Research Report

Distribution of ghrelin-immunoreactive neuronal networks in the human hypothalamus

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ABSTRACT

Ghrelin has been discovered as the endogenous ligand of the growth hormone secretagogue receptor (GHS-R). It stimulates growth hormone secretion and also potently increases food intake. To date, ghrelin is the only known peripheral orexigenic hormone. Recent studies have demonstrated that in addition to peripheral organs, ghrelin is also synthesized in the hypothalamus. In the present study, we examined the distribution of the ghrelin-immunoreactive (IR) elements in the human hypothalamus. Ghrelin-IR fibers were widely distributed throughout the hypothalamus. Based on the thickness of fibers, major subtypes of ghrelin-IR axons were observed: thick fibers with large varicosities and very fine axons with or without small varicosities. Dense networks of ghrelin-IR axons were observed in the hypothalamic suprachiasmatic, paraventricular, supraoptic, dorsomedial, ventromedial and infundibular nuclei and in the periventricular area. Ghrelin-IR axons also appeared in the external layer of the pituitary stalk. Ghrelin-IR cell bodies were not detected. Since hypothalamic regions innervated by ghrelin-IR axons also take part in the regulation of food intake and energy balance, the centrally synthesized ghrelin may play a major role in the central regulation of energy metabolism in humans.

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1. Introduction

Ghrelin, a 28 amino acid peptide with an n-octanoyl modification in the Ser3 was described as the endogenous ligand of growth hormone (GH) secretagogue receptors (GHS-R) (Kojima et al., 1999). Ghrelin regulates the release of GH in the pituitary in a dose-dependent manner (Takaya et al., 2000a), and it also increases the circulating levels of PRL, ACTH and cortisol (Nagaya et al., 2001a), whereas it has only a minor impact on the release of other hypophyseal hormones (Takaya et al., 2000b). Ghrelin-synthesizing cells were first described in the stomach (Kojima et al., 1999). While the fundus of stomach is...
the major source of circulating ghrelin, a substantially lower amount is synthesized in the bowel, kidney, placenta, blood cells, testicles, ovaries, pancreas, pituitary and the hypothalamus (Gnanapavan et al., 2002; Kojima et al., 1999). In addition to its effects on the pituitary, ghrelin was also shown to have a potent orexigenic effect (Tschope et al., 2000; Wren et al., 2000). Administration of ghrelin increases the food intake and decreases the fat utilization in both rodent and human (Tschope et al., 2000; Wren et al., 2001). Circulating levels of ghrelin are elevated during fasting, cachexia or anorexia nervosa (Ariyasu et al., 2001; Nagaya et al., 2001b) and decreased after feeding (Tschope et al., 2001), suggesting further its role in the regulation of energy homeostasis. 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 this brain region in the mediation of orexigenic effects induced by ghrelin (Zigman et al., 2006).

In addition to being 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 et al., 2003; Sato et al., 2005). Ghrelin-immunoreactive (IR) perikarya were observed in a unique location delineating an area among the ventromedial, dorsomedial and paraventricular nuclei (Cowley et al., 2003). Ghrelin-IR neurons of rodents project to several hypothalamic nuclei involved in the regulation of energy homeostasis, including the arcuate nucleus, the paraventricular nucleus (PVN), the lateral hypothalamus and the hypothalamic dorsomedial nucleus (DMN). Furthermore, fasting has also been shown to regulate ghrelin synthesis in the hypothalamus (Cowley et al., 2003).

To reveal whether the central ghrelin-synthesizing system is also a constituent of the human brain, we examined the distribution of ghrelin-immunoreactive elements in the human hypothalamus using immunohistochemistry on post-mortem human brain samples.

2. Results

Dense networks of ghrelin-immunoreactive fibers were observed in several areas of the human hypothalamus (Figs. 1 and 2). 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 suprachiasmatic nucleus (Figs. 1E and 2F), in the paraventricular (Figs. 1D and 2) and suprachiasmatic nuclei (Figs. 2A, B) and in the paraventricular part of the paraventricular nucleus (Figs. 1A–B and 2C–H). A loose network of very fine fibers was detected in the other parts of the paraventricular nucleus (Figs. 2C–H), in the ventral perifornical region (Fig. 2D) and in the rostral part of suprachiasmatic nucleus (Figs. 2C–E). The paraventricular 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. 1G–H and 2D–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. 2–I).

Ghrelin-immunoreactive cell bodies were not detected in the processed human hypothalami.

3. Discussion

Currently ghrelin is considered as the only peripheral orexigenic hormone (Horvath et al., 2003). While the ability of ghrelin to cross the blood–brain barrier has been demonstrated (Banks et al., 2002), central administration of ghrelin causes a significantly higher increase of food intake and body weight gain than its peripheral delivery (Tschope 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 (Tschope 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 et al., 2003) 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 et al. (2003). 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 extra-hypothalamic 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
Fig. 1 – 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). 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.
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 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 et al., 1999). Since peripheral ghrelin administration induces c-fos activation in these neurons (Horvath et al., 2001; Wang 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 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 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 et al., 2005).

In addition to the abovementioned feeding-related regions, the presence of ghrelin-IR fibers was observed in the
periventricular, suprachiasmatic and supraoptic nuclei, suggest-
ging 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 the present study
was highly reminiscent to the localization of GHS-R in the
rodent brain (Mitchell et al., 2001; Zigman 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 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 et al. (2005) who demonstrated that BIM-28163, a
GHS-R antagonist, blocks only the growth hormone secreta-
gogue effect of ghrelin but has no influence on the feeding
stimulatory effect of ghrelin treatment.

In conclusion, our present data demonstrated the dense
ghrelin-IR innervation of multiple hypothalamic nuclei includ-
ing the feeding-related infundibular, paraventricular and
dorsomedial nuclei. The findings suggest a definite role of the
hypothalamic ghrelin-IR neuronal circuits in central regu-
lation of energy balance in humans.

4. Experimental procedures

4.1. Preparation of human hypothalamic tissues

Hypothalami of three adult human individuals with no history
of neurological or endocrinological impairment were obtained
from autopsy. Tissue samples were taken within 24 h after
death in accordance with the permission and regulations of the
Regional Committee of Science and Research Ethics, Buda-
pest, Hungary (permission number TUKEB 49/1999).

The hypothalamic blocks were fixed in a mixture of 4%
acrolein and 2% paraformaldehyde for 48 h at 4 °C, then cryo-
protected in 30% sucrose and frozen on dry ice. Serial 30-μm-
thick coronal sections were cut parallel to the lamina termi-
nalis with a freezing microtome and stored in freezing solution
(30% ethylene glycol; 25% glycerol; 0.05 M PB) at −20 °C until
used.

4.2. 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 were treated with 1% sodium borohy-
dride in distilled water for 30 min and with 0.5% Triton X-100/
0.5% H2O2 in PBS for 15 min. To reduce nonspecific antibody
binding, the sections were treated with 2% normal horse serum
in PBS for 20 min. After pretreatment as described above,
sections were incubated in rabbit anti-ghrelin serum (Cowley
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, Burling-
game, 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% H2O2 in 0.05 M Tris buffer, pH 7.6, and
intensified using the Gallyas silver intensification technique to
yield a black precipitate (Liposits et al., 1994). The sections were
mounted onto glass slides, air-dried, dehydrated in ascending
series of ethanol and coverslipped with DPX (Fluka, Ronkon-
koma, NY).

The specificity of ghrelin immunostaining was earlier
demonstrated by showing the lack of immunoreaction signal
in ghrelin-KO mice (Cowley 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)
(Fig. 1C).

Photomicrographs were taken with a Zeiss Axiophot micro-
scope equipped with a RT Spot digital camera.

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