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.


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 Ca2+ 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 (–Trp(6))-GnRH has only one substitution while the GnRH antagonist cetorelix acetate (–Ac–d–Na(2))1, –d–Phe(4 Cl)2, –d–Pal3, –d–Cit6, –d–Ala10)-GnRH differs from the native decapeptide in five positions (Haviv et al. 1998). In contrast to agonists, antagonistic GnRH analogs such as cetorelix 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 food 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 cetorelix (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 pituitary, 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 containing the optimal tracer was established. The antibody to the fraction 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 ovarietomized 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.
was possible. Extracted total RNA was measured with a UV spectrophotometer at A 260 nm and each sample was diluted with \( H_2O \) 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 SUPERSCRIPT 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 × reaction buffer, 10 mM dithiothreitol, 500 nM deoxy-NTPs, 200 U Superscript RNase H\(^-\) 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′ exorugene 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

### 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)**

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Amount of total RNA (ng)</th>
<th>Amount of mutant cRNA (fg)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GnRH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′ ACTATGGTCCACCAGCGGG</td>
<td>Pituitary: 150</td>
<td>Pituitary: 10</td>
<td>Pituitary: 31</td>
</tr>
<tr>
<td>3′ TGCCCAGCTTCCTCTCAAT</td>
<td>Ovary: 500</td>
<td>Ovary: 10</td>
<td>Ovary: 38</td>
</tr>
<tr>
<td>3′TCATCAGTGGTATGCTGAGAGT</td>
<td>Uterus: 500</td>
<td>Uterus: 10</td>
<td>Uterus: 50</td>
</tr>
<tr>
<td><strong>GnRH-receptor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′ TCAGTGGTATGCTGAGAGT</td>
<td>Pituitary: 150</td>
<td>Pituitary: 100</td>
<td>Pituitary: 24</td>
</tr>
<tr>
<td>3′ATATAAGTGAGGTCGAAGC</td>
<td>Ovary: 100</td>
<td>Ovary: 100</td>
<td>Ovary: 22(+5)</td>
</tr>
</tbody>
</table>

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

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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 (Rn). The ΔRn represents Rn minus the baseline signal established in the first 3–15 cycles of the PCR reaction. Reactions are characterized by their threshold cycle (Ct) values, Ct being the cycle at which the baseline signal is exceeded. The Ct 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 µl) contained 12·5 µl TaqMan buffer, 300–900 nM of each primer, 225 nM hybridization probe and 4 µl 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 Ct value. To calculate the relative expression levels of the different samples, the sample-Ct values were interpolated against the standard curve.

**GnRH**

Sense primer: 5’-GCAGAACCCCAAGAATTCCA-3’; anti-sense primer: 5’-TGCCACGCTTCTCTTTC

**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-TCTGCGAGGCTCTT-3’.

**GnRH-receptor**

Sense primer: 5’-AGGGATGATGAACAGGCAGC-3’; anti-sense primer: 5’-TCTCGCAATGTGTGACC-3’; TaqMan hybridization probe: 5’-FAM-TTCATGCCACCATTGCGGAAGCTG-TAMRA-3’.

**Statistical evaluation and mathematical calculations**

Unless otherwise stated, results are presented as means ± 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 \((P<0.001, \text{log rank test, Statistica, StatSoft, Tulsa, OK, USA})\). 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 \(\mu\text{g/day}\) treatment and significantly higher after both TRIP treatments (10 and 100 \(\mu\text{g/day}\)) as compared with controls. LH and in particular FSH release was stimulated more markedly by the low dose of 10 \(\mu\text{g/day}\) TRIP than by the high dose of 100 \(\mu\text{g/day}\) 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 \(\mu\text{g/day}\) (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, \((C_v \text{value } 30 \text{ cycles})\) 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 \(\mu\text{g/day}\) 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 \(\mu\text{g/day}\) but inhibited after CET 100 \(\mu\text{g/day}\) (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 \(\mu\text{g/day}\) and CET 100 \(\mu\text{g/day}\) (Fig. 4d). In contrast, the uterine expression of GnRH-receptor was strongly stimulated by treatment with CET (100 \(\mu\text{g/day}\)) and TRIP (100 \(\mu\text{g/day}\)); the increase induced by the antagonist CET (100 \(\mu\text{g/day}\)) was significantly higher compared with the agonist TRIP (100 \(\mu\text{g/day}\)) (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 \(\mu\text{g/day}\) group was measured while CET 100 \(\mu\text{g/day}\) 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 \(\mu\text{g/day}\) 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 vivo 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
The transition from prepubertal to pubertal rats (Roth et al. 1998). The differences between the in vitro and 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
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

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**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 10, §P<0.05 vs CET 10.
r 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 µg/day 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 (Lomaye et al. 1982, Tsutsumi et al. 1995, Haisenleder et al. 1998). On the other hand missing exposure to 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–receptor 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 rats 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...
bioinactivity 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 immunooassays 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). Analog of GnRH reverse the growth stimulatory effect of epidermal growth factor (EGF)-α and insulin-like growth factor and block the EGF-induced mitogen-activated protein kinase (MAPK) activity of ovarian and endometrial cancer cells (Srkalicov 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 promotor 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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