; Implications for LPS-induced regulation of energy homeostasis  
Journal of Neuroendocrinology, 2008 Sep;20(9):1058-66.
5. G. Wittmann, T. Füzesi, Z. Liposits, R.M. Lechan, C. Fekete  
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Distribution of Hypophysiotropic Thyrotropin-Releasing Hormone (TRH)-  
Synthesizing Neurons in the Hypothalamic Paraventricular Nucleus of the Mouse  
Journal of Comparative Neurology, manuscript accepted (2010).

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