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INTIMATE ASSOCIATIONS BETWEEN THE ENDOGENOUS OPIATE SYSTEMS AND THE GROWTH HORMONE-RELEASING HORMONE SYSTEM IN THE HUMAN HYPOTHALAMUS

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Abstract—Although it is a general consensus that opioids modulate growth, the mechanism of this phenomenon is largely unknown. Since endogenous opiates use the same receptor family as morphine, these peptides may be one of the key regulators of growth in humans by impacting growth hormone (GH) secretion, either directly, or indirectly, via growth hormone-releasing hormone (GHRH) release. However, the exact mechanism of this regulation has not been elucidated yet. In the present study we identified close juxtapositions between the enkephalinergic/endorphinergic/dynorphinergic axonal varicosities and GHRH-immunoreactive (IR) perikarya in the human hypothalamus. Due to the long *post mortem* period electron microscopy could not be utilized to detect the presence of synapses between the enkephalinergic/endorphinergic/dynorphinergic and GHRH neurons. Therefore, we used light microscopic double-label immunocytochemistry to identify putative juxtapositions between these systems. Our findings revealed that the majority of the GHRH-IR perikarya formed intimate associations with enkephalinergic axonal varicosities in the infundibular nucleus/median eminence, while endorphinergic-GHRH juxtapositions were much less frequent. In contrast, no significant dynorphinergic-GHRH associations were detected. The density of the abutting enkephalinergic fibers on the surface of the GHRH perikarya suggests that these juxtapositions may be functional synapses and may represent the morphological substrate of the impact of enkephalin on growth. The small number of GHRH neurons innervated by the endorphin and dynorphin systems indicates significant differences between the regulatory roles of endogenous opiates on growth in humans.

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Abbreviations: DAB, diaminobenzidine; G-DAMME, D-ala2,MePhe4-Met-enkephalin-(o)-ol; GH, growth hormone; GHRH, growth hormone-releasing hormone; hpGRF, human pancreatic growth hormone releasing factor; IR, immunoreactive.

Key words: hypothalamus, enkephalin, endorphin, dynorphin, GHRH, growth.

INTRODUCTION

Opioids are ligands of the family of G-protein-coupled opioid receptors. It is a general consensus that opioids, including endogenous opiates, affect growth. Although intrauterine growth restriction (IUGR) that can be frequently observed in pregnancies of opioid-dependent mothers (Liu et al., 2010) may indicate an inhibitory effect of opiates on growth hormone (GH) secretion, numerous studies reported stimulatory effects of opioids on growth. Indeed, opioid receptor blockade by naloxone exerts an inhibitory role on growth hormone-releasing hormone (GHRH)-induced GH secretion (De Marinis et al., 1989; Villa et al., 1997).

Despite the accumulating evidence of the impact of opioids on growth, the mechanism of this phenomenon is largely unknown. Although the met-enkephalin analog D-ala2,MePhe4-Met-enkephalin-(o)-ol (G-DAMME) appears to stimulate GH secretion via inhibiting somatostatin release in humans and not via stimulating GHRH release (Delitala et al., 1989; Fanciulli et al., 1996), it has been hypothesized that opiates exert their regulatory action on growth, at least partially, by modulating GHRH release. Indeed, met-enkephalin analogs fail to alter the high circulating GH levels in acromegalics, indicating that by a negative feedback effect, high levels of circulating GH may disrupt the function of the hypothalamic opioid or the GHRH systems through which opioids exert their action (Ganzetti et al., 1987). The central role of GHRH in the opioid-modulated growth is supported by the finding that GHRH itself preferentially binds to delta opiate receptors (Codd et al., 1988). Since endogenous opiates, such as endorphin, enkephalin or dynorphin are ligands to the same opiate receptor family used, among others, by morphine, these peptides may be one of the key regulators of growth in humans by impacting GH secretion, either directly, or indirectly, via GHRH release.

We have previously described the morphology and distribution of the enkephalin, β-endorphin and GHRH systems in the human hypothalamus (Dudas and Merchenthaler, 2006; Deltondo et al., 2008). Dynorphin-immunoreactive (IR) structures have been reported by others in rats (Watson et al., 1981, 1982a,b; Khachaturian et al., 1982; Weber et al., 1982; Weber

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and Barchas, 1983; Simerly et al., 1988) and in primates (Khachaturian et al., 1985). Moreover, we have previously reported on intimate associations between the enkephalinergic/endorphinergic and luteinizing hormone-releasing hormone (LHRH)-IR neurons (Dudas and Merchenthaler, 2003, 2004), suggesting that opioids can affect gonadal regulation via direct synaptic contacts. Due to the long *post mortem* period and consequent lack of optimal preservation of the cell membranes in the brain, electron microscopy could not be utilized to detect the presence of synapses between the enkephalin/endorphin/dynorphin and GHRH neurons. Therefore, in the present study, we identified putative juxtapositions between the enkephalinergic/endorphinergic/dynorphinergic axonal varicosities and GHRH-IR perikarya utilizing double-label immunocytochemistry combined with high magnification light microscopy. It is generally accepted that these putative associations may indicate the presence of functional synapses and may represent the morphological substrate of the endogenous opioid-influenced GH release.

EXPERIMENTAL PROCEDURES

Tissue samples

Tissue blocks containing half of the hypothalamus split by the midsagittal line (one man and three adult women, 75–84 years of age) were harvested from autopsies at less than 24-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 hypothalami were fixed by immersion in 0.1 M phosphate-buffered (pH 7.4; PB) 4% formaldehyde at 4 °C for 2–8 weeks. Each block contained half of the hypothalamus 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 30-μm intervals in coronal planes. The sections were collected in antero-posterior order in three series of wells of plastic 24-compartment plates with PBS containing 0.2% sodium-azide, and stored at 4 °C until processing.

Immunohistochemistry

Immunohistochemistry was carried out using streptavidin–biotin (ABC) methods described previously (Dudas and Merchenthaler, 2006). Simultaneous detection of the enkephalinergic/endorphinergic/dynorphinergic and GHRH-IR structures was performed using double-label immunohistochemistry. The GHRH-containing structures were identified using a rabbit anti-GHRH serum (Chemicon [Millipore], Temecula, CA, USA) at a dilution of 1:8000 and the enkephalinergic/endorphinergic/dynorphinergic structures were visualized with rabbit anti-leu-enkephalin (Chemicon [Millipore], Temecula, CA; 1:1000), rabbit anti-β-endorphin (Chemicon [Millipore], Temecula, CA; dilution 1:1000) or rabbit anti-dynorphin A sera (ABCAM, Cambridge, MA, USA; dilution 1:500), respectively. The first signal of the immunohistochemistry was visualized by using the black diaminobenzidine (DAB)/nickel chromogen (0.05% diamino-benzidine, 0.125% nickel-ammonium-sulfate and 0.005% hydrogen-peroxide in 0.1 M Tris–HCl [pH 7.6]), and then the second signal was revealed with the brown DAB chromogen.

The antiserum against GHRH (AB1751, Chemicon [Millipore]) was raised in a rabbit against the full length sequence of the synthetic human pancreatic growth hormone releasing factor (hpGRF) (1-40) NH_2. The antiserum recognizes the primate sequence and does not cross-react with rat, mouse or ox sequences (Bloch et al., 1983). This antiserum stains the human brain in a pattern consistent with previous results (Bloch et al., 1983; Leidy and Robbins, 1986; Deltondo et al., 2008; Anderson et al., 2010; Rotoli et al., 2011). The antiserum against β-endorphin (AB5028, Chemicon [Millipore]) was raised in a rabbit against the full length sequence of the synthetic human β-endorphin conjugated to thyroglobulin. The antiserum recognizes the primate, rat and mouse sequences and has only minimal cross-reactivity with leu-enkephalin (0.03%) and beta-lipotropin (0.34%). This antiserum stains the human brain in a pattern consistent with previous results (Haynes et al., 1982; Bloom and Battenberg, 1983; Khachaturian et al., 1984; Pilcher et al., 1988; Bethea and Widmann, 1996). The antiserum against leu-enkephalin (AB5024, Chemicon [Millipore]) was raised in a rabbit against the full length sequence of the synthetic human leu-enkephalin conjugated to bovine serum albumin. The antiserum recognizes the primate, rat and mouse sequences and has only minimal cross-reactivity with met-enkephalin (0.93%) and β-endorphin (0.01%), and beta-lipotropin (90.01%) (Stengaard-Pedersen and Larsson, 1981; Palkovits, 1984; Kalivas, 1985; Wang and Larsson, 1985; Moore and Black, 1991). This antiserum stains the human brain in a pattern consistent with previous results (Dudas and Merchenthaler, 2003).

The antiserum against Dynorphin-A (AB11134, ABCAM) was raised in a rabbit against the full length sequence of the synthetic pig dynorphin. The antiserum recognizes the pig, primate, rat and mouse sequences. The staining with this antiserum cannot be prevented with its absorption with related antigens however, dynorphin 1-18 and 1-8 completely abolishes the staining.

In control sections, the primary antibodies were omitted or replaced by non-immune rabbit serum at the dilution of the used primary antibodies. No immunoreaction was observed in these control sections.

Computer assisted mapping and microscopic analysis

Following mounting and coverslipping the hypothalamic sections were scanned and maps depicting the enkephalinergic/endorphinergic and GHRH neurons