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ORIGINAL ARTICLE

Substance P appears to affect growth via growth hormone-releasing hormone (GHRH) neurons in the human hypothalamus

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Abstract

Substance P is an eleven-amino acid neuropeptide (undecapeptide) with multiple effects on the gastrointestinal, cardiovascular, and urinary systems as well as complex central nervous system functions such as pain, learning, memory, and sexual homeostasis. Previous studies also revealed that substance P exhibits regulatory effects on growth possibly via influencing hypothalamic GHRH release in human. However, the morphological substrate of this phenomenon has not been elucidated yet. In the present study, we examined the putative presence of juxtapositions between the substance P- and GHRH-immunoreactive (IR) systems using double-label immunocytochemistry. High-magnification light microscopy with oil immersion was used to identify putative juxtapositions between these systems. Our studies revealed substance P-IR fiber network abutting on the surface of the majority of GHRH-immunoreactive neurons in the human hypothalamus. These fiber varicosities often cover a significant surface area on the GHRH-IR neurons, forming basket-like encasements with multiple en passant type contacts. The majority of these densely innervated GHRH-IR neurons were found in the infundibular nucleus/median eminence, while substance P-IR fibers often abut on the GHRH-IR neurons in the periventricular zone and basal perifornical area of the tuberal region and in the dorsomedial subdivision of the ventromedial nucleus. The posterior hypothalamus did not contain observable substance P-GHRH associations. The density and the morphology of these intimate associations suggest that substance P influences growth by regulating hypothalamic GHRH release by direct synaptic contacts.

Keywords Substance P · GHRH · Juxtaposition · Immunohistochemistry · Human hypothalamus

Introduction

Substance P is an undecapeptide (von Euler and Gaddum 1931) that in addition to its effects on gastrointestinal, cardiovascular, and urinary systems, also functions as a neurotransmitter and neuromodulator. Substance P belongs to the tachykinin neuropeptide family with neurokinin A (NKA) and neurokinin B (NKB) (Dam and Quirion 1994). Substance P is primarily involved in nociception (Zubrzycka and Janecka 2000); however, it also has other neuromodulatory functions in the central nervous system (CNS), including neurogenesis (Park et al. 2007), regulation of anxiety, stress (Ebner and Singewald 2006), reinforcement (Huston et al. 1993), respiratory rhythm (Bonham 1995), neurotoxicity and nausea/emesis (Hesketh 2001). Substance P-induced inflammatory processes [for review see (O'Connor et al. 2004)] via neurokinin 1 receptors are believed to play a role in the pathogenesis of Parkinson's disease (Thornton and Vink 2015). Moreover, substance P is an active vasodilator (Meeking et al. 2000; Schrauwen and Houvenaghel 1980; Bossaller et al. 1992) and causes rapid intestinal contraction (von Euler and Gaddum 1931).

The vast majority of the substance P-immunoreactive (IR) elements of the hypothalamo-hypophyseal axis are located in the hypothalamus, although substance P has been also described in the anterior hypophysis using radioimmunoassay (Aronin et al. 1984; DePalatis et al. 1984). Substance P primarily targets neurokinin 1 receptor with high affinity, while it also binds to neurokinin 2 and neurokinin 3 receptors in significantly lower extent. Since tachikinin binding sites have been described in the diencephalon of

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several species using autoradiography (Dam and Quirion 1994; Petitet et al. 1993), and neurokinin 1 and neurokinin 3 receptor immunoreactivity has been found in rat and human throughout the CNS (Mileusnic et al. 1999; Nakaya et al. 1994), it seems feasible that substance P may play a key role in the neuroendocrine functions of the hypothalamo-hypophyseal axis. This paradigm has been supported by the findings that substance P is abundant in the basal hypothalamus (Chawla et al. 1997; Dudas and Merchenthaler 2002, 2006). Indeed, we have previously reported that substance P-IR elements form close associations with gonadotropin-releasing hormone (GnRH) neurons in the human diencephalon (Dudas and Merchenthaler 2002), indicating that substance P participates in the central regulation of gonadal functions.

There is a vast amount of evidence that substance P is involved in the regulation of growth. In addition to the role of substance P in stimulating DNA synthesis, cell growth and proliferation (Reid et al. 1993), it is a general consensus that substance P affects growth primarily by controlling growth hormone (GH) secretion in numerous species including humans [for review see (Aronin et al. 1986)]. Indeed, increase of GH serum levels has been reported after intravenous administration of substance P to anesthetized rats (Kato et al. 1976) and rats (Rivier et al. 1977).

Although previously published data appear to be sometimes contradictory and rather difficult to interpret, there is a general agreement that substance P influences GH release via modulating the hypothalamic growth hormone-releasing hormone (GHRH) secretion. This hypothesis has been supported by previous studies that reported neurokinin 1 receptor activity in the arcuate and periventricular areas of the guinea pig where GHRH neurons cluster (Yip and Chahl 2000, 2001). Indeed, intravenously administered substance P enhances serum GH levels and augments the GHRH-induced GH response in humans, indicating the involvement of GHRH in the regulatory role of substance P in GH release (Coiro et al. 1992). Moreover, the GH release of dispersed anterior pituitary cells is not affected by incubating the cells with synthetic substance P (Arisawa et al. 1989), further supporting the common consensus that substance P regulates GH secretion at the hypothalamic level, probably via GHRH secretion. While there is evidence for the involvement of GHRH in the modulatory action of substance P on GH release (Lemamy et al. 2012), the role of substance P in somatostatin and subsequent GH release is disputed. Some studies support the role of substance P in somatostatin release (Chihara et al. 1978; Sheppard et al. 1979), while others report unaffected somatostatin levels in the portal blood following intraventricular administration of substance P in anesthetized rats (Abe et al. 1981). These seemingly contradictory data indicate that the effect of the administered substance P, especially in case of intraventricular administration, may depend on multiple factors, including age, stress, gender, stage of circadian rhythm as well as the anesthesia of the animals, in addition to species differences [for review see (Aronin et al. 1986)]. Nevertheless, since intravenous administration of substance P augments GHRH-stimulated GH secretion in human subjects with a mean peak almost 12 times higher, it is feasible that substance P exerts its stimulatory effect on GH release, at least in human, primarily via the GHRH system (Coiro et al. 1992). Therefore, in the present study, we have examined the putative substance P-GHRH associations in the human hypothalamus using double-label immunohistochemistry. These juxtapositions may be functional synapses and may represent the morphological substrate of the impact of substance P system on growth in humans.

Materials and methods

Tissue samples

Hypothalamic samples (one man and three adult women, 75–87 years of age) were harvested from autopsies at less than 12 h post mortem period, in accordance with the regulations of the Institutional Review Board of Lake Erie College of Osteopathic Medicine (LECOM). The clinical records of the individuals did not indicate any neurological or neuroendocrinological disorders.

Tissue preparation

The harvested tissue blocks were fixed by immersion in 0.1 M phosphate-buffered (pH 7.4; PB) 4% formaldehyde at 4 °C for 2–8 weeks, then trimmed and the hypothalamus was divided in the midsagittal line. The samples were cryoprotected with 30% sucrose in phosphate buffer containing 0.9% sodium chloride (PBS) supplemented with 0.15% sodium-azide and then sectioned on a freezing microtome at 35 µm intervals in coronal planes. The sections were collected in series of wells of plastic 24-compartment plates with PBS containing 0.2% sodium-azide, and stored at 4 °C until processing. The adjacent sections were processed as follows: (1) single-label immunohistochemical detection of substance P (2) single-label detection of GHRH (3) double-label immunohistochemical detection of substance P (first label) and GHRH (second label).

Immunohistochemistry, single labeling

Following blocking (suppression of non-specific staining) with 2.5% normal horse serum (NHS), the sections were incubated in one of the primary antisera: rabbit anti-GHRH antiserum (Chemicon, Temecula, CA; dilution 1:8000) and rabbit anti-substance P (Millipore Sigma, Burlington, MA;

dilution 1:1000). Thereafter, the sections were incubated in a secondary antiserum containing peroxidase-labeled ready-to-use horse anti-rabbit IgG for 1 h (Vector Laboratories, Burlingame, CA; ImPRESS HRP reagent kit). Finally, the sections were placed into the chromogen solution until the sufficient staining was achieved (Vector SG chromogen kit; Vector Laboratories, Burlingame, CA).

Immunohistochemistry, double labeling

Simultaneous detection of the substance P- and GHRH-IR structures was performed using double-label immunohistochemistry. The first signal of the immunohistochemistry (substance P immunoreactivity) was visualized using the black Vector SG chromogen (Vector Laboratories, Burlingame, CA), and then the second signal (GHRH immunoreactivity) was revealed with the NovaRed (Vector Laboratories, Burlingame, CA). We selected the use of these chromogens instead of fluorescence markers for these studies as these chromogens are stable and the immunostained slides can be stored for prolonged time period. No immunoreaction was observed in control sections, where the primary antibodies were omitted or replaced by non-immune rabbit serum at the dilution of the used primary antibodies or by increasing the dilutions of the primary antisera until the staining disappeared.

Computer-assisted mapping and microscopic analysis

Maps depicting the substance P- and GHRH-IR neurons in the human hypothalamus were used to reveal the overlapping areas between these two neurotransmitter systems. These maps were also described in previous studies (Chawla et al. 1997; Dudas and Merchenthaler 2002, 2006). The accuracy of the distribution of substance P- and GHRH-IR elements has been confirmed, and no significant inconsistency has been observed between the previous and the present data. The maps were created as follows: the mounted and coverslipped hypothalamic sections were scanned and the substance P-IR and GHRH-IR neurons were denoted on these sections using Olympus BX45 microscope with camera lucida, and Adobe Photoshop software (Adobe Creative Suite 2.0; CS2). These maps were utilized to create a 3D model of the human substance P-IR and GHRH-IR systems by the computer-generated stacking of the consecutive sections using VoxBlast NT/9 × Version 3.0 Light (Vaytek, Image Analysis Facility, University of Iowa). Following the 3D modeling, the substance P- and GHRH-IR maps were superimposed to identify the overlapping areas.

The micrographs illustrating the juxtapositions were taken by Olympus BX45 microscope equipped with a digital camera and with 100× oil immersion objective. If the neurons depicted on the micrographs were larger than the frame of the camera, composite images were created from the consecutive micrographs using Adobe Photoshop software (Adobe Creative Suite 2.0; CS2). The GHRH-IR neurons were counted in a representative coronal section from each sample containing the GHRH neuronal subgroups populating the infundibular nucleus, the basal part of the periventricular area, the dorsomedial subdivision of the ventromedial nucleus, as well as the basal perifornical area of the tuberal region. The level of the coronal section was identical in each sample. The counted neurons (over 1500 in the 4 hypothalamic samples) were subdivided into three subclasses: densely innervated (more than five contacting substance P fiber varicosities), lightly innervated (1–5 contacts) and not innervated cells.

Results

Diencephalic substance P-IR elements

The morphology of the substance P-IR system has been described by our previous studies (Chawla et al. 1997; Dudas and Merchenthaler 2002, 2006). Briefly, substance P-IR perikarya are confined almost exclusively to the tuberal region of the human hypothalamus (Fig. 1); the periventricular zone of the preoptic area but the basal part of the posterior hypothalamus also contains few scattered substance P-IR neurons. In the tuberal region, the substance P-IR neurons form several subgroups; clusters of perikarya can be observed in the infundibular nucleus/median eminence, the basal part of the periventricular area, the dorsomedial subdivision of the ventromedial nucleus, as well as the basal perifornical area of the tuberal region (Fig. 1).

Morphologically, the vast majority of the substance P-IR neurons are fusiform-shaped with two processes emanating from the opposite poles of the spindle-shaped perikaryon; however, numerous multipolar cells can be observed in the dorsomedial subdivision of the ventromedial nucleus. Substance P-IR fibers are typically periventricularly arranged at the preoptic and tuberal regions. A dense network of substance P-IR fiber varicosities surrounds the portal vessels of the infundibulum/hypophyseal stalk. Substance P-IR fibers can also be observed passing over the optic tract in the lateral part of the basal hypothalamus. Few substance P-IR axon varicosities surround the anterior commissure and the fornix, and pass along the diagonal band of Broca. Occasional fibers can be detected in the paraventricular nucleus. Substance P-IR perikarya frequently receive contacting substance P-IR fiber varicosities in the basal hypothalamus that appear to be synapses. Moreover, substance P-IR fibers often surround fusiform neurons in the periventricular area that are apparently not substance P-IR.

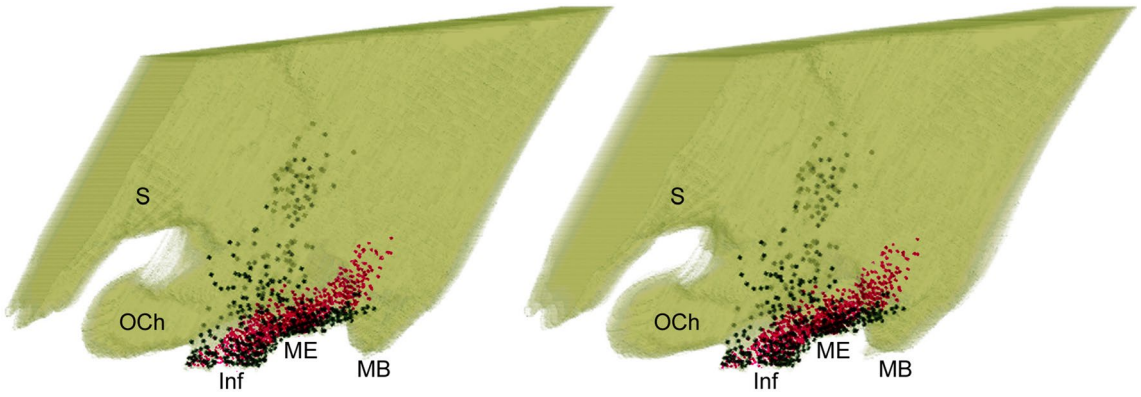

Fig. 1 Stereoscopic images of the human diencephalon denoting GHRH-IR perikarya (red dots) and substance P-IR perikarya (black dots). Stereoscopic images can be seen using U or parallel vision. The eyes are relaxed to look into the distance until the pair of the images fuses, and then refocused by the brain. With this technique a 3D hypothalamus can be seen on the figure, appearing to float in front of the paper, with the immunolabeled perikarya (marked by blue and red dots) discernible at different depths. *Inf* infundibulum, *MB* mamillary body, *ME* median eminence, *OCh* optic chiasm, *S* septal area

Human GHRH system

GHRH-IR neurons form a dense, well-defined cell cluster in the basal part of the infundibular region, as we have previously described (Chawla et al. 1997; Dudas and Merchenthaler 2002, 2006). In this region, four distinct subdivisions are formed by the perikarya: (1) the majority of the GHRH-IR perikarya can be observed in the infundibulum/median eminence and (2) in the basal part of the periventricular zone. (3) A subgroup of neurons populates the dorsomedial subdivision of the ventromedial nucleus and (4) the basal perifornical area of the tuberal region. Occasional GHRH-IR perikarya can be found in the medial preoptic area and in the posterior hypothalamus. In the basal part of the infundibulum and the periventricular area, GHRH-IR fibers form a dense network.

Substance P-GHRH associations

Dense substance P-IR fiber network often abuts on the surface of the GHRH-IR neurons in the infundibulum/median eminence (Fig. 2a–e, k–l). Here, substance P-IR axon varicosities typically form en passant-type associations, where fibers make multiple contacts with GHRH-IR perikarya and the emanating GHRH-IR axons/dendrites while passing by, characteristically following the contours of the GHRH-IR neurons. In multiple times (27% of the counted neurons), these fiber varicosities cover a significant amount of area on the surface of GHRH-IR neurons, forming basket-like encasements with more than 5 contacts/cell (Fig. 2).The remainder of the GHRH-IR neurons were lightly innervated (38% of the counted neurons), receiving 1–5 contacting substance P-IR axon varicosities, or did not appear to have any contacts with substance P-IR fibers (35% of the counted neurons). The majority of the densely innervated GHRH-IR neurons can be observed in the infundibular nucleus/median eminence (Fig. 2a–e, k–l), while numerous substance P-GHRH juxtapositions are located in the periventricular zone of the tuberal area (Fig. 2h), in the dorsomedial subdivision of the ventromedial nucleus (Fig. 2g) and in the basal perifornical area of the tuberal region (Fig. 2i, j). Juxtapositions were not detected around the occasional GHRH-IR neurons in the posterior hypothalamus. Close examination of these juxtapositions with high-magnification utilizing oil-immersion objectives did not reveal any gaps between the contacting elements. No significant gender/age differences were observed between the patterns of innervation of the individuals.

Discussion

There is a general consensus that substance P affects hypothalamic functions including, among others, gonad regulation and growth. However, the exact mechanism of substance P-modulated GH secretion remains elusive and appears to depend on several factors including dose, age, gender and species differences that often result in rather contradictory data. While somatotropes cosecrete substance P (Arita et al. 1994), there is no convincing evidence that substance P modulates GH release directly at the hypophyseal level (Houben and Denef 1993). Basal or stimulated GH release from rat primary pituitary cells in vitro is unaffected by substance P (Cheng et al. 1997), suggesting that substance P exerts its action primarily at the hypothalamic level (Lemamy et al. 2012).

Experimental data indicate that substance P increases GH secretion primarily via the GHRH system in humans

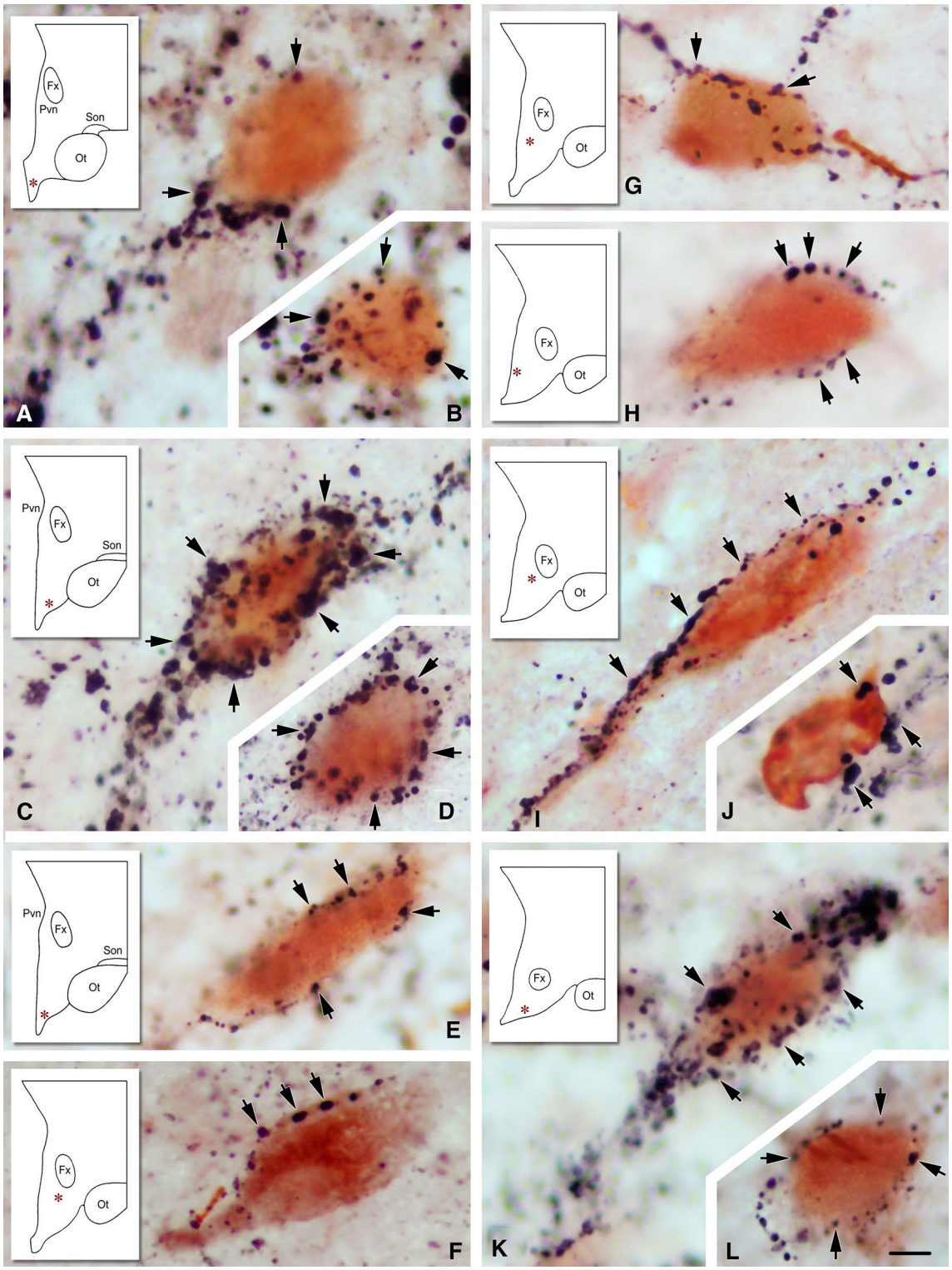

Fig. 2 Double-label immunohistochemistry illustrates juxtapositions between the substance P-IR (black) and GHRH-IR (brown) neural elements in the human diencephalon. The substance P-IR fiber varicosities often cover a significant surface area of the GHRH neurons forming numerous en passant-type contacts. The positions of the demonstrated GHRH-IR neurons are marked by asterisks on the coronal sections of the diencephalon in the corners of the micrographs. The thickness of the sections is 30 µm. Scale bar: 20 µm. *Fx* fornix, *Pvn* paraventricular nucleus, *Ot* optic tract, *Son* supraoptic nucleus

(Coiro et al. 1992). Indeed, in our present study we have shown that substance P-IR axon varicosities form numerous en passant-type associations with the GHRH-IR neurons in the infundibulum/median eminence where fibers make multiple contacts with the GHRH-IR perikarya while passing by. The density and the morphology of these intimate juxtapositions suggest that substance P influences growth by regulating hypothalamic GHRH release by direct synaptic contacts. In addition to the effect of substance P on GH release, substance P-IR axons often contact substance P-IR perikarya in the human basal hypothalamus, indicating an autoregulatory mechanism in substance P release.

The morphology and distribution of the substance P-IR and GHRH-IR neuronal elements are in good consensus with our previous studies (Chawla et al. 1997; Dudas and Merchenthaler 2002, 2006). A comparable distribution of the hypothalamic substance P neuronal system has also been previously described by different authors using in situ hybridization (Chawla et al. 1997).

Although the morphology of the substance P-GHRH associations reported in the present paper appears to support our hypothesis that these juxtapositions represent functional synapses, due to the significant post mortem time of the hypothalamic samples, the verification of the synaptic structures by double-label immunohistochemistry evaluated with electron microscopy was not possible. However, the density of the contacting substance P axonal varicosities surrounding the GHRH neurons strongly indicates that these neurotransmitter systems indeed communicate at certain hypothalamic regions. Although no significant differences were observed between the patterns of innervation of the individual samples, the relatively small sample size does not allow us to draw any conclusions regarding the putative gender/age differences.

GH secretion is inhibited by somatostatin that is also abundant in the basal hypothalamus. However, the role of substance P in somatostatin release and in the subsequent GH release remains uncertain, since some studies report unaffected somatostatin levels in the portal blood after intraventricular administration of substance P in anesthetized rats (Abe et al. 1981). These data, coupled by our findings in the present paper suggest that substance P exerts its regulatory action on GH secretion primarily via the GHRH system in humans. Indeed, the modulatory action of substance P on GHRH release has been supported by previous data (Lemamy et al. 2012; Coiro et al. 1992).

In conclusion, the present study has revealed a dense substance P-IR fiber network abutting on the surface of the majority of GHRH-IR neurons located in the infundibular and periventricular regions of the human hypothalamus, further emphasizing the importance of these neuronal subpopulations in the substance P-controlled GHRH secretion. The observed substance P-GHRH juxtapositions may represent the morphological substrate of interaction between these two systems, and consequently the morphological basis of regulation of growth by substance P. The morphology and density of these intimate associations indicate that substance P may have a pivotal impact on growth by modulating hypothalamic GHRH release via direct synaptic contacts. In addition, numerous neurotransmitter systems may exert regulatory role on the hypophyseal GH secretion by modulating the central substance P release. The findings that substance P-IR fibers often surround fusiform neurons in the periventricular area that are apparently not substance P-IR, coupled with the apparent close associations of the substance P-IR fiber varicosities with the portal vessels of the hypophyseal stalk indicates that substance P may play a pivotal role in regulating other neuroendocrine functions of the hypothalamo-hypophyseal axis in addition to growth.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants The brain utilized in these studies was harvested 12 h post mortem period in accordance with the regulations of the Institutional Review Board of Lake Erie College of Osteopathic Medicine (LECOM). See "Materials and methods".

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