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Expression and distribution of GnRH, LH, and FSH and their receptors in gastrointestinal tract of man and rat

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ABSTRACT

Background: Gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) regulate the reproductive axis. Their analogs have been found to influence gastrointestinal activity and enteric neuronal survival. The aims of the study were to investigate expression and cellular distribution of GnRH, LH, and FSH and their receptors in human and rat gastrointestinal tract.
Methods: Bioinformatic analysis of publicly available microarray gene expression data and Real-Time PCR mRNA quantification were used to study mRNA expression levels of hormones and receptors in human intestinal tissue. Full-thickness sections of human ileum and colon, and rat stomach, ileum, and colon, were used for immunocytochemistry. Antibodies against human neuronal protein HuC/D (HuC/D) were used as general neuronal marker. LH and FSH, and GnRH-, LH-, and FSH receptor immunoreactive (IR) neurons were evaluated.
Results: GnRH1 mRNA was detected in both small and large intestine, whereas GnRH2 was mainly expressed in small intestine. Approximately 20% of both submucous and myenteric neurons displayed LH receptor immunoreactivity in human ileum and colon. In rat, 4%–9% of all enteric neurons in fundus and ileum, and 13% of submucous neurons and 21% of myenteric neurons in colon were LH receptor-IR. Neither mRNA (man) nor the fully expressed proteins (man and rat) of LH and FSH, or GnRH and FSH receptors, could be detected.
Conclusions: GnRH1 and GnRH2 mRNA are expressed in human intestine. LH receptor-IR enteric neurons are found along the entire gastrointestinal tract in both man and rat.

1. Introduction

The reproductive axis is controlled by gonadotropin-releasing hormone (GnRH), which is produced in hypothalamic neurons and secreted in a pulsatile fashion. Via the portal circulation it reaches the anterior pituitary and stimulates secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) through GnRH receptor (GnRH-R) activation [1,2]. The gonadotropins target the gonads and regulate secretion of steroid hormones, like estrogen and progesterone [3]. During the last years, several studies have described effects by these hormones also on the gastrointestinal tract. GnRH analogs have been shown to inhibit gastric secretion and gastrin release in rat and dog [4,5], to inhibit cell proliferation of gastric epithelium [6] and smooth muscle cells [7], and to induce apoptosis and inhibit cell proliferation in gastric cancer cells [3,8]. Both GnRH analogs and LH have been shown to affect gastrointestinal motility in rat and man; GnRH analogs by stimulation of gastrointestinal motility and LH by prolonging and fragmenting of the phase III migrating motor complex (MMC) [9–11].

The question remains whether the effects observed on the gastrointestinal tract by GnRH analogs are exerted directly by GnRH-R activation or indirectly through the pituitary gland. It needs to be determined whether the GnRH-induced effects are executed by activation of intestinal GnRH receptors, or if it is executed by way of systemic LH release and subsequently due to stimulation of LH receptors (LH-R). Before we can start to speculate on the mechanisms behind, we need to recognize possible tissue expression of the peptides of interest. Recently, our research group described GnRH expression in human submucous and myenteric neurons in both small and large intestines [12]. In rat, presence of GnRH and GnRH-R have been suggested in several cell types of the gastrointestinal tract [5,7,13,14], whereas such expression could not be confirmed by our group [15]. Notably, LH-R was found to be expressed in enteric neurons in rat stomach, small and large intestines [15]. The aim of the present study was to study the possible expressions of GnRH, LH, and FSH as well as their receptors in man and rat, by retrieving microarray data from public repositories, by using quantitative Real-Time polymerase chain reaction (qRT-PCR), and by immunocytochemistry, with particular focus on the gastrointestinal tract.

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2. Material and methods

2.1. Microarray and qRT-PCR analysis

The Web-based software tool Genevestigator was used to evaluate gene expression across a variety of human tissues for all genes of interest [16]. The Human Multiple Tissue cDNA (MTC) panel I and the Human Digestive System MTC panel from Clontech (Clontech, Mountain View, CA, USA) were used to study mRNA expression levels of hormones and receptors in brain and intestinal tissues. Messenger RNA (mRNA) expression levels were measured by performing quantitative Real-Time PCR in an ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with TaqMan fast universal PCR master mix according to the manufacturer's instructions. Real-time PCR reactions were performed in triplicate on each sample with TaqMan assays for human GnRH 1(*GNRH1*) (Hs00171272_m1), human GnRH 2 (*GNRH2*) (Hs01122823_m1), human GnRH-R (*GNRHR*) (Hs00171248_m1 and Hs00369692_m1, respectively for isoform 1 and 2), human LH (*LHB*) (Hs00751207_s1), human LH-R (*LHCGR*) (Hs00896336_m1), human FSH (*FSHB*) (Hs00174919_m1), human FSH-R (*FSHR*) (Hs00174865_m1), and human GAPDH as housekeeping gene (Hs00193002_m1). After normalization to the internal endogenous controls GAPDH, mRNA expression levels for each gene in each sample were determined by the comparative CT method of relative quantification, and expressed in arbitrary units relative to the expression detected in the brain (set = 1). To avoid spurious, unreliable results, samples/tissues with corresponding average CT values above 35 were not taken into consideration, and the expression level is reported as not detected (nd).

2.2. Gastrointestinal tissue sampling

Patients were treated according to the Helsinki declaration and the studies were approved by the Ethics Committee, Lund/Malmö. Written, informed consent was obtained from the patients prior surgery. One male and ten female Sprague–Dawley rats (170–300 g), purchased from Charles River, Sulzfeld, Germany, were used. The rats were allowed to acclimatize to the climate- and light-controlled animal facility for at least five days prior experimentation. Standard rat chow and water were supplied at all times. The experimental design was approved by the animal ethics committee, Lund and Malmö, Sweden. Animals were used in accordance with the European Communities Council Directive (2010/63/EU) and the Swedish Animal Welfare Act (SFS 1988:534).

2.2.1. Man

Paraffin sections of ileum from one man (20 years of age) diagnosed with enteric inflammatory neuropathy [12,17] and from one woman (30 years of age) diagnosed with enteric degenerative neuropathy [12,18] were used for immunocytochemistry. In addition, ileum tissue was collected from one woman (84 years of age) undergoing gastrointestinal surgery due to sigmoideum diverticulosis, and colonic tissue from one woman (65 years of age) with non-obliterating adenocarcinoma of rectosigmoideum and one woman (68 years of age) due to sigmoideum diverticulosis. The latter three patients had no history of gastrointestinal dysmotility, and samples were taken from macroscopic normal areas 10 cm above the tumor, or in a diverticulosis free area. Tissue segments from both ileum and colon were rinsed in saline and fixed in Stefanini's fixative (a mixture of 2% formaldehyde and 0.2% picric acid in phosphate buffer, pH 7.2) or in 4% paraformaldehyde in 0.1 M phosphate buffer for 22 h at 4 °C.

2.2.2. Rat

Rats were euthanized under deep anesthesia and the pituitary, stomach, ileum, colon, ovary, and testis were removed. The gut segments were rinsed in saline, opened and pinned flat on balsa wood. One portion of each tissue segment or organ was fixed in Stefanini's fixative for 22 h at 4 °C. The other portion was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 22 h at 4 °C.

2.3. Immunocytochemistry

Stefanini-fixed specimens were rinsed in Tyrode's solution containing 10% sucrose before orientated for longitudinal and cross sectioning and mounted in Tissue-Tek (Sakura, Histolab, Göteborg, Sweden), frozen on dry ice, and sectioned (10 μm). Paraformaldehyde-fixed specimens were dehydrated in ethanol, cleared in xylene, orientated for longitudinal and cross sectioning, embedded in paraffin and sectioned (5 μm). All sections were processed for immunocytochemistry.

Antibodies against human neuronal protein HuC/D (HuC/D) were used as general neuronal marker. Possible presence of GnRH-R, LH, LH-R, FSH, and FSH-R immunoreactive (IR) submucous and myenteric neurons in rat stomach and in man and rat small and large intestine were evaluated. Cryo sections were washed in PBS containing 0.25% triton and incubated with antibodies against HuC/D in combination with antibodies against GnRH-R, LH or LH-R in a moist chamber at 4 °C overnight. Paraffin sections were deparaffinized, hydrated, and subjected to antigen retrieval by boiling in citrate acid buffer (0.01 M, pH 6) in a microwave oven (650 W) for 2 × 8 min. The sections were cooled and washed in distilled water followed by PBS/triton before incubation with antibodies against HuC/D in combination with antibodies against GnRH-R, LH-R, FSH or FSH-R in a moist chamber at 4 °C overnight. Details on the antibodies are given in Table 1.

The site of the antibody-antigen reactions were visualized by exposure to a mixture of DyLight TM 488-conjugated goat anti-mouse IgG antiserum and Alexa Fluor TM 594-conjugated donkey anti-rabbit IgG or a mixture of DyLight TM 488-conjugated donkey anti-goat IgG and DyLight TM 594-conjugated donkey anti-mouse IgG antiserum (all diluted 1:1000 and from Jackson ImmunoResearch laboratories inc. Novakemi AB, Stockholm, Sweden) for 1 h in room temperature (RT) and then mounted in phosphate buffer:glycerol 1:1.

To aid characterization on the cellular localization of GnRH-R, LH, LH-R, FSH, and FSH-R, positive control tissues from rat were used. Cryo- and paraffin sections from pituitary, ovary or testis were exposed to antibodies against GnRH-R, LH, LH-R, FSH or FSH-R. All positive controls displayed intense immunoreactivity. In the immunocytochemical procedures, absorption controls were performed. LH antibodies were inactivated by the addition of an excess amount of lutropin alfa (dilution 1 μM, Luveris®, MerckSerono, London, Great Britain). Antibodies against GnRH-R, LH-R, FSH, and FSH-R were inactivated by the addition of an excess amount of antigen (10–100 μg of synthetic peptide/ml diluted antiserum) before used in immunostaining. Except for the GnRH-R antibodies raised in rabbit (90217.01), which was not inactivated by its antigen, none of the negative controls exhibited any immunostaining. Since synthetic antigens for testing the specificity of antibodies against HuC/D are not commercially available, omission of primary antibodies were used as control.

Relative numbers of LH-R-IR enteric neurons were quantified. HuC/D-IR neurons also IR for LH-R were counted in cross and longitudinally cut, whole wall sections from fundus (only rat), ileum and colon (both man and rat). From rat fundus (n = 5), ileum (n = 5), and colon (n = 10), 6–9 sections, each 7–10 mm long, cut at different depths were quantified. From man (n = 3), 2 sections, each 15–20 mm long from each gut region, cut at different depths were quantified. The results are expressed in percentage of HuC/D-IR neurons.

3. Statistical analyses

Results of the immunocytochemistry are presented as medians and spreads, expressed as 25th and 75th percentile.