Elucidation of feeding-related neuronal networks involved in the regulation of the hypophysiotropic thyrotropin-releasing hormone- and corticotropin-releasing hormone-synthesizing neurons in the rat

Ph.D. Thesis

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Budapest
2007

Introduction

The hypothalamic paraventricular nucleus (PVN) contains hypophysiotropic neuron populations that control the hormone secretion of the anterior pituitary gland. The hypophysiotropic thyrotropin-releasing hormone (TRH)-synthesizing neurons are located in the periventricular and medial parvocellular subdivisions of the PVN, and govern the hypothalamic-pituitary-thyroid (HPT) axis. The hypophysiotropic corticotropin-releasing hormone (CRH)-synthesizing neurons, located laterally from the TRH neurons in the medial parvocellular subdivision, are the central regulators of the hypothalamic-pituitary-adrenocortical (HPA) axis. Hypophysiotropic TRH and CRH neurons integrate a wide variety of humoral and neural signals and serve as final common pathways in the regulation of the HPT and HPA axes, respectively. These signals set the activity of the hypophysiotropic neurons to adapt to the altered external or internal environment. The achieved alterations in the peripheral levels of thyroid hormones and corticosterone play essential role for promoting survival during the periods of challenges, such as fasting or stress.

Both the HPT and HPA axes play critical role in maintaining energy homeostasis and normal metabolic processes. During fasting, a period of energy restriction, synthesis of TRH and CRH in the PVN are inhibited. This inhibition is caused primarily by the reduced circulating level of leptin, a white-adipose tissue-derived hormone. The effects of leptin on hypophysiotropic TRH and CRH neurons are mainly indirect, involving two separate cell groups in the hypothalamic arcuate nucleus: the cell group that produces the anorexigenic peptides alpha-melanocyte stimulating hormone (α-MSH) and cocaine- and amphetamine-regulated transcript (CART); and the neurons that synthesize the orexigenic peptides agouti-related protein (AGRP) and neuropeptide Y (NPY). Both types of neurons express leptin receptors, but their neuropeptide transmitter synthesis is oppositely regulated by leptin: α-MSH and CART gene expressions are increased, while AGRP and NPY expressions are inhibited by leptin administration. Conversely, during fasting, AGRP and NPY syntheses are increased, whereas α-MSH and CART syntheses are inhibited. Both α-MSH/CART and AGRP/NPY neurons send afferents to innervate hypophysiotropic TRH and CRH neurons. Since α-MSH and CART stimulate, while NPY and AGRP inhibit TRH synthesis, during fasting the decrease of stimulatory and increase of inhibitory signaling on TRH neurons result in an inhibition of TRH

production in hypophysiotropic neurons. Although it has not been so thoroughly studied yet, several data support the hypothesis that suppression of CRH synthesis in fasting animals is elicited similarly by arcuate nucleus α-MSH/CART and AGRP/NPY neurons.

Among the four arcuate nucleus-derived neuropeptides described above, the AGRP and α-MSH innervation of hypophysiotropic TRH and CRH neurons originate exclusively from the arcuate nucleus. In contrast, morphologic studies suggested that NPY- and CART-containing axons originating outside the arcuate nucleus also substantially contribute to the innervation of hypophysiotropic TRH and CRH neurons. A main focus of this Ph.D. thesis was to identify the sources of these NPY- and CART-containing axons. In addition, we also examined whether NPY and CART mRNA expressions are regulated by fasting in brainstem adrenergic neurons that co-synthesize these peptides and have been shown to innervate the hypophysiotropic TRH and CRH neurons. Finally we investigated whether galanin and galanin-like peptide (GALP), two closely related orexigenic peptides are also present in axons innervating hypophysiotropic TRH neurons.

Specific Aims

1. Determine whether adrenergic NPY-containing axon terminals innervate hypophysiotropic TRH neurons
2. Delineate the precise location of CART-containing cell populations that innervate the PVN
3. Reveal the contribution of adrenergic CART-containing fibers to the CART-immunoreactive innervation of TRH neurons
4. Demonstrate the presence of synaptic associations between CART-containing axon terminals and CRH neurons
5. Estimate the proportion of CART-containing afferents to CRH neurons that originate from the arcuate nucleus and adrenergic neurons of the medulla

6. Elucidate whether NPY and CART mRNA levels are changed during fasting in the C1-C3 regions
7. Examine whether galanin- and GALP-containing axons innervate TRH neurons in the PVN

Materials and Methods

Animals

The experiments were carried out on adult male Wistar (TOXI-COOP KKT, Budapest, Hungary) and Sprague-Dawley (Taconic Farms, Germantown, NY) rats weighing 200-500g. The animals were kept under standard environmental conditions (light between 06:00-18:00 h, temperature 22±1°C, rat chow and water *ad libitum*). All experimental protocols were reviewed and approved by the Animal Research Committees at the Institute of Experimental Medicine of the Hungarian Academy of Sciences and Tufts-New England 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. PNMT/NPY-immunoreactive (IR) innervation of proTRH-IR neurons in the PVN	Triple-labeling immunofluorescence
2. CART-IR innervation of the PVN	Retrograde neuronal tract-tracing, double-and triple-labeling immunofluorescence
3. CART/ PNMT-IR innervation of proTRH mRNA-containing perikarya in the PVN	Combined *in situ* hybridization and double labeling immunofluorescence
4. Ultrastructural examination of CART-IR innervation of CRH-containing neurons in the PVN	Double-labeling immunocytochemistry at electron microscopic level
5. CART/PNMT-IR and CART/α-MSH-IR innervation of CRH neurons in the PVN	Quadruple-labeling immunofluorescence, confocal-microscopic analysis
6. Expression of NPY and CART mRNA in medullary C1-C3 areas in fasting rats	Quantitative isotopic *in situ* hybridization
7. Galanin- and GALP-IR innervation of proTRH neurons in the PVN	Double-labeling immunocytochemistry at light- and electron microscopic level

Retrograde tract tracing (in study 2)

The retrogradely transported cholera toxin β subunit (CTB, List Biological Laboratories) was injected into 10 animals by iontophoresis (7.0 μamps for 15 min, pulsed at 7 second intervals) through a stereotaxically positioned glass micropipette (tip diameter 20 μm) targeting the parvocellular PVN, based on the coordinates of the atlas of Paxinos and Watson. Transport time of CTB was 6–7 days.

Light microscopic immunocytochemistry (in studies 1,2,5,7)

Animals received colchicine intracerebroventricularly ranging from 40-100μg for optimal detection of CART, CRH and proTRH in perikarya, and 20 hours later were transcardially perfused with fixative containing acrolein and paraformaldehyde. Sections were cut on a Leica freezing microtome, processed for immunocytochemistry and incubated in dilutions of specific primary antisera. For immunofluorescence, antigens were detected by AMCA-, FITC-, CY3- and CY5-conjugated secondary antibodies (Jackson ImmunoResearch), or by peroxidase-reaction catalyzed biotinylated tyramide amplification followed by AMCA- or FITC-conjugated avidin (Vector). Immunofluorescent specimens were analyzed with a Zeiss epifluorescent microscope or Bio-Rad Radiance 2000 confocal microscope. In study 7, antigens were detected by peroxidase reaction: galanin was labeled by silver intensified Ni-diaminobenzidine (Ni-DAB) precipitate yielding black color, proTRH was labeled by brown DAB precipitate.

Fluorescent in situ hybridization and immunofluorescence (in study 3)

Sections of paraformaldehyde-fixed brains were cut on a freezing microtome and were hybridized with a 1241 base long digoxigenin-11-UTP (Roche)-labeled antisense riboprobe for proTRH. Digoxigenin-labeled riboprobe was detected by peroxidase-conjugated anti-digoxigenin antibody (Roche), followed by biotinylated tyramide amplification and AMCA-conjugated avidin. Immunofluorescence detection of CART and PNMT was performed after *in situ* hybridization.

Electron microscopic immunocytochemistry (in study 4 and 7)

Animals were treated with colchicine (60μg, icv.) and perfused with a fixative containing acrolein and paraformaldehyde. Sections were cut on a vibratome and processed for immunocytochemistry. CART and galanin were detected with immunoperoxidase method yielding DAB precipitate. CRH and proTRH were detected using colloidal gold-conjugated secondary antibodies (Electron Microscopy

Sciences), followed by silver intensification with IntenSe silver enhancement reagents (GE Healthcare). Sections were osmificated, contrasted with uranyl-acetate and flat-embedded in Durcupan epoxy resin (Fluka). Ultrathin sections were cut with a Leica ultramicrotome, and examined with a Hitachi electron microscope.

Isotopic in situ hybridization (in study 6)

Animals were fed *ad libitum* or fasted for 64 hours, then perfused transcardially with paraformaldehyde. Sections of the medulla were cut on a cryostat (Leica), mounted on Superfrost Plus glass slides and hybridized with S^{35}-UTP (PerkinElmer)-labeled antisense riboprobes for NPY and CART. Slides were dipped in Kodak NTB autoradiography emulsion, and after 8d (NPY) or 10d (CART) of exposure, the autoradiograms were developed. Hybridized sections were examined under darkfield illumination and analyzed by quantitative densitometry using ImageJ software.

Statistics

All data are presented as mean ± SEM. Densitometric data of CART and NPY hybridization signals of fed and fasted animals were compared by Student's t-test.

Results

1. Contribution of adrenergic NPY-containing axons to the innervation of TRH neurons

Since adrenergic fibers densely innervate the hypophysiotropic TRH neurons, and the majority of adrenergic neurons in the C1-C3 areas co-express NPY, we determined the ratio of adrenergic NPY-containing axon terminals in the total NPY innervation of TRH neurons. Immunoreactivity for phenylethanolamine-N-methyltransferase (PNMT), the enzyme synthesizing adrenaline, was used as a marker of adrenergic axons. The majority (74.8 ± 1.1%) of PNMT-IR varicosities in contact with proTRH-IR neurons also contained NPY-immunoreactivity, while PNMT-immunoreactivity was found in 26.6 ± 2.2% of NPY-IR axon varicosities in close apposition to proTRH neurons.

2. Origin of CART-IR innervation of the PVN

As a first step to identify putative CART-expressing cell groups outside the arcuate nucleus that innervate TRH and CRH neurons, we injected the retrograde tracer CTB into the PVN, to map the origin of CART innervation of the PVN. Three

brains with CTB injection sites centered in the parvocellular parts of the PVN were used to map the CART-IR neurons projecting to the PVN. The distribution of double-labeled CART/CTB neurons was similar in all three brains. Numerous double-labeled CART/CTB-IR neurons were found in the retrochiasmatic area, arcuate nucleus, zona incerta, lateral hypothalamus/perifornical area, C1-C3 areas of the brainstem, and medial subnucleus of the nucleus tractus solitarius (NTS).

Colocalization of CART with other neurotransmitters in neurons projecting to the PVN

Triple immunofluorescent labeling revealed that in the zona incerta, lateral hypothalamus, and perifornical area, all neurons co-containing CTB and CART also expressed melanin-concentrating hormone (MCH). The majority of CART neurons throughout the C2 and C3 areas contained the adrenergic marker enzyme PNMT, whereas in the C1 area the colocalization of CART and PNMT was found only in its rostral and medial portions; no CART-IR neurons were observed in the caudal part of the C1 region. Of the neurons in the C1–3 regions that co-contained CART and PNMT, the majority also contained CTB. In contrast, none of the CART/CTB-containing neurons in the medial NTS contained PNMT.

3. Contribution of adrenergic fibers to the CART innervation of TRH neurons

In the periventricular and medial parvocellular subdivisions, where hypophysiotropic TRH neurons are located, both CART and PNMT-IR axon varicosities were found in close juxtaposition to nearly all proTRH mRNA-containing cell bodies. PNMT-immunoreactivity was present in 44.0 ± 3.6% of CART-IR axon varicosities in close apposition to proTRH neurons in the PVN. Conversely, approximately half of the PNMT-IR varicosities in contact with proTRH mRNA-containing neurons also contained CART-immunoreactivity in both the periventricular (51.3 ± 5.6%) and medial (53.2 ± 1.1%) parvocellular subdivisions.

4. Ultrastructural examination of CART-IR innervation of CRH neurons in the PVN

By ultrastructural analysis, DAB-labeled CART-IR terminals were seen to establish synapses on CRH neurons, the latter identified by the presence of the highly electron-dense silver particles. Tracing the juxtaposed CART-IR terminals and CRH-

IR neurons through a series of ultrathin sections, both axo-somatic and axo-dendritic synaptic specializations were observed.

5. Origin of CART-IR innervation of CRH neurons in the PVN

We used α-MSH and PNMT as markers to identify CART-IR axons arising from the arcuate nucleus and C1-C3 adrenergic cell groups, respectively. All α-MSH-containing axons co-contained CART in the PVN, as we have previously described, while CART was present in the vast majority of PNMT-IR fibers. CART/PNMT-IR axon varicosities were found in juxtaposition to nearly all (95.00 ± 1.53%) CRH neurons. An average of 5.45 ± 0.14 CART/PNMT boutons per CRH cell were observed. CART/α-MSH-containing boutons were also found in close proximity to CRH neurons but not as frequently as CART/PNMT boutons. CART/α-MSH fibers were juxtaposed to 58.67 ± 3.71% of CRH neurons, and an average of 2.73 ± 0.13 CART/α-MSH boutons were found on the surface of the innervated cells. Of all CART-containing axon varicosities located on the surface of CRH neurons, 59.60 ± 2.10% contained PNMT, whereas only 18.47 ± 1.55% contained α-MSH. An additional 21.93 ± 1.98% of CART-IR varicosities were exclusively single-labeled, suggesting that these axons originate from loci other than the arcuate nucleus and the C1-3 areas. These single-labeled CART-IR varicosities were juxtaposed to 68.33 ± 4.91% of CRH neurons.

6. Effect of fasting on NPY and CART mRNA levels of the medullary C1-C3 areas

The intensity of NPY mRNA hybridization in the C1-C3 areas was similar between fed and fasted animals. Densitometric analysis of the middle portion of the C1 area (approximately between -12.8 and -12.5 mm from the Bregma), and C2 and C3 regions did not reveal significant differences between the fed and fasted group in either the number of NPY mRNA expressing cells, or in the intensity of hybridization signals of individual neurons in each regions.

Quantitative densitometry of the middle portion of the C1 area revealed no differences in the number of CART mRNA expressing cells, and in the area and integrated density values of CART mRNA positive cells between fed and fasted animals, while the mean gray value of hybridized C1 neurons was slightly but

significantly reduced in fasted animals compared to fed controls (fasted vs. fed: 73.07 ± 1.25 vs. 77.01 ± 1.07, p=0.023). We did not detect significant differences between fed and fasted animals in any of the measured parameters in the C3 region. Since we could not identify unambiguously C2 neurons in our sections due to the close location of a large population of CART neurons in the medial NTS that are not adrenergic, we did not analyze the CART mRNA expression of the C2 area.

7. Galanin- and GALP-IR innervation of proTRH-containing neurons in the PVN

Galanin-IR axon varicosities were closely apposed to the majority of TRH neurons: in the periventricular and medial parvocellular subdivisions, 75.8 ± 6.7% and 61.6 ± 3.4% of the proTRH containing neurons were in juxtaposition with galanin-IR axon terminals, respectively.

By ultrastructural analysis, DAB labeled galanin-IR terminals were seen to establish synapses on proTRH neurons, the latter recognized by the presence of the highly electron-dense silver particles. In all instances, the synapses were found to be of the symmetric type.

GALP-IR fibers were found in juxtaposition to only a small number of proTRH-IR neurons in the PVN. In the periventricular and medial parvocellular subdivisions, only 9.6 ± 1.0% and 2.1 ± 0.2% of the proTRH-IR neurons were in juxtaposition with GALP-IR fibers.

Conclusions

1. In addition to the arcuate nucleus, adrenergic neurons in the C1-C3 regions that co-express both CART and NPY, represent another major source of NPY and CART innervation of hypophysiotropic TRH and CRH neurons. In contrast to the arcuate nucleus where NPY and CART mRNA levels change robustly during fasting, we found that NPY and CART mRNA levels in the C1-C3 regions are practically unchanged during starvation. Thus, NPY and CART expressed by adrenergic neurons probably do not contribute to the suppression of TRH and CRH synthesis in the PVN during fasting. Medullary adrenergic neurons have been implicated in the activation of CRH neurons in states of hypoglycemia and infection/inflammation, while adrenergic stimulation of TRH neurons is suggested in cold exposure. CART and NPY may have important roles in eliciting or modulating these effects on hypophysiotropic TRH and CRH neurons.

2. Our results indicate that CRH neurons receive additional CART-IR innervation from outside the arcuate nucleus and C1-C3 areas, from either one or more of the CART cell groups located in the zona incerta and lateral hypothalamus, the NTS and locally in the PVN. These cell populations may contribute to the innervation of hypophysiotropic TRH neurons as well.

3. Finally, galanin but not GALP, is another orexigenic peptide that is in anatomic position to regulate TRH neurons.

We conclude that TRH and CRH neurons are innervated by axons containing feeding-related peptides that arise from multiple sources and differentially regulate these hypophysiotropic neurons.

Acknowledgements

I express my deep gratitude to Csaba Fekete, my tutor. He devoted so much time and attention to teach me scientific research from the years I was a graduate student at university. I thank him that he has fully promoted my professional development.

I am very grateful to Professor Zsolt Liposits, my co-tutor and Head of the Laboratory of Endocrine Neurobiology, who has provided me absolute support to progress in scientific research.

My sincere thanks to Professor Ronald M. Lechan at Tufts University in Boston, for his important contribution to the papers of this PhD thesis.

Thanks to Summit Sarkar who performed the GALP-TRH immunocytochemistry at Tufts University.

I wish to express my thanks to our assistants whom I worked together with: Zsuzsanna Fodor, Éva Laki, Andrea Kádár and Ágnes Simon. I appreciate their careful and precise work that was a great help of me.

Special thanks to my closest colleagues Tamás Füzesi and Judit Menyhért for succesful cooperation and the joyful hours spent together, and to Gergely Túri and Barbara Vida for their fellowship.

I would like to thank all of my colleagues in the Laboratory of Endocrine Neurobiology for the many pieces of advice and technical helps, and for their great companionship: Bekó Norbertné, Levente Deli, Orsolya Dohán, Márton Doleschall, Imre Farkas, Balázs Gereben, Vivien Hársfalvi, Erik Hrabovszky, Csilla Jekkel, Edit Juhász, Imre Kalló, Miklós Sárvári, Patrícia Varjú, Anikó Zeöld.

I would also like to express my thanks to the members of the Medical Gene Technological Unit, especially to Balázs Bényei, head of the Unit, and to Mária Szűcs and Rozália Szafner for having always been helpful when I worked in the animal facility.

List of publications underlying the thesis

1. Wittmann G., Liposits Z., Lechan R.M., Fekete C.
   Medullary adrenergic neurons contribute to the neuropeptide Y-ergic innervation of hypophysiotropic thyrotropin-releasing hormone-synthesizing neurons in the rat
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2. Fekete C., Wittmann G., Liposits Z., Lechan R.M.
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List of publications related to the subject of the thesis

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