

# Autoregulation of the gonadotropin-releasing hormone (GnRH) system during puberty: effects of antagonistic versus agonistic GnRH analogs in a female rat model

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## Abstract

To address whether gonadotropin-releasing hormone (GnRH) regulates its own expression and the expression of its receptor in the hypothalamus and ovary, we treated five groups of prepubertal/peripubertal female rats from postnatal days 25–36 with either the GnRH agonist triptorelin (TRIP) or the GnRH antagonist cetrorelix (CET), each 10 or 100 µg/day, or a placebo. We compared their effects regarding pubertal development, serum gonadotropins and the expression of GnRH and GnRH-receptor in the hypothalamus, pituitary, ovary and uterus. Onset of puberty was determined by vaginal opening, and expression levels of GnRH and GnRH-receptor were determined using either quantitative real-time PCR or competitive RT-PCR. Onset of puberty was retarded by both analogs but CET (100 µg/day) inhibited while TRIP (10 and 100 µg/day) stimulated serum gonadotropins ( $P < 0.05$ ). The expression of GnRH in the preoptic area did not show significant differences among the treatment groups but ovarian GnRH mRNA levels were significantly stimulated by CET (100 µg/day). GnRH mRNA could not be detected in the uterus by either real-time PCR or competitive RT-PCR. The GnRH-receptor

expression in the hypothalamus (preoptic area and medio-basal hypothalamus) did not vary among any of the groups, whereas in the pituitary GnRH-receptor mRNA levels were stimulated by TRIP (10 µg/day) but inhibited by CET (100 µg/day). In contrast, in the ovary GnRH-receptor mRNA levels were inhibited by both TRIP (100 µg/day) and CET (100 µg/day). Interestingly, the GnRH-receptor was even expressed in the uterus where it was strongly stimulated by both CET and TRIP in a dose-related manner. This shows that in addition to their different pituitary effects, the GnRH analogs cetrorelix and triptorelin exert different actions at the hypothalamic, ovarian and uterine level. This study also demonstrates an organ-specific regulation of GnRH and GnRH-receptor gene expression which is likely part of a local autoregulatory system. We conclude that the ovarian and uterine effects of GnRH analogs must be considered in addition to their known pituitary effects when deciding which GnRH analog is most suitable for treating precocious puberty.

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## Introduction

The decapeptide gonadotropin-releasing hormone (GnRH) is a key molecule of sexual maturation and reproductive functions in mammals. GnRH binds with high affinity to the GnRH receptor (GnRH-receptor) on the cell surface of pituitary gonadotropins (Norwitz *et al.* 1999). Here, it activates intracellular signal transduction pathways to effect both the synthesis and intermittent release of the gonadotropins. The GnRH-receptor is a G-protein coupled receptor which activates phospholipase C. In the pituitary, via the generation of several second

messenger molecules such as diacylglycerol and inositol, this leads to activation of protein kinase C and release of  $Ca^{2+}$  from intracellular pools which results in secretion and synthesis of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Ortmann & Diedrich 1999). It is well documented that the expression of GnRH-receptor in the pituitary is regulated by GnRH itself and gonadal steroids (Norwitz *et al.* 1999). GnRH receptors have also been characterized in several extrapituitary tissues such as the gonads, placenta and various brain tissues as well as in immortalized hypothalamic neurons (Stojilkovic *et al.* 1994). There is evidence that GnRH and

its binding sites are also expressed in the ovary (Peng *et al.* 1994). GnRH may affect ovarian steroidogenesis possibly as a local autocrine/paracrine regulator by modulation of the gonadotropin-stimulated steroidogenesis in the ovary (Peng *et al.* 1994, Vaananen *et al.* 1997). It has also been shown that in the rat ovary local actions of GnRH are predominantly inhibitory (Nathwani *et al.* 2000).

Activation of the pituitary–gonadal axis depends upon the GnRH pulse generator (Bourguignon *et al.* 1990). This neuronal network causes the phasic release of GnRH and subsequently controls pulsatile gonadotropin release which is the prerequisite of gonadal activation (Wildt *et al.* 1980). In the rat, cell bodies of the GnRH neurons are located in the preoptic area of the anterior hypothalamus, while their axons are mainly projecting into the median eminence of the mediobasal hypothalamus (Merchenthaler *et al.* 1989). In humans, prolonged exposure of pituitary gonadotrophins to non-pulsatile GnRH secretion leads to desensitization and/or down-regulation of pituitary GnRH receptors (Plosker & Brogden 1994) and consequently to suppressed LH and FSH secretion. This phenomenon is the basis for clinical use of GnRH agonists in the treatment of central precocious puberty due to premature release of GnRH (Oostdijk *et al.* 1991) and of steroid-dependent tumors such as prostate cancers (Lamharzi *et al.* 1998, Schally 1999a).

Synthetic GnRH analogs contain substitutions of the native decapeptide. Depending on the substitutions, the analogs have GnRH agonistic or antagonistic properties. The GnRH agonist triptorelin (D-Trp<sup>6</sup>)-GnRH has only one substitution while the GnRH antagonist cetrorelix acetate (Ac-D-Nal(2)<sup>1</sup>, D-Phe(4 Cl)<sup>2</sup>, D-Pal<sup>3</sup>, D-Cit<sup>6</sup>, D-Ala<sup>10</sup>)-GnRH differs from the native decapeptide in five positions (Haviv *et al.* 1998). In contrast to agonists, antagonistic GnRH analogs such as cetrorelix act by competitive binding to the pituitary GnRH receptors thereby preventing the action of endogenous GnRH (Beckers *et al.* 1995). Little is known about the use of GnRH antagonists and their potential advantages compared with GnRH agonists regarding pituitary hormone secretion to control the onset of puberty (Schally 1999b).

To examine the potential role of GnRH as an autocrine regulator during puberty relative to these divergent analogs, we investigated the expression of GnRH and its receptor in the pituitary, hypothalamus, ovary and uterus of peripubertal female rats under the influence of either agonistic or antagonistic GnRH analogs at two different dose potencies.

## Materials and Methods

### Animals

This study was approved by the local ethical committee for animal experiments (No. 509-42502/01–3-00). Female Sprague Dawley rats were housed under standardized

conditions (lights on from 0700 h to 1900 h, 25 °C room temperature, 10 animals per cage, free access to water and feed available *ad libitum*). Animals were randomized into five groups, each consisting of 12–16 animals. Each animal was marked with a code on the tail for identification purposes. After randomization and marking, the animals from all treatment groups were housed together in each cage. They were weighed and monitored for changes to the external and internal genitalia, vaginal opening marking the first estrous cycle and the beginning of puberty (according to our experience this occurred in controls at postnatal day 33–38).

### Drugs and serum hormones

Drugs were diluted with 0.9% NaCl to a volume of 0.4 ml for each injection. From day 25 to day 36 (12 days) rats were intraperitoneally injected once per day at 1800 h with either 1 × 0.4 ml 0.9% NaCl (placebo group), 1 × 100 µg or 1 × 10 µg cetrorelix (CET 100 or CET 10 group) or 1 × 100 µg or 1 × 10 µg triptorelin (TRIP 100 or TRIP 10 group). On day 37, rats of all five groups were decapitated in the morning 14 h after the last injection of drugs. Brains, anterior pituitaries, ovaries and uteri were rapidly removed and frozen on dry ice for mRNA studies.

Serum levels of LH, FSH and estradiol were determined using established RIA methods. Estradiol was measured by using a sensitive commercial RIA (Diagnostic Systems Laboratories, Webster, TX, USA) with a lower detection limit of 1.2 pg/ml. Reference preparations were RP2 for both LH and FSH. For iodination, LH I-6 and FSH I-7 were used. To achieve maximum sensitivity, 100 µl serum were used for LH and FSH measurements. Each assay was performed with freshly prepared tracer which had been purified by FPLC-column chromatography on Superdex 75 column (Amersham Pharmacia Biotech, Freiburg, Germany). In pilot experiments, fractions were tested for maximum sensitivity. Based on these data, an elution pattern of the chromatography with regard to the fraction containing the optimal tracer was established. The antibodies were NIADDK rat-S7 (LH) and NIADDK rat-S11 (FSH). The sensitivity limits were 0.1 ng/ml (LH) and 0.4 ng/ml (FSH). The intra- and interassay coefficients of variation from replicates of pooled samples from ovariectomized rats were 7.5% and 11.5% (LH) and 8.5% and 13.5% (FSH).

### RNA preparation

RNA isolation from the anterior pituitary, micropunches of the preoptic area (POA) and tissues of the mediobasal hypothalamus (MBH), ovary and uterus of each animal was carried out with the RNeasy Total RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Uterine tissue had to be homogenized using QIAshredder columns (Qiagen) before RNA isolation

**Table 1** Primer pairs for competitive PCR for encoding mRNA sequences according to Kaiser *et al.* (1992) (GnRH-receptor) and Bond *et al.* (1989) (GnRH)

	Primer sequence	Amount of total RNA (ng)	Amount of mutant cRNA (fg)	No. of cycles
<b>Probe</b>				
GnRH	5' ACTATGGTCACCAGCGGG 3' TGCCCAGCTTCCTCTCAAT	Pituitary: 150 Ovary: 500 Uterus: 500	Pituitary: 10 Ovary: 10 Uterus: 10	Pituitary: 31 Ovary: 38 Uterus: 50
GnRH-receptor	5' TCAGTGGTATGCTGGAGAGT 3' ATATAAGTGGGTCGAAGCAC	Pituitary: 150 Ovary: 100	Pituitary: 100 Ovary: 100	Pituitary: 24 Ovary: 22(+5)

(+5), additional cycles for competitive PCR only for GnRH-receptor in the ovary.

was possible. Extracted total RNA was measured with a UV spectrophotometer at  $\lambda$  260 nm and each sample was diluted with H<sub>2</sub>O to achieve a concentration of 100–500 ng RNA/ $\mu$ l for the reverse transcription (RT). The mRNA levels were quantified using competitive RT-PCR (GnRH: ovary; GnRH-receptor: pituitary, ovary) or real-time RT-PCR (GnRH: POA; GnRH-receptor: POA, MBH, uterus) or both methods (GnRH: pituitary, uterus).

#### Competitive RT-PCR

For generation of mutant RNA templates, composite primers were designed for each probe. An RT-PCR reaction was carried out with composite primer plus the respective sense or antisense primer and the resulting PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands). After sequencing, the mutant RNA was reverse transcribed with the Reverse Transcription System purchased from Promega (Mannheim, Germany). RT-PCR was conducted using SUPERSRIPT for RT and SUPERMIX for PCR (both purchased from Gibco-BRL, Karlsruhe, Germany). The RT reaction was carried out with 100 ng (ovarian GnRH-receptor) – 500 ng (ovarian and uterine GnRH) total RNA (10 min at 22 °C, 50 min at 42 °C, 10 min at 95 °C). Prior to RT-PCR reaction various amounts (10–100 fg) of mutant RNA were added to all reaction vials. The concentration of this cRNA was evaluated for each investigated gene by pilot titration experiments. For PCR reaction, 2–4  $\mu$ l cDNA and 25 pM primer were added to the SUPERMIX solution. PCR procedure was performed in a Biometra automated thermocycler (1 min 94 °C, 1 min 60 °C, 2 min 72 °C). Table 1 shows primer pairs, amount of total RNA and mutant cRNA as well as numbers of cycles used for RT-PCR. Different numbers of PCR cycles were tested in preceding experiments. To prevent or destroy secondary structures, the PCR was continued with 5 cycles of 1 min at 78 °C, 1 min at 60 °C and 2 min at 72 °C followed by a final

step at 79 °C for 3 min (only for GnRH-receptor). The amplified PCR products were size-fractionated by electrophoresis in a 1.5% agarose gel in Tris borate ethylene diamine tetracetic acid (TBE) buffer and photographed after staining with ethidium bromide. Intensities of bands were evaluated with the Kodak DC 1D program. The possibility of contamination was ruled out as no PCR products were observed or detected in negative controls.

#### Real-time RT-PCR

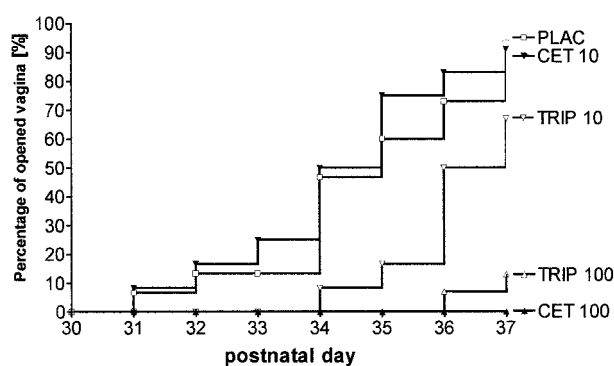
The RT-reaction proceeded at 22 °C for 10 min and 42 °C for 50 min with 15 ng (GnRH POA), 250 ng (GnRH pituitary), 250 ng (GnRH uterus), 15 ng (GnRH-receptor POA), 15 ng (GnRH-receptor MBH) or 250 ng (GnRH-receptor uterus) total RNA. The reaction mixture contained 5 ng/ $\mu$ l random primers, 1  $\times$  reaction buffer, 10 mM dithiothreitol, 500 nM deoxy-NTPs, 200 U Superscript RNase H<sup>-</sup> reverse transcriptase (all reagents from GibcoBRL) and 10 U ribonuclease inhibitor (Promega) in a final volume of 20  $\mu$ l. At the end of incubation, the samples were heated at 95 °C for 10 min to inactivate the enzyme and denature RNA–cDNA hybrids. Real-time PCR reactions were performed using the 5' exonuclease activity of the DNA polymerase (TaqMan). Gene specific PCR primer pairs and the TaqMan hybridization probe were designed using the Primer Express software (PE Applied Biosystems). The encoding sequences for GnRH-receptor were published by Kaiser *et al.* (1992) and the ones for GnRH were published by Bond *et al.* (1989). The hybridization probe is linked at the 5'-end to FAM (6-carboxy-fluorescein) as fluorogenic reporter dye and at the 3'-end to the fluorogenic group TAMRA (6-carboxy-tetramethyl-rhodamine), which quenches the FAM emission spectrum. To prevent probe extension, the 3' end of the probe is blocked by a phosphate group. When the hybridization probe is intact, the reporter fluorescence is absorbed by the quenching dye. During the extension phase of a PCR cycle, the Taq DNA polymerase cleaves the TaqMan

hybridization probe by its exonuclease activity resulting in a release of reporter dye and fluorescence emission. This increase of fluorescence intensity, detected by an automated sequence detector combined with special software (ABI Prism 7700 Sequence Detection System; Applied Biosystems, Perkin Elmer, Foster City, CA, USA), depends directly on the accumulation of PCR product. The fluorescence signal is normalized for changes in concentration or volume by dividing the emission intensity of the reporter dye by the emission intensity of an internal reference dye (ROX, 6-carboxy-X-rhodamine). This ratio is defined as the normalized reporter ( $R_n$ ). The  $\Delta R_n$  represents  $R_n$  minus the baseline signal established in the first 3–15 cycles of the PCR reaction. Reactions are characterized by their threshold cycle ( $C_t$ ) values,  $C_t$  being the cycle at which the baseline signal is exceeded. The  $C_t$  value is directly dependent on the initial number of target molecules and is defined as the fractional cycle number at which the reporter group fluorescence generated by the cleavage of the hybridization probe exceeds the ten times standard deviation of baseline emission.

Each PCR run included 7 points of the standard curve (see below), a no template control and the sample cDNAs. Amplification reactions (25  $\mu$ l) contained 12.5  $\mu$ l TaqMan buffer, 300–900 nM of each primer, 225 nM hybridization probe and 4  $\mu$ l cDNA. Reagents were from Perkin Elmer and Applied Biosystems, Weiterstadt, Germany. The cycle conditions were 2 min at 50 °C for eliminating carryover PCR products by uracil DNA glycosylase treatment, 10 min at 95 °C for activation of the AmpliTaq Gold DNA polymerase (Perkin Elmer) followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each PCR product was cloned into the pCR II-TOPO plasmid using the TOPO TA Cloning Kit following the manufacturer's instructions (Invitrogen). After confirming the sequence identity and the orientation of the cloned PCR product by commercial sequencing, the plasmid construct was linearized with the appropriate restriction endonuclease and purified using the Wizard DNA Clean-Up System (Promega) as preparation for the *in vitro* transcription reaction. RNA was synthesized using the RiboMAX Large Scale RNA Production System-SP6 or -T7 for GnRH and GnRH-receptor respectively (Promega). Seven serial dilutions of the RNA generated by *in vitro* transcription were reverse transcribed as described above. Resulting cDNAs were subjected to real-time PCR and a standard curve was generated by plotting the relative amount of these DNAs against their corresponding  $C_t$  value. To calculate the relative expression levels of the different samples, the sample- $C_t$  values were interpolated against the standard curve.

### GnRH

Sense primer: 5'-GCAGAACCCAGAACTTCGA-3';  
anti-sense primer: 5'-TGCCAGCTTCCTCTTC



**Figure 1** Percentage of pubertal rats with opened vaginas on various postnatal days up to day 37. The pubertal development was inhibited in a dose-related manner by treatment with both GnRH analogs, as determined by the lower percentage of vaginal opening at any given pubertal age compared with controls (PLAC, placebo).

AAT-3'; TaqMan hybridization probe: 5'-FAM-TCT GCGAGGAGCTCTGGAACGTCTG-TAMRA-3'.

### GnRH-receptor

Sense primer: 5'-AGGGATGATGAACAGGCAGC-3';  
anti-sense primer: 5'-TCTCGCAATGTGTGACC CAC-3'; TaqMan hybridization probe: 5'-FAM-TTC ATGCCACCATTGCGGAAAGCTG-TAMRA-3'.

### Statistical evaluation and mathematical calculations

Unless otherwise stated, results are presented as means  $\pm$  s.e.m. All data from the RIA and PCR analysis were statistically evaluated with one-way ANOVA. The Kruskal-Wallis test was used if normal distributions could not be assumed. Differences were considered significant if  $P$  was less than 0.05. The analysis was performed by using the Prism program (GraphPad Software, Inc., San Diego, CA, USA).

## Results

### Pubertal development and serum hormone levels

At the end of the treatment at pubertal age (postnatal day 37) the vagina was open in nearly all of the control rats (14/15); in the low-dose-treated rats, 8/12 TRIP 10 rats and 11/12 CET 10 rats had an open vagina. In the high-dose-treated groups, the vagina was open in only 2/15 TRIP 100 rats and in the group of CET 100 rats pubertal development was completely abolished (Fig. 1). The percentage of rats with opened vagina in the CET 100 group and in the TRIP 100 group was significantly lower than in controls ( $P < 0.001$ ). There were also significant differences found between the CET 100 and CET 10

groups, the TRIP 100 and the TRIP 10 groups, the CET 100 and the TRIP 10 groups as well as the CET 10 and the TRIP 100 groups ( $P < 0.001$ , log rank test, Statistica, StatSoft, Tulsa, OK, USA). The body weight gain and the weights at day 37 were similar for all groups (data not shown). Serum LH and FSH levels were significantly lower under CET 100  $\mu\text{g}/\text{day}$  treatment and significantly higher after both TRIP treatments (10 and 100  $\mu\text{g}/\text{day}$ ) as compared with controls. LH and in particular FSH release was stimulated more markedly by the low dose of 10  $\mu\text{g}/\text{day}$  TRIP than by the high dose of 100  $\mu\text{g}$  TRIP. Serum estradiol values at the time of decapitation, measured in trunk blood in the morning and not adjusted to the estrous cycle, were low in all groups. Estradiol was significantly lower in the CET 100-treated rats compared with the CET 10 group, but no significant differences were found among the other groups (Fig. 2a,b,c).

#### *Gene expression of GnRH in different tissues*

The expression of GnRH in the preoptic area varied in the five groups but there were no significant differences (real-time PCR: Fig. 3a). In contrast, ovarian GnRH mRNA levels were significantly stimulated by treatment with CET 100  $\mu\text{g}/\text{day}$  (competitive RT-PCR: Fig. 3b). Among the treatment groups there were no significant differences in pituitary GnRH mRNA levels. The pituitary GnRH mRNA could be detected using the real-time PCR ( $C_t$  value 30 cycles) but not by the competitive RT-PCR (31 cycles). Despite the presence of GnRH-receptor mRNA in the uterus (see below), expression of the ligand GnRH could not be detected. Neither the real-time PCR nor the competitive RT-PCR yielded a positive signal of GnRH mRNA in the uterus despite the use of up to 40 or 50 cycles respectively.

#### *GnRH-receptor expression in different tissues*

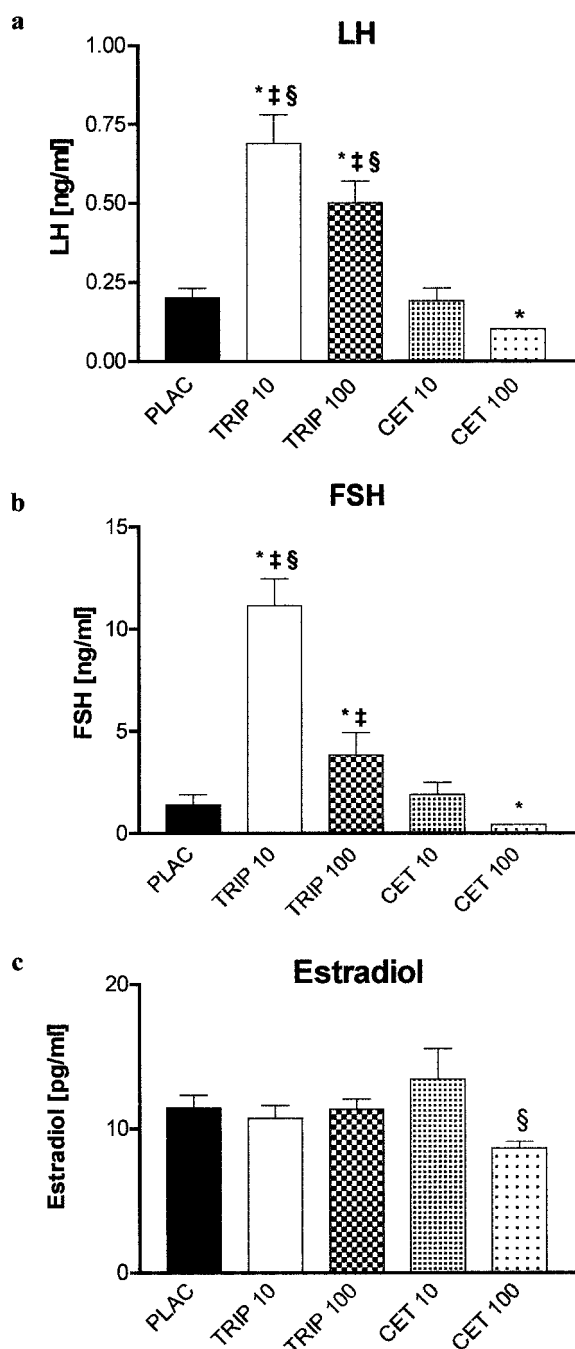
Except for a lower GnRH-receptor expression of the TRIP 100  $\mu\text{g}/\text{day}$  group in the preoptic area, there were no differences among the treatment groups in the preoptic area nor in the mediobasal hypothalamus (Fig. 4a,b). In the pituitary, GnRH-receptor mRNA levels mirrored the pattern of gonadotropin levels, i.e. they were significantly stimulated after treatment with TRIP 10  $\mu\text{g}/\text{day}$  but inhibited after CET 100  $\mu\text{g}/\text{day}$  (Fig. 4c). In the ovary, the GnRH-receptor mRNA levels were affected by the antagonist and the agonist in the same manner: they were inhibited significantly by both TRIP 100  $\mu\text{g}/\text{day}$  and CET 100  $\mu\text{g}/\text{day}$  (Fig. 4d). In contrast, the uterine expression of GnRH-receptor was strongly stimulated by treatment with CET (100  $\mu\text{g}/\text{day}$ ) and TRIP (100  $\mu\text{g}/\text{day}$ ); the increase induced by the antagonist CET (100  $\mu\text{g}/\text{day}$ ) was significantly higher compared with the agonist TRIP (100  $\mu\text{g}/\text{day}$ ) (Fig. 4e).

## Discussion

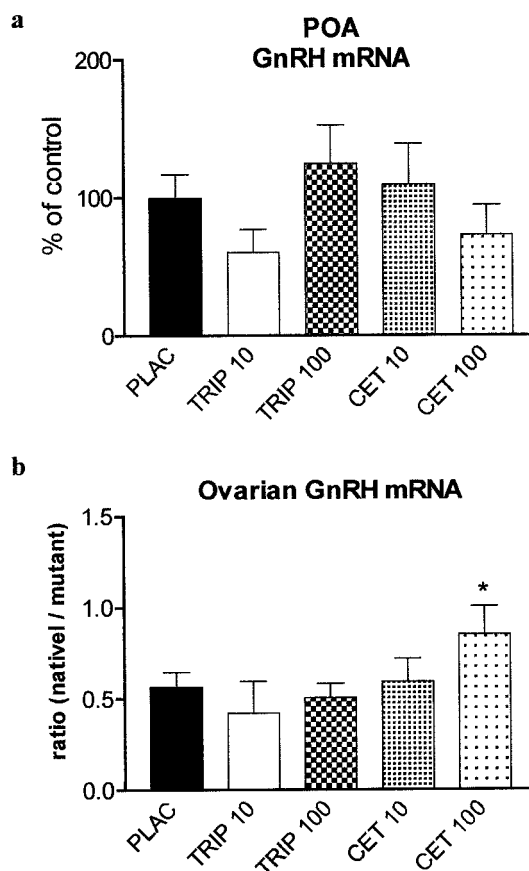
The present study demonstrates that CET and TRIP influence GnRH and GnRH-receptor expression in a different manner, indicating that agonistic and antagonistic GnRH analogs involve different mechanisms of action. This study also demonstrates that agonistic and antagonistic GnRH analogs differ greatly in how they regulate the GnRH system at the hypothalamic, pituitary, ovarian and uterine level in peripubertal female rats. Both GnRH analogs inhibit puberty in a dose-related manner but only CET inhibits gonadotropin secretion in peripubertal female rats.

In our experiments we first used the competitive RT-PCR to quantify mRNAs as this method does not require costly equipment and the results are reliable. However, after establishing real-time RT-PCR for several genes, this technique became the favored method as it is a more rapid and sensitive technique. In the pituitary, GnRH mRNA could be detected by real-time RT-PCR but not by competitive PCR. In addition to its superior speed and sensitivity, real-time RT-PCR is an important development for studies like ours of gene expression in the brain micropunches of single rats where RNA is not available in abundant quantities. To examine whether both techniques revealed identical results, we analyzed the relative amounts of GnRH-receptor mRNA in the pituitary. With both RT-PCR methods, a twofold increase of GnRH-receptor mRNA in the TRIP 10  $\mu\text{g}/\text{day}$  group was measured while CET 100  $\mu\text{g}/\text{day}$  treatment resulted in a 50% reduction. The extent of the changes was similar in both assays (data not shown). Thus, with both methods, the real-time PCR and the competitive PCR, mRNA levels could be quantified in our experiments.

In the present study the hypothalamic GnRH-receptor expression in the preoptic area was affected by treatment with TRIP 100  $\mu\text{g}/\text{day}$  but not by CET. In a recent study with perfused hypothalamic cells, antagonistic and agonistic GnRH analogs exerted different effects on neuronal GnRH release (Krsmanovic *et al.* 2000). This study demonstrated that GnRH-receptor activation is required for pulsatile GnRH release *in vitro* and that the GnRH-receptor activation on GnRH neurons is involved in the pulse generation of GnRH, possibly due to an ultrashort-loop autocrine feedback mechanism (Feleder *et al.* 1996, Krsmanovic *et al.* 2000). In our study the unchanged GnRH-receptor mRNA levels after treatment with CET were surprising as we expected reduced hypothalamic GnRH-receptor expression following cetrorelix administration due to disruption of the integrated control of GnRH secretion from the hypothalamus. GnRH mRNA levels were analyzed in the POA where the perikarya of GnRH neurons are located. After treatment with GnRH analogs GnRH mRNA levels were also unchanged. This is similar to a previous study of ours where no changes of GnRH expression in the preoptic area were found in the



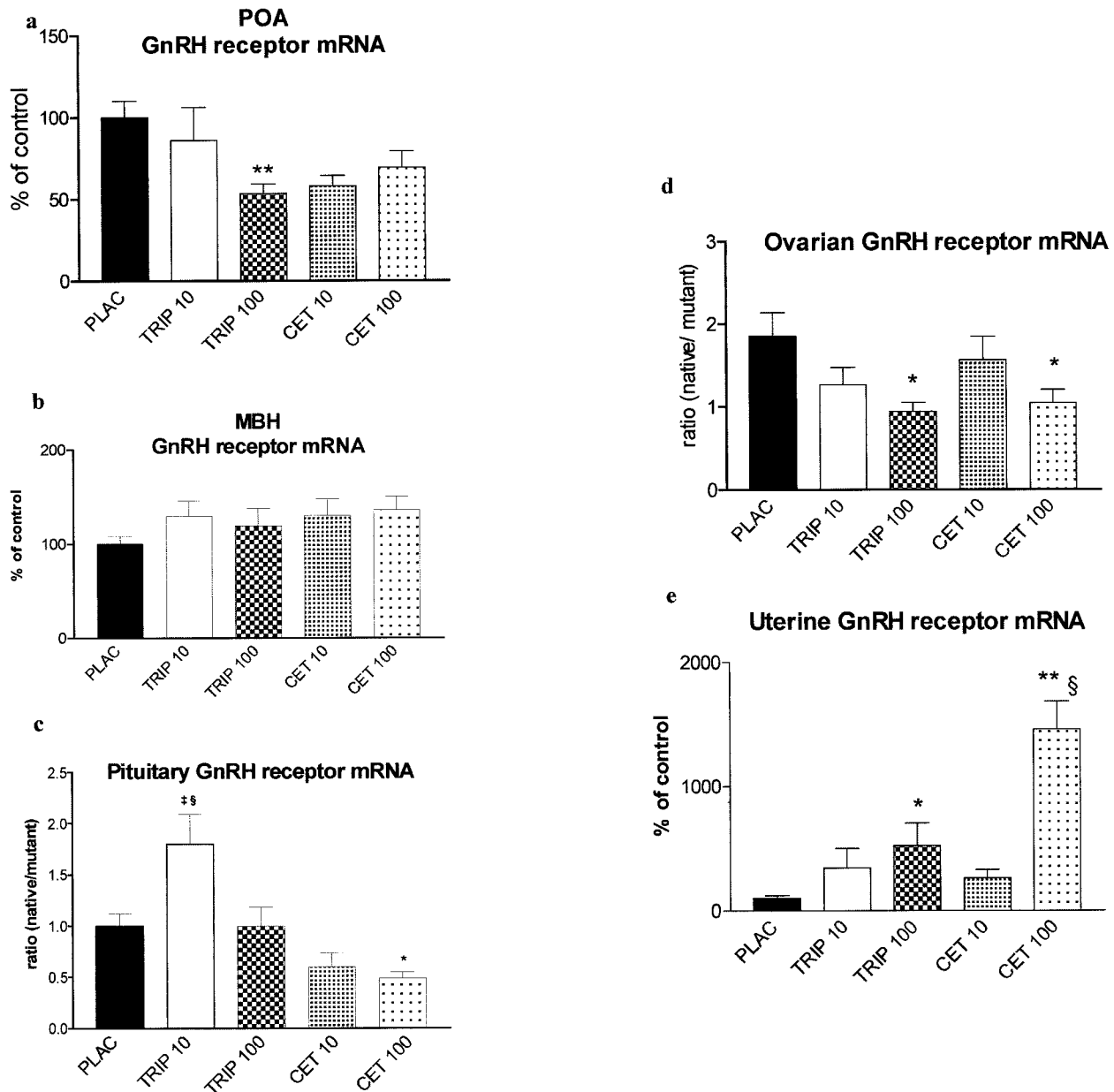
**Figure 2** Serum gonadotropins and estradiol levels at the end of the treatment with CET, TRIP or placebo (PLAC; controls). (a) Serum LH and (b) FSH levels were lower in the CET 100 group but were stimulated in the TRIP-treated rats (both 10 µg/day and 100 µg/day) compared with controls. LH and FSH levels in TRIP (10 µg/day, 100 µg/day)-treated rats were significantly higher than in CET (100 µg/day)-treated rats. (c) Morning estradiol values, measured in trunk blood not adjusted to the estrous cycle, were relatively low in all groups. Rats in the CET 100 group had significantly lower estradiol levels than those in the CET 10 group. \* $P < 0.05$  vs controls, † $P < 0.001$  vs CET 100, § $P < 0.05$  vs CET 10.



**Figure 3** The GnRH expression in the preoptic area (POA) of the hypothalamus and in the ovary. (a) In the POA, mRNA levels, which were detected by real-time PCR and related to 100% in the controls, did not show significant differences between the treatment groups. (b) The GnRH expression in the ovary was determined by competitive RT-PCR. The ratio of native to mutant DNA fragments is shown. Ovarian GnRH mRNA levels were significantly stimulated by treatment with CET 100 µg/day. \* $P < 0.05$  compared with controls (PLAC, placebo).

transition from prepubertal to pubertal rats (Roth *et al.* 1998). The differences between the *in vitro* and the *in vivo* data in which GnRH analogs were investigated may be explained, at least in part, by the blood–brain barrier, i.e. it is not yet known how much of the GnRH analogs reaches the GnRH perikarya and axon terminals. *In vivo* effects with regard to an auto-regulation of GnRH are only achieved upon intracerebroventricular injection (DePaolo *et al.* 1987). Therefore, the missing effects in the present study may be explained by the route of application. However, we purposely chose the intraperitoneal application because this treatment is more closely related to the clinical use of GnRH drugs than is intracerebroventricular application.

At the pituitary level, the GnRH signal is mediated through a transmembrane GnRH-receptor located on the



**Figure 4** The GnRH-receptor expression in the preoptic area (POA), mediobasal hypothalamus (MBH), pituitary, ovary and uterus. (a and b) In the POA and MBH, mRNA levels were determined by real-time PCR and were related to 100% in the controls. The values did not show significant differences between the treatment groups except for the lower values of the TRIP 100 µg/day group in the preoptic area (c and d) The expression of the pituitary and ovarian GnRH-receptor was determined by competitive RT-PCR and shown by the ratio of native to mutant DNA fragments. The pituitary GnRH-receptor mRNA levels were stimulated after treatment with TRIP 10 µg/day and were inhibited after CET 100 µg/day (c). Ovarian GnRH-receptor mRNA levels were inhibited by both TRIP 100 µg/day and CET 100 µg/day (d). (e) The uterine expression of GnRH-receptor determined by real-time PCR was strongly stimulated by treatment with CET 100 µg/day and by TRIP 100 µg/day. \* $P < 0.05$ , \*\* $P < 0.01$  compared with controls (PLAC, placebo), † $P < 0.001$  vs CET 100, § $P < 0.05$  vs CET 10.

surface of gonadotropins. Binding of GnRH to GnRH-receptor initiates several intracellular signaling pathways, i.e. the classical GnRH receptor transduction pathway via

phospholipase-C, protein kinase C and adenylate cyclase (Ortmann & Diedrich 1999). In our study the pituitary GnRH-receptor expression of CET 100 µg/day-treated

rats was significantly lower compared with controls and TRIP 10 µg/day-treated rats, indicating the interruption of the physiological stimulation by GnRH. This is in contrast to a study with infantile female rats in which a three-day treatment with a GnRH antagonist did not lead to significant changes of GnRH-receptor mRNA levels in the gonadotroph (Wilson & Handa 1997). It is important to note that in our study low dose TRIP (10 µg/day)-treated rats showed significantly higher GnRH-receptor mRNA levels than saline-injected rats despite a treatment period of 12 days. Among the four treatment groups, the greatest stimulation of the receptor was achieved with low doses of the agonist TRIP. We determined the mRNA levels only at one time point after the 12-day treatment with GnRH analogs. In a previous study, we determined the gonadotropin levels at various time points just after the beginning and after different intervals following the injections of GnRH analogs (Roth *et al.* 2000). From that study we know that TRIP activates LH and FSH secretion permanently throughout the 12-day treatment. Presumably, in the present study the receptor activation lasted during the entire treatment period as the pituitary GnRH receptor activation determined by mRNA levels was in line with the high gonadotropin levels seen in the TRIP 10 group. This conclusion will be verified in future experiments.

It is known that GnRH-receptor itself is subject to regulation by GnRH. In pituitary cultures, pulsatile stimulation by GnRH results in an increase of GnRH-receptor mRNA, but after prolonged exposure to GnRH the number of GnRH receptors declines (Loumaye *et al.* 1982, Tsutsumi *et al.* 1995, Haisenleder *et al.* 1998). On the other hand missing exposure of GnRH to its ligand causes down-regulation of GnRH-receptor and a rapid reduction of LH levels (Seong *et al.* 1995). Taken together, low doses or pulsatile treatment of GnRH up-regulates its receptor, whereas high doses, continuous treatment or no exposure down-regulates the GnRH-receptor in the pituitary. In line with this conclusion are the data of the CET 100 group showing a clear reduction of GnRH-receptor mRNA levels in the pituitary due to the antagonist. In a study with adult male rats it has been shown that a reduction of the number of GnRH receptor binding sites is associated with reduced GnRH-receptor gene expression (Pinski *et al.* 1996). Changes in the GnRH-receptor mRNA level have been explained as at least part of the mechanisms underlying up- and down-regulation of GnRH receptor numbers. However, decreased efficiency of GnRH-receptor mRNA translation also contributes to the GnRH-receptor down-regulation on gonadotrophs following prolonged exposure to high concentrations of GnRH (Tsutsumi *et al.* 1995). In this study with a gonadotroph cell line, after exposure to GnRH the number of GnRH-receptor binding sites decreased but the levels of GnRH-receptor mRNA were not affected, showing an 'uncoupling' of mRNA levels and binding

sites. In our study with peripubertal female rats it is, however, very likely that the changes of GnRH-receptor mRNA in the pituitary reflect the number of GnRH-receptors because the pattern of changes of gonadotropin and GnRH-receptor mRNA levels are similar.

The number of GnRH receptors correlates with the sensitivity of the pituitary to GnRH (Norwitz *et al.* 1999). Estradiol is known as a positive regulator of GnRH receptor gene expression in the preovulatory period. An increased serum concentration of estradiol probably causes elevation of pituitary GnRH receptor mRNA which precedes maximal numbers of GnRH receptors before the LH surge. Through this mechanism maximal sensitivity of gonadotrophs to GnRH is achieved (Turzillo & Nett 1999). Therefore, it is highly likely that both GnRH and ovarian factors control the synthesis and secretion of the gonadotropins. In our experiments the influence of ovarian steroids at the pituitary level in both CET and TRIP high-dose treatment groups should be similar as a clear inhibition of puberty appeared in both treatment groups. It seems that ovarian steroids might not be responsible for the different GnRH-receptor mRNA levels which are possibly due to different hypothalamic and pituitary mechanisms of the two GnRH analogs. We conclude that the different actions of these analogs on the pituitary GnRH-receptor are caused by different receptor/postreceptor effects rather than by different gonadal steroid levels.

Even though GnRH is primarily known for its role in the regulation of gonadotropin secretion from the pituitary, GnRH also modulates gonadotropin-stimulated ovarian steroidogenesis, demonstrating a direct action of the peptide in the ovary (Peng *et al.* 1994, Anderson *et al.* 1996, Vaananen *et al.* 1997, Kang *et al.* 2000, Nathwani *et al.* 2000). In a recent study, GnRH mRNA levels in human granulosa-luteal cells were reduced by treatment with estradiol for 24 h, whereas the GnRH receptor mRNA levels were increased by short-term treatment (6 h) but diminished by long-term estradiol treatment (48 h) indicating that estradiol regulates the expression of GnRH and its receptor in the ovary (Nathwani *et al.* 2000). Furthermore, it has already been shown that in rat as well as in human ovary, LH/human chorionic gonadotropin (hCG) down-regulates GnRH mRNA levels (Peng *et al.* 1994, Olofsson *et al.* 1995). These results provide evidence for an interaction between gonadotropins, GnRH and steroids at the ovarian level. Furthermore, GnRH is known to exert antigonadotrophic effects by inhibiting granulosa cell differentiation, and it directly induces apoptotic cell death of granulosa cells (Nathwani *et al.* 2000). Therefore, locally produced GnRH seems to be an autocrine/paracrine regulator of ovarian function in addition to its well established role as neuroendocrine regulator of the anterior pituitary.

In our study we observed that the vaginal opening is delayed by TRIP treatment despite elevated gonadotropin levels. The most likely explanation is that TRIP causes



bioactivity of the gonadotropins. This can be due to the release of immature LH and FSH molecules lacking the appropriate glycosylation. These molecules do not act in the ovary because they cannot activate their receptors. This possibility is supported by data that GnRH agonist treatment leads to secretion of bioinactive LH molecules into the circulation which are detected by RIAs but not by immunoassays based on monoclonal antibodies (Jaakkola *et al.* 1990, Kwekkeboom *et al.* 1990, Uemura *et al.* 1992). The second possibility is that TRIP stimulates the release of bioactive LH and FSH molecules whose action is counteracted by direct effects of TRIP in the ovary due to an interference with a local ovarian autoregulatory GnRH system. Regardless which explanation is correct, the biological consequence is the retardation of puberty.

In a study using rat ovarian granulosa cells, the effects of GnRH were determined by the state of granulosa cell development with mainly inhibitory actions in immature cells and stimulatory actions in differentiated mature cells. In granulosa cells, the signaling is associated with the activation of the phospholipase C inositol phosphate and calcium transduction pathway similar to that which occurs in the pituitary. But, in contrast to the biphasic calcium response in the pituitary, both mature and immature rat ovarian granulosa cells showed a monophasic calcium response after binding of GnRH (Anderson *et al.* 1996). In our study with peripubertal female rats, GnRH expression could clearly be detected in the ovarian tissue after PCR amplification with 38 cycles. CET-treated rats (100 µg/day) had significantly higher GnRH mRNA levels compared with both the controls and the TRIP-injected groups. In human granulosa-luteal cells GnRH-receptor gene expression is up-regulated by GnRH and down-regulated by hCG (Peng *et al.* 1994) which may be explained by a positive auto-regulation of GnRH-receptor expression by GnRH. The slightly upregulated GnRH mRNA as seen in our CET (100 µg/day)-treated animals may be the consequence of low serum LH levels where they were not sufficiently increased to maintain GnRH-receptor expression. However, this explanation is somewhat unlikely as GnRH mRNA levels in the TRIP 100 group were unchanged compared with controls. In our experiments CET 100 µg/day-treated rats had the lowest estradiol serum levels. It is known that treatment with estradiol reduces GnRH mRNA levels in human granulosa-luteal cells (Nathwani *et al.* 2000). Therefore, in our experiments the possibility exists that upregulated GnRH mRNA levels of the CET 100 group are the consequence of low estradiol levels. The low GnRH-receptor mRNA levels seen in both CET and TRIP high dosage-treated rats might be the consequence of direct inhibitory actions of agonistic and antagonistic GnRH analogs in the ovary.

GnRH analogs have proven to be effective in treating GnRH-receptor-bearing tumors, including carcinomas of the ovary, breast, and endometrium (Harris *et al.* 1991,

Emons *et al.* 1993a,b). It has been suggested that the well established GnRH-receptor signaling mechanism via activation of phospholipase C and protein kinase C is probably not involved in the GnRH effects on tumor cells (Emons *et al.* 1998). Analogs of GnRH reverse the growth stimulatory effect of epidermal growth factor (EGF)- $\alpha$  and insulin-like growth factor and block the EGF-induced mitogen-activated protein kinase (MAPK) activity of ovarian and endometrial cancer cells (Srkalovic *et al.* 1990, Emons *et al.* 1998). The MAPK cascade is involved in protein kinase C independent signal transduction pathways and the regulation of the GnRH-receptor promoter activity in response to GnRH (Han & Conn 1999, Lin & Conn 1999). Taken together, these data indicate that at least in tumor cells of the ovary and endometrium the dichotomy of GnRH agonists and antagonists may not be valid because apparently both types of analogs activate signal transduction cascades in the same manner. Therefore, agonistic and antagonistic properties may be defined only for actions in the pituitary.

In human endometrium, GnRH and GnRH-receptor mRNAs can be detected and show dynamic changes throughout the menstrual cycle (Raga *et al.* 1998). GnRH agonists have been found to directly inhibit the proliferation of human endometrial cancer cells (Emons *et al.* 1993a,b) and also of rat uterine cells (Sakamoto *et al.* 1993). In early pregnant rats and in estrogen plus progesterone-treated mice, GnRH mRNA could be seen in uterine stromal cells by *in situ* hybridization (Ikeda *et al.* 1996). In our study with peripubertal rats we could not detect GnRH mRNA in uterine tissue by sensitive real-time PCR nor competitive RT-PCR even with an amplification of up to 50 cycles. This is probably because in these animals with low estradiol levels GnRH expression is extremely low. Interestingly, our results showed that both the analogs TRIP and CET induced very high GnRH-receptor mRNA levels, in contrast to their inhibitory effects on the ovarian GnRH-receptor, indicating that these analogs also exhibit their actions directly on the uterine tissue.

Our study demonstrates that the two GnRH analogs CET and TRIP exert different actions at the hypothalamic, pituitary, ovarian and uterine levels – in particular expression of the GnRH-receptor is differentially regulated. Besides indirect actions via altered pituitary hormone release, GnRH analogs might directly influence the gonadal hormone production via gonadal GnRH receptors which are part of a local autoregulatory system. We conclude that there is an organ-specific regulation of GnRH and GnRH-receptor gene expression possibly due to different transcription factors or different postreceptor signal pathways. These data indicate that the direct ovarian and uterine effects of GnRH analogs have to be considered in addition to their known pituitary effects when deciding which GnRH analog is most suitable for treating precocious puberty.

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## References

- Anderson L, Hillier SG, Eidne KA & Miro F 1996 GnRH-induced calcium mobilisation and inositol phosphate production in immature and mature rat ovarian granulosa cells. *Journal of Endocrinology* **149** 449–456.
- Beckers T, Marheineke K, Reiländer H & Hilgard P 1995 Selection and characterization of mammalian cell lines with stable over-expression of human pituitary receptors for gonadoliberin. *European Journal of Biochemistry* **231** 535–543.
- Bond CT, Hayflick JS, Seeburg JH & Adelman JP 1989 The rat gonadotropin-releasing hormone: SH locus: structure and hypothalamic expression. *Molecular Endocrinology* **3** 1257–1262.
- Bourguignon JP, Gerard A & Franchimont P 1990 Maturation of the hypothalamic control of pulsatile gonadotropin-releasing hormone secretion at onset of puberty. II. Reduced potency of an inhibitory autofeedback. *Endocrinology* **127** 2884–2890.
- DePaolo LV, King RA & Carrillo AJ 1987 *in vivo* and *in vitro* examination of an autoregulatory mechanism for luteinizing hormone-releasing hormone. *Endocrinology* **120** 272–279.
- Emons G, Ortmann O, Becker M, Irmer G, Springer B, Laun R, Holzel F, Schulz KD & Schally AV 1993a High affinity binding and direct antiproliferative effects of LHRH analogues in human ovarian cancer cell lines. *Cancer Research* **53** 5439–5446.
- Emons G, Schröder B, Ortmann O, Westphalen S, Schulz KD & Schally AV 1993b High affinity binding and direct antiproliferative effects of luteinizing hormone-releasing hormone analogs in human endometrial cancer cell lines. *Journal of Clinical Endocrinology and Metabolism* **77** 1458–1464.
- Emons G, Müller V, Ortmann O & Schulz KD 1998 Effects of LHRH analogues on mitogenic signal transduction in cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **65** 199–206.
- Feleder C, Jarry H, Leonhardt S, Moguilevsky JA & Wuttke W 1996 Evidence to suggest that gonadotropin-releasing hormone inhibits its own secretion by affecting hypothalamic amino acid neurotransmitter release. *Neuroendocrinology* **64** 298–304.
- Haisenleder DJ, Cox ME, Parsons SJ & Marshall JC 1998 Gonadotropin-releasing hormone pulses are required to maintain activation of mitogen-activated protein kinase: role in stimulation of gonadotrope gene expression. *Endocrinology* **139** 3104–3111.
- Han XB & Conn PM 1999 The role of protein kinases A and C pathways in the regulation of mitogen-activated protein kinase activation in response to gonadotropin-releasing hormone receptor activation. *Endocrinology* **140** 2241–2251.
- Harris N, Dutlow C, Eidne K, Dong KW, Roberts J & Millar R 1991 Gonadotropin-releasing hormone gene expression in MDA-MB-231 and ZR-75-1 breast carcinoma cell lines. *Cancer Research* **51** 2577–2581.
- Haviv F, Bush EN, Knittle J & Greer J 1998 LHRH antagonists. *Pharmaceutical Biotechnology* **11** 131–149.
- Ikeda M, Taga M, Sakakibara H, Minaguchi H, Ginsburg E & Vonderhaar BK 1996 Gene expression of gonadotropin-releasing hormone in early pregnant rat and steroid hormone exposed mouse uteri. *Journal of Endocrinological Investigation* **19** 708–713.
- Jaakkola T, Ding YQ, Kellokumpu-Lehtinen P, Valavaara R, Martikainen H & Tapanainen J 1990 The ratios of serum bioactive/immunoreactive luteinizing hormone and follicle-stimulating hormone in various clinical conditions with increased and decreased gonadotropin secretion: re-evaluation by a highly sensitive immunometric assay. *Journal of Clinical Endocrinology and Metabolism* **70** 1496–1505.
- Kaiser UB, Zhao D, Cardona GR & Chin WW 1992 Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. *Biochemical and Biophysical Research Communications* **189** 1645–1652.
- Kang SK, Choi KC, Cheng KW, Nathwani PS, Auersperg N & Leung PC 2000 Role of gonadotropin-releasing hormone as an autocrine growth factor in human ovarian surface epithelium. *Endocrinology* **141** 72–80.
- Krsmanovic LZ, Martinez-Fuentes AJ, Arora KK, Mores N, Tomic M, Stojilkovic SS & Catt KJ 2000 Local regulation of gonadotroph function by pituitary gonadotropin-releasing hormone. *Endocrinology* **141** 1187–1195.
- Kwekkeboom DJ, Lamberts SW, Blom JH, Schroeder FH & De Jong FH 1990 Prolonged treatment with the GnRH analogue buserelin does not affect alpha-subunit production by the pituitary gonadotroph. *Clinical Endocrinology* **32** 443–451.
- Lamharzi N, Schally AV & Koppan M 1998 Luteinizing hormone-releasing hormone (LH-RH) antagonist Cetrorelix inhibits growth of DU-145 human androgen-independent prostate carcinoma in nude mice and suppresses the levels and mRNA expression of IGF-II in tumors. *Regulatory Peptides* **77** 185–192.
- Lin X & Conn PM 1999 Transcriptional activation of gonadotropin-releasing hormone (GnRH) receptor gene by GnRH: involvement of multiple signal transduction pathways. *Endocrinology* **140** 358–364.
- Loumaye E, Naor Z & Catt KJ 1982 Binding affinity and biological activity of gonadotropin releasing hormone agonists in isolated pituitary cells. *Endocrinology* **111** 730–736.
- Merenthaler I, Setalo G, Petrusz P, Negro-Vilar A & Flerko B 1989 Identification of hypophysiotropic luteinizing hormone-releasing hormone (LHRH) neurons by combined retrograde labeling and immunocytochemistry. *Experimental and Clinical Endocrinology* **94** 133–140.
- Nathwani PS, Kang SK, Cheng KW, Choi KC & Leung PC 2000 Regulation of gonadotropin-releasing hormone and its receptor gene expression by 17 beta-estradiol in cultured human granulosa-luteal cells. *Endocrinology* **141** 1754–1763.
- Norwitz ER, Jeong KH & Chin WW 1999 Molecular mechanisms of gonadotropin-releasing hormone receptor gene regulation. *Journal of the Society for Gynecologic Investigation* **6** 169–178.
- Olofsson JI, Conti CC & Leung PC 1995 Homologous and heterologous regulation of gonadotropin-releasing hormone receptor gene expression in preovulatory rat granulosa cells. *Endocrinology* **136** 974–980.
- Oostdijk W, Drop SL, Odink RJ, Hummelink R, Partsch CJ & Sippell WG 1991 Long-term results with a slow-release gonadotrophin-releasing hormone agonist in central precocious puberty. Dutch-German Precocious Puberty Study Group. *Acta Paediatrica Scandinavica Supplement* **372** 39–45.
- Ortmann O & Diedrich K 1999 Pituitary and extrapituitary actions of gonadotrophin-releasing hormone and its analogues. *Human Reproduction* **14** 194–206.
- Peng C, Fan NC, Ligier M, Vaananen J & Leung PC 1994 Expression and regulation of gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells. *Endocrinology* **135** 1740–1746.
- Pinski J, Lamharzi N, Halmos G, Groot K, Jungwirth A, Vadillo-Buenfil M, Kakar SS & Schally AV 1996 Chronic administration of the luteinizing hormone-releasing hormone

- (LHRH) antagonist cetrorelix decreases gonadotrope responsiveness and pituitary LHRH receptor messenger ribonucleic acid levels in rats. *Endocrinology* **137** 3430–3436.
- Plosker GL & Brogden RN 1994 Leuprorelin – a review of its pharmacology and therapeutic use in prostatic cancer, endometriosis and other sex hormone-related disorders. *Drugs* **48** 930–967.
- Raga F, Casan EM, Kruessel JS, Wen Y, Huang HY, Nezhat C & Polan ML 1998 Quantitative gonadotropin-releasing hormone gene expression and immunohistochemical localization in human endometrium throughout the menstrual cycle. *Biology of Reproduction* **59** 661–669.
- Roth C, Leonhardt S, Theiling K, Lakomek M, Jarry H & Wuttke W 1998 Ontogeny of the GnRH-, glutaminase- and glutamate decarboxylase-gene expression in the hypothalamus of female rats. *Developmental Brain Research* **110** 105–114.
- Roth C, Leonhardt S, Seidel C, Luft H, Wuttke W & Jarry H 2000 Comparative analysis of different puberty inhibiting mechanisms of two GnRH agonists and the GnRH antagonist cetrorelix using a female rat model. *Pediatric Research* **48** 468–474.
- Sakamoto S, Tajima M, Sawaki K, Suzuki S, Kudo H, Sassa S, Kuwa K, Sugiura Y, Kasahara N & Nagasawa H 1993 Effects of luteinizing hormone-releasing hormone analogue on DNA synthesis in rat prostate and uterus. *In Vivo* **7** 13–16.
- Schally AV 1999a Luteinizing hormone-releasing hormone analogs: their impact on the control of tumorigenesis. *Peptides* **20** 1247–1262.
- Schally AV 1999b LH-RH analogues. I. Their impact on reproductive medicine. *Gynecological Endocrinology* **13** 401–409.
- Seong JY, Jarry H, Kuhnemuth S, Leonhardt S, Wuttke W & Kim K 1995 Effect of GABAergic compounds on gonadotropin-releasing hormone receptor gene expression in the rat. *Endocrinology* **136** 2587–2593.
- Srkalovic G, Wittliff JL & Schally AV 1990 Detection and partial characterization of receptors for [p-Trp6]-luteinizing hormone-releasing hormone and epidermal growth factor in human endometrial carcinoma. *Cancer Research* **50** 1841–1846.
- Stojilkovic SS, Reinhardt J & Catt KJ 1994 Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. *Endocrine Reviews* **15** 462–499.
- Tsutsumi M, Laws SC, Rodic V & Sealfon SC 1995 Translational regulation of the gonadotropin-releasing hormone receptor in alphaT3-1 cells. *Endocrinology* **136** 1128–1136.
- Turzillo AM & Nett TMJ 1999 Regulation of GnRH receptor gene expression in sheep and cattle. *Journal of Reproduction and Fertility (Suppl)* **54** 75–86.
- Uemura T, Yanagisawa T, Shirasu K, Matsuyama A & Minaguchi H 1992 Mechanisms involved in the pituitary desensitization induced by gonadotropin-releasing hormone agonists. *American Journal of Obstetrics and Gynecology* **167** 283–291.
- Vaananen JE, Tong BL, Vaananen CM, Chan IH, Yuen BH & Leung PC 1997 Interaction of prostaglandin F2 alpha and gonadotropin-releasing hormone on progesterone and estradiol production in human granulosa-luteal cells. *Biology of Reproduction* **57** 1346–1353.
- Wildt L, Marshall G & Knobil E 1980 Experimental induction of puberty in the infantile female rhesus monkey. *Science* **207** 1373–1375.
- Wilson ME & Handa RJ 1997 Ontogeny of gene expression in the gonadotroph of the developing female rat. *Biology of Reproduction* **56** 563–568.

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