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Expression of the gene for the receptor of gonadotropin-releasing hormone in the rat mammary gland

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Abstract Recent findings have demonstrated that the GnRH gene is expressed in the mammary gland of pregnant and lactating rats but not of virgin rats. Indeed, significant concentrations of biologically active GnRH have been found in milk of human, cow, sheep and rat. We have, therefore, looked for expression of the GnRH receptor in the rat mammary gland. By reverse transcription (RT)-PCR amplification, we have demonstrated the presence of GnRH receptor mRNA in mammary gland samples derived from virgin, pregnant and lactating rats. The GnRH receptor transcript cloned from the mammary gland was sequenced and found to have an identical coding region to the one cloned from the pituitary gland. In addition, we have found that the mammary gland, as the pituitary gland, contains at least two transcripts having the same coding region but different 5′ non-coding regions. Binding studies, however, could demonstrate only low-affinity binding sites. These results, therefore, suggest that the regulation of the GnRH receptor occurs posttranscriptionally rather than at the level of transcription.

Key words: Mammary gland (rat); LHRH/GnRH receptor transcript

1. Introduction

Gonadotropin-releasing hormone (GnRH) is the primary regulator of the reproductive cycle. This neurohormone is synthesized in hypothalamic neurosecretory cells, released in a pulsatile pattern and triggers the secretion of the gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. However, continuous stimulation of the pituitary by either GnRH or its long-acting agonists results in desensitization of the gonadotropes and thereby in reduced gonadotropin secretion and suppression of gonadal activity.

Breast cancer is one of the most common cancers in women. GnRH agonists are being tested in the clinic as one of the means for the treatment of breast cancer. It is generally believed that the main therapeutical effect of the GnRH analogs is due to desensitization of the pituitary gland resulting from their continuous administration. However, the therapeutical effects of GnRH analogs on breast tumor regression in postmenopausal women cannot be explained on the basis of suppression of gonadal activity, since their serum gonadal steroid levels are already reduced [1,2]. Indeed, there are several studies describing direct effects of GnRH analogs on the growth of mammary tumor cells in culture [3–5].

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Recently [6], we have demonstrated that the GnRH gene is expressed in the mammary gland of the rat. The GnRH mRNA was present in the mammary gland of pregnant and lactating rats but not of virgin rats implying that expression of the GnRH gene is activated during pregnancy. These findings have complemented our initial findings [7,8] which demonstrated the existence of considerable concentrations of biologically active GnRH in milk of several species such as human, cow, sheep and rat. GnRH synthesized by the mammary gland may function as a paracrine/autocrine agent within the breast itself. Such an effect is dependent, however, on the existence of GnRH receptors in the mammary gland.

The cDNA encoding the pituitary GnRH receptor of several species has been recently cloned, sequenced and characterized [9–14]. These studies made it possible to investigate the GnRH receptor gene expression in the mammary gland. Although conventional binding studies are unable to detect high-affinity binding sites in the rat mammary gland, we have found that at least two different transcripts of GnRH receptor mRNA are expressed in this tissue.

2. Materials and methods

2.1. Animals

Randomly cycling Wistar-derived female rats (2–3 months old) from the departmental colony were used. Rats were housed under controlled conditions of temperature and light. Food and water were supplied ad libitum. Rats were decapitated, tissues were collected and were either used immediately or kept at −140°C until use.

2.2. RT-PCR analysis of GnRH receptor transcripts

Rat pituitaries and mammary glands were homogenized by a polytron and total RNA was extracted using the guanidinium isothiocyanate-CsCl method [15]. After DNAse treatment, 10 μg of the total RNA extracts were reverse-transcribed, using 0.5 μg oligo $(dT)_{15}$ as primer together with 200 U of Moloney-murine leukemia virus reverse transcriptase in a total volume of 50 μl, according to the manufacturer's specifications (USB, USA). 1 μl of the cDNA was amplified by PCR using specific primers to the previously published rat GnRH receptor cDNA sequence [11]. (Primer A) 5′-GACTCTTGAAGCCCGTCCTTGGAG-3′ and (Primer B) 5′-CGGAAAGCTGCAGTGGGTCACAC-3′ corresponding to nucleotides −28 to −5 (sense) and 584 to 606 (antisense), respectively. The predicted fragment size was 634 bp. PCR amplification was performed in a total volume of 50 μl using Vent polymerase. Amplification was carried out for 32 cycles; denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min. 10-μl aliquots of each PCR reaction were analysed by electrophoresis and identified by hybridization with a ^{32}P-labeled GnRH receptor cDNA probe. In parallel experiments, actin mRNA levels were assessed using specific actin primers [16], 5′-GAGACCTTCAACACCCCAGCC-3′ (sense) and 5′-GGCCATCTCTTGCTCGAAGTC-3′ (antisense).

For cloning the GnRH receptor cDNA from the rat mammary gland, Primer A was used together with Primer C (5′-ACAAGTGCTTGG-

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GTAGTCTCCC-3′, 987 to 1008; antisense) [11]. PCR amplification was then carried out for 35 cycles; denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and extension at 72°C for 1.5 min. These two primers spanned the entire coding region and yielded a fragment of 1036 bp, which was subsequently purified and subcloned into the *Sma*I site of the pBluescript KS. The sequence of the cDNA was determined by an automated DNA sequencer (Applied Biosystems, model 373A).

Two pituitary GnRH receptor cDNAs having different 5′ non-coding regions were cloned recently [12,13]. To look for different transcripts of the GnRH receptor in the mammary gland, we used two sense primers that are specific for the 5′ non-coding region of each type of the pituitary receptor cDNA. For Type I: [12] (primer D) 5′-GCCGGTTTCCAGCCGCAGTTT-3′ (−170 to −150; sense) and for Type II: [13] (primer E) 5′-GGCACGCTCGAACTCTTTGTAGG-3′ (−196 to −174; sense) together with primer B. PCR amplification was then carried out for 32 cycles; denaturation at 94°C for 45 s, annealing at 62°C for 45 s and extension at 72°C for 1 min. The predicted fragment sizes were 776 bp for Type I and 802 bp for Type II.

2.3. Southern blot analysis

The DNAs were electrophoretically transferred from 0.75% agarose gel onto Gene Screen Plus membrane (DuPont, USA) according to manufacturer's instructions. After transfer, the membrane was baked for 2 h at 80°C and prehybridized for 3 h at 42°C with a solution containing 2 × SSC, 1% SDS, 10% dextran sulfate, 50% formamide, 1 × Denhardt's and 100 μg/ml denatured salmon sperm DNA. The GnRH receptor cDNA probe was prepared by excision from the plasmid using *Eco*RI and was purified by electroelution. The cDNA was labeled by using a random-primed DNA-labeling kit (Boehringer Mannheim, Germany). The ^{32}P-radiolabeled cDNA probe was then added to the prehybridization solution and the membranes were hybridized for 18 h at 42°C. The membrane was washed as follows: (1) 2 × SSC, 20°C, 10 min; (2) 2 × SSC, 1% SDS, 42°C, 30 min; (3) 0.2 × SSC, 1% SDS, 42°C, 30 min. Each step was repeated twice. The membrane was then exposed for autoradiography (10–30 min at −70°C).

2.4. Binding assays

The procedures for the preparation of the radioiodinated ligand and the binding assay have been published [17]. In brief, pituitary (40 μg/protein) or mammary gland (140 μg/protein) membranes were incubated with 50,000–100,000 cpm of [^{125}I][DTrp^6]GnRH in a total volume of 0.5 ml (10 mM Tris-HCl containing 0.1% BSA) for 90 min at 4°C. The reaction was terminated by adding cold PBS and filtration under vacuum through Whatman GF/C filters. Pituitary glands were collected from proestrus rats whereas mammary glands were taken from proestrus, pregnant or lactating rats.

3. Results

3.1. GnRH receptor expression in rat mammary gland

RT-PCR analysis using GnRH receptor-specific primers (A and B, see section 2) yielded a fragment of 634 bp in mammary gland samples derived from pregnant and lactating rats (Fig. 1A, lanes 2 and 3). The primers encompassed the first exon–intron boundary which is located between nucleotides 522–523 [18]. A fragment of identical size was obtained in pituitary gland cDNA samples which were used as positive control (Fig. 1A, lane 4). Control reactions in which reverse transcriptase was omitted during the RT step did not yield any PCR product, ruling out the possibility of plasmid or genomic DNA contamination (Fig. 1A, lane 5). To examine whether the appearance of GnRH receptor transcripts coincides with that of GnRH [6], we used cDNA samples from virgin, 6 and 18 days pregnant, 6 and 7 days lactating rats. A fragment of similar size was detected in all the samples examined. The amplified fragment was identified as GnRH receptor cDNA, by direct sequencing of the PCR product, and by hybridization with a GnRH receptor cDNA probe (Fig. 1B).

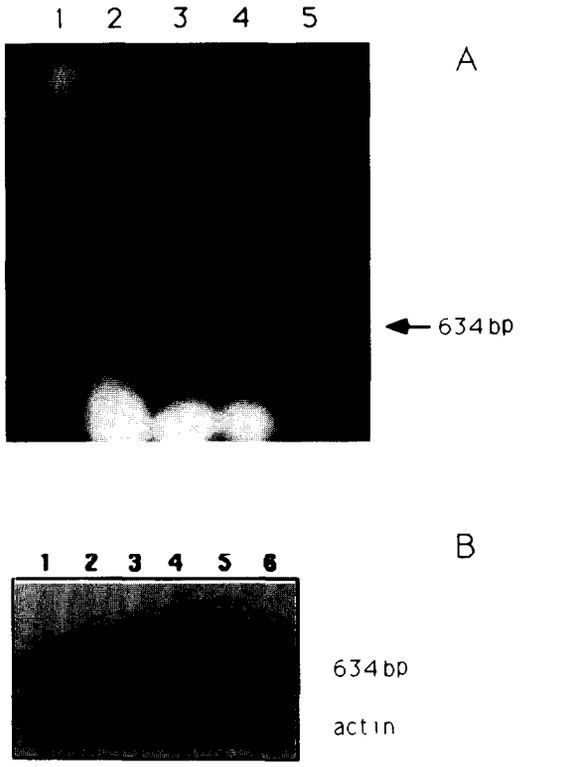

Fig. 1. GnRH receptor transcripts. Panel A: ethidium bromide staining of PCR products resolved on 1% agarose gel. For experimental details, see section 2. Lanes: (1) kb ladder; (2) mammary gland from 18-day pregnant rats; (3) mammary gland from 7-day lactating rats; (4) pituitary gland from virgin rats; (5) mammary gland control sample. All samples except the control, in which the reverse transcriptase was omitted (lane 5), yielded a fragment of 634 bp as predicted. Panel B: Southern blot analysis of GnRH receptor. PCR products from mammary gland and pituitary gland were hybridized to a ^{32}P-labeled rat GnRH receptor cDNA probe. Lanes: (1) mammary gland of virgin rats; (2) mammary gland of 6-day pregnant rats; (3) mammary gland of 18-day pregnant rats; (4) mammary gland of 6-day lactating rats; (5) mammary gland of 7-day lactating rats; (6) pituitary gland from virgin rats. The inset contains ethidium bromide staining of actin transcripts amplified from the same cDNA samples.

For complete sequence analysis, a fragment of 1036 bp spanning the entire coding region of the GnRH receptor (981 bp) was amplified from the mammary gland cDNA of virgin rats using primers (A and C). Sequence analysis showed (Fig. 2) that the coding region of the receptor cloned from the mammary gland, was identical with the one cloned from the pituitary gland [11].

3.2. GnRH-binding sites

The GnRH receptor cloned from the mammary gland and the one cloned from the pituitary gland had identical coding regions. However, specific high-affinity binding sites could be demonstrated in the pituitary gland, but not in the mammary gland. Displacement curves (Fig. 3) show that the mammary gland contains specific low-affinity binding sites for GnRH.

3.3. Different GnRH receptor transcripts in the mammary gland

The abovementioned findings suggested that the receptor from the mammary gland is regulated differently from that of the pituitary gland. Recently, a GnRH receptor cDNA having

a different 5′ non-coding region was cloned from a rat pituitary library. We have examined the expression pattern of the two types of GnRH receptor cDNA in the mammary gland. Since amplification of the coding region could not differentiate between the two types of cDNA, we used separate forward primers (D or E) specific for the 5′ non-coding region of each type and a common antisense primer (B). The PCR products were identified as GnRH receptor transcripts both by sequencing and by hybridization. Fig. 4 shows the hybridization of the RT-PCR products with the GnRH receptor cDNA. Type I [12] (Fig. 4A) and Type II [13] (Fig. 4B) transcripts were both expressed in the pituitary gland (lane 1) as well as in the mammary gland (lanes 2–5) cDNA samples.

4. Discussion

In this study, we have used the RT-PCR to amplify the levels of endogenous GnRH receptor mRNA that may be present in rat mammary gland. We have demonstrated the expression of mRNA for GnRH receptor in the breast of virgin, pregnant and lactating rat (Fig. 1A,B). Samples in which the reverse transcriptase was omitted have served as a control for genomic DNA contamination (Fig. 1A) and confirmed that the fragments that were amplified originated from RNA. Unlike the transcript for GnRH that was evident in mammary glands of pregnant and lactating but not of virgin rats [6], the transcript for the GnRH receptor was present in the mammary glands of all animal models that were examined including the virgin rat. The mRNA for the GnRH receptor has also been detected in the breast of human [19] but not in that of mouse [20], suggesting a species-specific regulation for this gene.

Although GnRH receptor transcript is present in the rat mammary gland we could not demonstrate specific high-affinity binding of GnRH analogs to mammary gland preparations. Only low-affinity binding could be demonstrated (Fig. 3). Parallel experiments in which pituitary gland preparations were used have demonstrated the expected high-affinity binding sites. These findings are in agreement with other reports that

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                                    gactcttgaagcccgtccttggagAAAT   -1
ATGGCTAACAATGCGTCTCTTGAGCAGGACCAAAATCACTGCTCAGCCAT              50
CAACAACAGCATCCCCCTGACACAGGGCAAGCTCCCGACTCTAACCTTAT             100
CTGGAAAGATCCGAGTGACGGTGACTTTCTTCCTTTTCCTACTCTCTACT             150
GCCTTCAATGCCTCTTTCTTGGTAAAGCTGCAGAGGTGGACCCAGAAGAG            200
GAAGAAAGGAAAAAAGCTCTCAAGGATGAAGGTGCTTTTAAAGCATTTGA            250
CCTTAGCCAACCTCCTTGAGACTCTAATCGTCATGCCGCTGGATGGGATG            300
TGGAACATCACTGTTCAGTGGTATGCTGGAGAGTTCCTTTGCAAAGTTCT            350
CAGCTATCTGAAGCTCTTCTCTATGTATGCCCCAGCCTTCATGATGGTGG            400
TGATTAGCCTGGATCGCTCCCTGGCCGTCACTCAGCCCTTAGCTGTCCAA            450
AGCAAGAGCAAGCTTGAACGGTCTATGACCAGCCTGGCCTGGATTCTCAG            500
CATTGTCTTTGCGGGACCACAGTTATATATCTTCAGGATGATCTACCTAG            550
CCGACGGCTCTGGGCCAGCAGTTTTCTCGCAATGTGTGACCCACTGCAGC            600
TTTCCGCAATGGTGGCATGAAGCCTTCTACAACTTTTTCACCTTCAGCTG            650
CCTGTTCATCATCCCTCTTCTCATCATGCTAATCTGCAATGCCAAAATCA            700
TCTTCGCCCTCACACGAGTCCTTCATCAGGACCCACGCAAACTACAGCTG            750
AATCAATCCAAGAATAATATCCCAAGAGCACGGCTGAGAACTCTAAAGAT            800
GACAGTGGCATTTGCCACCTCCTTTGTCATCTGCTGGACTCCCTACTACG            850
TCCTAGGAATCTGGTACTGGTTTGATCCGGAAATGTTAAACAGGGTGTCA            900
GAGCCAGTCAATCACTTCTTCTTTCTCTTTGCTTTTCTAAACCCGTGCTT            950
CGACCCACTTATATATGGGTATTTCTCTTTGTAATTgggagactacccaa           1000
gcacttgt                                                     1008
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Fig. 2. Nucleotide sequence of the rat mammary gland GnRH receptor. The primers used for the amplification of the cDNA span the entire coding region and are shown in lower case letters.

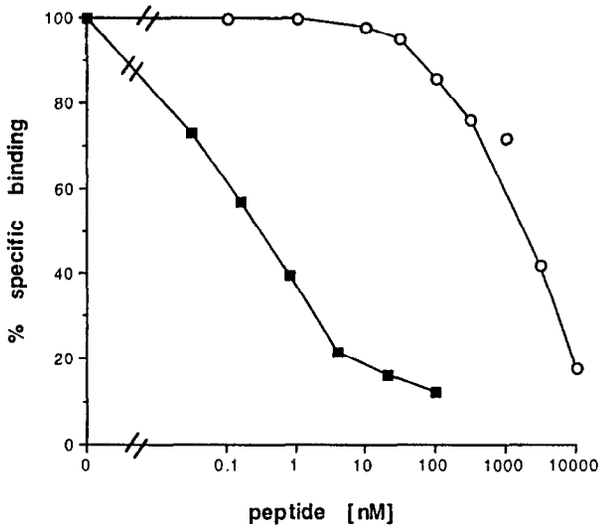

Fig. 3. Displacement of [^{125}I][DTrp^{6}]GnRH binding to rat pituitary (black squares) or mammary gland (open circles) membranes by the unlabeled GnRH analog. Each point represents the mean of triplicate determinations of a representative experiment. Pituitary gland of proestrus rats and mammary gland of 8-day lactating rats were used in this experiment. Maximal binding to the pituitary gland and the mammary gland membrane preparations was 40% and 13%, respectively.

failed to demonstrate high-affinity binding of GnRH to non-carcinogenic mammary tissue [21,22]. It was suggested that this discrepancy might be due to activation of some genes as a result of carcinogenic transformation. However, our results suggest that the regulation of GnRH receptor occurs posttranscriptionally rather than at the level of transcription.

Recently, the extrapituitary GnRH receptor cDNA has been cloned from a human breast cancer cell line MCF-7 and from rat gonads [19,23]. In both reports, the extrapituitary GnRH receptor transcripts were found to have nucleotide sequence identical with that of the pituitary gland. Although GnRH analogs have been demonstrated to exhibit direct effects on growth rate of MCF-7 cells [3,4,24], the affinity and even the detection of specific binding sites for GnRH in this cell line is still a matter of controversy [21,22,24].

Heterogeneity of receptors has been recognized to be an efficient way for obtaining different biological responses to a single hormone stimulation. Different isoforms of rat TRH receptors produced by alternative splicing of the receptor RNA [25] have recently been isolated. To examine whether a similar event occurs with the GnRH receptor, we amplified the entire coding region and analysed its sequence. The results demonstrate that the sequence of the GnRH receptor cloned from the rat mammary gland is identical with that of the pituitary gland (Fig. 2).

It is also possible that a certain protein-coding sequence is linked to different 5′ non-coding sequences, as demonstrated for the M_2 muscarinic receptor [26]. In such events, the differential regulation of transcription is achieved by tissue-specific promoters, response elements or by transcription factors. In fact, different 5′ non-coding regions of the rat pituitary GnRH receptor cDNA have been demonstrated [12,13]. Therefore, it was of interest to investigate whether transcripts with different 5′ non-coding regions are also present in the mammary gland, or that there is some tissue specificity. For this purpose, we

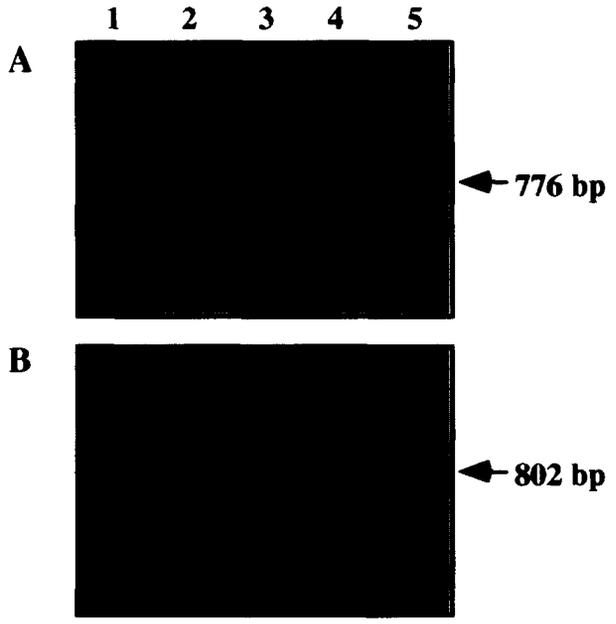

Fig. 4. Hybridization of the different transcripts with the GnRH receptor cDNA. RT-PCR products which were amplified using sense primers (D and E) specific to the 5′ non-coding region of each transcript and a common antisense primer (B), were hybridized with a ^{32}P-labeled pituitary GnRH receptor cDNA probe. Hybridization was carried out with the Type I transcript (panel A) and with the Type II transcript (panel B). For further details, see section 2. Lanes: (1) pituitary gland; (2) mammary gland from virgin rats; (3) mammary gland from 6-day pregnant rats; (4) mammary gland from 18-day pregnant rats; (5) mammary gland from 7-day lactating rats. The sizes of the hybridization products were 776 bp for Type I and 802 bp for Type II.

have used specific primers for the 5′ non-coding regions of the two types of the GnRH receptor transcripts. Amplification of pituitary and mammary gland cDNAs with these primers has shown that the two types of GnRH receptor transcripts are present in both tissues (Fig. 4A,B), thus, excluding the possibility of tissue-specific regulation for these two transcripts. Although the methodology used in these experiments was not quantitative, there were no striking differences in the pattern of the transcripts in relation to the physiological status of the animal. The relative amount of the two transcripts did not differ in virgin, pregnant and lactating rats, suggesting that the regulation of these two transcripts in the mammary gland is similar. In these transcripts, the sequence difference starts at −32 and goes upstream whereas all the downstream nucleotides including the 3′ non-coding region are identical.

Some members of the G protein-coupled receptor family have been demonstrated to be encoded by more than one gene. The different subtypes of somatostatin receptor, for example, have distinct tissue distribution and are encoded by different genes [27]. Some other members of this family such as the D_2 dopamine receptor [28] are products of alternative splicing which again generates transcripts with differences in the amino acid sequence. In the M_2 muscarinic receptor subtype, similar to the GnRH receptor, an identical protein-coding region is preceded by different regulatory sequences [26]. However, unlike the GnRH receptor, each transcript generated by alternative splicing, is present in a different tissue, indicating a tissue-specific regulation. The functional importance of two different transcripts encoding the same protein in the same tissue is not known. A recent study has suggested that the downregulation of the GnRH receptor in the αT_3-1 gonadotrope cell line, is accompanied by a decreased efficiency of GnRH receptor mRNA translation, without any change in GnRH receptor mRNA levels [29].

Differential posttranslational modifications of the GnRH receptor in the pituitary gland, in the breast as well as in other tissues such as the brain, may alter the conformation of the receptor leading to changes in ligand affinity and/or specificity. Thus, although GnRH administered directly into the mid-brain central grey induced mating behavior [30,31], there are no reports demonstrating the existence of high-affinity binding sites in this brain region. Moreover, sexual behavior can be induced not only by GnRH but also by some GnRH fragments [31] or analogs [30] that are devoid of any agonistic activity at the pituitary level. Similarly, it has been demonstrated [32] that the affinity of [D-Trp^6]GnRH to human breast cancer membranes was at least 10-fold higher than that of buserelin. The difference in the affinity of these two GnRH analogs is surprising in view of their activity at the anterior pituitary level. It seems, therefore, that the currently available GnRH analogs have been designed and selected so as to achieve maximal bioactivity at the pituitary gland. GnRH receptors may be present in other organs but due to different receptor conformation resulting from distinct posttranslational modifications, may exhibit intrinsic binding characteristics.

A considerable amount of GnRH is synthesized by the breast [6]. GnRH produced by the mammary gland may act locally in a paracrine and/or an autocrine way. Locally produced hormones may reach concentrations that will be sufficient for binding and activation of their receptor. It is known, for example, that the affinity of various ligands to the muscarinic cholinergic receptor is in the μM range [33,34]. Therefore, it is possible that adequate concentrations of GnRH are present in the breast tissue to activate low-affinity binding sites. The finding that there were no apparent differences in the expression of the GnRH receptor mRNA in the mammary gland of intact, pregnant or lactating rats imply that activation of the breast receptor is regulated by the availability of GnRH. This hormone is produced during lactation but not in the virgin cycling female rat. During the last decade, however, analogs of GnRH are being widely used for various purposes and indications such as for treatment in programs of in vitro fertilization, for endometriosis, precocious puberty, etc. Since GnRH receptors are present in the breast of these patients, it is possible that they will be activated by administration of the exogenous GnRH analogs. The consequences of such an unexpected stimulation are still unknown.

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