Distribution and relationship of neurons containing feeding-related neuropeptides in the human hypothalamus

Ph.D. Thesis

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List of abbreviations

α-MSH	-	alpha-melanocyte stimulating hormone
ACTH	-	adrenocorticotrop hormone
AGRP	-	agouti-related protein
ARC	-	arcuate nucleus
BAT	-	brown adipose tissue
BBB	-	blood-brain barrier
CART	-	cocaine- and amphetamine-regulated transcript
CCK	-	cholecystokinin
CNS	-	central nervous system
CRH	-	corticotropin-releasing hormone
CVO	-	Circumventricular organ
DAB	-	diaminobenzidine
DMN	-	hypothalamic dorsomedial nucleus
FITC	-	fluorescein isothiocyanate
GABA	-	gamma-aminobutyric acid
GFP	-	green fluorescent protein
GHS-R	-	growth hormone secretagogue receptor
GLP1	-	glucagon-like peptide 1
HPA axis	-	hypothalamic-pituitary-adrenocortical axis
HPT axis	-	hypothalamic-pituitary-thyroid axis
icv.	-	intracerebroventricular
INF	-	infundibular nucleus
IR	-	immunoreactive

MC3-R, MC4-R	-	melanocortin 3 and 4 receptors
MCH	-	melanin-concentrating hormone
MTII	-	melanotan II (MC3R and MC4 agonist)
Ni-DAB	-	nickel-diaminobenzidine
NPY	-	neuropeptide Y
NTS	-	nucleus of the solitary tract
Ob-Rb	-	long form of leptin receptor
PBS	-	phosphate buffered saline
POMC	-	proopiomelanocortin
PP	-	pancreatic polypeptide
PV	-	periventricular nucleus
PVN	-	hypothalamic paraventricular nucleus
PYY	-	peptide YY
SEM	-	standard error of the mean
SOCS 3	-	suppressor of cytokine signaling 3
SHU9119	-	MC3R and MC4 agonist
TRH	-	thyrotropin-releasing hormone
VMN	-	hypothalamic ventromedial nucleus
Y1-5	-	NPY receptors 1-5

Introduction

The energy homeostasis depends on the balance of energy intake and energy expenditure. This balance is very tightly regulated by the interaction of the peripheral organs and the central nervous system. The white adipose tissue, pancreas and the gastrointestinal tract send information to the brain about the actual conditions of the energy depots and food consumption partly through the vagus nerve, but also by secreting hormones into the peripheral circulation (Schwartz, Woods et al. 2000).

The first reported adiposity signal was insulin, which originates from the pancreas and has a potent anorexigenic effect (Schwartz, Figlewicz et al. 1992). Plasma insulin levels correlate to the amount and distribution of body adiposity with a key role of visceral fat, and increase rapidly after meals. Insulin has a physiological role in the control of food intake, fat mass and hepatic functions (Porte and Woods 1981). Besides its direct effects on the liver, insulin also alters the glucose output level of the liver through its central action (Plum, Belgardt et al. 2006; Koch, Wunderlich et al. 2008). Both the basal and meal induced insulin secretion are elevated, when body adiposity is increased, as a compensatory mechanism to cope with the insulin resistance to keep normal glucose homeostasis (Bagdade, Bierman et al. 1967). When this adaptive increase is failed to be maintained, hyperglycaemia occurs that leads to type II diabetes. Central insulin injection into animals and humans results in a dose-dependent decrease of food intake which in long term results in decreased fat accumulation (Woods, Lotter et al. 1979; Air, Strowski et al. 2002). Insulin can cross the BBB in proportional to its plasma levels via a saturable, receptor-mediated process (Duffy and Pardridge 1987).

The discovery of leptin in 1994 (Zhang, Proenca et al. 1994) drew scientific attention to the importance of humoral communication between the white adipose tissue and the brain in the regulation of the energy homeostasis (Frederich, Hamann et al. 1995; Zhang, Matheny et al. 2004). Leptin is synthesized and secreted to the circulation by the white adipocytes. The level of leptin in the serum is proportional to the size of energy stores (Frederich, Hamann et al. 1995; Zhang, Matheny et al. 2004). Thus during fasting, leptin levels are decreased, while obese animals have increased levels of leptin (Frederich, Hamann et al. 1995). Exogenous administration of leptin markedly inhibits the food intake and increases the energy expenditure resulting in a loss of body weight

(Campfield, Smith et al. 1995; Halaas, Gajiwala et al. 1995; Pelleymounter, Cullen et al. 1995). In contrast, the absence of leptin in the ob/ob mice or absence of leptin receptor in the db/db mice results in morbid obesity, type II diabetes, and a reduction in activity, metabolism, and body temperature (Pelleymounter, Cullen et al. 1995) (Montague, Farooqi et al. 1997). Leptin resistance can develop due to obesity, but high-fat diet per se can also cause leptin insensitivity (Frederich, Hamann et al. 1995). Transgenic overexpression of leptin in hypothalamic nuclei leads to reduced food intake and decreased energy expenditure (Ogus, Ke et al. 2003). Leptin also acts on the brainstem causing similar anorexigenic effects by reducing food intake.

In addition to insulin and leptin, a long list of peripheral hormones including cholecystokinin (CCK), pancreatic polypeptide (PP), peptide (PYY) and glucagon-like peptide 1 (GLP1) inhibit food intake and increase the energy expenditure (Morton, Cummings et al. 2006). Currently, however, only one circulating orexigenic hormone is known: ghrelin is synthesized in the wall of the stomach, exert orexigenic effect and its level is increased during fasting (Tschop, Smiley et al. 2000; Morton, Cummings et al. 2006). The primary central target of most of these hormones is the hypothalamic arcuate nucleus (Schwartz, Seeley et al. 1996; Hewson and Dickson 2000; Tso, Sun et al. 2004; Ballantyne 2006; Valassi, Scacchi et al. 2008).

The arcuate nucleus is one of the most important metabolic sensor areas of the brain (Morton, Cummings et al. 2006). Ablation of the arcuate nucleus by monosodium glutamate treatment results in obesity and leptin resistance (Olney 1969; Bakke, Lawrence et al. 1978; Dawson and Lorden 1981; Dawson, Pelleymounter et al. 1997). The arcuate nucleus contains at least two, distinct populations of neurons that are directly regulated by peripheral feeding related signals (Schwartz, Seeley et al. 1996; Fan, Boston et al. 1997; Schwartz, Seeley et al. 1997; Mizuno, Murakami et al. 1998; Mizuno, Makimura et al. 1999), and involved in the regulation of feeding behavior (Fan, Boston et al. 1997; Rossi, Kim et al. 1998). The first is a medially located neuronal group that synthesizes and co-contains the two, potent, orexigenic peptides, neuropeptide Y (NPY) and agouti-related protein (AgRP) that are inhibited by leptin and stimulated by ghrelin. The second is a more laterally located neuronal group that synthesizes and co-contains the anorectic peptides, alpha-melanocyte-stimulating

hormone (α-MSH) and cocaine- and amphetamine-regulated transcript (CART), that are stimulated by leptin and inhibited by ghrelin.

Blood-Brain Barrier

The brain, in order to be protected against infections and toxins is separated from the bloodstream by the blood-brain barrier (BBB) (Begley 1996). The tight junctions between the endothelial cells of central nervous system (CNS) vessels also surrounded with astrocyte end feet results in a highly restricted permeability. Lipid-soluble molecules can diffuse across cell membrane depending on their size, but polar molecules only can cross by transport mechanisms. Circumventricular organs (CVOs) can be found at distinct sites of the ventricular system of the brain. Having an incomplete BBB, these areas are able to receive direct information about peripheral concentrations of certain proteins. Out of the seven CVOs, median eminence lies at the base of the third ventricle and has a prominent role in regulating energy homeostasis. Median eminence acts as a link between pituitary and hypothalamus, and also has a role as an afferent sensory organ, located adjacent to important feeding-related nuclei, including the hypothalamic arcuate and ventromedial (VMN) nuclei (Broadwell, Balin et al. 1983).

Role of the AGRP/NPY neurons in the regulation of energy homeostasis

The orexigenic neurons of the arcuate nucleus are located in the ventromedial part of the nucleus and express NPY and AGRP (Hahn, Breininger et al. 1998). The importance of this neuronal group in the regulation of food intake is clearly demonstrated by the data showing that selective ablation of the NPY/AGRP neurons in adult mice results in profound hypophagia and weight loss (Gropp, Shanabrough et al. 2005; Luquet, Perez et al. 2005).

NPY, a 36-amino-acid amidated peptide and member of the pancreatic polypeptide family (Tatemoto 1982), is one of the most potent orexigenic substances. Central administration of NPY results in a marked increase of food intake and weight gain (Stanley and Leibowitz 1985). In addition, a recent study where NPY expression in the ARC was inhibited by a gene therapy approach showed that the treated animals released 50% less NPY, gained less weight and ate less than the controls up to 50 days after

treatment (Gardiner, Kong et al. 2005). Since third ventricular administration of NPY potently stimulate food intake, it is believed that the orexigenic effect of NPY is primarily exerted through the hypothalamus (Stanley, Chin et al. 1985). Indeed, focal administration of NPY into several hypothalamic nuclei results in increased food intake, however, NPY is the most potent when it is injected into the PVN or into the neighboring perifornical area (Stanley, Chin et al. 1985; Stanley and Leibowitz 1985; Stanley, Magdalin et al. 1993).

In addition to its effect on food intake, NPY also inhibits the energy expenditure while simultaneously inducing lipogenic enzymes in the liver and white adipose tissue (Billington, Briggs et al. 1991). The inhibitory effects of NPY on the energy expenditure are partly mediated through the inhibition of the sympathetic nervous system (Egawa, Yoshimatsu et al. 1991). NPY decreases sympathetic activity in the brown adipose tissue (BAT) in a dose dependent fashion after injection into the third ventricle or into the hypothalamic paraventricular nucleus (PVN). Since the thermogenesis of BAT is an important component of the energy expenditure, the inhibition of BAT thermogenesis through the sympathetic nervous system is important in the regulation of the energy homeostasis of the animals. NPY also influences the energy expenditure through the regulation of the hypothalamic-pituitary-thyroid (HPT) axis (Fekete, Kelly et al. 2001; Fekete, Sarkar et al. 2002). NPY neurons of the arcuate nucleus directly innervate the hypophysiotropic TRH neurons and decrease the TRH synthesis in these cells (Legradi and Lechan 1998). Therefore, increased NPY synthesis and release inhibit the hypothalamic-pituitary-thyroid axis (Fekete, Kelly et al. 2001). Since hypothyroidism reduces the basal metabolic rate by 30% (Franco, Arciuch et al. 2006), the fall of thyroid hormone levels induced by the central NPY treatment may be an important component of the effect of NPY on the energy homeostasis.

NPY binds to at least five different G_i protein-coupled receptors of the PP family, including the Y1, Y2, Y4, Y5, and Y6 receptors (Schwartz, Sheikh et al. 1987; Bard, Walker et al. 1995; Hu, Bloomquist et al. 1996; Weinberg, Sirinathsinghji et al. 1996). However, NPY can exert its central effects only through the Y1, Y2, and Y5 receptors in the rat, as the Y6 receptor is not expressed in the rat (Widdowson, Buckingham et al. 1997), and NPY has negligible affinity for the Y4 receptor, which is primarily a PP receptor (Bard, Walker et al. 1995). Of these three NPY receptors, only the Y1 and Y5

receptor subtypes are believed to postsynaptic NPY receptors (Gerald, Walker et al. 1996). Activation of both Y1 and Y5 receptors influences the same intracellular pathway, as both receptor subtypes decrease intracellular levels of cAMP *in vitro* (Michel, Beck-Sickinger et al. 1998).

AGRP, the second orexigenic peptide of the arcuate nucleus neurons was discovered based on an interesting genetic anomaly: in the agouti mice, ectopic overexpression of agouti-peptide in the central nervous system results in obesity despite the fact that agouti-peptide is not expressed in the brain of wildtype mice. Sequence similarity was used to identify AGRP, a protein that is 25% identical with agouti and expressed in the brain (Ollmann, Wilson et al. 1997). Within the central nervous system, AGRP is synthesized exclusively in the NPY/AGRP neurons of the arcuate nucleus.

Similarly to NPY, centrally administered AGRP increases food intake and decreases energy expenditure (Ollmann, Wilson et al. 1997; Small, Liu et al. 2003). Long term central administration results in hyperphagia and obesity (Hagan, Rushing et al. 2000; Tallam, Kuo et al. 2004; Tang-Christensen, Vrang et al. 2004). In addition, transgenic overexpression of human AGRP in mice also causes obese phenotype (Graham, Shutter et al. 1997). The effect of AGRP on body weight gain is only partly mediated through the increased food intake, because AGRP treated animals that are *pair fed* to controls, also have increased body weight gain and increased fat accumulation (Small, Kim et al. 2001; Small, Liu et al. 2003). These data indicate that independently from its effect on food intake, AGRP decreases the energy expenditure. Indeed, both acute and chronic central administration of AGRP decreases the oxygen consumption and the activity of brown adipose tissue even in *pair fed* animals (Small, Liu et al. 2003).

AGRP exerts its effect in the central nervous system as a potent selective endogenous antagonist of melanocortin signaling on the melanocortin 3 and 4 receptors (MC3R and MC4R)(Fan, Boston et al. 1997; Rossi, Kim et al. 1998).

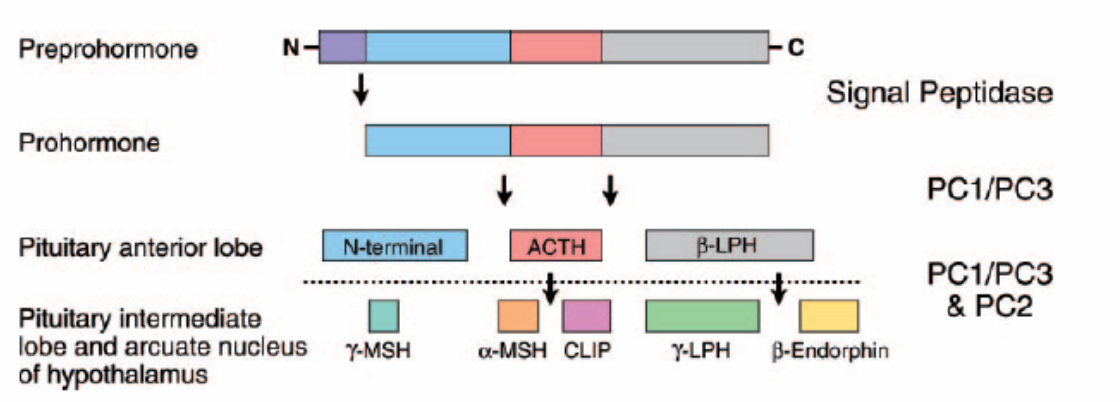
The activity of the orexigenic AGRP/NPY neurons is tightly controlled by peripheral satiety signals. These neurons are known to express the long form of leptin receptor, insulin receptor and also the receptor of the orexigenic hormone, ghrelin. Fasting results in a marked activation of the synthesis of both AGRP and NPY. Both peripheral and central administration of leptin inhibit the synthesis of these peptides and can

completely reverse the fasting induced increase of the AGRP and NPY gene expression. In contrast, administration of ghrelin stimulates these orexigenic neurons.

The AGRP/NPY neurons send axonal projections to the feeding related centers of the brain including the hypothalamic dorsomedial and paraventricular nuclei. Through these projections, the AGRP/NPY neurons can mediate the effects of the changes of peripheral satiety related signals toward other metabolic centers of the brain.

Melanocortins

Melanocortins are deriving from the posttranslational processing of the proopiomelanocortin protein (POMC). Derivatives of POMC, like ACTH and MSH-peptides are ligands of melanocortin receptors, and have important role in the regulation of energy homeostasis. The schematic figure illustrates the posttranslational processing and the products of POMC.



From Cone et al. (Cone 2006)

Figure 1: POMC protein goes through tissue-specific posttranslational processing. The first step of processing in all tissues is the proteolitic cleavage of the N-terminal signal peptide by members of the subtilisin-like prohormone convertase family. The two prohormone convertase enzymes, PC1/3 and PC2, that expressed in the brain has major role in processing POMC prohormone. PC1/3 and PC2 has a different and well-defined distribution pattern in the brain and pituitary which allows producing various proteins in different tissues. In the corticotropes of pituitary anterior lobe, PC1/ PC3 is the predominant convertase, cleaving the prohormone into ACTH and b-lipotropin. With the absence of PC2, ACTH remains unclevad and α-MSH is not produced. In the arcuate nucleus and in the melanotropes of the intermediate lobe of the pituitary both PC2 and PC1/3 is expressed. In these locations, PC2 further cleaves ACTH into α-MSH and CLIP (corticotropinlike intermediate peptide).

Melanocortin receptors.

Melanocortin receptors are seven transmembrane domain containing G-protein coupled receptors. When a melanocortin ligand binds to the receptor, G protein is released and activates adenylate cyclase, which leads to the increase of cAMP in the cell. The five melanocortin receptors have different tissue distribution and different ligand specificity detailed in Table 1.

Table 1

name	tissue	role	Order of potency	Antagonists
MC1R	melanocytes, pituitary and leukocytes (Chhajlani 1996)	Pigmentation (regulation of the eumelanin-pheomelanin switch)	α-MSH >> γ-MSH	Agouti
MC2R	adrenal cortex	Adrenocortical steroidogenesis	ACTH	
MC3R	brain, gut, placenta, pituitary	Energy homeostasis, natriuresis	γ-MSH = α-MSH = β-MSH	AgRP
MC4R	brain, pituitary, osteoblasts	Energy homeostasis, erectile function , bone remodeling, blockade of ethanol intake (Navarro, Cubero et al. 2005) blood pressure (Greenfield, Miller et al. 2009) HPA axis activity, emotional states, pain, drug addiction	α-MSH = β-MSH >> γ-MSH	AgRP, Agouti
MC5R	brain, exocrine glands, pituitary, skin, stomach, lung, spleen	Synthesis and secretion of exocrine gland products	α-MSH > β-MSH > γ-MSH	

Modified from Rana et al. (Obici, Zhang et al. 2002; Rana 2003; Zhang, Hupfeld et al. 2005).

Action of peripheral hormones on melanocortin receptors depending on the feeding status

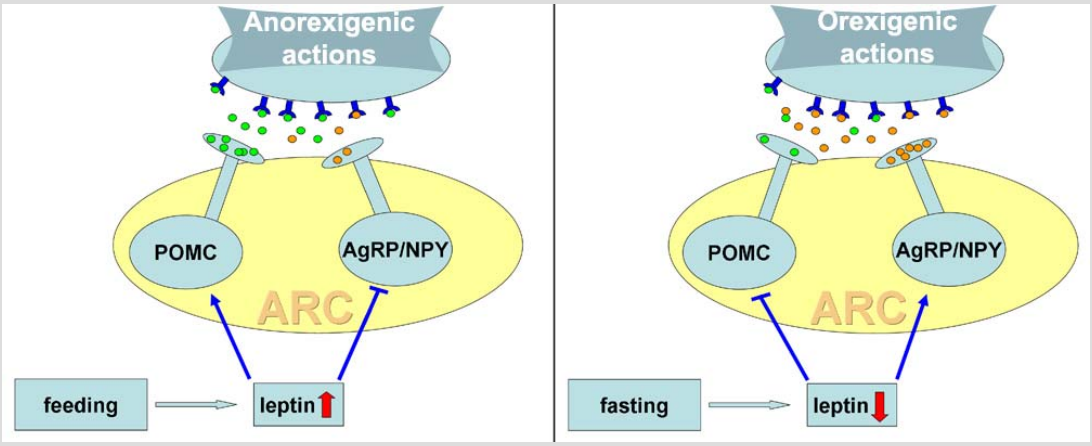


Figure 2

In response to feeding, circulating leptin levels are increased and activate α-MSH/CART expressing neurons and inhibit NPY/AgRP producing cells in the arcuate nucleus. α-MSH activates melanocortin receptors 3 and 4 that leads to inhibition of feeding. During fasting, α-MSH secretion is decreased and the release of melanocortin antagonist AGRP is increased. These changes together result in decreased activation of melanocortin receptors and therefore increased food intake.

Role of the POMC/CART neurons in the regulation of energy homeostasis

The anorexigenic neurons are located in the lateral part of the arcuate nucleus in rodents. These neurons synthesize proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Elias, Lee et al. 1998; Cowley, Smart et al. 2001). The posttranslational processing of POMC results in several biologically active peptides including the melanocortin agonist α-MSH, β-MSH, γ-MSH and adrenocorticotropin (ACTH) and the opioid receptor agonist β-endorphin (Liotta, Loudes et al. 1980; Gee, Chen et al. 1983; Liotta, Advis et al. 1984; Pritchard and White 2007). Among the POMC derived peptides, the role of the α-MSH is the best understood in the field of the regulation of energy homeostasis. However, α-MSH is a ligand of all melanocortin receptors except the MC2R, the primary central actions of α-

MSH are exerted through the MC3R and MC4R (Mountjoy, Mortrud et al. 1994; Schaffer and Bolognia 2001). Central administration of α-MSH or the MC3/4R agonist melanotan II (MTII) reduce food intake in both wildtype and leptin-deficient *ob/ob* mice (Fan, Boston et al. 1997). In contrast, the administration of the MC3/4R antagonist SHU9119 alone causes increase of nocturnal and fasting induced food intake, indicating that the melanocortin system exerts a tonic inhibitory effect on the food intake (Fan, Boston et al. 1997; Huszar, Lynch et al. 1997; Giraudo, Billington et al. 1998). Genetic ablation of the POMC gene or the MC4R results in an obesity syndrome that is highly similar to the obesity phenotype of the agouti mouse (Huszar, Lynch et al. 1997). These obesity syndromes together are called melanocortin obesity syndromes and characterized by hyperphagia, hypometabolism, hyperinsulinemia and increased linear growth (Huszar, Lynch et al. 1997; Farooqi, Keogh et al. 2003). The MC4R knockout mice have been shown to be highly susceptible for increased fat content of diet (Butler, Marks et al. 2001). Wildtype mice initially increase the caloric intake on moderate fat diet, but then reduce the food intake to normalize the caloric intake (Butler, Marks et al. 2001). In contrast, MC4R knockout mice are unable to adjust to the increased fat content of food and remain hyperphagic and hypometabolic even on moderate fat diet that results in profound accumulation of body fat (Samama, Rumennik et al. 2003). Mutation of MC4R also results in a similar phenotype in humans and can be found in 4-6% of morbidly obese individuals (Farooqi, Yeo et al. 2000; Farooqi, Keogh et al. 2003). Deletion of the MC3R results in a markedly different type of obesity (Butler, Kesterson et al. 2000; Chen, Marsh et al. 2000). Despite the fact that the MC3R knockout mice has normal body weight and eat normal amount of food, the fat content of these mice is increased by approximately 40% (Chen, Marsh et al. 2000). In addition, no differences of resting and basal metabolic rate can be measured, but these mice have reduced activity suggesting that reduced energy expenditure may contribute to the increased fat content of these animals (Butler, Kesterson et al. 2000; Chen, Marsh et al. 2000).

CART synthesized in the anorexigenic neurons of the arcuate nucleus is also an anorexigenic peptide (Elias, Lee et al. 1998; Fekete, Mihaly et al. 2000; Fekete, Wittmann et al. 2004). Acute administration of CART into the cerebral ventricles induces c-fos in many feeding related areas (Vrang, Larsen et al. 2000), decreases food

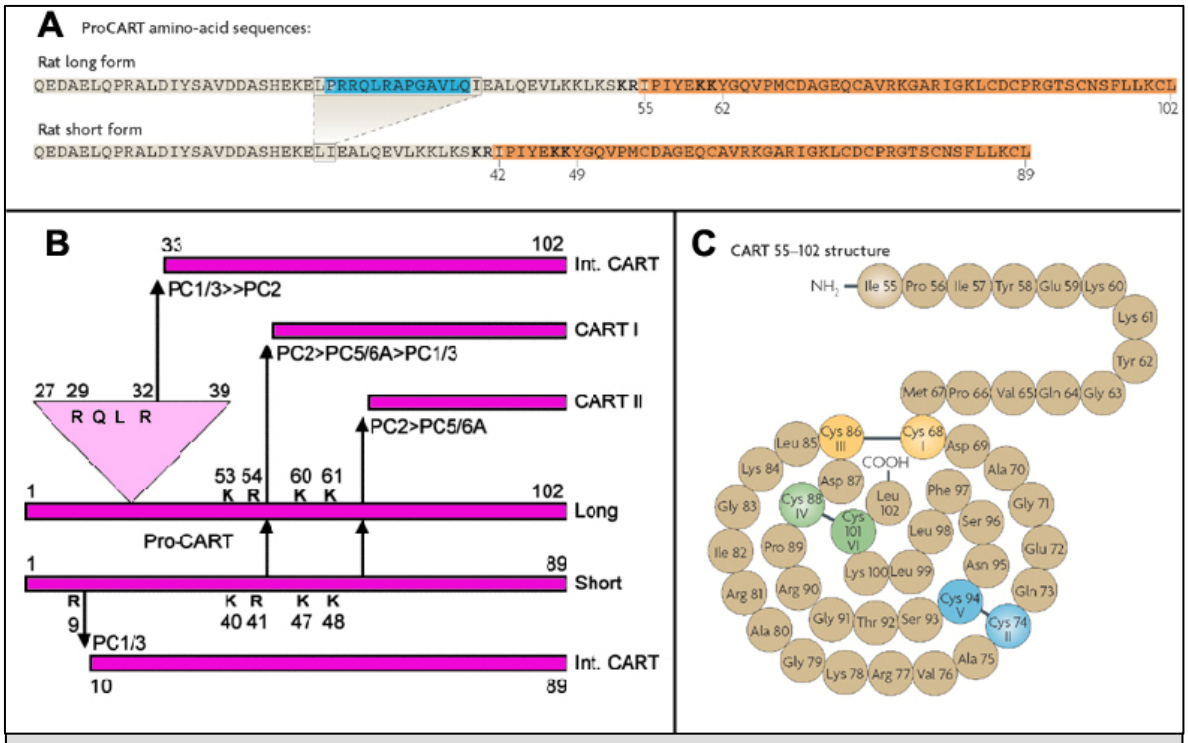
intake (Kristensen, Judge et al. 1998), and inhibits the orexigenic effects of NPY (Clark, Kalra et al. 1984; Volkoff and Peter 2000), whereas chronic CART administration induces significant weight loss (Larsen, Vrang et al. 2000; Rohner-Jeanrenaud, Craft et al. 2002). Conversely, the central administration of CART antiserum increases food intake (Levine and Morley 1984; Kristensen, Judge et al. 1998; Lambert, Couceyro et al. 1998).

While there is general agreement that icv administration of CART has anorexigenic effects, some discrepancies have been reported. Wang et al. (Wang, Billington et al. 2000) observed inhibition of food intake when CART is injected directly into the PVN, whereas Abbott et al. (Abbott, Rossi et al. 2001) and Smith et al. (Smith, Gardiner et al. 2008) observed increased food intake after focal injection of CART into either the PVN or arcuate nucleus. Nevertheless, consistent with the role of CART as an anorexic peptide, CART deficient transgenic mice develop obesity when placed on a high caloric diet (Asnicar, Smith et al. 2001), and polymorphisms of the 5' flanking region of the CART promoter are associated with obesity (Yamada, Yuan et al. 2002).

CART is produced in different feeding related hypothalamic nuclei, including the arcuate nucleus, PVN, DMN, VMN and the lateral hypothalamus (LHA) (Douglass, McKinzie et al. 1995; Gautvik, de Lecea et al. 1996; Koylu, Couceyro et al. 1997). In the hypothalamus, CART colocalizes with several neuropeptides, like melanin-concentrating hormone (MCH) in the LHA, cotricotropin-releasing hormone (CRH) in the PVN and α-MSH in the ARC (Elias, Saper et al. 1998; Broberger 1999; Vrang, Larsen et al. 1999; Sarkar, Wittmann et al. 2004). Colocalization with both orexigenic and anorexigenic peptides suggests a modulatory role in feeding regulation.

In the arcuate nucleus, the synthesis of both POMC and CART is highly regulated by circulating levels of leptin (Cheung, Clifton et al. 1997; Kristensen, Judge et al. 1998; Cowley, Smart et al. 2001; van Dijk 2001). Exogenous administration of leptin or elevated leptin levels induced by high fat diet-induced obesity, increases both POMC and CART mRNA in the arcuate nucleus (Rohner-Jeanrenaud, Craft et al. 2002; Vrang, Kristensen et al. 2002). Conversely, both POMC and CART gene expression is decreased in the arcuate nucleus of leptin resistant fa/fa Zucker rats and leptin deficient ob/ob mice (Kristensen, Judge et al. 1998). In the later animal model, POMC and CART mRNA levels can be normalized by leptin replacement .

The actions of leptin is believed to be exerted directly on POMC/CART-producing neurons of the arcuate nucleus, as these neurons express leptin receptors and show evidence of SOCS 3 expression following leptin administration (Bjorbaek, Elmquist et al. 1998; Elmquist 2001). The POMC/CART-synthesizing arcuate nucleus neurons project directly to the PVN (Baker and Herkenham 1995; Fekete, Mihaly et al. 2000; Jobst, Enriori et al. 2004) and the intermediolateral column of the spinal cord (Kiss, Cassell et al. 1984; Elias, Lee et al. 1998), thereby coordinating the effects of α-MSH and CART to simultaneously regulate food intake and energy metabolism.



From Rogge et al., (Rogge, Jones et al. 2008)(A and C) and Dey et al., (Dey, Xhu et al. 2003) (B)

Figure 3: Structure and isoforms of cocaine- and amphetamine regulated peptide (CART). Alternative splicing of CART mRNA results in two different proCART peptides: long CART isoform (102 amino acids) and short isoform (89 amino acids) (Fig. 4 A). The proCART has to be further processed by prohormone convertase enzymes PC1/3 and PC2. In the rat hypothalamus, two forms of active CART peptides are present: CART(42–89) and CART(49–89). In contrast to rodents, only the short form exists in humans.

Recently Stein and his group found that PC5/6A also takes part in processing CART, cleaving both prohormone isoforms, and generating both CART(42–89) and CART(49–89).

Interaction of NPY/AGRP and POMC/CART neurons

The two functionally antagonistic neuronal populations of the arcuate nucleus, the AGRP/NPY and POMC/CART neurons, interact with each other at the level of the arcuate nucleus, but also in the terminal field of these neurons.

Morphological studies demonstrated a direct innervation of the POMC/CART neurons of the arcuate nucleus by NPY-IR terminals (Csiffary, Gorcs et al. 1990; Cowley, Smart et al. 2001). However, there is no report about the innervation of the AGRP/NPY neurons by α-MSH-containing axons. The asymmetric nature of the interconnection of the two cell types were also suggested by patch-clamp electrophysiological studies using transgenic mice that express GFP either in the POMC neurons or in the AGRP and NPY neurons (Roseberry, Liu et al. 2004). In these studies, while NPY strongly inhibited POMC neurons via the Y1 receptor, the α-MSH analog MTII had no effect on activity of NPY neurons in the arcuate nucleus (Roseberry, Liu et al. 2004). Identification of MC3 receptor on AGRP neurons, however, may provide evidence that α-MSH/CART neurons may also influence the AGRP/NPY cells (Bagnol, Lu et al. 1999).

Interestingly, the terminal field of the orexigenic and anorexigenic neuron populations highly overlap innervating the same feeding related neuronal groups both inside and outside of the hypothalamus (Horvath 2005). This indicates that the two antagonistic neuronal populations may innervate the very same neurons. Interacting NPY/AgRP and POMC/CART neurons of the arcuate nucleus are known to be the key component of the regulation of metabolism. Indeed, our laboratory has shown that all hypophysiotropic TRH neurons in the PVN that are innervated by the α-MSH/CART neurons are also innervated by axons of the AGRP/NPY neurons of the arcuate nucleus. In addition, the transmitters of the two neuronal populations interact at receptorial or postreceptorial levels. AGRP acts as an antagonist of α-MSH on the MC3R and MC4R, while NPY can prevent the α-MSH induced CREB phosphorylation.

The strong, multilevel interaction of the two oppositely regulated functionally antagonistic neuronal groups may be critical in the fine tuning of the activity of the feeding related neuronal groups of the brain (Bray 2000).

NPY/AGRP and α-MSH-containing neurons also form two distinct neuronal populations in the infundibular nucleus, the homolog of the rodent arcuate nucleus, in the human hypothalamus (Mihaly, Fekete et al. 2000; Goldstone, Unmehopa et al. 2002) and densely innervate the PVN of the human hypothalamus (Mihaly, Fekete et al. 2000). In addition, both neuron populations densely innervate the infundibular nucleus (Mihaly, Fekete et al. 2000), but it is unknown whether an interconnection of these cells exist in the human brain. In addition, it is unknown whether CART and α-MSH are expressed in the same neuronal population of the infundibular nucleus.

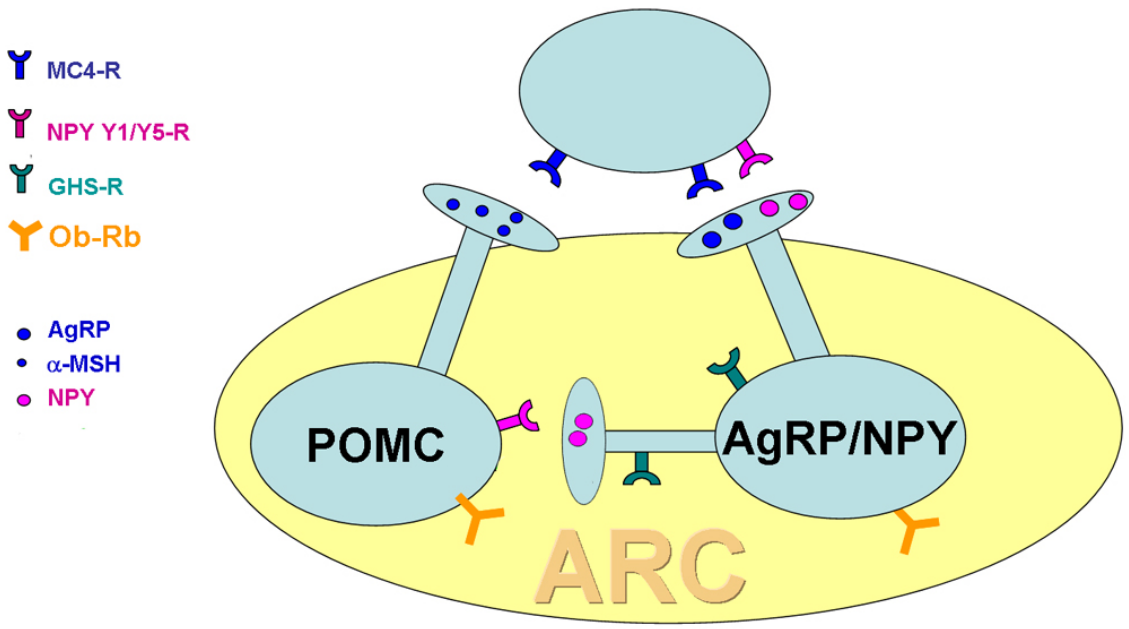
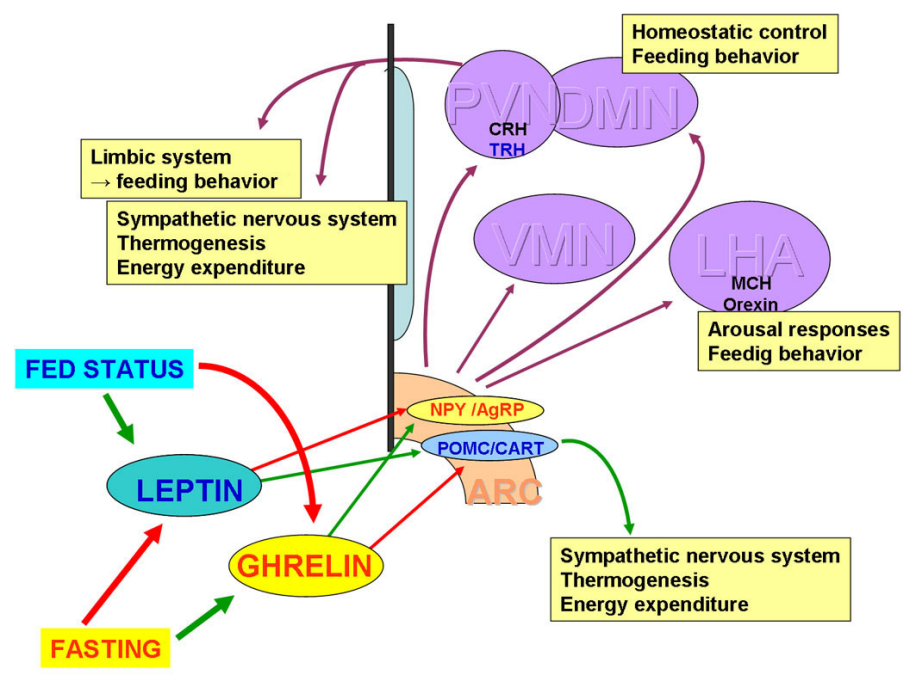


Figure 4: Schematic illustration of the two major feeding related neuronal populations of the arcuate nucleus, AgRP/NPY and POMC/CART producing neuronal groups in the arcuate nucleus. Receiving signals from the peripheral energy stores, these antagonistic cell populations have a pivotal role in maintaining the energy homeostasis. Anorexigenic POMC/CART neurons express Y1 (NPY) and Ob-Rb (leptin), while AgRP/NPY cells express ghrelin receptor (GHS-R) and Ob-Rb, thus the orexigenic NPY/AgRP cell population can regulate the anorexgenic POMC/CART cells. Both populations project to other feeding-related nuclei in the brain.

MCH

Melanin-concentrating hormone (MCH) was first described in fish, where similarly to α-MSH, MCH also regulates the pigmentation (Kawauchi, Kawazoe et al. 1983), however, the two peptides have an opposing effect . In addition to peripheral organs, MCH is also expressed in the hypothalamus in mammals. MCH expressing neurons are located in the lateral hypothalamus and the perifornical area (Inui 2000).MCH has a potent orexigenic effect when administered centrally (Qu, Ludwig et al. 1996). Focal administration of MCH into the arcuate, paraventricular and dorsomedial nuclei markedly increases food intake, while administration to other nuclei (supraoptic nucleus, lateral hypothalamic area, medial preoptic area, anterior hypothalamus and the ventromedial nucleus) did not influence the amount of food consumed by the animals (Abbott, Kennedy et al. 2003). In hypothalamic slices, MCH activates orexigenic NPY/AgRP neurons, and inhibits the expression and release of the anorexigenic α-MSH and CART in the arcuate nucleus (Abbott, Kennedy et al. 2003). In vivo, icv administration of MCH stimulates the HPA axis (Jezova, Bartanusz et al. 1992). These data suggest that the orexigenic effects of MCH are mediated primarily by neurons of the arcuate, dorsomedial and paraventricular nuclei of the hypothalamus. Leptin negatively regulates the synthesis of MCH in the lateral hypothalamus and perifornical area. In contrast, fasting increases the MCH expression in these neurons. The fasting induced changes of MCH synthesis can be restored by leptin administration.



Modified from Crowley et al., (Crowley, Yeo et al. 2002)

Figure 5: Influences of peripheral feeding related hormones on the hypothalamic neuronal circuits. Green arrows represent stimulating effects, while red arrows shows inhibiting effects. Purple arrows indicate that the action is depending on which neuronal population is activated in the arcuate nucleus. Peripheral hormones, such as leptin and ghrelin, mediate the information about the body energy stores toward the brain. The main central site of action of these hormones is the arcuate nucleus, where two feeding related neuronal groups are found: the orexigenic NPY/AgRP and the anorexigenic POMC/CART neurons. The circulating level of the adipocyte derived leptin is increased when the lipid content of the energy stores is increased. The elevated leptin level activates the anorexigenic POMC/CART neuron population while inhibits the NPY/AgRP cells. These changes result in decreased food intake and slower gastric emptying. With the activation of POMC/CART neurons, sympathetic nervous system is stimulated. In response to fasting, leptin secretion is inhibited, but ghrelin expression is increased. Ghrelin acts oppositely to leptin by inhibiting POMC/CART cells and stimulating the orexigenic NPY/AgRP neurons. Arcuate nucleus neurons project to other feeding-related hypothalamic nuclei and mediate the effects of the peripheral satiety hormones

The role and regulation of feeding-related hypothalamic neuropeptides are summarized in Table 2.

Table 2: Role and regulation of feeding-related hypothalamic neuropeptides.

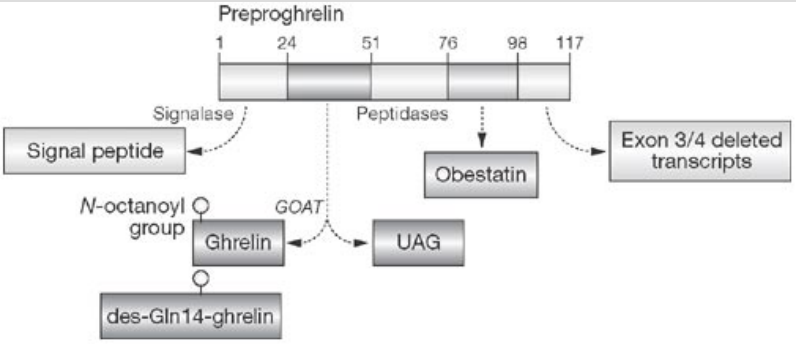
Peptides	Action	Stimulating Neuropeptides / hormones	Effect of fasting	Inhibited by
NPY	Orexigenic	Ghrelin	upregulates	α-MSH, CART, leptin, insulin, PYY
AgRP	Orexigenic	Ghrelin	upregulates	α-MSH, CART, leptin, insulin, PYY
Ghrelin	Orexigenic		upregulates	
α-MSH	Anorexigenic	Leptin, insulin	downregulates	AgRP, NPY,
CART	Anorexigenic	Leptin, insulin	downregulates	AgRP, NPY,
MCH	Orexigenic		upregulates	

Ghrelin and the ghrelin-IR neuronal system of the hypothalamus

Ghrelin, a 28 amino acid peptide with an n-octanoyl modification in the Ser3 was described as the endogenous ligand of growth hormone secretagogue receptor (GHS-R) (Kojima, Hosoda et al. 1999). Ghrelin-synthesizing cells were first described in the rat stomach. While the fundus of stomach is the major source of circulating ghrelin, substantially lower amount is synthesized in many tissues including the bowel, kidney, placenta, blood cells, testicles, ovaries, pancreas, pituitary and the hypothalamus (Kojima, Hosoda et al. 1999; Gnanapavan, Kola et al. 2002). Ghrelin is more potent GH secretagogue than growth hormone-releasing hormone (GHRH), and exerts its effect in a dose-dependent manner (Takaya, Ariyasu et al. 2000). Ghrelin also increases the circulating levels of prolactin, ACTH and cortisol (Nagaya, Kojima et al. 2001), whereas it has only a negligible impact on the release of other hypophyseal hormones (Takaya, Ariyasu et al. 2000). In addition to its effects on the pituitary, ghrelin was also shown to have a potent orexigenic effect and so far ghrelin is the only known peripheral orexigenic peptide, reviewed in (Bowers 2005). Circulating levels of ghrelin are elevated during fasting, cachexia or anorexia nervosa (Ariyasu, Takaya et al. 2001; Nagaya, Uematsu et al. 2001), and decreased after feeding (Tschop, Weyer et al. 2001).

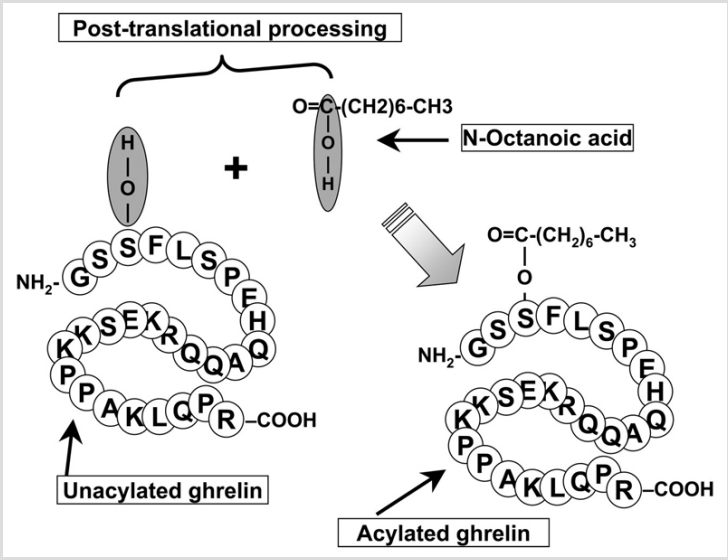
Furthermore, fasting has also been shown to regulate ghrelin synthesis in the hypothalamus (Cowley, Smith et al. 2003). Administration of ghrelin directly activates the NPY producing neurons in the arcuate nucleus, and can antagonize the effects of leptin on these cells (Nakazato, Murakami et al. 2001). In contrast, ghrelin inhibits POMC/CART neurons, but this effect of ghrelin is indirect and mediated primarily through a gabaergic pathway (Cowley and Grove 2004). Therefore, ghrelin is considered as a "meal-initiating" signal. Peripheral ghrelin may access the brain through vagal afferents, as GHS receptors are expressed in the sensory neurons of the vagus, but ghrelin also reach arcuate nucleus directly. GHS-receptors are also widely distributed in the rodent brain. The presence of ghrelin receptor in feeding-related nuclei of the hypothalamus and activation of these regions after ghrelin administration indicate a major role of these brain regions in the mediation of orexigenic effects induced by ghrelin. In addition to be regulated by peripherally synthesized ghrelin, the ghrelin-responsive neurons may also be influenced and regulated by brain-born ghrelin, as it is suggested by the presence of ghrelin mRNA and mature ghrelin in the rodent hypothalamus (Cowley, Smith et al. 2003; Sato, Fukue et al. 2005). Ghrelin-immunoreactive perikarya were described in several parts of the rodent hypothalamus (Kojima, Hosoda et al. 1999; Lu, Guan et al. 2002), including a unique location delineating an area among the ventromedial, dorsomedial and paraventricular nuclei (Cowley, Smith et al. 2003). RT-PCR studies show ghrelin expression in the arcuate nucleus (Mondal, Date et al. 2005), but no Western-blot and in situ hybridization results are reported. Ghrelin-IR neurons of rodents project to several hypothalamic nuclei involved in the regulation of energy homeostasis, including the arcuate nucleus, the PVN, the lateral hypothalamus and the hypothalamic dorsomedial nucleus (Cowley, Smith et al. 2003). However, the presence of ghrelin-IR neurons has been demonstrated in the human infundibulum (Korbonits, Bustin et al. 2001), the projection fields of ghrelin-synthesizing neurons are unknown in the human hypothalamus.

Structure of ghrelin gene and maturation process of ghrelin



From Manuel Tena-Sempere et al., (Tena-Sempere 2008)

Figure 6: Human ghrelin gene consists of 4 exons and 3 introns, the mature protein is encoded by exons 1 an 2. Ghrelin promoter is activated by glucagon and cAMP. hnRNa of the transcript goes through alternative splicing which results in two different mature peptides: the ghrelin precursor and the des-Gln 14-ghrelin, that are both biologically active. Ghrelin precursor protein, preproghrelin consists of 117 amino acids, is further processed by proteolytic enzymes, giving rise to the 28 amino acid ghrelin and the 23 amino acid obestatin. Before ghrelin is secreted, ghrelin precursor is further processed by an enzymatic acylation in the cytoplasm, which results in the n-octanoyl modification at Ser 3. This modification is crucial for the efficiency of ghrelin in feeding regulation. Interestingly, despite that, this octanoylated ghrelin is the active form, 80-90% of circulating ghrelin is non octanoylated.



From van der Lely, (van der Lely, Tschop et al. 2004)

Specific Aims

1. To reveal the interconnection between orexigenic NPY- and anorexigenic α-MSH producing cells in the human hypothalamus

2. To determine the presence of CART in the orexigenic and anorexigenic cell populations of the human hypothalamus

3. To map the distribution of ghrelin-immunoreactive elements in the human hypothalamus

Materials and Methods

Human tissue preparation

Diencephalic samples of four adult human individuals with no history of neurological or endocrinological disorders were obtained at autopsy (Table 3). Tissue samples were taken within 6-24 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-µm 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.

Table 3: Summary of human cases

Gender	Age	Cause of death
Male	39	self-hanging
Male	54	sudden cardiac death
Male	29	self-hanging
Male	45	sudden cardiac death

Animal controls

The rats 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.

To test the effect of postmortem time and the immersion fixation on the colocalization of CART and α-MSH, three adult male Wistar rats (TOXI-COOP KKT, Budapest, Hungary) were euthanized with nembuthal (70 mg/kg BW). Then, the carcasses were stored at 4°C for 6h. The brains were removed from the skull and processed as described above for human tissues.

Pretreatment of hypothalamic sections for immunofluorescent labelling

Sections were pretreated with 1% sodium borohydride in distilled water for 30 min. After rinsing in phosphate-buffered saline, pH 7.4 (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, Fekete 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 antibodies against the examined neuropeptides (see detalies below).

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. After the above described pretreatment, sections were immersed 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 a 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).

Specificity of the used primary antisera

In most cases, specificity controls of used antisera were described previously, that are referred. Immunsera are listed in alphabetical order.

sheep anti-α-MSH serum (gift of Dr. Jeffrey B. Tatro): Described in detail in ref. (Elias, Saper et al. 1998). Briefly, prior to immunocytochemical studies, antiserum specificity was determined with radioimmunoassay by using iodine 125 (125I)-α-MSH as tracer. The antiserum is highly specific for α-MSH (with no apparent cross reactivity with MCH) and is dependent on the amidated C-terminal region for recognition. For immunocytochemical control, all specific staining was abolished when rat hypothalamic sections were incubated in α-MSH antiserum that had been preadsorbed with α-MSH (50 μg/ml of diluted antisera).

murine monoclonal antibody against CART (gift of Dr. Jes Thorn Clausen): As shown by HPLC, the monoclonal antibody reacts with CART 42–89 and CART 49–89 peptides, isolated from extracts of rat hypothalamus (Thim, Kristensen et al. 1999).

rabbit anti-MCH serum (gift of Dr. Eleftheria Maratos-Flier): All specific staining was abolished when rat hypothalamic sections were incubated in antiserum that had been preadsorbed with MCH (50 μg/ml of diluted antisera) (Elias, Saper et al. 1998).

sheep anti-NPY serum (gift of Dr. István Merchenthaler): The specificity of immunostaining with NPY antiserum was demonstrated by the loss of immunoreactivity after preadsorption of the diluted antiserum with an excess of synthetic NPY peptide (1 μg/ml) (data not shown).

Image analysis

The relationship of the two peptidergic systems was analyzed using a confocal laserscanning 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 μm thick, and the series of optical slices were recorded with a 0.6 μm 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.

Double-labeling immunofluorescence studies in the human hypothalamus to study the interrelationship of CART with other feeding related neuropeptides

Double-labeling fluorescence immunocytochemistry was performed to study the colocalization of CART with α-MSH-, NPY-, AgRP- and MCH-immunoreactive (IR) elements in the infundibular nucleus and the CART innervation of the three feeding related neuronal groups. Human hypothalamic sections were pretreated as described above, and then incubated in the following mixtures: 1) murine monoclonal antibody against CART 1:9,000 (gift from Dr. Jes Thorn Clausen, Novo Nordisk A/S, Bagsvaerd, Denmark) and sheep anti-α-MSH serum 1:6000 (gift from Jeffrey B. Tatro, Tufts- New England Medical Center, Boston, MA), 2) murine monoclonal antibody against CART 1:9,000 and sheep anti-NPY serum 1:16,000 (gift from István Merchenthaler, University of Maryland, Baltimore, MD), 3) murine monoclonal antibody against CART 1:9,000 and rabbit anti-AgRP serum 1:1,500 (Phoenix Pharmaceuticals Inc, Burlingame, CA), 4) murine monoclonal antibody against CART 1:9,000 and rabbit anti-MCH 1:6,000 (gift from Eleftheria Maratos-Flier, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA) for 48 h at 4°C. After further washes in PBS, tissues were immersed overnight in one of the following cocktails: 1) Cy3-conjugated donkey anti-mouse IgG for the detection of CART-IR elements (1:200; Jackson Immunoresearch, West Grove, PA) and FITC-conjugated donkey anti-sheep IgG for detection of α-MSH and NPY 2) Cy3-conjugated donkey anti-mouse IgG to detect CART and FITC-conjugated donkey anti-rabbit IgG for detection of AgRP and MCH (1:100; Jackson Immunoresearch). The sections were mounted and then coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Omitting any of the primary antibodies from the double-label immunostainings resulted in loss of the immunostaining specific for the omitted primary antibody demonstrating the lack of the cross reactivity in the double-labeling immunofluorescence technique.

Image Analysis

Double-labeled preparations were analyzed using a confocal laser microscope (Bio-Rad Laboratories, Hemel Hempstead, UK). At least three sections were analyzed from each brain, from different rostro-caudal levels of the infundibular nucleus, PVN and lateral hypothalamus. For quantification 296 X 296 μm areas of the entire infundibular nucleus, PVN or lateral hypothalamus were recorded with a 40X oil immersion objective. For illustrations, the images were taken with 40X or 60X oil immersion objectives. The fluorochromes were detected with the following laser lines and filters: 488 nm for FITC and 543 nm for CY3 and Dichroic/emission filter 560 nm/500–530 nm for FITC and 560–625 for CY3. Pinhole sizes were set to obtain optical slices less than 0.7 μm thick, and the series of optical slices were recorded with a 0.6 μm Z step. The series of optical sections were merged and displayed with Laser Vox software and an IBM compatible personal computer. Co-localization of CART and the other peptides were examined in perikarya and fibers. All single- and double labeled perikarya were counted on 5 sections from different rostrocaudal levels from each brain to determine the degree of co-localization between CART and the other examined neuropeptides (α-MSH, NPY, AgRP, MCH).

Detection of ghrelin immunoreactivity in the human hypothalamus

Series of hypothalamic sections were immunostained for ghrelin, while on subsequent sections Nissl counterstaining was performed to facilitate the localization of ghrelin-IR elements. The sections for immunocytochemistry were treated with 1% sodium borohydride in distilled water for 30 min and with 0.5% Triton X-100/ 0.5% H_2O_2 in PBS for 15 min. To reduce nonspecific antibody binding, the sections were treated with 2% normal horse serum in PBS for 20 min. The sections, then, were incubated in rabbit anti-ghrelin serum (Cowley, Smith et al. 2003) at 1:3000 for 2 days at 4 °C. After rinses in PBS, the sections were incubated in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at 1:500 dilution for 2 h and ABC Elite complex (Vector Laboratories, Burlingame, CA, 1:1000) for 1 h. Tissue sections were then rinsed in PBS, and the immunoreaction product was developed with 0.05% diaminobenzidine (DAB), 0.15% nickel ammonium sulfate and 0.005% H_2O_2 in 0.05 M Tris buffer, pH. 7.6, and intensified using the Gallyas silver intensification technique to

yield a black precipitate (Liposits, Setalo et al. 1984). The sections were mounted onto glass slides, air-dried, dehydrated in ascending series of ethanol and coverslipped with DPX (Fluka, Ronkonkoma, NY). The specificity of ghrelin immunostaining was earlier demonstrated by showing the lack of immunoreaction signal in ghrelin-KO mice (Cowley, Smith et al. 2003). In addition, we have demonstrated the specificity of the immunostaining in human tissues by showing the total loss of immunolabeling after preabsorption of the primary antisera with the excess of 10^{-5}M synthetic ghrelin (Phoenix Pharmaceuticals, Inc, Belmont, CA). Photomicrographs were taken with a Zeiss Axiophot microscope equipped with a RT Spot digital camera.

Results

1. Interconnection between α-MSH- and NPY-IR neurons in the hypothalamic infundibular nucleus in humans

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 ± 0.5 μm) and displayed fusiform or multipolar shapes. The α-MSH-IR cells were significantly larger (26.1 ± 0.6 μm; $P > 0.001$) 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 ± 1.00%, was contacted by NPY-containing axon varicosities (Figs. 7A, B). In some instances, the NPY-IR varicosities encircled the α-MSH-IR cell bodies (Fig. 7B). An average of 6.56 ± 1.32 NPY-IR boutons was found on the surface of an α- MSH-IR perikaryon. α-MSH-IR axon varicosities contacted 79.67 ± 8.33% of NPY-IR neurons. However, only an average of 2.27 ± 0.10 α-MSH-IR varicosities was observed in juxtaposition to an NPY-IR neuron (Fig. 7C).

Table 4: Summary of semiquantitative analyses of the interconnection of NPY and α-MSH neurons in the human infundibular nucleus

	Percentage of contact	Number of boutons/cell
α-MSH cells having NPY contacts	97.00 ± 1.00%,	6.56 ± 1.32
NPY cells having α-MSH contacts	79.67 ± 8.33%	2.27 ± 0.10

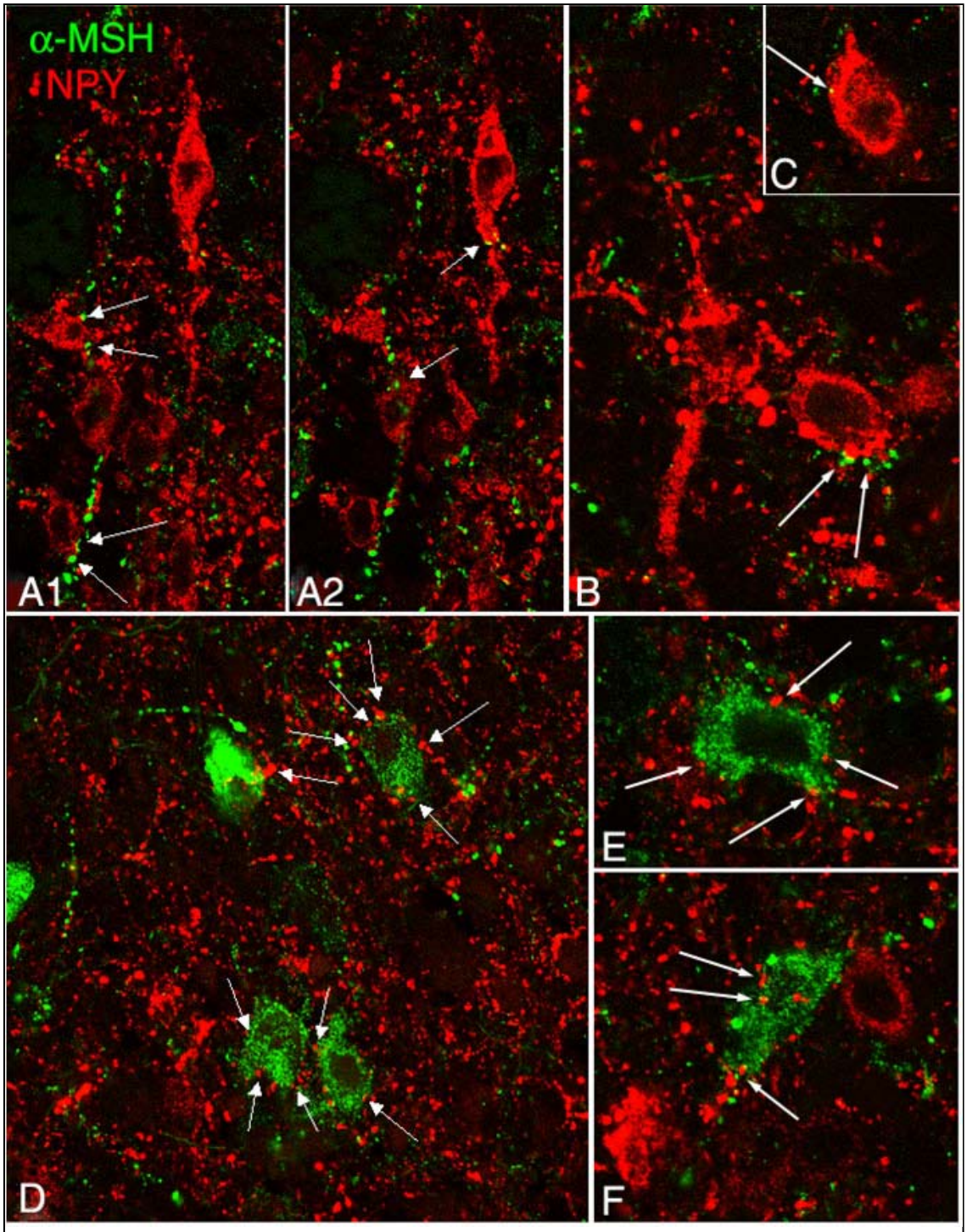


Figure 7 : Interconnection of α-MSH and NPY-IR neuron populations in the infundibular nucleus of the human hypothalamus. Legend on next page.

Figure 7 : Interconnection of α-MSH and NPY-IR neuron populations in the infundibular nucleus of the human hypothalamus. **A-C**: High power magnification images show α-MSH-IR (green) varicosities in juxtaposition to NPY-IR neurons (arrows). **A1-A2**: Images indicate the same NPY-IR neurons in different focal plane. **D-F** images demonstrate NPY-IR varicosities in the close proximity of α-MSH-IR neurons. Note the high number of NPY-IR varicosities in juxtaposition to the α-MSH-IR perikarya. 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 NPY cells. The thickness of optical slices were 0.7 μm.

2. Colocalization of CART with NPY, α-MSH, AgRP and MCH peptides in the human hypothalamus

In the infundibular nucleus of the human hypothalamus, numerous α-MSH-IR neurons and relatively less CART-IR neurons were found in a dense network of axons containing α-MSH or CART-immunoreactivity (Fig. 8A, B). No co-localization of these peptides was observed in neurons of the infundibular nucleus (Fig. 8A, B). In addition, only single labeled α-MSH and CART axons were observed in both the infundibular nucleus and the PVN (Fig. 8A-C). In contrast, in the rat brain processed similar to the human hypothalamic samples, the majority of α-MSH-IR neurons in the arcuate nucleus and α-MSH-IR axons in the arcuate and PVN also contained CART-immunoreactivity (Fig. 8D, E). CART-immunoreactivity was present in a population of NPY-IR perikarya in the human infundibular nucleus (Fig. 9A). Similarly, CART-immunoreactivity was also observed in neurons containing AGRP (Fig. 9D), known to be co-expressed with NPY in the human infundibular nucleus (Goldstone, Unmehopa et al. 2002). Semiquantitative analyses demonstrated CART-immunoreactivity in 35.7±2.2% of NPY-IR cell-bodies in the infundibular nucleus, whereas CART/NPY neurons formed 54.1±4.6% of CART–IR neurons. CART also co-localized with both NPY and AGRP in numerous axons in the infundibular nucleus and PVN of the human hypothalamus (Fig. 9G-M). In the perifornical region of the lateral hypothalamus, colocalization of CART- and MCH-immunoreactivity was observed in both perikarya and axonal processes (Fig.

10). While 56.7±3.4% of CART-IR neurons co-contained MCH, CART was present in 44.9±7.1% of MCH neurons in this brain region. Examination of double labeled preparations also revealed that the feeding-related neurons of the infundibular nucleus and the lateral hypothalamus are frequently contacted by CART-IR varicosities (Fig. 8F-I; 9B, C; E, F; 10D-F). The vast majority of α-MSH, NPY/AGRP and MCH neurons were juxtaposed by CART-IR axons. In most cases, CART-IR varicosities encircled the immunolabeled neurons. All studied parameters were highly similar in the four studied cases independently from the
cause of death.

Table 5: Summary of semiquantitative analyses of CART colocalizations with feeding-related peptides

NPY neurons containing CART	35.7±2.2%
CART neurons containing NPY	54.1±4.6%
MCH neurons containing CART	44.9±7.1%
CART neurons containing MCH	56.7±3.4%

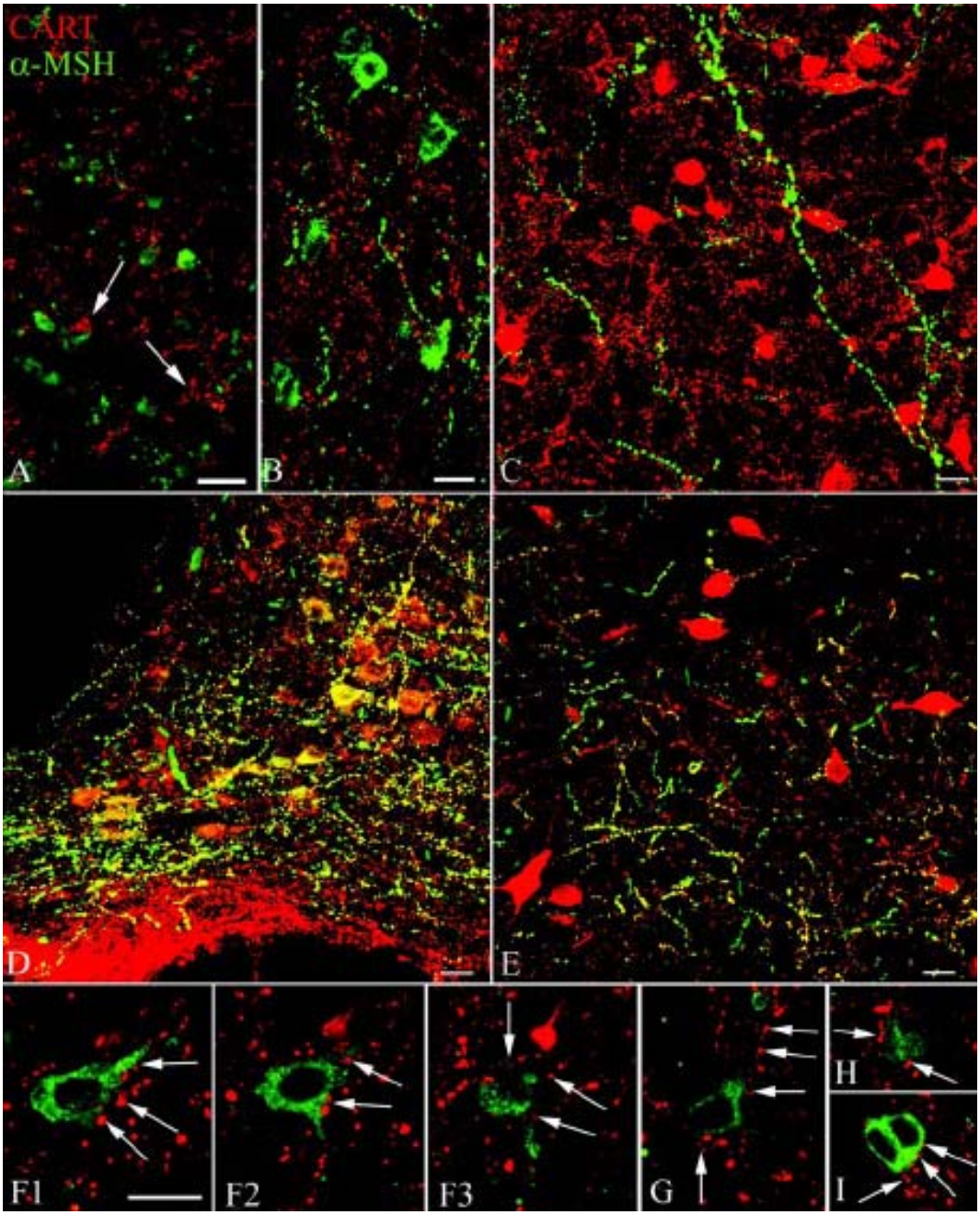


Figure 8: Relationship of CART- (red) and α-MSH-IR neuronal elements (green) in the human hypothalamus. Low (A) and medium power micrographs (B) illustrate the distribution of CART- and α- MSH-IR elements in the human infundibular nucleus. CART is present both in the perikarya (arrows, A) and axons in the infundibular nucleus. There is no apparent colocalization of the two peptides. The PVN is densely innervated by both CART- and α-MSH-IR fibers, but the two peptides are observed in separate populations of axons (C). *(Continued on next page)*

While α-MSH-immunoreactivity is present only in fibers in the PVN, CART-immunoreactivity can be observed in both perikarya and axons (C). In contrast, in the rat arcuate nucleus, CART is present in nearly all α-MSH-IR perikarya and axons (D; Yellow profiles). In addition, all α- MSH-IR axons contain CART in the PVN of rats (E). High magnification micrographs illustrate the juxtaposition of CART-IR terminals to α-MSH-IR neurons in the human infundibular nucleus (arrows) (FI). Unlike rodents, no CART/ α-MSH-colocalization was observed in the infundibular nucleus in humans, but numerous CART-IR boutons were seen in juxtaposition to α-MSH perikarya (arrows). F1-3 represents the same neuron in different confocal plans. Scale bar on A= 40μm, scale bars on B and F=20μm.

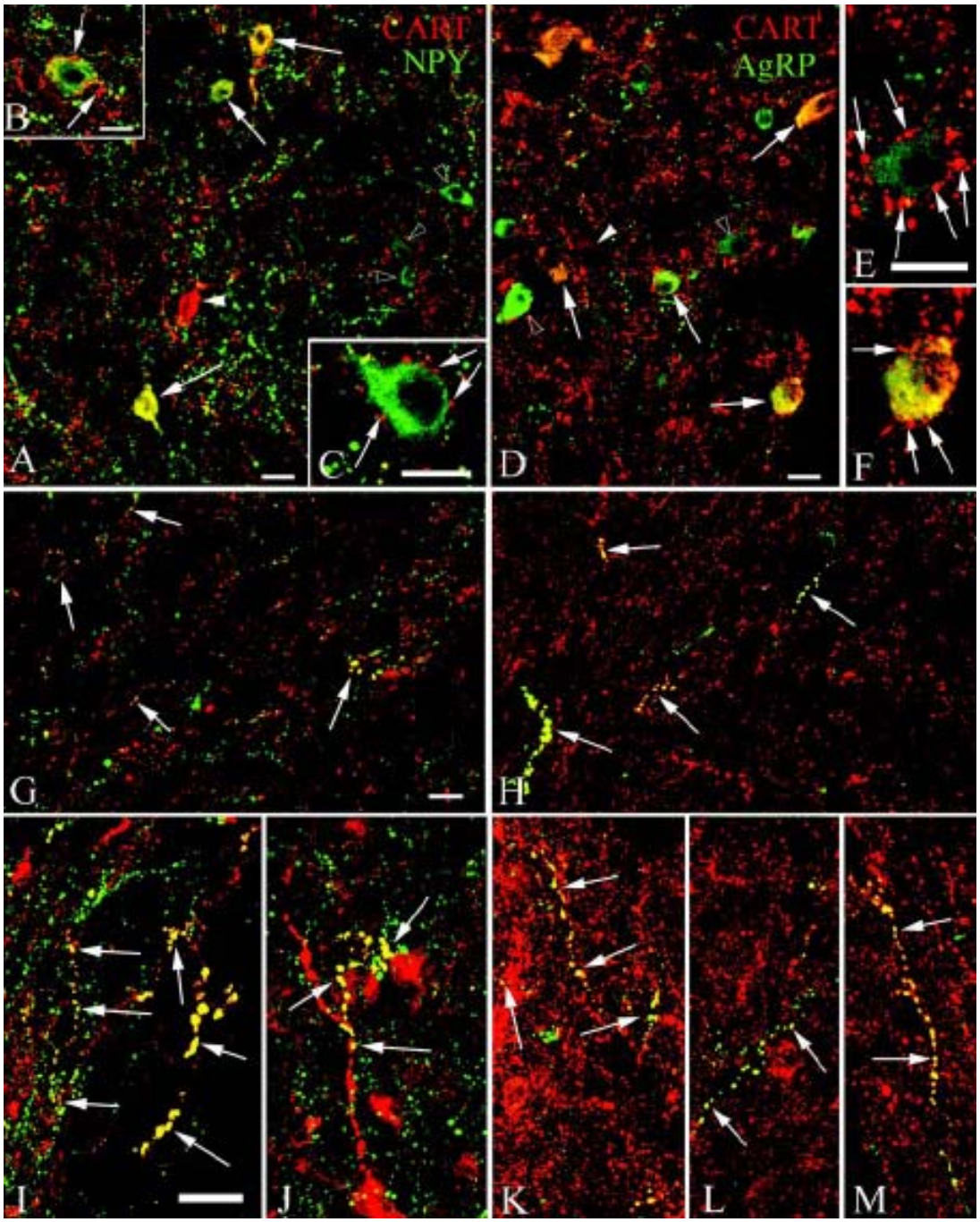


Figure 9: Relationship of CART- and NPY- and CART- and AgRP-IR elements in the human hypothalamus. Legend on next page.

Figure 9: Relationship of CART- (red) and NPY- (green; A-C, G, I, J) and CART- (red) and AGRP-IR elements (green; D-F, H, K-M) in the human hypothalamus. Colocalization of CART and NPY in neurons of the infundibular nucleus (A). Cells containing both CART and NPY appear in yellow color (arrows). Single-labeled CART (arrowhead) and single labeled NPY neurons (open arrowhead) can also be seen in the infundibular nucleus. CART-IR boutons are present in juxtaposition to both NPY/CART (B) and single labeled NPY neurons (C, arrows). CART and NPY are also colocalized in axons in the infundibular nucleus (G; arrows) and in the PVN (I, J; arrows). Colocalization of CART and AGRP in the perikarya of the infundibular nucleus (D). Double-labeled AGRP/CART cells (arrows), single labeled CART (arrowhead) and single labeled AGRP neurons (arrowheads) are seen in the human infundibular nucleus. Both AGRP (E) and AGRP/CART neurons (F) are surrounded by CART-IR varicosities in the infundibular nucleus (arrows). Colocalization of CART and AGRP is also detected in axons within the infundibular nucleus (H; arrows) and the PVN (K-M; arrows). Scale bars =20 μm; scale bar on E corresponds to E, F; scale bar on G corresponds to G, H; scale bar on I corresponds to I-M.

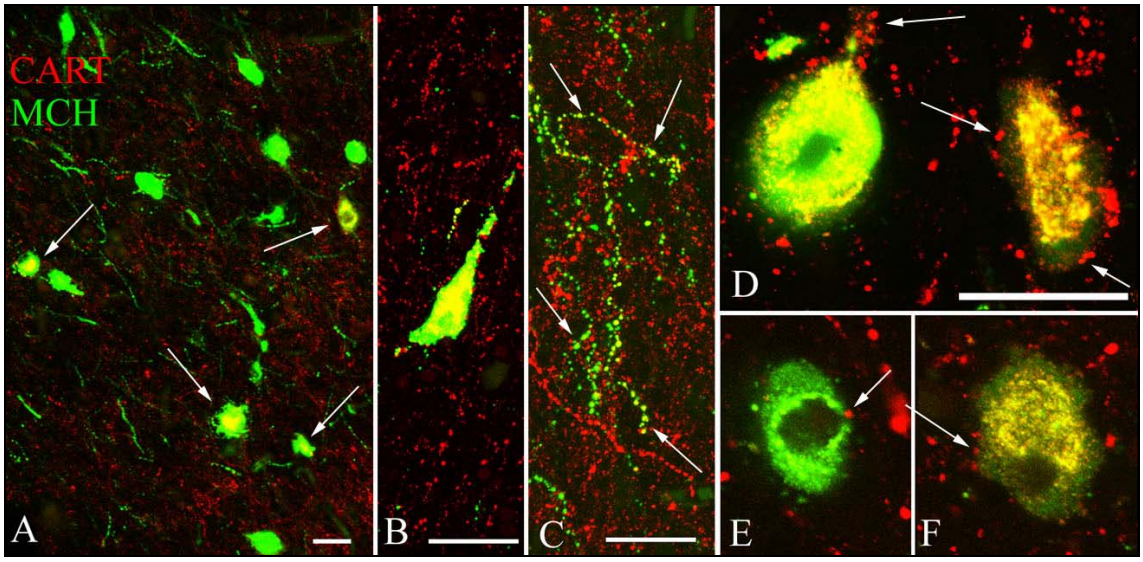


Figure 10: Relationship of CART- (red) and MCH-IR elements (green) in the human hypothalamus. Low magnification micrograph illustrates that CART-immunoreactivity is present in a population of MCH-IR neurons (arrows) in the perifornical area (A). Medium power micrograph shows colocalization of CART and MCH in a neuron from the perifornical region (B). A portion of CART-IR axons in the infundibular nucleus contains MCH-immunoreactivity (arrows) indicating the lateral hypothalamic origin of these fibers (C). High magnification micrographs illustrate the juxtaposition of CART-IR axon varicosities (arrows) to the perikarya of CART/MCH neurons (D, F) and a single labeled MCH cell (E) in the perifornical area. Scale bars= 40 μm; Scale bar on D corresponds to D-F.

3. Distribution of ghrelin immunoreactive elements in the human hypothalamus

Dense networks of ghrelin-immunoreactive fibers were observed in several areas of the human hypothalamus (Figs. 11 and 12). Based on the thickness of fibers, two major types of ghrelin-IR axons were seen in the hypothalamus; i.e., thick fibers with large varicosities and very fine fibers with or without small varicosities. A dense network of thick fibers with large varicosities was observed in the caudal part of supraoptic nucleus (Figs. 11E and 12F), in the periventricular (Figs. 11D and 12) and suprachiasmatic nuclei (Figs. 12A, B) and in the periventricular part of the paraventricular nucleus (Figs.

11A–B and 12C–H). A loose network of very fine fibers was detected in the other parts of the paraventricular nucleus (Figs. 12C–H), in the ventral perifornical region (Fig. 12D) and in the rostral part of supraoptic nucleus (Figs. 12C–E). The periventricular nucleus also contained very fine fibers with small varicosities, in addition to the thick varicose fibers. A network of thick fibers was observed along the medial and lateral zones of the infundibular nucleus, whereas bundles of thick fibers were intermingled with a dense network of thin axons in the central part of the nucleus (Figs. 11G–H and 12D–H). The dorsomedial and the ventromedial nuclei were filled with a dense network of very fine fibers. Varicose immunolabeled fibers were also detected in the external layer of the pituitary stalk. While most regions of the mammillary complex lacked ghrelin-IR elements, a few thin fibers were observed in the ventromedial part of the mammillary nucleus (Figs. 12J–L). Ghrelin-immunoreactive cell bodies were not detected in the processed human hypothalami.

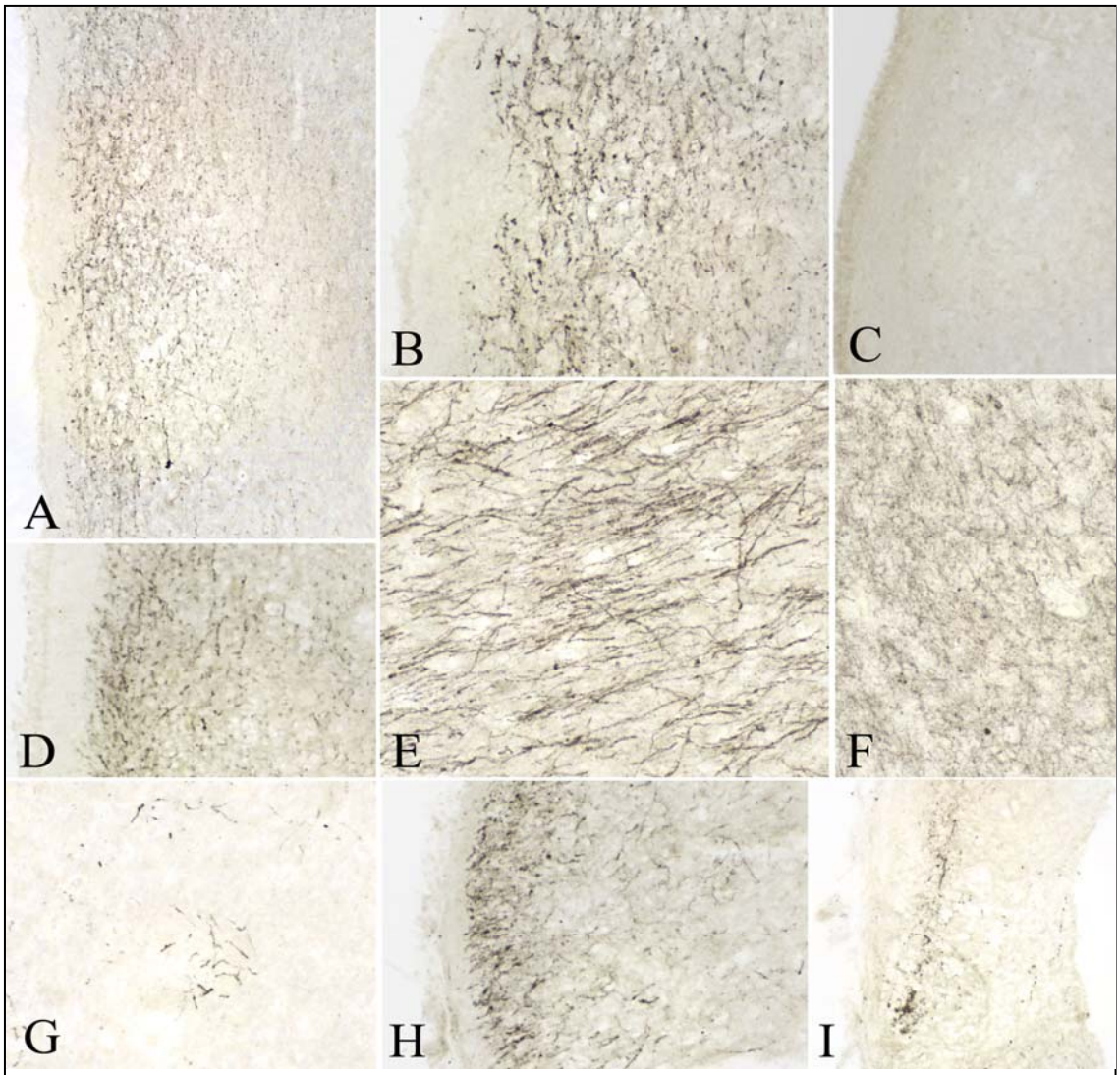


Figure 11: Immunohistochemical detection of ghrelin-immunoreactivity in the human hypothalamus. The hypothalamic paraventricular nucleus (PVN) contains a dense network of ghrelin-IR fibers (A). While thick varicose fibers are present in the medial portion of the nucleus, predominantly thin fibers can be observed in the lateral part of the PVN (A). High magnification image illustrates the network of varicose ghrelin-IR axons in the medial portion of the PVN (B). The ghrelin-IR fiber network is completely absent from the PVN after using primary antibody preabsorbed with synthetic ghrelin (C). Thick ghrelin-IR fibers running parallel to the ventricular wall in the periventricular nucleus (D). *(Continued on next page)*

Figure 11 The highest density of thick ghrelin-IR fibers can be observed in the supraoptic nucleus. Most of these axons run parallel to the ventral surface of the hypothalamus (E). The ventromedial nucleus (VMN) contains a very dense network of thin ghrelin-IR fibers (F). In the central part of the infundibular nucleus, 'islands' of ghrelin-IR fibers are present (G). Ghrelin-IR fibers with large varicosities are located in the lateral part of the infundibular nucleus (H) and within external third of the pituitary stalk (I). Scale bar=100 μm. Abbreviations: PVN: paraventricular nucleus; PV: periventricular nucleus; SON: supraoptic nucleus; VMN: ventromedial nucleus; INF C: central part of the infundibular nucleus; INF L: lateral part of the infundibular nucleus; PS: pituitary stalk; III: third ventricle.

Table 6: Description of hypothalamic regions containing ghrelin-IR axons

	Thick fibers with large varicosities	Fine fibers with small or without varicosities
Dense network	Supraoptic nucleus (SON), periventricular nucleus (PV), suprachiasmatic nucleus (SCh), paraventricular nucleus (PVN)	Dordomrdisl nucleus (DMN), ventromedial nucleus (VMN)
Loose network	Infundibulum, pituitary stalk	Paraventricular nucleus (PVN), supraoptic nucleus (SON), perifonical region (pFx), periventricular nucleus (PV), mamillary body (Mb)

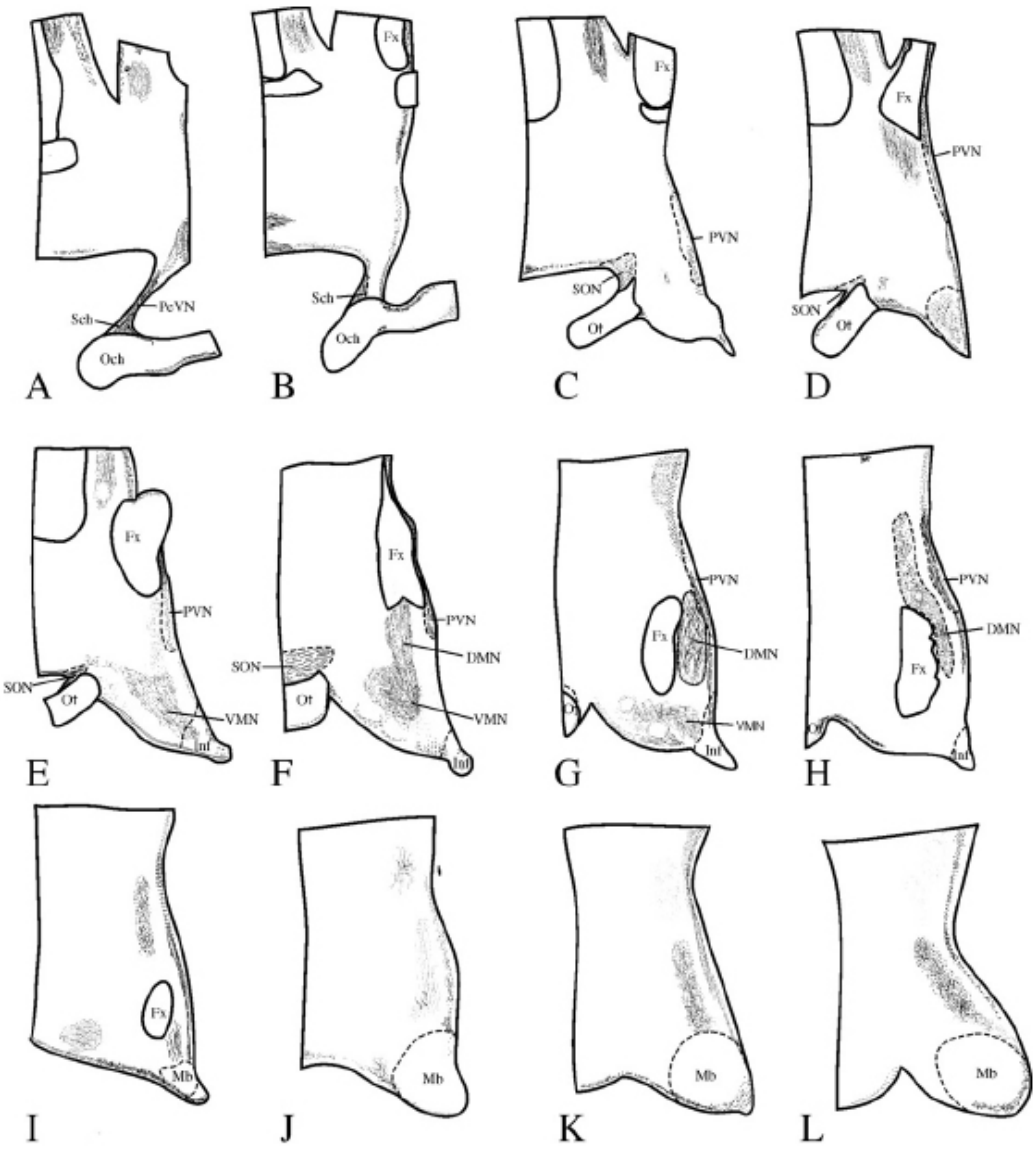


Figure 12: Schematic maps of the distribution of ghrelin-immunoreactive fibers through the rostrocaudal extent of human hypothalamus. Abbreviations: DMN, dorsomedial nucleus; Fx, fornix; Mb, mammillary body; Och, optic chiasm; Ot, optic tract; PVN, paraventricular nucleus; Sch, suprachiasmatic nucleus; Son, supraoptic nucleus; VMN, ventromedial nucleus.

Discussion

Interconnection of NPY- and α-MSH- producing neurons in the human hypothalamus

In accordance with earlier studies from our group (Mihaly, Fekete et al. 2000), we have found that similarly to rodents, 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 (Csiffary, Gorcs et al. 1990; Horvath, Naftolin et al. 1992; Cowley, Smart et al. 2001). 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, Yamano et al. 1985; Sawchenko, Swanson et al. 1985). The catecholaminergic neurons also coexpress vesicular glutamate transporter 2, in addition to NPY (Hokfelt, Lundberg et al. 1983; Sawchenko, Swanson et al. 1985; Stornetta, Sevigny et al. 2002), and establish asymmetric synapses (Liposits, Phelix et al. 1986) suggesting a glutamatergic phenotype of these neurons. The innervation of the α-MSH neurons by GABA-ergic, NPY-containing terminals (Cowley, Smart et al. 2001) indicates that the arcuate nucleus, where NPY colocalizes with the GABA synthesizing enzyme glutamic acid decarboxylase (Horvath, Bechmann 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, Liu et al. 2004) and decreases the α-MSH level in the hypothalamus (Blasquez, Jegou et al. 1995). Cowley et al. (Cowley, Smart 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, Giraud 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, Liu 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, Lu et al. 1999), Roseberry et al. (Roseberry, Liu 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, Matheny 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, Lu et al. 1999). Therefore, the MC3 receptor-containing NPY cells could have been underrepresented in the in vitro study (Roseberry, Liu 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 CART and vesicular glutamate transporter 2 (Elias, Lee et al. 1998; Collin, Backberg et al. 2003), 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.

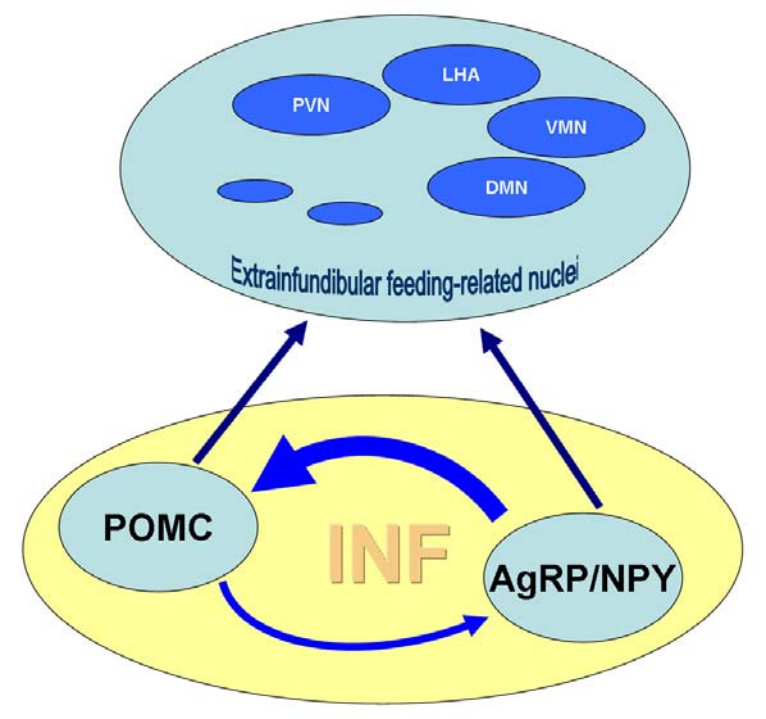


Figure 13: Schematic illustration of the interrelationship between the two major feeding-related neuronal populations of the infundibular nucleus. A main difference between rodent and human NPY- α-MSH system in the ARC is the bilateral connection between these populations. However, POMC producing anorexigenic neurons have nearly three times more NPY/AgRP-containing varicosities than the number of α-MSH containing boutons on the surface of the NPY cells.

CART may play a different role in humans than described in rodents

In the present study, we demonstrate that contrary to the findings observed in rats and mice, CART and α-MSH do not colocalize in the human infundibular nucleus. Since rapid axonal transport of CART into the axon terminals of α-MSH neurons could explain the absence of CART in the perikarya of these neurons, we also determined whether CART and α-MSH were present in axons of two brain regions known to be heavily innervated by α-MSH fibers of arcuate nucleus origin, the infundibular nucleus, itself, and the hypothalamic paraventricular nucleus (Fekete, Mihaly et al. 2000). While CART and α-MSH fibers densely innervated both regions, the two peptides were present in separate populations of axons without any apparent co-localization. To exclude the possibility that the discrepancies between the rodent and human data are due to different processing of the brain tissues, we performed double-labeling immunocytochemistry on rat hypothalamic tissues processed similar to the human diencephalic blocks. In rat hypothalamic samples that were immersion fixed 6h after death, the vast majority of the α-MSH-IR elements also contained CART-immunoreactivity. Together, these data suggest that in contrast to the rodent brain, the expression of CART in α-MSH neurons of the infundibular nucleus is not necessary to mediate the effects of peripheral satiety signals in the human hypothalamus. Similar observations were made by Grayson and co-workers in the hypothalamus of non-human primates (Grayson, Allen et al. 2006). While CART expression is not observed in NPY/AGRP neurons in the rodent hypothalamus (Vrang, Larsen et al. 1999), approximately one third of the NPY/AGRP neurons detected by the immunofluorescent labeling were found to contain CART in the human samples, and co-localization of CART with NPY and AGRP was also observed in axons in the known projection fields of infundibular NPY neurons. While CART/NPY fibers may originate from both the arcuate nucleus and adrenergic neurons of the rodent the brainstem that synthesize both CART and NPY and project to the PVN (Sawchenko, Swanson et al. 1985; Wittmann, Liposits et al. 2002; Fekete, Wittmann et al. 2004; Wittmann, Liposits et al. 2005), the exclusive source of CART/AGRP fibers are NPY/AGRP neurons is the infundibular nucleus, because AGRP is synthesized exclusively in the arcuate/infundibular nucleus (Broberger, Johansen et al. 1998; Hahn, Breininger et al. 1998). The preferential co-

localization of CART with orexigenic peptides in the human hypothalamus raises the possibility that contrary to the rodent, CART may play orexigenic role in the human infundibular nucleus or subserves other functions. The potential orexigenic role of CART is supported by data that CART injection to the PVN increases food intake even in rodents (Abbott, Rossi et al. 2001; Smith, Gardiner et al. 2008). Alternatively, infundibular CART neurons may have a role in prolactin regulation. We have previously demonstrated that CART is capable of inhibiting TRH-induced prolactin secretion (Raptis, Fekete et al. 2004; Sanchez, Fekete et al. 2007), and Baranowska et al (Baranowska, Wolinska-Witort et al. 2003) have shown direct prolactin inhibiting effects of CART in cell culture. While we presume that the origin of CART neurons involved in prolactin regulation in the rat derives primarily from the hypothalamic PVN (Fekete and Lechan 2006), this may not be the case in the human brain. Nevertheless, there is some physiological evidence that CART may have an anorexigenic role in the human brain despite the observation that CART does not colocalize with α-MSH. A point mutation of the CART gene that leads to altered processing and decreased activity of CART peptides, is associated with severe obesity in humans (Yanik, Dominguez et al. 2006), suggesting that CART exerts a constant anorexigenic tone in the human brain. Indeed, starvation decreases CART mRNA levels in the arcuate nucleus of monkeys (Van Vugt, Lujan et al. 2006), where the α-MSH neurons also do not synthesize CART (Grayson, Allen et al. 2006), suggesting that infundibular CART is regulated like an anorexigenic peptide in primates. The co-expression of CART with NPY and AGRP in the human brain is distinctly different from the rodent where these peptides do not colocalize in the arcuate nucleus. The explanation for this difference is not clear but by itself, does not exclude the possibility that CART exerts anorexic actions. There are many examples where neurons synthesize substances that exert opposite actions on target neuronal populations. For example, the two, main, excitatory and inhibitory neurotransmitters, glutamate and GABA, respectively, have been shown to co-localize in nearly all neurons of the anteroventral periventricular area (Ottem, Godwin et al. 2004) and in hippocampal mossy fibers of young animals (Safiulina, Fattorini et al. 2006). In addition, both CART and NPY are synthesized by adrenergic neurons of the C1-3 area in the rat brain (Sawchenko, Swanson et al. 1985; Wittmann, Liposits et al. 2002; Fekete, Wittmann et al. 2004; Wittmann, Liposits et al. 2005) and contained in

adrenergic axons innervating the hypophysiotropic TRH neurons (Wittmann, Liposits et al. 2002; Wittmann, Liposits et al. 2005). While CART stimulates TRH gene expression, NPY exerts potent inhibitory effect on TRH-synthesis in the same neurons (Fekete, Kelly et al. 2001). Presumably, differential regulation of the opposing transmitters may enable the neurons to regulate their postsynaptic targets in a more precise manner. As noted previously, it is also possible that CART synthesized in NPY neurons of the infundibular nucleus is not involved in the regulation of food intake, but rather regulates other neuronal functions. Indeed, fasting has no effect on the NPY expression in the arcuate nucleus of the monkey hypothalamus (Van Vugt, Lujan et al. 2006). Since both CART and NPY are known to stimulate the hypothalamic-pituitary-adrenal (HPA) axis through hypophysiotropic CRH neurons, and these paraventricular neurons are innervated by AGRP/NPY fibers (Mihaly, Fekete et al. 2002), it is feasible that the AGRP/NPY/CART neurons of the human infundibular nucleus may be involved in the regulation of the HPA axis under certain stress conditions. In contrast to the infundibular nucleus, the colocalization of CART with MCH in the lateral hypothalamus is similar to that observed in the rodent brain (Elias, Lee et al. 2001). Unfortunately, little is known about the role of CART synthesized in this neuronal population. Though it is well-known that MCH gene expression is markedly upregulated by fasting (Kokkotou, Tritos et al. 2001) and MCH has a potent orexigenic effect (Ludwig, Mountjoy et al. 1998), CART gene expression seems to be unaltered by changes in energy homeostasis in the MCH neurons of rodents (Yang and Shieh 2005). These data suggest that MCH and CART may be involved in the regulation of different physiological functions executed by the same neurons. Indeed, it has been suggested that CART synthesis in lateral hypothalamic neurons is involved in the regulation of dopaminergic reward pathways (Philpot and Smith 2006). Lateral hypothalamic CART neurons are known to project to the ventral tegmental area where CART exerts psychostimulant-like effects through the stimulation of dopaminergic neurons (Jaworski, Vicentic et al. 2003; Dominguez, Vicentic et al. 2004; Kuhar, Jaworski et al. 2005). Dense CART innervation of feeding related neurons in both the infundibular nucleus and in the lateral hypothalamus suggest that CART, originating from multiple brain sources, may be involved in the control of food intake through the regulation of these neuronal groups. In summary, in contrast to the rodent brain, CART is synthesized

in a population of NPY/AGRP neurons and it is absent from the α- MSH-synthesizing neurons in the infundibular nucleus of the human hypothalamus. These data indicate that there are important differences between the hypothalamic feeding regulatory systems of the humans and rodents, and raise the possibility that CART may play a different role in the regulation of feeding in humans than described in rodents.

Ghrelin is widely distributed in the human hypothalamus

Currently ghrelin is considered as the only peripheral orexigenic hormone (Horvath, Castaneda et al. 2003). While the ability of ghrelin to cross the blood–brain barrier has been demonstrated (Banks, Tschop et al. 2002), central administration of ghrelin causes a significantly higher increase of food intake and body weight gain than its peripheral delivery (Tschop, Smiley et al. 2000). These data suggest that although ghrelin of peripheral origin controls feeding through central pathways, the centrally synthesized ghrelin also has a major contribution to the regulation of feeding (Tschop, Smiley et al. 2000). In the hypothalamus, we have observed two major subtypes of the ghrelin-IR fibers: thick fibers with large varicosities and thin fibers with or without small varicosities. However, the importance of this morphological feature of ghrelin-IR fiber network is currently unknown, one may raise the possibility that the two types of fibers originate from two separate cell populations. On the other hand, we cannot exclude the possibility that the thick fibers form a major trajectory from a single ghrelin-IR cell population and the thin fiber network represents the arborization of thick ghrelin-IR axons. While earlier studies described the presence of ghrelin-IR neuronal cell bodies in the human infundibular nucleus (Korbonits, Bustin et al. 2001) without the presence of ghrelin-IR fibers, in the current study we have observed dense networks of ghrelin-IR axonal profiles in multiple regions of the hypothalamus without detectable levels of ghrelin-immunoreactivity in perikarya of this brain region. The reason for this discrepancy in the two studies is not known. The potential changes of the epitopes of the ghrelin molecule during the maturation of the peptide and the likely different epitope-binding preferences of the used antibodies may explain the discrepancies. This hypothesis is further supported by the fact that ghrelin immunoreactivity was localized in a small perinuclear region of the infundibular neurons and did not fill the entire

cytoplasm of the perikarya in the publication by Korbonits (Korbonits, Bustin et al. 2001). This intracellular localization is reminiscent of the localization of Golgi apparatus, suggesting that the antibody used in that study recognized a relatively immature form of ghrelin. In addition, similarly to the results of this human study, we have also observed the preferential detection of axon profiles without the recognition of ghrelin-IR perikarya in the brain of rodents, if colchicine pretreatment was not used (unpublished data). Further studies using in situ hybridization are needed to clarify the hypothalamic and potential extrahypothalamic distribution of ghrelin-synthesizing neurons in the human brain. In the present study, we described the wide distribution of ghrelin-IR fibers in the human hypothalamus. Dense networks of ghrelin-IR axons were detected in the paraventricular, dorsomedial, ventromedial and infundibular nuclei, regions known to have a principal role in the regulation of food intake and energy balance. These regions innervated by ghrelin-IR fibers also express growth hormone secretagogue receptor (GHS-R), the only currently known receptor of ghrelin in the rodent brain (Zigman, Jones et al. 2006). In the hypothalamic arcuate nucleus, the rodent homologue of the infundibular nucleus, neurons synthesizing the orexigenic peptide neuropeptide Y (NPY) and agouti-related protein (AGRP) also express GHS receptor and are highly innervated by ghrelin-IR axons (Willesen, Kristensen et al. 1999). Since peripheral ghrelin administration induces c-fos activation in these neurons (Horvath, Diano et al. 2001; Wang, Saint-Pierre et al. 2002), the NPY/AGRP neurons seem to be under the control of ghrelin originating from both peripheral visceral and central, neuronal sources. Ghrelin has also been shown to have a potent orexigenic effect when injected directly into the paraventricular nucleus (Olszewski, Grace et al. 2003). Since the effects of ghrelin injected in the PVN are very similar to those of NPY on food intake and energy metabolism regulation (Tucci, Rogers et al. 2004), and ghrelin binds to NPY-IR axons of the PVN, it has been suggested that the effect of ghrelin in the PVN may be mediated through an increased NPY release (Currie, Mirza et al. 2005). In addition to the above mentioned feeding-related regions, the presence of ghrelin-IR fibers was observed in the periventricular, suprachiasmatic and supraoptic nuclei, suggesting the putative role of these fibers in the regulation of GH secretion, water and salt balance and circadian rhythms in humans. The presence of ghrelin-immunoreactive axons in the external zone of the pituitary stalk raises the possibility of

ghrelin synthesis in some hypophysiotropic neurons and the release of the peptide into the portal circulation, whereby central ghrelin might influence anterior pituitary functions as well. The distribution of ghrelin-IR fibers in our study was highly reminiscent to the localization of GHS-R in the rodent brain (Mitchell, Bouret et al. 2001; Zigman, Jones et al. 2006). The only exception was the supraoptic nucleus, where dense ghrelin-IR fiber network was detected in the human hypothalamus, but GHS-R has not been revealed in the rodent brain (Mitchell, Bouret et al. 2001). However, the possibility exists that GHS-R may be present in the SON of the human. In addition, the presence of another yet unknown ghrelin receptor in the human SON cannot be excluded. It is worth mentioning that the existence of an additional ghrelin receptor is supported by the recent data of (Halem, Taylor et al. 2005) who demonstrated that BIM-28163, a GHS-R antagonist, blocks only the growth hormone secretagogue effect of ghrelin but has no influence on the feeding stimulatory effect of ghrelin treatment.

In conclusion, our data demonstrated the dense ghrelin-IR innervation of multiple hypothalamic nuclei including the feeding-related infundibular, paraventricular and dorsomedial nuclei.

Conclusions

We conclude that unlike in rodents, 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 in the human hypothalamus.

In contrast to the rodent brain, CART is synthesized in a population of NPY/AGRP neurons and it is absent from the α-MSH-synthesizing neurons in the infundibular nucleus of the human hypothalamus. These data indicate that there are important differences between the hypothalamic feeding regulatory systems of humans and rodents, and raise the possibility that CART may play a different role in the regulation of feeding in humans than described in rodents.

Mapping the ghrelin-IR elements in the human hypothalamus revealed a dense ghrelin-IR innervation of multiple hypothalamic nuclei including the feeding-related infundibular, paraventricular and dorsomedial nuclei. The findings suggest a definite role of the hypothalamic ghrelin-IR neuronal circuits in central regulation of energy balance in humans.

Summary

The maintenance of energy homeostasis is regulated by the cooperation of the brain and the peripheral endocrine systems. The main hypothalamic sensor area of the peripheral feeding related hormones is the hypothalamic arcuate nucleus. In rodents, this nucleus contains two neuronal groups that play critical role in the regulation of energy homeostasis: the orexigenic NPY/AGRP and the anorexigenic α-MSH/CART synthesizing neurons. An other, recently recognized feeding related system of the hypothalamus is the hypothalamic ghrelin-synthesizning neuronal group. To understand whether the experimental data obtained from rodents may be valid for humans, we have studied the feeding related neuronal groups of the human infundibular nucleus, the analogue of the rodent arcuate nucleus, and the ghrelin-IR system of the human hypothalamus

We have found that there is a bilateral, but asymmetric connection between the NPY and α-MSH-synthesizing neurons of the human infundibulum. Three times more NPY boutons were observed on the surface of the anorexigenic α-MSH cells, than the number of α-MSH varicosities on the NPY neurons. The reason of this phenomenon can be due to evolutionary advantages of preferring feeding stimulating pathways contra feeding inhibiting, but may be also other compensatory mechanisms present.

In rodents, CART is described as an anorexigenic peptide. However, we have observed that CART is not synthesized in the anorexigenic α-MSH neurons of the human infundibular nucleus, but rather CART is present in a population of orexigenic NPY neurons in the human hypothalamus, raising the question whether CART is orexigenic or anorexigenic peptide in the human brain.

Using single-labeling immunocytochemistry, we mapped the distribution of ghrelin-IR neuronal elements in the human hypothalamus. Ghrelin-IR axons were found in almost all feeding related nuclei of the human hypothalamus, including the paraventricular nucleus (PVN), periventricular nucleus (PeVN), the infundibular nucleus, the supraoptic nucleus (SON), VMN and also in the medial part of the hypophyseal stalk.

Our data indicate that despite the similarities of the rodent and human feeding related circuits, some aspects of this regulators system underwent major changes during the evolution.

Összefoglaló

Az energiahomeosztázist az agy és a perifériás endokrin rendszerek együttműködése szabályozza. A perifériáról érkező táplálkozással kapcsolatos hormonális jelek egyik legfőbb érzékelő központja az arcuatus mag. A rágcsálók arcuatus magjában két, egymással ellentétes hatású neuroncsoport található, a táplálékfelvételt serkentő NPY-t és AgRP-t termelő sejtek, illetve a táplálékfelvételt gátló hatású α-MSH és CART peptideket termelő neuronok. A táplálkozás szabályozásában részt vesz egy újabban felfedezett rendszer is, a hipotalamusz ghrelint-termelő sejtcsoportja. Vizsgálatainkban arra kerestük a választ, hogy vajon a rágcsálókban leírt rendszerek azonos módon működnek-e az ember esetében is. Kísérleteink fő tárgya a rágcsálók arcuatus magjának megfelelő emberi infundibularis mag, iletve a ghrelin-tartalmú rendszer vizsgálata volt.

Eredményeink az infundibuláris mag két táplálkozást szabályozó sejtcsoportja között kölcsönös, kétirányú, ám aszimmetrikus kapcsolatrendszert igazoltak. A táplálkozást gátló α-MSH sejtek felszínén háromszor annyi NPY-tartalmú axont észleltünk, mint az NPY sejtek felszínén megfigyelt α-MSH varikozitások száma. E jelenség egyik magyarázata lehet, hogy a táplálék felvételére ösztönző folyamatok evolúciós előnyt jelentettek a táplálkozást gátlókkal szemben; ugyanakkor nem zárhatjuk ki egyéb kompenzációs folyamatok meglétét sem.

Rágcsálókban a CART fehérjét mint táplálkozást gátló anyagot írták le. Kísérleteink feltárták, hogy az emberi hipotalamuszban az α-MSH tartalmú idegsejtek és axonok nem tartalmaznak CART peptidet, ugyanakkor az NPY-t termelő sejtek egy csoportjában CART termelődés figyelhető meg. Habár van rá példa, hogy ellentétes hatású molekulák termelődjenek egyazon sejtben, eredményeink felvetik a kérdést, hogy emberben a CART táplálkozást gátló vagy táplálkozást serkentő hatású peptid-e.

Az emberi hipotalamusz ghrelin tartalmú idegelemeinek feltérképezése során megfigyeltük, ghrelin-tartalmú idegrostok előfordulását a humán hipotalamusz minden táplálkozás szabályozásban szerepet játszó területén, mint például a paraventrikuláris, periventrikuláris, infundibuláris, supraopticus and ventromedialis magokban, illetve a hipofízisnyél középső részében is.

Eredményeink rámutatnak, hogy a rágcsálókban leírt szabályozó rendszerekkel való nagyfokú hasonlóság ellenére e rendszerek is változtak az evolúció során.

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List of publications underlying the thesis

1. Menyhért J., Wittmann G., Hrabovszky E., Keller É., Liposits Z., Fekete C.
   Interconnection between orexigenic neuropeptide Y- and anorexigenic alpha-melanocyte stimulating hormone-synthesizing neuronal systems of the human hypothalamus
   Brain Res, 1076 (2006) 101-105

2. Menyhért J., Wittmann G., Hrabovszky E., Szlávik N., Keller É., Tschöp M., Liposits Z., Fekete C.
   Distribution of ghrelin-immunoreactive neuronal networks in the human hypothalamus
   Brain Res, 1125 (2006) 31-36

3. Menyhért J., Wittmann G., Lechan R.M., Keller É., Liposits Z., Fekete C.
   Cocaine- and amphetamine regulated transcript (CART) is colocalized with the orexigenic NPY and AGRP and absent from the anorexigenic α-MSH neurons in the infundibular nucleus of the human hypothalamus
   Endocrinology. 2007 Sep;148(9):4276-81.

List of other publications

1. Zeöld A, Doleschall M, Haffner MC, Capelo LP, Menyhért J, Liposits Z, da Silva WS, Bianco AC, Kacskovics I, Fekete C, Gereben B.
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