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Sand, Elin; Bergvall, Monika; Ekblad, Eva; D'Amato, Mauro; Ohlsson, Bodil

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PO Box 117  
221 00 Lund  
+46 46-222 00 00

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

Elin Sand<sup>1,2\*</sup>, Monika Bergvall<sup>3\*</sup>, Eva Ekblad<sup>1</sup>, Mauro D'Amato<sup>3</sup>, Bodil Ohlsson<sup>2</sup>

\* = Contributed equally

<sup>1</sup>Department of Experimental Medical Science, Neurogastroenterology, Lund University,  
Sweden

<sup>2</sup>Department of Clinical Sciences, Division of Internal Medicine, Skåne University Hospital,  
Malmö, Lund University, Lund, Sweden

<sup>3</sup>Department of Bioscience and Nutrition, Karolinska Institutet, Stockholm, Sweden

Corresponding author: Bodil Ohlsson  
Skåne University Hospital  
Inga Marie Nilssons street 46  
205 02 Malmö, Sweden  
bodil.ohlsson@med.lu.se

## 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 receptor, 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.

Key words: follicle-stimulating hormone (FSH), gastrointestinal tract, gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), man, rat

## 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, 10, 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 intestine [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 intestine [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.

## Material and methods

### ***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).

### ***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).

### *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.

### *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.

### ***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 $\mu$ M, Luveris<sup>®</sup>, 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  $\mu$ g 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.

### **Statistical analyses**

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

### **Results**

#### ***Quantification of gene expression in human tissues and the gastrointestinal tract***

An initial characterization of mRNA expression was undertaken by using the web-based tool Genevestigator, examining the expression of human genes coding for GnRH 1 and 2 (*GNRH1* and *GNRH2*), GnRH-R (*GNRHR*), LH (*LHB*), LH-R (*LHCGR*), FSH (*FSHB*), and FSH-R (*FSHR*). The results of this analysis, which includes published microarrays and gene expression data from more than 730 studies and 53,000 samples of human origin, are shown in Supplementary Figure S1-S6. In this analysis, all examined genes in the present study were found to be expressed at low or very-low level in all human tissues, with the exception of *LHB* and *FSHB* in the pituitary gland, in which high expressions were detected.

In order to consolidate and expand these results, we also characterized the mRNA expression of all the above mentioned genes by qRT-PCR in a series of human tissues from different intestinal locations, including duodenum, jejunum, ileum, ileocecum, cecum, ascending and descending colon, and rectum, as well as brain as a tentative positive control. No mRNA expression could be detected for *GNRHR*, *LHCGR* and *FSHR* in any of the tissues tested under our experimental conditions, while *FSHB* and *LHB* were found to be expressed, respectively, at low and very low levels only in the brain. Notably is that GnRH 1 and 2 (*GNRH1* and *GNRH2*) were both detected in the human intestine, although with varying levels of expression along the gastrointestinal tract (Figure 1). Overall, the results from gene expression analyses suggest that GnRH-, LH-, and FSH receptors are expressed at extremely low levels, if at all, in the human gastrointestinal tract, while GnRH 1 and 2 can be found in detectable amounts. However, this analysis is only partially informative, because it does not allow cell-type specific analyses. Therefore, we set to further investigate this with immunocytochemistry.

### ***Enteric neurons immunoreactive for the peptides and their receptors***

#### ***GnRH receptor***

No GnRH-R-IR nerve cell bodies or fibers were detected in human or rat gastrointestinal tract. In the rat pituitary, used as positive control for GnRH-R, the cell membrane of gonadotrophs displayed GnRH-R immunoreactivity.

#### *LH and LH receptor*

No LH-IR nerve cell bodies or fibers were detected in human or rat gastrointestinal tract.

In the rat pituitary, used as positive control for LH, the gonadotrophs' cytoplasm displayed intense LH immunoreactivity. Rat ovary, used as positive control for LH-R, displayed numerous LH-R-IR granulosa cells.

LH-R immunoreactivity was found in enteric neurons in human ileum and colon and was quantified in three patients (Figure 2). In the patient diagnosed with inflammatory neuropathy with infiltration of lymphocytes (man 20 years of age), 29% of the submucous and 17% of the myenteric neurons in ileum displayed LH-R immunoreactivity. In ileum from a patient diagnosed with degenerative neuropathy (woman 30 years of age), the submucous neurons was too few to allow any quantification, while 11% of the myenteric neurons displayed LH-R immunoreactivity. In colon taken from one patient undergoing gastrointestinal surgery (woman 68 years of age), 17% of both submucous and myenteric neurons displayed LH-R immunoreactivity.

Intense LH-R immunoreactivity was found in enteric neurons throughout the rat gastrointestinal tract (Figure 2). The IR material was mainly located in nerve cell bodies. Few LH-R-IR fibers were found in the smooth muscle layers of fundus, ileum, and colon. In fundus, LH-R-IR submucous neurons were too few to allow quantification, but 8% of the myenteric neurons were LH-R-IR (Figure 2). In ileum, 4% of the submucous and 9% of the myenteric neurons, and in colon, 13% of submucous and 21% of myenteric neurons, displayed LH-R immunoreactivity, respectively (Figure 2).

*FSH and FSH receptor*

No FSH-IR or FSH-R-IR nerve cell bodies or fibers were detected in human or rat gastrointestinal tract. In the rat pituitary, used as positive control for FSH immunoreactivity, the gonadotrophs' cytoplasm displayed intense FSH immunoreactivity. Rat testis, used as positive control for FSH-R, displayed numerous FSH-R-IR sertoli cells.

## Discussion

GnRH 1 and 2 mRNA were most abundantly expressed in the small intestine in the present study. The detection of intestinal mRNAs suggests a local translation of these peptides. Of the peptides (GnRH-R, LH, LH-R, FSH, and FSH-R) tested, only LH-R immunoreactivity was detected in rat stomach and small and large intestine from rat and man.

Both GnRH 1 and 2 are decapeptides and discriminated by different amino acids in position 5 to 8. The two peptides are identical in rat and man [19]. GnRH1 is a hormone which regulates the hypothalamic-pituitary axis and gonadotropin production [1, 2, 3], whereas GnRH2 is a neuropeptide expressed in the brain, in particular hind brain and spinal cord, hence being extra-hypothalamic [20]. GnRH2 is believed to participate in the reproduction and sexual behavior through neuro-modulation [19, 21]. We do not know the role of GnRH 1 and 2 in the gastrointestinal tract. It may be that one acts as a hormone secreted into the blood circulation and one as a neuropeptide, in analogy with that found in brain, but this has to be further studied. Different receptors for GnRH 1 and 2 have been described in mammals although GnRH-R2 is not expressed as a fully functional receptor, due to a genomic stop sequence/frame shift [22, 23]. However, both GnRH1 and GnRH2 are able to signal through the type 1 receptor [19]. GnRH1 has a short half-life of 2–4 minutes, which, in combination with its secretion into the anatomically inaccessible portal circulation of the pituitary, has rendered sampling of GnRH very complicated [1, 2]. Instead, the downstream-secreted LH is studied.

Previously, we reported expression of GnRH in submucous and myenteric neurons in human small and large intestine [12, 18], while GnRH and GnRH-R-IR enteric neurons were absent in rat [15]. In contrast, other groups have found GnRH and GnRH-R immunoreactivity in rat enteric neurons, smooth muscle cells, and on parietal cells in the epithelium [5, 6, 7, 13]. Our antibodies were raised against synthetic GnRH1; possible cross-

reactivity to GnRH2 has not been able to evaluate. Antibodies raised against GnRH2 are not commercially available. The GnRH antibodies used by others were produced by the groups themselves, and may differ in their affinity for GnRH2 and/or GnRH1 [5, 6, 7, 13]. The different results between our and others' result may be explained by different affinity of antibodies to GnRH2. However, the PCR results in our present study suggest predominance of GnRH1 rather than GnRH2, at least in the human gut. Future efforts using in situ hybridization and/or other detection methods may further aid in revealing the cellular expressions and distributions of the peptides.

The GnRH-R and LH-R both have more than 80% amino acid homology between rat and man [24, 25]. LH-R has been demonstrated in submucous and myenteric neurons in small and large intestine, glia cells and endothelial cells in man [17], while in rat LH-R-IR was exclusively confined to enteric neurons [15]. In the former publication, no absorption controls were performed in man, but our present findings confirm LH-R on submucous and myenteric neurons.

Our present and previous findings of GnRH and LH-R in human and LH-R in rat gastrointestinal tract [12, 15, 17, 18], may provide an explanation for the small intestinal dysmotility noted after administration of GnRH analogs or LH [9, 10, 11]. Patients with enteric dysmotility and chronic intestinal pseudo-obstruction exhibit disturbances in small intestinal motility patterns [26, 27]. We have observed that such disorders can manifest themselves after repeated treatment with high doses of GnRH analogs, along with reduced number of GnRH-IR myenteric neurons and appearance of antibodies against GnRH in serum [12, 18]. Furthermore, intermittent administration of the GnRH analog buserelin to rats rendered up to 50% reduction of enteric neurons, accompanied with a down-regulation of the neuronal expression of LH-R [15]. Taken together, these findings raise the suspicion that GnRH and/or LH play pivotal roles in intestinal motility regulation.

Hormonal influence on gastrointestinal functions may explain a female predominance with respect to functional and motility disorders of the bowel [26, 27, 28]. Furthermore, high titers of GnRH antibodies have been described in patients with such diseases [29]. Continuous treatment with the GnRH analog leuprolide was shown to improve symptoms in patients with functional bowel disorders [30, 31]. The mechanism behind is enigmatic, but may be due to down-regulation of the GnRH-R in the pituitary gland and subsequent depletion of gonadotropins and sex hormones [3], suggested to influence gastrointestinal motility [32, 33]. Another plausible explanation to the leuprolide-induced effects is by activation of intestinal GnRH-R. Although we could not demonstrate any GnRH-R expression in our samples, this does not exclude low levels of expression in subsets of intestinal cells.

Neither FSH nor FSH-R were detected in the gastrointestinal tract of man or rat, by any method used, why the role of FSH on the gastrointestinal function can be anticipated to be of minor or no importance.

## Conclusions

GnRH1- and GnRH2 mRNA are expressed in human intestine. LH-R-IR enteric neurons are found along the entire gastrointestinal tract in both man and rat. GnRH or its receptor are not found in rat gastrointestinal tract, and neither FSH or its receptor is found in the gastrointestinal tract.

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Table 1 Details on antibodies used in immunocytochemistry

Raised against	Code no.	Host	Working dilution cryo sections	Working dilution paraffin sections	Supplier	References
human neuronal protein (HuC/D)	A-21271	mouse	1:800		Molecular Probes, Eugene, OR, USA	Lin et al. 2003 (35)
Gonadotropin-releasing hormone receptor (GnRH-R)	Sc-8682	goat	1:800		Santa Cruz Biotech Inc. CA, USA	Sand et al. 2013 (15)
GnRH-R	90217.01	rabbit		1:50	Innovagen, Sweden	Roth et al. 2013 (34)
Luteinizing hormone (LH)	K104		1:1600		Eurodiagnostica, Sweden	
LH / Choriogonadotropin receptor (LH-R, N-terminal)	L6792	rabbit	1:1600		Sigma-Aldrich Sweden	Sand et al. 2013 (15)
LHR-R (C-terminal)	ABIN 122503	rabbit		1:1600	Antibodies-online GmbH, Germany	Sand et al. 2013 (15)
Follicle stimulating hormone (FSH)	Sc-7797	goat	1:200		Santa Cruz Biotech Inc.	Kubosaki et al. 2006 (36)
Follicle stimulating hormone receptor (FSH-R)	Sc-7798	goat	1:200		Santa Cruz Biotech Inc.	Ponglowhap an et al. 2007 (37)

## Legends of figures

### Figure 1

The relative expression of mRNA (arbitrary units) for gonadotropin-releasing hormone 1 and 2 (GnRH1 and GnRH2) in brain and human intestinal tract. Nd = non-detected.

### Figure 2

Paraffin sections of colon myenteric ganglia (MG) from man (a, b) and rat (c, d). Sections are doubled immunostained with primary antibodies raised against the pan-neuronal marker protein HuC/D (a, c) and against the luteinizing hormone (LH) receptor (b, d). Arrows indicate myenteric neurons immunoreactive (IR) for LH receptors. Magnification bars = 25µm. Relative numbers of LH receptor-IR neurons in MG of rat fundus and submucous ganglia (SG) and MG of rat ileum and colon (e). Cell counting was performed on longitudinally cut whole wall sections. Results are presented as medians and spreads expressed as 25<sup>th</sup> and 75<sup>th</sup> percentile.



