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Research Report

Interconnection between orexigenic neuropeptide Y- and anorexigenic α -melanocyte stimulating hormone-synthesizing neuronal systems of the human hypothalamus

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ABSTRACT

Peripheral feeding-related hormones such as leptin, insulin, and ghrelin exert their main central effects through neuropeptide Y- (NPY) synthesizing and α -melanocyte-stimulating hormone- (α -MSH) synthesizing neurons of the hypothalamic arcuate nucleus. In rodents, recent reports have described an asymmetric signaling between these neuron populations by showing that while NPY influences α -MSH-synthesizing neurons, the melanocortin-receptor agonist Melanotan II (MTII) does not modulate the electrophysiological properties of NPY neurons. The functional neuroanatomy of the relationship between these cell populations is unknown in humans. The aim of the current study was to analyze the putative relationship of the orexigenic NPY and anorexigenic α -MSH systems in the infundibular nucleus of the human hypothalamus, the analogue of the rodent arcuate nucleus. Double-labeling fluorescent immunocytochemistry for NPY and α -MSH was performed on postmortem sections of the human hypothalamus. The sections were analyzed by confocal laser microscopy. Both NPY- and α -MSH-immunoreactive (IR) neurons were embedded in dense, intermingling networks of NPY- and α -MSH-IR axons in the human infundibular nucleus. NPY-IR varicosities were observed in juxtaposition to all α -MSH-IR neurons. The mean number of NPY-IR axon varicosities on the surface of an α -MSH-IR neuron was approximately six. The majority of NPY-IR neurons were also contacted by α -MSH-IR varicosities, although, the number of such contacts was lower (two α -MSH-IR varicosities per NPY neuron). In summary, the present data demonstrate that these two antagonistic, feeding-related neuronal systems are interconnected in the infundibular nucleus, and the neuronal wiring possesses an asymmetric character in the human hypothalamus.

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1. Introduction

The balance of food intake and energy expenditure is tightly regulated by the brain (Friedman, 1997; Schwartz et al., 2000). The hypothalamus is a prominent constituent of this central regulatory system (Friedman, 1997; Schwartz et al., 2000). It integrates feeding-related neuronal inputs derived from multiple loci of the central nervous system (CNS), senses peripheral metabolic signals such as leptin, insulin, and ghrelin, and regulates the energy homeostasis via its efferent neuroendocrine and autonomic neuronal pathways (Schwartz et al., 2000). Peripheral hormones act as mediators between the energy stores of the body and the brain (Flier,

1998; Schwartz et al., 2000). Although leptin, insulin and ghrelin are secreted by different tissues (Kojima et al., 1999; Schwartz et al., 2000), their circulating levels are related to the amount of stored energy (Benoit et al., 2004; Cummings et al., 2005).

The hypothalamic arcuate nucleus plays a primary role in the signaling of these peripheral satiety-related hormones (Hewson et al., 2002). Chemical ablation of the arcuate nucleus results in an obese phenotype and severe leptin resistance (Dawson et al., 1997). The arcuate nucleus contains two antagonistic neuronal populations that are sensitive to all three peripheral hormones (Hewson et al., 2002; Schwartz et al., 2000). A medially located cell population expresses the potent orexigenic peptide,

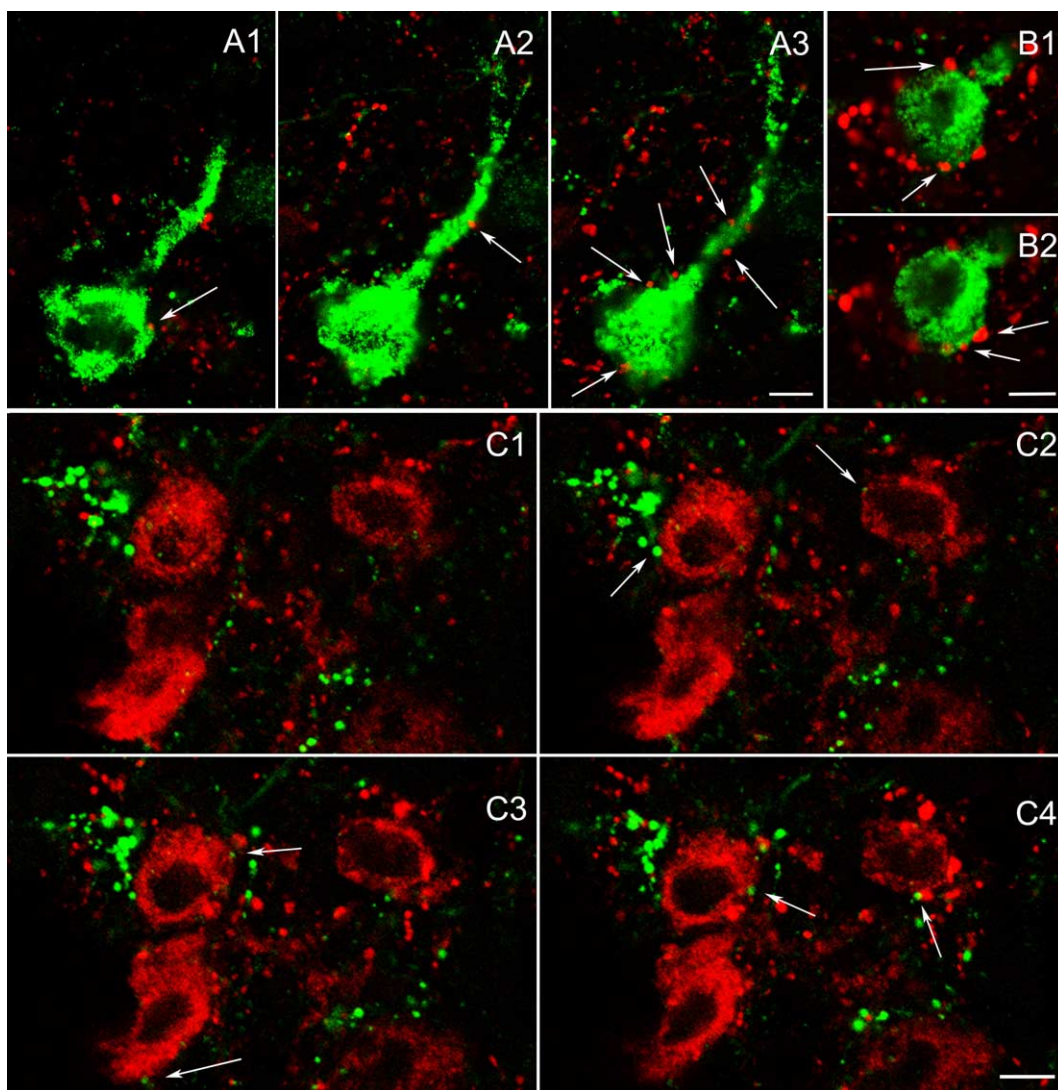


Fig. 1 – Interconnections of α -MSH- and NPY-IR neuron populations in the infundibular nucleus of the human brain. (A–B) High power magnification images show NPY-IR varicosities (red, arrows) in juxtaposition to α -MSH-IR (green) neurons. (A1–A3, B1–B2) Images of single optical slices illustrate two α -MSH-IR neurons in different focal planes. Note the high number of NPY-IR varicosities in juxtaposition to the α -MSH-IR perikarya. (C1–C4) High power magnification images of the same field in different focal planes demonstrate α -MSH-IR varicosities in contact with NPY-IR neurons (arrows). α -MSH varicosities are juxtaposed to most of the NPY cell bodies; however, only one to three α -MSH-IR varicosities are in contact with the NPY cells. The thickness of optical slices is 0.7 μ m. Scale bars in panels A–C are 10 μ m.

neuropeptide Y (NPY), while the laterally located cell group synthesizes the anorexigenic peptide, α -melanocyte stimulating hormone (α -MSH) (Schwartz et al., 2000). Both neuron populations are regulated by peripheral metabolic signals and send projections to other feeding-related centers of the brain, where they regulate the activity of the second order neurons of the feeding circuits, like the thyrotropin-releasing hormone (TRH)- and corticotropin-releasing hormone (CRH)-synthesizing neurons of the hypothalamic paraventricular nucleus (Mihaly et al., 2002; Mihaly et al., 2000; Schwartz et al., 2000).

For the maintenance of the normal body weight, an accurately regulated balance between the activity of NPY and α -MSH systems is required. In addition to the central effects of hormones regulating feeding and metabolism, the direct interaction of the orexigenic and anorexigenic neuronal populations may also contribute to the regulatory mechanisms by coordinating and modulating the gene expression and the electrophysiological activity of the two functionally antagonistic systems (Cowley et al., 2001; Roseberry et al., 2004). In the rat, an asymmetric interplay has been revealed between NPY and POMC neurons by patch clamp electrophysiology (Cowley et al., 2001; Roseberry et al., 2004). These data implicate that the orexigenic NPY-ergic system exerts a constant inhibitory tone over the POMC/ α -MSH neurons (Roseberry et al., 2004).

In the present study, we have examined the anatomical basis of a putative communication between NPY and α -MSH neurons located in the infundibular nucleus of the human hypothalamus, the homologous structure of rodent's arcuate nucleus.

2. Results

NPY- and α -MSH-IR neurons were present in the entire length of the infundibular nucleus of the human hypothalamus. NPY-IR cells were of small to medium size in a range of 14–30 μm (average size: $21.0 \pm 0.5 \mu\text{m}$) and displayed fusiform or multipolar shapes. The α -MSH-IR cells were significantly larger ($26.1 \pm 0.6 \mu\text{m}$; $P > 0.001$) in a range of 16–36 μm and multipolar in shape. While the two neuronal populations were intermingled in the infundibular nucleus, colocalization of the two peptides was not observed. NPY and α -MSH-IR axons and their terminals were numerous in the infundibular nucleus. The two axon systems were interwoven in this region and formed reticular networks that hosted the NPY- and α -MSH-IR cell bodies and dendrites.

The α -MSH-IR neurons were intensely surrounded by NPY-IR varicosities. The vast majority of the α -MSH-IR cells, $97.00 \pm 1.00\%$, was contacted by NPY-containing axon varicosities (Figs. 1A, B). In some instances, the NPY-IR varicosities encircled the α -MSH-IR cell bodies (Fig. 1B). An average of 6.56 ± 1.32 NPY-IR boutons were found on the surface of an α -MSH-IR perikaryon.

α -MSH-IR axon varicosities contacted $79.67 \pm 8.33\%$ of NPY-IR neurons. However, only an average of 2.27 ± 0.10 α -MSH-IR

varicosities were observed in juxtaposition to an NPY-IR neuron (Fig. 1C).

3. Discussion

In accordance with earlier studies from our group (Mihaly et al., 2000), we have found that NPY- and α -MSH-synthesizing neurons form separate neuronal populations in the infundibular nucleus of the human hypothalamus and densely innervate this brain region. To explore whether the two antagonistic neuronal populations are interconnected with each other, we have performed double label immunocytochemistry combined with confocal microscopic analysis on postmortem human hypothalamic tissue samples.

We have found that the majority of α -MSH-synthesizing neurons were heavily contacted by NPY-IR varicosities. An average of 6 NPY varicosities were in juxtaposition to the cell surface of the α -MSH-IR neurons. This innervation pattern is highly similar to what has been observed in rats by several groups (Cowley et al., 2001; Csiffary et al., 1990; Horvath et al., 1992). However, the origin of the NPY-IR innervation of the α -MSH-IR neurons has not been studied yet. In rats, the NPY-IR innervation of the hypothalamus derives from two major loci: the arcuate nucleus and catecholaminergic cell groups of the brainstem (Bai et al., 1985; Sawchenko et al., 1985). The catecholaminergic neurons also coexpress vesicular glutamate transporter 2, in addition to NPY (Hokfelt et al., 1983; Sawchenko et al., 1985; Stornetta et al., 2002), and establish asymmetric synapses (Liposits et al., 1986) suggesting a glutamatergic phenotype of these neurons. The innervation of the α -MSH neurons by GABA-ergic, NPY-containing terminals (Cowley et al., 2001) indicates that the arcuate nucleus, where NPY colocalizes with the GABA synthesizing enzyme, glutamic acid decarboxylase (Horvath et al., 1997), is a major source of the NPY innervation to α -MSH neurons.

It has been shown that NPY exerts inhibitory effects on the α -MSH-synthesizing neurons. NPY hyperpolarizes the α -MSH neurons through Y1 receptors (Roseberry et al., 2004) and decreases the α -MSH level in the hypothalamus (Blasquez et al., 1995). Cowley et al. (2001) have demonstrated that GABA also exerts an inhibitory tone on the POMC neurons suggesting that GABA and NPY may act in concert to potentiate the direct, inhibitory effects of the fasting induced fall of peripheral leptin levels on the α -MSH-synthesizing neurons.

In the human brain, the NPY and α -MSH neurons establish reciprocal connections. As we have demonstrated in this study, NPY-IR neurons are also contacted by α -MSH-IR varicosities in the infundibular nucleus. Since there are only two possible sources of the α -MSH-IR fibers, the arcuate nucleus and the nucleus of the solitary tract (NTS) (Cone, 2005), and the latter seems to play only a minor role in the innervation of the hypothalamus (Eskay et al., 1979), the α -MSH-IR innervation of NPY neurons is likely to originate from the α -MSH neurons of the arcuate nucleus. Our present results suggest that NPY- and α -MSH-IR neurons located in the infundibular nucleus form an interconnected network in humans, where the output of the two antagonistic neuronal populations is regulated and synchronized not only by the peripheral metabolic hormonal inputs, but also by the

bidirectional neuronal communication established between these neuron populations. While NPY neurons have been demonstrated to exert direct inhibitory effect on the α -MSH neurons (Roseberry et al., 2004), the role of α -MSH-synthesizing neurons is controversial in the regulation of NPY-producing cells. Despite the presence of melanocortin 3 receptors in the NPY neurons of the rat (Bagnol et al., 1999), Roseberry et al. (2004) have found that melanotan II (MTII), an agonist of α -MSH on the melanocortin 3 and 4 (MC3 and 4) receptors, has no discernible effect on the electrophysiological activity of NPY neurons in the arcuate nucleus. In contrast, central administration of MTII inhibits NPY gene expression in the arcuate nucleus (Zhang et al., 2004). In the rostral arcuate nucleus, 55% of NPY cells express MC3 receptor, but only 28% of the NPY neurons synthesize MC3 receptor in the caudal part of the arcuate nucleus (Bagnol et al., 1999). Therefore, the MC3 receptor containing NPY cells could have been underrepresented in the *in vitro* study (Roseberry et al., 2004), raising the possibility that α -MSH may influence at least a subpopulation of the NPY neurons. Moreover, the α -MSH neurons also express cocaine- and amphetamine-regulated transcript (CART) and vesicular glutamate transporter 2 (Collin et al., 2003; Elias et al., 1998), suggesting that the α -MSH neurons may influence those NPY neurons that are not responsive to MTII by CART, glutamate, or other yet unknown cotransmitters.

While we have found evidence for a reciprocal connection between the NPY and α -MSH neurons of the infundibular nucleus, this interaction seems to be asymmetrical. α -MSH neurons receive at least 3 times more NPY-IR contacts than the number of α -MSH varicosities contacting the surface of NPY cells. However, it is possible that the evolutionally conserved importance of the energy accumulation over the maintenance of an optimal body weight is behind the asymmetric connection of the two systems. Finally, the possibility also exist that brainstem or other NPY neuronal groups significantly contribute to the innervation of α -MSH neurons accounting partly for the asymmetry we describe here. Therefore, further studies are needed to elucidate the relative contribution of different NPY-synthesizing neuron populations of the brain to the innervation of α -MSH neurons.

In summary, we conclude that α -MSH and NPY neurons are anatomically interconnected in the infundibular nucleus of the human hypothalamus. However, in contrast to the very dense NPY-IR innervation of α -MSH-IR neurons, NPY neurons are less frequently contacted by α -MSH-IR varicosities. These data suggest an asymmetric communication between these functionally antagonistic neuron populations.

4. Experimental procedures

4.1. Tissue preparation

Diencephalic samples of three adult human individuals with no history of neurological or endocrinological disorders were obtained at autopsy. Tissue samples were taken within 24 h after death in accordance with the permission and regulations of the Regional Committee of Science and Research Ethics, Budapest, Hungary (permission number TUKEB 49/1999). The diencephalic blocks

were fixed in a mixture of 4% acrolein and 2% paraformaldehyde for 48 h at 4 °C, then cryoprotected in 30% sucrose and frozen on dry ice. Serial 30- μ m thick coronal sections were cut parallel to the lamina terminalis with a freezing microtome (Leica Microsystem, Nussloch GmbH, Germany) and stored in a freezing solution (30% ethylene glycol; 25% glycerol; 0.05 M PB) at -20 °C until used.

4.2. Double-labeling immunofluorescence of NPY- and α -MSH-IR elements in the infundibular nucleus

Double-labeling fluorescence immunocytochemistry was performed to study the interrelationship of α -MSH and NPY-IR elements in the infundibular nucleus. Sections were pretreated with 1% sodium borohydride in distilled water for 30 min. After rinsing in phosphate-buffered saline (PBS), the sections were transferred to 0.5% hydrogen peroxide and 0.5% Triton X-100 in PBS for 15 min. Then, they were immersed in 1% sodium acetate for 1 min, treated in graded dilutions of acetone (50, 70, 90, 100, and 90%) for 5 min each, washed in 70% ethanol, and then treated with 0.3% Sudan black in 70% ethanol for 30 min to reduce autofluorescence (Mihaly et al., 2000). Following differentiation in 70% ethanol, sections were washed in PBS, pretreated with 2% normal horse serum in PBS, and then incubated in a mixture of rabbit anti- α -MSH serum 1:1000 (Chemicon International Inc., Temecula, CA) and sheep anti-NPY serum 1:16,000 (gift from István Merchenthaler, School of Medicine University of Maryland at Baltimore, Baltimore, USA) for 48 h at 4 °C. After further washes in PBS, tissues were immersed in the cocktail of fluorescein isothiocyanate (FITC)-conjugated donkey-anti-rabbit IgG (1:50; Jackson ImmunoResearch, West Grove, PA) and Cy3-conjugated donkey-anti-sheep IgG (1:100; Jackson ImmunoResearch) overnight. The sections were mounted and then coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

The specificity of immunostaining with α -MSH antiserum was demonstrated by the loss of immunoreactivity after preabsorption of the diluted antiserum with an excess (10^{-5} M) of synthetic α -MSH peptide (Bachem, Bubendorf, Switzerland). Characterization of NPY antiserum has been characterized as described elsewhere (Wittmann et al., 2002).

The relationship of the two peptidergic systems was analyzed using a confocal laser microscope (Bio-Rad Laboratories, Hemel Hempstead, UK) and the following laser excitation lines: 488 nm for FITC and 543 nm for CY3 and Dichroic/emission filters 560 nm/500–530 nm for FITC and 560–625 for CY3. Pinhole sizes were set to obtain optical slices less than 0.7 μ m thick, and the series of optical slices were recorded with a 0.6 μ m Z step. The series of optical sections were merged and displayed with Laser Vox software and an IBM compatible personal computer. The largest diameter of the NPY- and α -MSH-IR perikarya was measured using Laser Vox software.

While tracing individual neurons through the series of optical sections, NPY-IR axon varicosities were counted on the surface of α -MSH-IR cells and also the number of α -MSH-IR boutons juxtaposed to NPY-IR perikarya and dendrites was counted to obtain semiquantitative estimate of the degree and intensity of the interneuronal communication between NPY- and α -MSH-IR elements in the infundibular nucleus.

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