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Anterior Pituitary Gene Expression with Reproductive Aging in the Female Rat[1]

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ABSTRACT

Although reproductive aging in women is classically attributed to loss of ovarian follicles, recent data have suggested that the entire hypothalamic-pituitary-ovarian axis undergoes functional changes with time. The aim of this study was to characterize age-related changes in pituitary gene expression for factors with known importance for gonadotroph function, including 1) steroid hormone receptors (*Esr* and *Pgr*), 2) orphan nuclear receptors [*Nr5a1* (steroidogenic factor-1) and *Nr5a2* (liver receptor homologue-1)], and 3) pituitary-derived polypeptides (activin, inhibin, and follistatin), as well as 4) gonadotropin subunits and 5) GnRH receptors. We chose to utilize a middle-aged rat model for these studies. Young (Y; 3-mo-old) and middle-aged (MA; 9- to 12-mo-old) rats were ovariectomized, primed with estradiol, and injected with progesterone to induce an LH surge. The mRNA levels for the gonadotropin subunits and GnRH receptors were decreased in middle-aged females relative to young animals. *Nr5a1* and follistatin mRNA levels were significantly greater in Y versus MA animals following ovariectomy. Furthermore, steroid-induced regulation of these genes was lost in the MA animals. Regulation of the *Nr5a2*, *Inhba*, and *Inhbb* transcripts was also limited to the young animals. In contrast, there were no significant differences in the mRNA levels of *Esr* or *Pgr* family members between age groups at any time point. Although this in vivo model normalizes ovarian steroid levels, it does not control for potential differences in GnRH stimulation with aging. Therefore, in a second set of experiments, we used an in vitro perifusion system to compare the effects of pulsatile GnRH in the two age groups. *Nr5a1* mRNA expression was greater in Y than MA animals and was significantly decreased by GnRH pulses in both age groups. Follistatin mRNA levels increased significantly with GnRH treatment in Y animals but were not significantly changed in the MA females. Taken together, these data demonstrate gene-specific blunting of pituitary gene expression postovariectomy and during the steroid-induced surge in middle-aged rats. We propose that age-related changes in pituitary physiology may contribute to reproductive senescence.

aging, follistatin, gene regulation, pituitary, steroid hormone receptors

INTRODUCTION

Reproductive aging in females is associated with the progressive decline and ultimate cessation of ovarian function. Accumulating evidence from both women and animal models suggests that this decline may be partially due to changes at the hypothalamic-pituitary level of the reproductive axis in addition to the better appreciated intrinsic failure of the ovary [1–3]. Prior to obvious changes in the reproductive cycle, middle-aged (MA) rats (generally defined as 8–14 mo of age) demonstrate delayed and attenuated luteinizing hormone (LH) surges relative to young (Y) females on proestrus and following ovariectomy and steroid priming [4, 5]. Aging females also demonstrate a blunted and delayed postovariectomy rise in gonadotropin levels [6]. This abnormal response persists in animals downregulated with continuous GnRH prior to ovariectomy, suggesting primary neuroendocrine dysfunction [7]. Studies have also demonstrated that the ability of estrogen to modulate LH release in ovariectomized (OVX) rats is attenuated in these MA animals [8, 9]. In addition to secretory changes, middle-aged animals also demonstrate impaired gonadotropin biosynthesis. *Lhb* mRNA levels are decreased at noon on proestrus in middle-aged rats with an attenuated preovulatory surge [10]. Similarly, persistent estrus rats express decreased *Lhb* mRNA levels and demonstrate a blunted increase in all three gonadotropin subunits following ovariectomy [6]. Of note, it is currently not known whether these changes in gonadotroph secretion reflect altered hypothalamic input or are additionally due to primary dysfunction of the pituitary gonadotrophs.

A limited number of studies have suggested that intrinsic abnormalities in gonadotroph physiology occur with aging that appear to be independent of changes in hypothalamic input. In a study of older postmenopausal women, Rossmanith and colleagues observed blunted GnRH-induced increases in LH and FSH release [11]. The aging rat also secretes less LH in response to acute GnRH stimulation in both in vivo and in vitro models [12–14].

The maintenance of normal reproductive function is dependent upon precise temporal and quantitative interactions between the hypothalamus, anterior pituitary gland, and ovaries. The hypothalamus secretes pulsatile GnRH into the hypophyseal portal system to regulate the biosynthesis and secretion of the gonadotropins, LH, and FSH. Circulating gonadotropins act on the ovary to stimulate oocyte maturation as well as the biosynthesis and secretion of the primary ovarian steroids, estrogen and progesterone. The ovary also secretes the so-called "ovarian" peptides, activin, inhibin, and follistatin. These hormones act through positive and negative feedback loops to alter both hypothalamic and pituitary function [15, 16]. Thus, the anterior pituitary gland integrates signals from multiple tissues and possesses a central position within the reproductive axis.

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The ovarian steroids estrogen and progesterone exert genomic effects through interaction with their respective nuclear receptors. Three estrogen receptor (*Esr*) isoforms, *Esr1*, *Esr2*, and the dominant negative estrogen receptor *Terp1* (truncated estrogen receptor product-1), are expressed widely in the rat anterior pituitary [17, 18]. The mRNAs for the two progesterone receptor (*Pgr*) isoforms, *PgrA* and *PgrB*, have also been identified in the anterior pituitary gland, with abundant expression in the gonadotrophs and somatotrophs [19, 20].

Steroidogenic factor 1 (SF-1; *Nr5a1*) and liver receptor homologue-1 (LRH-1; *Nr5a2*) are two closely related nuclear receptors that evolved from the duplication of an ancestral gene, *Drosophila fushi tarazu* factor [21]. Originally considered to be orphan receptors, it has recently been reported that phospholipids, including phosphatidyl inositol, may act as ligands for these receptors [22]. NR5A1 is a key regulator of endocrine development and function and is believed to exert its effects alone or in conjunction with other transcription factors at multiple levels of the reproductive axis [23]. Pituitary-specific *Nr5a1* knockout mice show impaired expression of *Lhb*, *Fshb*, *Gnrhr*, and the common α-subunit of glycoprotein hormones (*Cga*) [24]. Within the anterior pituitary gland, *Nr5a1* expression is limited to the gonadotroph subpopulation, consistent with its critical role in the transcriptional regulation of the gonadotropin genes [25–27]. *Nr5a2* mRNA and protein have been identified in the anterior pituitary gland. Our laboratory has demonstrated that NR5A2 upregulates gonadotropin gene expression, acting via the previously described NR5A1 DNA-regulatory regions [28]. Furthermore, we have preliminary data that LRH-1 gene expression is stimulated by GnRH (data not shown). Although existing data is limited, these new results strongly support a role for NR5A2 in gonadotroph function.

Three polypeptide factors—activin, inhibin, and follistatin—were originally isolated from the gonads, but have subsequently been detected in the anterior pituitary gland, where they are believed to play an autocrine/paracrine role in the control of gonadotropin gene expression [29–32]. Inhibin is composed of an α-subunit linked to one of two β-subunits (βA and βB), whereas activin consists of β-subunit homodimers. Activin increases and inhibin decreases *Fshb* mRNA levels and secretion both in vivo and in vitro [33, 34]. Follistatin has been localized to all pituitary cell types, with high expression in the gonadotroph and folliculostellate subpopulation. Structurally unrelated to the inhibin/activin subunits, follistatin is believed to act via binding and subsequent inhibition of activin action [35].

For the studies reported here, we hypothesized that age-related changes in pituitary function contribute to loss of reproductive cyclicity and are not simply secondary to ovarian or hypothalamic deficits. Therefore, we chose to examine middle-aged female rats that exhibited regular estrous cycles at the time of ovariectomy (in vitro studies) or that were ovariectomized within 30 days after the cessation of estrous cyclicity (in vivo studies). For the in vivo experiments, we compared pituitary mRNA levels in young and middle-aged female rats both post-OVX and on the day of a steroid-induced LH surge. In a second set of experiments, we utilized a primary pituitary cell perifusion system to remove potential differences in GnRH and pituitary-derived factors between the two age groups. We then analyzed gene expression following treatment with pulsatile GnRH.

We chose to analyze receptors and peptide factors which are known to be critical mediators of gonadotroph physiology. Specifically, we expected to confirm previous reports that middle-aged animals experience a delayed and attenuated gonadotropin surge with a correlated decrease in *Cga*, *Lhb*, and *Fshb* mRNA levels as was reported on proestrus [5, 10]. We also predicted that *Gnrhr* levels would be decreased in the older animals relative to young animals based on two reports that middle-aged cycling female rats have a blunted response to GnRH in perifusion [13, 36]. The steroid receptors ESR and PGR are believed to be regulated by estradiol levels [37–39]. Therefore, we hypothesized that expression of these steroid receptors would be unchanged across age groups in animals postovariectomy or with equivalent steroid administration. As NR5A1 and NR5A2 stimulate gonadotropin expression, we predicted that mRNA levels of these orphan receptors would be decreased in the MA animals in which gonadotropin gene expression is blunted [25–27]. Along this same line of reasoning, we postulated that the stimulatory factor activin, derived from the inhibin-activin β-subunits, would be decreased, while follistatin and the unique inhibin subunit, inhibin α-, would be increased in the middle aged animals. Our results demonstrate specific deficits in anterior pituitary gene expression in middle-aged rats and suggest that intrinsic changes in pituitary function may contribute to the cascade of events that lead to reproductive decline in the female.

MATERIALS AND METHODS

Animals and Pituitary Tissue Collection

Female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (CRBL, Wilmington, MA) at 57–70 days of age. Middle-aged females for the in vitro studies were purchased as retired breeders at 10 mo. Animals were housed in the Tufts University and University of Texas Southwestern Medical Center Animal Resource centers on a 14L:10D light cycle with lights on at 0400 h and off at 1800 h. Food and water were available ad libitum. Vaginal smears were checked daily. Prior to ovariectomy (OVX), young virgin females (3 mo of age) and those middle-aged females used for the in vitro studies had exhibited 4- to 5-day estrous cycles. Middle-aged females that provided pituitaries for RNA extraction were OVX within 30 days of transitioning from estrous cyclicity to constant estrus (10–12 mo of age). Three weeks after OVX, rats treated with steroids received an injection of estradiol benzoate (EB, 4 μg/100 g body weight) at 1000 h (Day 21) followed by an injection of progesterone (P, 0.8 mg/100 g body weight) 48 h later (Day 23). This steroid priming regimen induces a robust LH surge in OVX female rats with peak LH levels at approximately 1600 h [40], as observed in Figure 1. Pituitary samples were harvested on the day of the steroid-induced LH surge (Day 23) at 1000 h, 1400 h, 1800 h, and 2000 h. Samples at 1000 h were collected prior to progesterone injection. Samples from rats without steroid priming were also collected at 1000 h. Animals were decapitated following a brief exposure to metofane anesthesia (Pitman Moore, Mudelein, IL) and the pituitaries were rapidly removed. Each pituitary was snap-frozen in methylbutane on dry ice and stored at −80°C. Trunk blood was collected for determination of LH levels. The in vivo data represent the results obtained from 3 to 7 animals/age group/time point. All animal procedures were performed in accordance with guidelines established by the University Animal Care and Use Committee at each institution and with the International Guiding Principles for Biomedical Research Involving Animals.

Plasma LH Levels

Plasma LH levels were measured by radioimmunoassay using materials provided by the NIDDK. The LH standard used was rat RP3 (lot No. AFP-7187B) and the antibody was antirat LH-S-11 antisera (lot No. AFP-C697071P). All plasma samples were run in a single assay. The intraassay variability, as determined from a series of pools dispersed throughout the assay, was 4.6%.

In Vitro Primary Pituitary Cell Perifusion

The anterior pituitaries were collected and dispersed by enzyme digestion as described by Wilfinger and colleagues with minor modifications [41]. Briefly, anterior pituitary glands were washed twice in Hanks balanced salts (HBSS) supplemented with 0.1% fetal bovine serum (FBS) and 15 mM hepes. The gland was cut into small blocks and suspended in 10 ml of dissociation medium

consisting of HBSS + 0.3% BSA + 0.3% trypsin (Sigma) + 2 μg/ml DNase and 15 mM hepes and 1.2 mM EDTA. The tissue and medium mixture was incubated in water bath at 30°C with shaking (~35 rev/min) for 20 min. The tissue blocks were gently triturated approximately 20 times with a disposable Pasteur pipette. The cell suspension was transferred to a new conical tube containing an equal volume of Dulbecco modified eagle medium (DMEM) with high glucose and 10% FBS, 23 mM hepes, 1 mM sodium pyruvate, and 1× penicillin/streptomycin (DMEM culture medium). The undissociated tissue was allowed to settle to the bottom of the tube, followed by repeat trituration as described above. The gland was fully dispersed after three dissociation periods. The individual cell suspensions obtained from each dissociation were centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cells were resuspended in DMEM culture medium.

The dispersed primary cells were counted and plated on coverslips (13 mm in diameter at a seeding density of 4×10^5 cells/cm^2). The cells were cultured overnight to allow them to attach to the coverslip. The coverslips were placed in the perifusion chambers (Minucells and Minutissue Vertriebs GmbH) and perifused at 9 ml/h with phenol red-free DMEM medium containing 10% charcoal-treated FBS for 24 h. This initial perifusion step, which achieves washout every 30 min, was designed to remove age-related differences in hormonal environment experienced by the cells when in vivo as well as to remove the effects of locally produced factors. Cells were stimulated with vehicle or 10 nM GnRH in DMEM culture medium for 10 pulses (3 min/pulse every 30 min) at the same perifusion rate and then subjected to total RNA isolation.

Isolation and Purification of Pituitary Total RNA

Total RNA was isolated from individual pituitaries and perifused primary pituitary cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA). RNA concentration was determined at 260 nm OD (SmartSpec 3000, BIO-RAD, Hercules, CA) and its integrity was confirmed by agarose gel eletrophoresis. To eliminate any contaminating DNA, DNA-free DNase Treatment & Removal Reagents (Ambion Inc., Austin, TX) were used to treat the RNA samples.

RNase Protection Protocol

Aliquots of 2 μg RNA/rat pituitary gland were used to measure mRNA levels of *Lhb* and *Cga*. Aliquots of 3 μg RNA/rat pituitary gland were used to assess *Fshb* mRNA levels and aliquots of 5 μg RNA/rat pituitary gland were used to assess *Gnrhr* mRNA levels. RNA samples were precipitated with NaCl, ethanol, and 20 μg tRNA, centrifuged and washed with 70% ethanol; the pellets were resuspended in 20 μl of hybridization solution (0.1 M EDTA, pH 8, and 4 M guanidine thiocyanate, pH 7.5).

Probe and Reference RNA Preparation

Five DNA subclones were used as templates for reference and probe RNAs. First was a PGEMT-T plasmid containing a 280-bp fragment of rat *Gnrhr* cDNA. This cDNA fragment was obtained by RT-PCR using two oligonucleotides located in exon 1 of the *Gnrhr* cDNA sequence. Second was a pBluescript SK (+/–) plasmid containing a 426-bp EcoRV-EcoRI fragment from the noncoding region of exon 3 of the rat *Fshb* cDNA sequence. This cDNA fragment was subcloned from ~1 Kb genomic HindIII-EcoRI clone, generously provided by Dr. W. Chin. Third was a pBluescript SK (+/–) plasmid containing a 223-bp fragment of rat *Lhb* cDNA spanning from PstI-ApaI restriction sites, generously provided by Dr. M. Jakubowski. Fourth was a pBluescript SK (+/–) plasmid containing a 505-bp EcoRI-HindIII fragment of rat *Cga* cDNA, generously provided by Dr. M. Jakubowski. Fifth was a linearized cyclophilin (*Ppia*) cDNA 103-bp clone spanning exons 1 and 2, which was purchased from Ambion, Inc. (Austin, TX).

Probes and reference RNA were transcribed in vitro from linearized template DNA using bacteriophage T7 or T3 RNA polymerase (39), and transcription was terminated by digesting the template DNA with RNase-free DNase I (Worthington, Freehold, NJ). Probe RNAs were purified by ethanol precipitation in the presence of 2.5 M ammonium acetate. Reference RNAs were phenol-chloroform extracted and purified through two sequential ethanol precipitations in the presence of 2.5 M ammonium acetate. The purified reference RNAs were quantified spectrophotometrically using the A_{260} reading, aliquoted, and stored at −80°C.

Solution Hybridization/RNase Protection Assay

RNA samples from each pituitary were incubated simultaneously with one of four probes: 1) 0.2-ng GnRH receptor complementary RNA probe labeled with [^{32}αP] uridine triphosphate (UTP) to high-specific activity (1600 cpm/pg) and 0.3 ng cyclophilin probe also labeled to high-specific activity (1500 cpm/pg), or 2) 1.5 ng *Lhb* complementary RNA probe labeled with [^{32}αP] uridine triphosphate (UTP) to low-specific activity (520 cpm/pg) and 0.3 ng cyclophilin probe labeled to high-specific activity (1500 cpm/pg), or 3) 1.5 ng *Fshb* complementary RNA probe labeled with [^{32}αP] uridine triphosphate (UTP) to low-specific activity (477 cpm/pg) and 0.3 ng cyclophilin probe labeled to high-specific activity (1300 cpm/pg), or 4) 3 ng *Cga* complementary RNA probe labeled with [^{32}αP] uridine triphosphate (UTP) to low-specific activity (252 cpm/pg) and 0.3 ng cyclophilin probe labeled to high-specific activity (1200 cpm/pg).

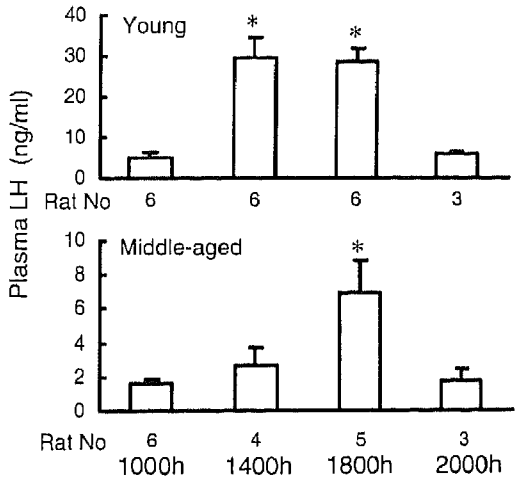

FIG. 1. Mean plasma LH levels in OVX, steroid-primed young and middle-aged rats on the day of an LH surge. The number below each bar indicates the number of rats used in each group.

The probes were incubated with RNA samples in a final volume of 25 μl hybridization solution for 16–18 h at 30°C. After hybridization, the samples were treated with 300 μl of RNase solution (10 mM Tris-HCl, pH 8, 300 mM NaCl, 40 μg/ml RNase A, 1 μg/ml RNase T1) for 1 h at 30°C, followed by proteinase K digestion (140 μg/ml) for 15 min at 45°C. Samples were extracted with 1 volume of phenol-chloroform (1:1), and the RNA was precipitated with ethanol, dried, dissolved in 7 μl gel-loading buffer (25% Ficoll, 0.2 M EDTA pH 8, 0.25% bromophenol blue, 0.25% xylene cyanol), and electrophoresed through a 5% nondenaturing polyacrylamide gel. Gels were mounted on 3M Whatman paper and dried. Protected bands were visualized by autoradiography (Fig. 2), and the density was analyzed by phosphoimaging technology and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) as described previously [39]. RNase protection data were expressed as phosphoimager units of the mRNA of interest (*Gnrhr*, *Lhb*, *Fshb*, or *Cga*) per phosphoimager unit of cyclophilin.

Reverse Transcription of cDNA

Two micrograms of purified total RNA was incubated with 1 μg of random hexamers in H_2O at 70°C for 10 min, 4°C for 2.5 min, and 25°C for 2 min. The remaining reaction components were added for a total volume of 40 μl containing 5 mM $MgCl_2$, 1X PCR buffer B (Promega, Madison, WI), 1 mM deoxyribonucleotides, 80 U RNase OUT (Invitrogen Life Technologies, Carlsbad, CA), and 20 U AMV reverse transcriptase (Promega, Madison, WI). Samples were incubated at 25°C for 10 min, 37°C for 60 min, and 95°C for 5 min, and stored at −20°C for use in PCR reactions. AMV reverse transcriptase was replaced with H_2O to generate negative control samples.

PCR Reactions

Semiquantitative PCR and real-time PCR were used. *Fst* transcripts were analyzed by both approaches, yielding similar results. Primer sequences and the expected product size for the semi-quantitative PCR reactions are listed in Table 1. Two pairs of primers were used to detect *PgrA+B* and *PgrB*. The first pair amplifies a 326-bp fragment from the ligand-binding domain common to the A and B isoforms, and the second pair amplifies a 221-bp sequence from the 5′ untranslated region of the rat *Pgr* cDNA, unique to the B isoform [20]. *Tubal* (α-tubulin) was used as a reference gene in the same PCR reaction for all products. PCR conditions were optimized for each pair of genes by adjusting

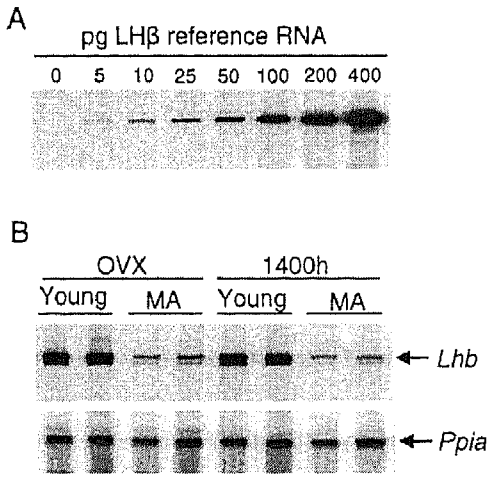

FIG. 2. Validation of RNase protection assays of *Lhb* mRNA expression. A) Different amounts of *Lhb* reference RNA were used to confirm the sensitivity of the assays. B) Two RNA samples from Y and MA rats at OVX and 1400 h, respectively, were measured for *Lhb* expression. *Ppia* (cyclophilin) expression was measured as the reference gene.

the Mg^{2+} concentration and the annealing temperature at 35 cycles. The PCR reactions were conducted in 50 μl volume containing 2.0–3.0 mM $MgCl_2$, 1× PCR buffer (Promega, Madison, WI), 200 μM dNTP, 400 nM of each primer, 12.5 to 100 ng cDNA, and 2 U Taq DNA polymerase (Promega, Madison, WI) in an Icycler (Biorad, Hercules, CA). PCR products were separated on 1.2% agarose gels containing ethidium bromide (1 μg/ml) in TAE buffer. Gels were photographed using a Kodak digital camera (DC 290), and the net density of signals was evaluated by Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY). The linear range of amplification was determined for each gene pair by changing the amount of cDNA input (6.25–50 ng for *Esr1*, *Pgr*, and *Terp1*, and 50–300 ng for *Esr2*) and the number of PCR cycles (20–40 cycles) (Fig. 3, Table 1, and data not shown). The amount of each target gene product was normalized to that of *Tuba1*. The constancy of *Tuba1* expression was validated under the experimental conditions used for this report. The average from the two cDNA inputs was used as the measurement for each pituitary sample.

The primers for real-time PCR were designed using the Primer Express Software (Applied Biosystems, Foster City, CA) (Table 2). Each real-time PCR reaction contained 25 ng of reverse-transcribed cDNA, 400 nM forward and reverse primers, and 1× SYBR Green PCR master mix in a final volume of 30 μl. PCR reactions were carried out in 96-well plates by using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). All reactions were performed in triplicate. For each target, validation experiments were conducted to confirm that the amplification efficiencies of the target and reference (*Tuba1*) genes were approximately equal, that the absolute values of the slope of log cDNA input vs. ΔC_T were all smaller than 0.1, and that there was only a single peak in the dissociation curve indicating generation of a single product. The comparative C_T method was utilized for mRNA quantification.

Statistical analysis

All results are presented as mean ± SEM. Two-way analysis of variance was performed with time and age as the variables. In all cases, overall significance was confirmed and the data was analyzed across time for each age group by ANOVA followed by post hoc analysis with the Tukey test for pairwise multiple comparisons using Sigmastat statistical software (SPSS Inc., Chicago, IL). Significance was set at $P < 0.05$.

RESULTS

Plasma LH Levels in OVX, Steroid-Primed Young and Middle-Aged Animals on the Day of an LH Surge

As shown in Figure 1, LH levels were significantly increased in young animals at 1400 h and 1800 h relative to 1000 h. In middle-aged females, LH levels at 1800 h (but not at 1400 h) were significantly elevated relative to 1000 h. At all time points, LH levels were lower in middle-aged females compared to young females.

Age-Related Changes in Expression of Gonadotropin Subunits and GnRH Receptor mRNA Levels

The expression levels of the mRNAs encoding the CGA, LHB, and FSHB as well as the GnRH receptor (GNRHR) were quantified 21 days after ovariectomy in females that received no steroid priming and in females that were treated with estrogen only or with estrogen and progesterone to induce an LH surge (Fig. 4). For all measurements, the two-way ANOVA revealed significant overall differences and significant differences by time and by age, but no significant interactions were observed.

The gonadotropin subunit and GnRH receptor transcripts were significantly lower in the MA rats than in the Y rats at most points examined and showed greater dynamic alterations in the Y animals than in the MA rats. In young animals, significant changes in mRNA levels were noted for *Lhb* and *Fshb* as well as *Gnrhr*. Middle-aged animals showed a significant change only in *Fshb* mRNA levels.

The mRNA levels of *Cga* and *Fshb* did not differ in young and middle-aged females following ovariectomy alone, but did differ in both cases following steroid treatment. *Lhb* and *Gnrhr* mRNA levels differed by age following ovariectomy alone and after steroid treatment. Only the 1000 h time point failed to show age-related differences in *Gnrhr* mRNA levels. Of note,

TABLE 1. Primers used in semiquantitative PCR.

Genes	Primers	Sequences (5′–3′)	Size (bp)	Complementary DNA inputs (ng)[a]	No. of PCR cycles
Esr1	Forward	CAG GTC CAA TTC TGA CAA TCG	321	12.5; 25	27
	Reverse	TTT CGT ATC CCG CCT TTC ATC			
Esr2	Forward	AAC ACT TGC GAA GTC GGC AG	327	50.0; 100	34
	Reverse	AAC CTC AAA AGA GTC CTT GGT GTG			
Terp1	Forward	CCA TTT CTT GAG CTT GTT GAA CAG	370	12.5; 25	29
	Reverse	AGT GTC TGT GAT CTT GTC CAG GAC			
PgrA+B	Forward	CCC ACA GGA GTT TGT CAA GCT C	326	12.5; 25	29
	Reverse	TAA CTT CAG ACA TCA TTT CCG G			
PgrB	Forward	GTG TGA GGA TTC TGC CTT TC	221	12.5; 25	31
	Reverse	CGC TCT CAG GAC TTC TTA CG			
Tuba1	Forward	CCA TCC ACC CGG CAG CCG	516		
	Reverse	CCG TAG TCG ACA GAG AGC C			

[a] Complementary DNA inputs were chosen within the linear range of the validation curve.

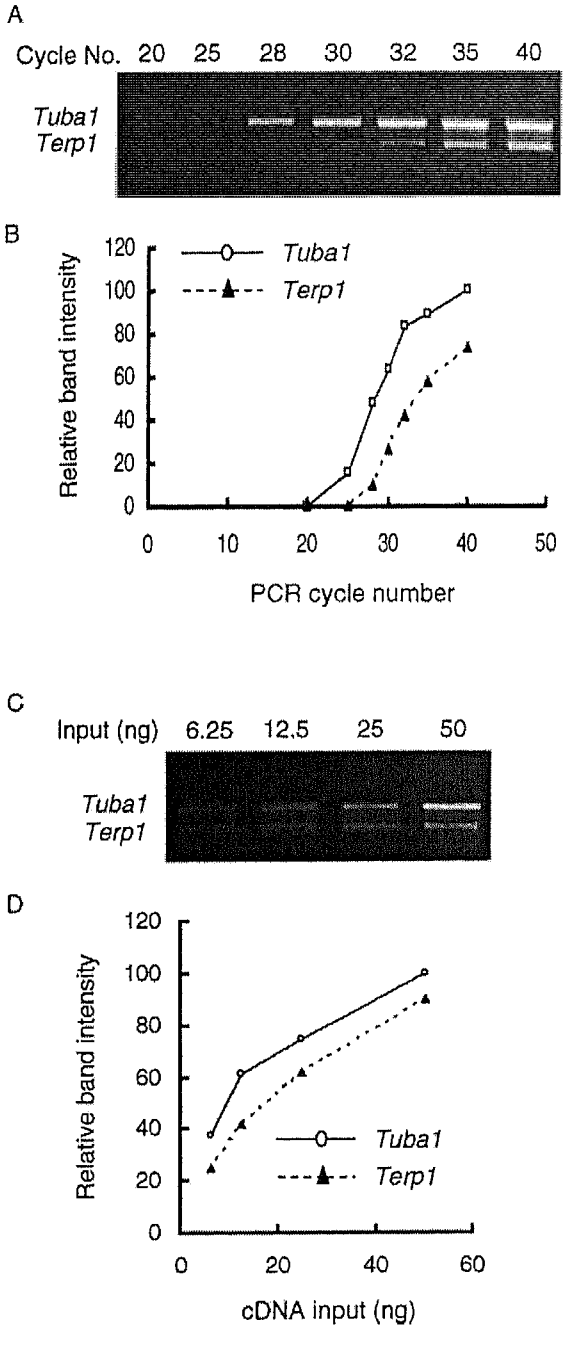

FIG. 3. Optimization of RT-PCR for semiquantification of mRNAs encoding the estrogen receptor (*Esr*) isoform, truncated *Esr* product-1 (*Terp1*). A) *Terp1* and *Tuba1* (α-tubulin) were amplified simultaneously for 20–40 cycles and the products separated on a 1.2% agarose gel. B) Quantification of product intensity using Kodak 1D image analysis software. Band intensity of *Tuba1* at 40 cycles was normalized to 100%. C) Optimization of cDNA inputs for coamplification of *Terp1* and *Tuba1* using 6.25–50 ng of cDNA template. D) Quantification of band intensity in C. Band intensity of *Tuba1* at 50 ng input was normalized to 100%.

the *Gnrhr* expression pattern that we observed in Y animals paralleled the pattern reported by Shirman-Hildesheim and colleagues in normal cycling rats, supporting the physiologic equivalency of our steroid protocol [42].

TABLE 2. Primers used in real-time PCR.

Genes	Primers	Sequences (5′–3′)
Nr5a1	Forward	ATC GAC AAG ACG CAG CGT AA
	Reverse	CCC ACC GTC AGG CAC TTC
Nr5a2	Forward	CCA GAC CCT GTT CTC CAT TGT T
	Reverse	CAT TTG GTC ATC AAC CTT CAG TTC
Fst	Forward	TCC CTT GTA AAG AAA CGT GTG AGA
	Reverse	TTC TTC TTG TTC ATT CGG CAC TT
Inha	Forward	TGG GAC CGC TGG ATC GTA
	Reverse	GCA TCC CGC AGC TAC CAT
Inhba	Forward	TGC AGG CTA GGC AGT CTG AAG
	Reverse	GAC CTT GCC ATC ACA CTC CAA
Inhbb	Forward	CAT CAG CTT TGC AGA GAC AGA TG
	Reverse	TTG CCT TCG TTA GAG ACG AAG AA
Tuba1	Forward	GCA ACC ATG CGT GAG TGT ATC T
	Reverse	TAG AGC TCC CAG CAG GCA TT

Esr *Transcripts in Young and Middle-Aged Animals*

At no time did expression of the three *Esr* isoforms differ between Y and MA animals (Fig. 5). *Esr1* and *Esr2* mRNAs were detected in the pituitary of both young and MA OVX rats. On the day of the steroid-induced LH surge, *Esr1* transcripts in Y females peaked prior to progesterone treatment and then showed minimal changes. *Esr2* levels in Y animals demonstrated a small, nonstatistically significant increase with steroid administration. *Terp1* transcripts were undetectable in both Y and MA animals after OVX. Following estrogen replacement (1000 h), *Terp1* mRNA levels were markedly induced in both age groups. In the Y females, *Terp1* transcripts decreased progressively on the day of the steroid-primed surge. A trend downwards was observed in the MA animals; however, these changes were not statistically significant. The marked inducibility of *Terp1* by steroids is in agreement with a previous report by Friend and colleagues [37].

Taken as a whole, these data suggest that *Esr* expression levels are essentially the same between Y and MA animals, although for the time points examined in this study, Y animals demonstrate greater sensitivity to steroid replacement.

Pgr *mRNA Levels in Young Versus Middle-Aged Rats*

Because *PgrA* is a truncated form of *PgrB*, it is not possible to distinguish *PgrA* from *PgrA+B* transcripts. We observed that *PgrA+B* and *PgrB* mRNA levels were significantly induced by estrogen-priming in both Y and MA females (Fig. 6). *Pgr* transcript number varied on the day of the LH surge with essentially the same pattern in both age groups. As observed for *Esr* family members, there were no statistical differences between the two age groups.

Age-Related Changes in the Expression of Nr5a1 *and* Nr5a2 *mRNA*

Both *Nr5a1* and *Nr5a2* mRNA were expressed in the anterior pituitary of young and MA OVX rats (Fig. 7). *Nr5a1* mRNA expression levels were decreased significantly in MA OVX rats compared with the Y OVX rats. *Nr5a1* expression in the MA animals was significantly lower than Y animals at all time points except 1800 h. Steroid priming produced a biphasic change in *Nr5a1* gene expression in the Y animals but not in the MA rats.

Unlike *Nr5a1*, there was no significant difference in *Nr5a2* expression between the Y and MA OVX rats prior to steroid treatment. As observed for *Nr5a1*, steroid treatment only regulated *Nr5a2* expression in the Y animals. These results

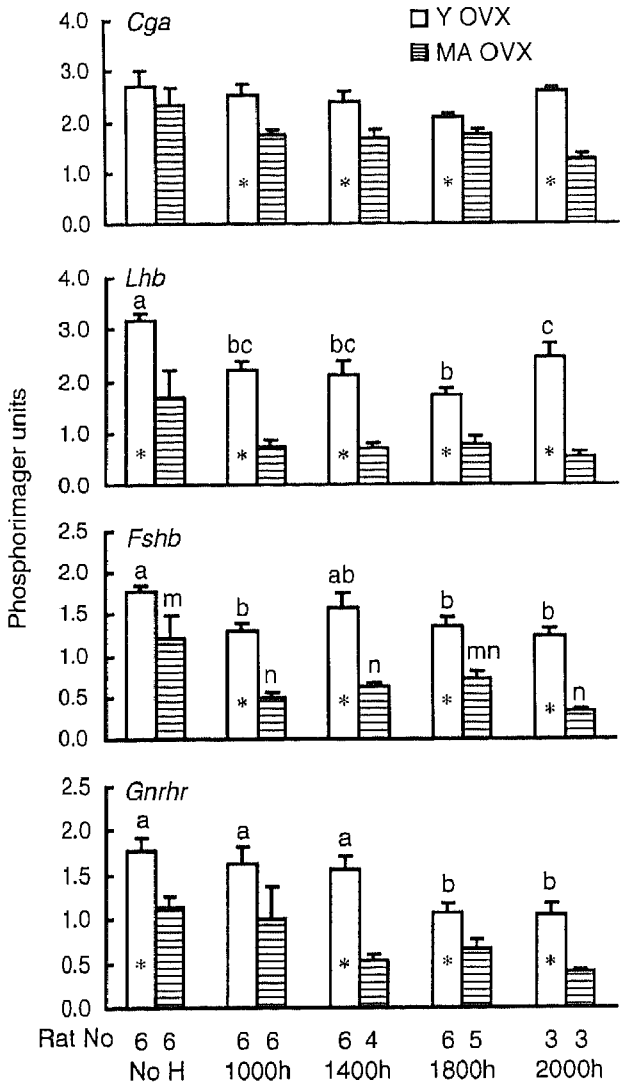

FIG. 4. Expression of glycoprotein hormones, α-subunit (*Cga*), *Lhb*, *Fshb*, and *Gnrhr* transcripts in the pituitary of young and middle-aged OVX rats across a steroid-induced gonadotropin surge determined by RNase protection assays. Bars represent the mean ± SEM within each group. Bars with different letters (a/b/c for Y and m/n for MA rats) vary significantly ($P < 0.05$). An asterisk represents a significant difference ($P < 0.05$) between the young and middle-aged rats at the same time point. The numbers above each time point represent the number of rats used in the corresponding Y and MA groups.

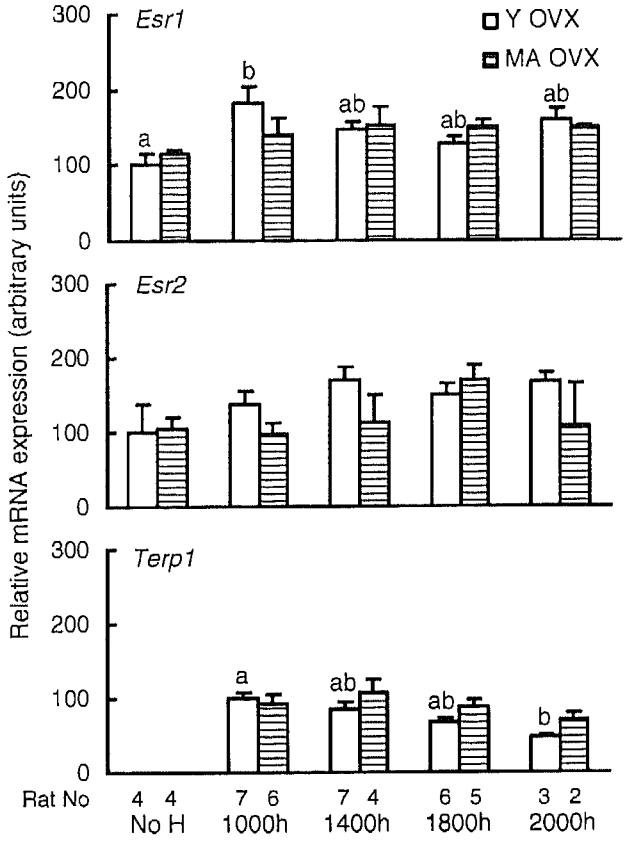

FIG. 5. *Esr1*, *Esr2*, and *Terp1* mRNA levels in the anterior pituitary of young and middle-aged rats. Bars, letters, and numbers represent the same as in Figure 4. No significant difference was observed between the young and middle-aged rats at any time point.

suggest a significant loss of pituitary expression and responsiveness in MA animals relative to Y animals.

Expression of Follistatin, Activin, and Inhibin mRNA Levels Differ Between Age Groups

Pituitary follistatin mRNA expression was significantly lower in MA versus Y rats following OVX (Fig. 8). On the day of the LH surge, follistatin mRNA expression varied significantly in the Y rats only. In contrast, *Fst* transcripts in the MA rats remained very low. These results indicate that aging results in a marked decrease in the expression and regulation of *Fst* gene expression.

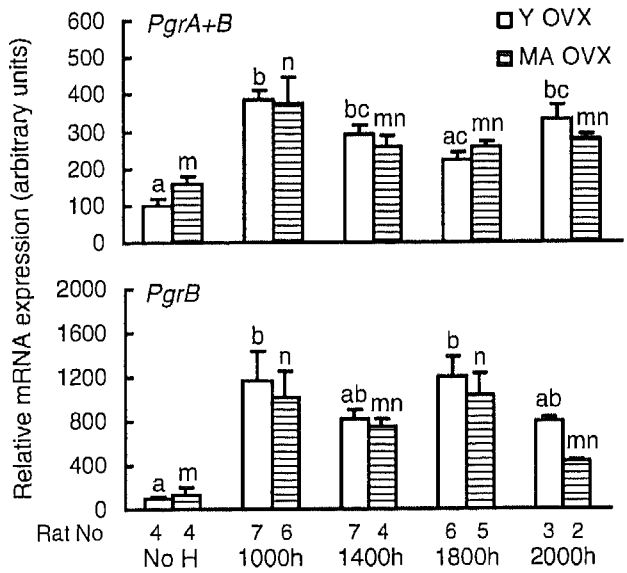

FIG. 6. Expression of *PgrA+B* and *PgrB* in the pituitary of young and middle-aged rats. Bars and numbers represent the same as in Figure 4. Bars with different letters (a/b/c for Y and m/n for MA rats) vary significantly ($P < 0.05$). No significant difference was observed between young and middle-aged rats at any time point.

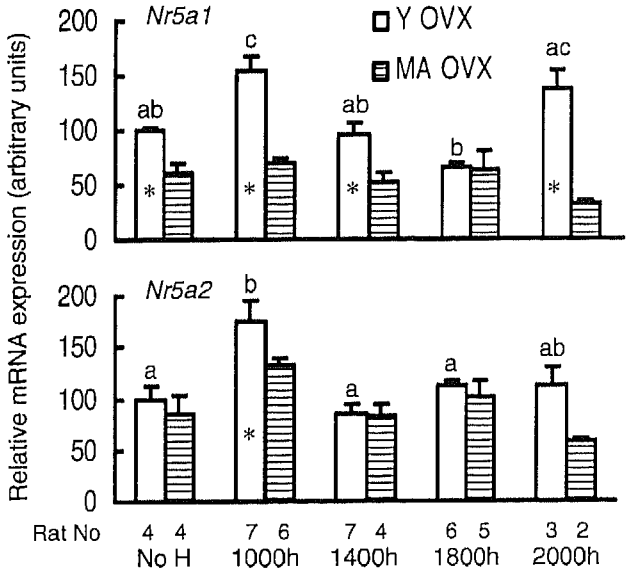

FIG. 7. Regulation of pituitary expression of *Nr5a1* and *Nr5a2* in young versus middle-aged rats undergoing a steroid-induced gonadotropin surge. Bars, letters, numbers, and asterisks indicate the same as in Figure 4.

Following OVX, mRNA expression of the inhibin α-subunits *(Inha)* was higher in MA relative to Y rats. Levels were also elevated in MA females at 1800 h relative to Y rats. Estrogen treatment significantly decreased *Inha* mRNA expression in MA rats but not in the Y animals. On the day of the LH surge, *Inha* mRNA displayed similar dynamic changes in both age groups. *Inhba* and *Inhbb* mRNA levels were the same in both Y and MA animals following OVX. Steroid treatment altered expression of these subunits in Y but not MA animals. The *Inhba* mRNA level in the young rats was significantly higher than that in the MA rats at 1000 h, 1400 h, and 2000 h. *Inhbb* mRNA levels in the young rats were significantly greater than that in the MA rats at 1000 h and 1400 h. Thus, *Inha* mRNA levels appear to be regulated in both age groups, while steroid-mediated *Inhba* and *Inhbb* changes are only observed in Y animals.

Follistatin and Nr5a1 *Expression in Primary Pituitary Cells Treated with Pulsatile GnRH*

The model that was utilized for the in vivo experiments controls for differences in circulating steroid levels in Y versus MA animals; however, this model does not control for indirect effects of these steroids on gonadotroph expression via alterations in hypothalamic GnRH. This is a potentially important deficiency as patterns of GnRH release are altered in MA relative to young females on the day of a steroid-induced LH surge [43]. Furthermore a decrease in the percent of activated GnRH neurons has been reported in MA rats in conjunction with the spontaneous or steroid induced LH surge [44, 45]. Therefore, we developed an in vitro primary pituitary cell perifusion system that allows investigation of intrinsic pituitary alterations with aging. This technique equalizes input of factors, such as GnRH, while removing endogenously produced substances, such as follistatin and activin/inhibin, which may modulate the response.

We chose to focus on three transcripts, *Gnrhr*, *Nr5a1*, and *Fst*, as these transcripts demonstrated marked differences in endogenous expression between Y and MA animals both post-

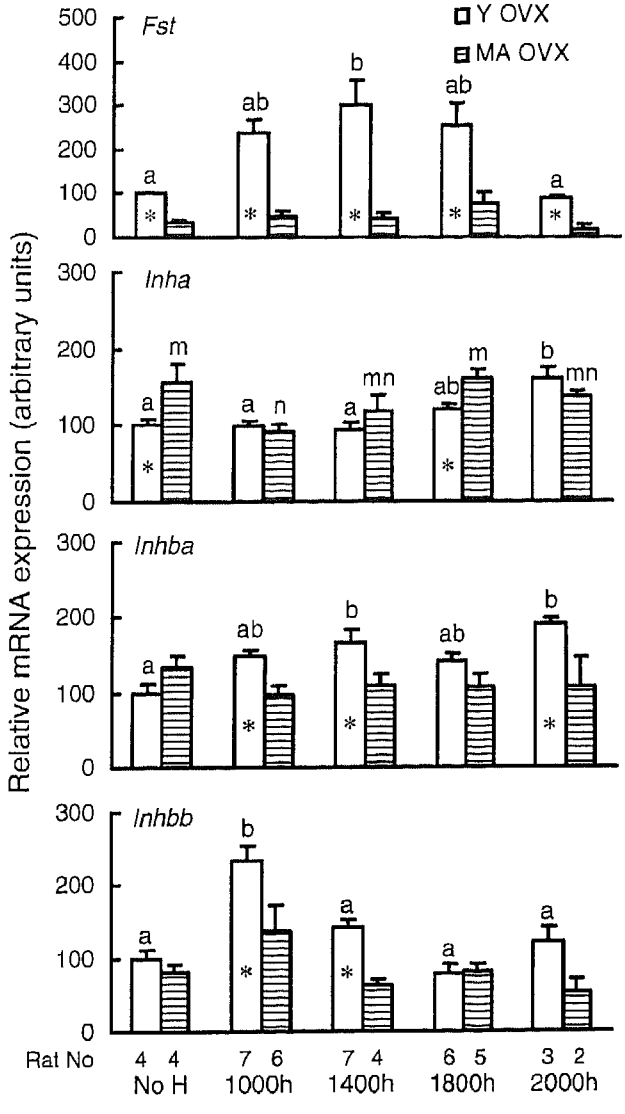

FIG. 8. Expression of pituitary *Fst*, *Inha*, *Inhba*, and *Inhbb* transcripts in the pituitary of young and middle-aged female rats on the day of steroid-induced gonadotropin surge. Bars, letters, numbers, and asterisks represent the same as in Figure 4.

OVX and during steroid-primed induction of the LH surge (Figs. 4, 7, and 8). Measurement of *Gnrhr* transcripts was also important as expression levels would impact the ability of the cells to respond to GnRH. Furthermore, *Gnrhr* responses to GnRH acted as a positive control in our system as their regulation by GnRH has been well-defined [46].

As observed in vivo, *Gnrhr* expression levels were lower in MA pituitary cells than in cells from Y animals, although this decrease did not achieve statistical significance (Fig. 9). Pulsatile GnRH treatment significantly increased *Gnrhr* transcripts in the young but not the older animals. *Gnrhr* levels in the Y pituitaries were significantly greater than in the MA pituitaries following GnRH treatment. These results are consistent with a loss of GnRH-responsiveness of this transcript with aging. Of note, other investigators previously have shown that GnRH induces *Gnrhr* transcripts in young animals [47]. Therefore, our results validate the efficacy of our perifusion model.

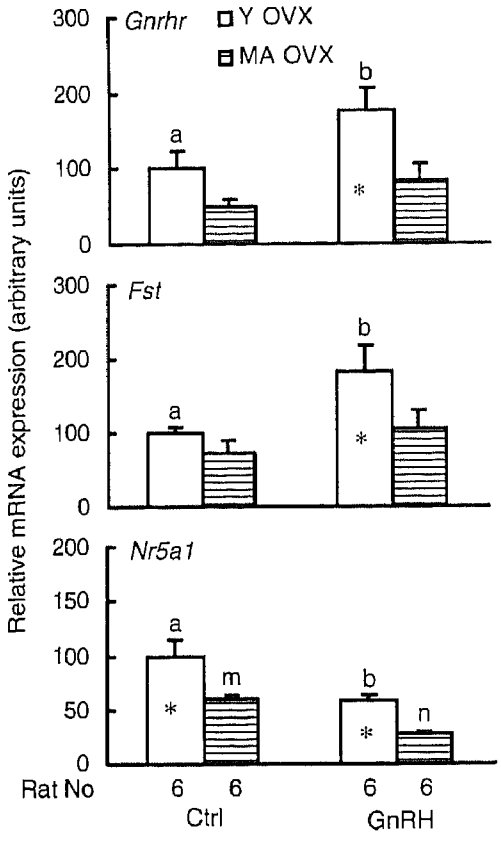

FIG. 9. Effect of pulsatile GnRH treatment on mRNA expression of *Gnrhr*, *Nr5a1*, and *Fst* in perifused primary pituitary cells from young and middle-aged female rats. Bars, letters, numbers, and asterisks represent the same as in Figure 4.

Fst mRNA expression was significantly increased by GnRH pulses in primary pituitary cells from the Y rats, but not the MA females. As observed for *Gnrhr* transcripts, *Fst* mRNA levels in Y pituitaries statistically exceeded MA levels in response to GnRH.

As observed in vivo, *Nr5a1* expression levels were significantly lower in MA pituitary cells than in pituitary cells from Y animals. GnRH decreased *Nr5a1* transcripts in both age groups, although expression remained greater in cells from the Y animals.

DISCUSSION

Data from many laboratories reveal that age-related changes in the hypothalamus play an important role in female reproductive aging [48, 49]. Additional evidence supports the concept that alterations at all levels of the hypothalamic-pituitary-ovarian axis occur with age and contribute to the cascade of effects that lead to the loss of the preovulatory LH surge, estrous cyclicity, and fertility [50]. The studies reported here focus specifically on the potential contribution of the pituitary to the age-related reproductive decline in middle-aged female rats. To date, relatively few studies of age-related changes at the pituitary level have been completed. Although data from a few studies suggest deficits in pituitary function, it is difficult to exclude the role of the hypothalamus in the majority of these reports.

TABLE 3. Age-related changes in pituitary gene expression.

		Steroid replacement			
		Estradiol[b]		Progesterone[b]	
Genes	OVX[a]	Young	MA	Young	MA
Cga	Y = MA	~	~	–	~
Lhb	Y > MA	–	~	~	~
Fshb	Y = MA	–	–	~	~
Gnrhr	Y > MA	~	~	–	~
Esr1	Y = MA	+	~	~	~
Esr2	Y = MA	~	~	~	~
Terp1	ND	+	+	–	~
PgrA+B	Y = MA	+	+	–	~
PgrB	Y = MA	+	+	~	~
Inha	Y < MA	~	–	+	+
Inhba	Y = MA	~	~	+	~
Inhbb	Y = MA	+	~	–	~
Fst	Y > MA	+	~	–	~
Nr5a1	Y > MA	+	~	–	~
Nr5a2	Y = MA	+	~	–	~

[a] Y, Young; MA, middle-aged; >, significantly greater than; <, significantly less than; =, no significant difference; ND, not detected.
[b] Symbols indicate: +, significant increase; –, significant decrease; ~, no significant change.

The present study was undertaken to determine the potential role of the pituitary in the age-related attenuation and eventual loss of the LH surge. Gonadotropin gene expression demonstrated both blunting and loss of dynamic changes in response to steroid administration with aging. Specifically, significant decreases in the expression of the gonadotropin subunits, *Gnrhr*, follistatin, and *Nr5a1* mRNA levels were observed in MA (OVX and OVX, steroid-treated rats) compared with Y animals. These data are summarized in Table 3.

The factors examined in our studies include the mRNAs for the *Gnrhr* and the gonadotropin subunits, each of which was significantly blunted in the MA rats compared with the Y rats (Fig. 4). GNRHR and the gonadotropins are directly involved in the preovulatory LH surge. However, synthesis and secretion of the pituitary gonadotropins are influenced by the parameters of pulsatile GnRH release [51] as is the level and density of GnRH receptors [52]. Therefore altered levels of these proteins, while suggestive of pituitary deficits, cannot distinguish between a central hypothalamic deficit and intrinsic pituitary deficits in middle-aged females. For this reason it is important to assess the ability of controlled GnRH administration, such as achieved with a perifusion system, to influence gene expression in the aging pituitary. Using a perifusion system, we have demonstrated that pituitary cells from MA animals lose GnRH-induced changes in *Fst* and *Gnrhr* but not *Nr5a1* transcripts at the pulse frequency and duration tested.

As we had predicted, we did not detect a difference in pituitary mRNA levels of the *Esr* isoforms or *Pgr* transcripts between the Y and MA rats (Figs. 5 and 6). *Esr* gene expression was unchanged with aging following OVX. However, the small degree of steroid-mediated modulation in transcript levels observed in Y animals was lost in the MA animals. A number of investigators have studied the effect of estrogen on the mRNA expression of *Esr* isoforms in the rat pituitary, with varying results [17, 18, 37]. In each of these studies, estrogen induced a marked increase in *Terp1* transcript number, consistent with our findings. The *Terp1* transcriptional start site is distinct from that of the *Esr1* and *Esr2* genes and is presumed to contain alternative regulatory elements that explain differences in steroid sensitivity [18]. Our results also

support the work of Friend and colleagues, who noted a small increase in *Esr1* mRNA levels 48 hours after estradiol injection [37]. Prior studies have found no significant effects of progesterone treatment, either alone or in combination with estrogen, on the mRNA levels of pituitary *Esr* isoforms [18, 37]. In this study, we did not observe any effects of progesterone on *Esr1* or *Esr2* mRNA, but we detected a significant decrease in *Terp1* mRNA levels in the young OVX steroid-treated rats at 2000 h. Schreihofer et al. also observed a decrease in *Terp1* mRNA levels on the afternoon of proestrous (i.e., during the endogenous gonadotropin surge), although their result did not achieve statistical significance [18].

Of note, the inability to detect significant changes in *Esr1* and *Esr2* expression with age or with endocrine manipulation may reflect contributions of pituitary cell types other than gonadotrophs as estrogen receptors are widely expressed in the rat pituitary [17]. Furthermore, levels of pituitary receptor number may not fully parallel our analysis of transcript expression. Nevertheless, the lack of substantial changes in estrogen and progesterone receptor transcripts within the pituitary suggests that loss of response to steroid treatment with aging may occur outside of the pituitary. The equivalent induction of *Pgr* transcripts by estrogen in both age groups suggests that the pituitary response to this target of estrogen action remains intact in middle-aged females.

To date, there is no evidence of an age-related decline in *Esr1* levels in brain regions known to regulate gonadotropin release, although there is evidence of a decline in alternative brain regions in middle-aged females [53]. In contrast, a decline in *Esr2* has been reported in the periventricular nucleus of middle-aged rats, a brain region implicated in the regulation of LH release [54, 55]. Furthermore, as observed here in the pituitary, aging does not appear to interfere with upregulation of *Pgr* mRNA by estrogen in any of the brain regions studied [56].

Our results demonstrate a marked induction of *Pgr* transcripts with estrogen replacement in both Y and MA OVX animals (Fig. 6). This response to estradiol is consistent with previous reports that estrogens are a potent stimulator of *Pgr* mRNA expression [20, 39, 57]. In a study by Schwartz and coworkers, maximal levels of the *Pgr* mRNAs were detected on the morning of proestrus, with a decline to nadir values by estrus [20]. Turgeon and coworkers reported a rapid and transient downregulation of *Pgr* mRNA by progesterone in vitro using estrogen-primed primary pituitary cells [39]. Our results indicate that this biphasic effect of progesterone on *Pgr* mRNA expression also occurs in vivo in estrogen-primed OVX rats.

We hypothesized that levels of the two stimulatory transcription factors, *Nr5a1* and *NR5a2*, would be blunted in the older animals. As shown in Figure 7, *Nr5a1* transcript levels in MA animals are significantly decreased relative to Y females both post-OVX and at the majority of time points across the steroid-induced surge. In contrast, *Nr5a2* mRNA levels did not appear to be influenced by age, except 48 hours after estrogen treatment when Y females expressed slightly greater levels. Interestingly, the ability of steroid treatment to modulate expression of both *Nr5a1* and *Nr5a2* was lost in the MA animals.

Prior reports regarding the regulation of *Nr5a1* expression by GnRH have apparent discrepancies [58–63]. Some in vivo data suggest that GnRH stimulates *Nr5a1* expression [61, 62], but this response was not observed in a third study [63]. In vitro studies with LβT2 and αT3 cells have not confirmed the ability of GnRH to increase *Nr5a1* biosynthesis [58–60]. In fact, although the results did not achieve statistical significance, Dorn and colleagues observed decreased expression of *Nr5a1* mRNA at multiple GnRH doses (0.1 to 30 nM) in gonadotroph cells treated at 24 hours and 8 hours prior to harvest [58]. As observed in vivo, *Nr5a1* expression was decreased in MA animals when measured in our perifusion model (Fig. 9). Furthermore, treatment with pulsatile GnRH significantly decreased *Nr5a1* mRNA expression in primary pituitary cells from both Y and MA animals. The combined results of our in vivo and in vitro data are consistent with a loss of *Nr5a1* basal expression and steroid responsiveness but not GnRH responsiveness in aging gonadotrophs. This age-dependent decrease in *Nr5a1* expression may provide a molecular mechanism by which gonadotropin biosynthesis decreases with age, leading to an attenuated LH peak in MA rats as reported previously [6, 10, 64].

Inhibin and activin are best known for their ability to alter FSH biosynthesis and secretion. Activin has subsequently been shown to regulate expression of *Lhb* and the *Gnrhr* genes [29–32, 65]. We now show that steroid-mediated regulation of the inhibin α-subunit is maintained in MA animals, but lost for the inhibin/activin β-subunits (Fig. 8). In a prior report, *Inha*, *Inhba*, and *Inhbb* were relatively stable across the endogenous estrous cycle [66]. Subtle changes in *Inha* and *Inhbb* mRNA and protein levels were subsequently reported following ovariectomy [67]. Our data demonstrate relatively small but statistically significant changes in *Inha* and *Inhbb* expression across the steroid-induced surge.

In one important study, Tebar and colleagues utilized an in vivo female rat model system in which the animals received a GnRH antagonist to remove any impact of steroids on hypothalamic function. In their study, progesterone decreased expression of *Inha*, *Inhba*, and *Inhbb* in the pituitary [68]. In our studies, the addition of progesterone similarly decreased *Inhbb* expression, but increased *Inha* expression. These differences are likely due to differences in the models utilized. For example, in our studies, progesterone was added in the presence of estradiol. Overall, these data suggest that MA animals may fail to achieve cycle-specific changes in the amount of locally produced inhibin or activin required for appropriate gonadotropin production.

In the present study, MA OVX rats expressed follistatin transcripts at a significantly lower level than observed for Y animals. Furthermore, estrogen and progesterone failed to modify follistatin expression in the older females (Fig. 8). Contrary to these results, we had predicted an increase in follistatin expression as follistatin inhibits *Fshb* expression. In light of our results, we have reconsidered our initial hypothesis. Dynamic changes in pituitary follistatin mRNA expression have been reported across the estrous cycle and under varying physiological paradigms [51, 66, 69–72]. Members of our group have previously demonstrated marked stimulation of follistatin mRNA levels following ovariectomy or coinciding with a spontaneous gonadotropin surge [66]. In vitro and in vivo experiments, including our data in perifusion (Fig. 9), have indicated that pulsatile GnRH treatment results in a significant elevation in pituitary follistatin mRNA levels [51]. GnRH expression is known to be blunted in aging animals and this change may explain, in part, the decrease in follistatin gene expression. While gonadotropin gene expression is decreased in the early stages of middle-age reproductive dysfunction, it is also known that perimenopausal women and middle-aged persistent-estrus rats ultimately experience an increase in plasma FSH levels [6, 73–77]. Therefore, the observed loss of follistatin expression and regulation may foreshadow the eventual increase in gonadotropin expression.

In the perifusion system, follistatin transcript levels were not statistically different between OVX Y and MA animals prior to pulsatile GnRH exposure. This result differs from the in vivo result and may be due to the washout of known stimulatory factors for follistatin, such as activin, in the perifusion system [78]. We also observed a GnRH-mediated increase in follistatin levels in pituitary cells from the Y animals (Fig. 9), but not in MA cells.

Our studies form a basis for the further exploration of the role of the gonadotroph in reproductive senescence. The lack of substantial changes in estrogen and progesterone receptor transcripts within the pituitary suggests that loss of response to steroid treatment with aging may not occur within the pituitary. As previously mentioned, levels of pituitary receptor number may not fully parallel our analysis of transcript expression. Alternatively, the loss of steroid response may be due to changes in *Esr* and *Pgr* expression at the level of the hypothalamus with subsequent changes in GnRH pulsatility.

While age-related hypothalamic changes may explain a subset of our results, they cannot easily explain all of the results reported. Changes in GnRH pulsatility could explain differential *Gnrhr* expression between age groups in vivo; however, the inability of the MA animals to mount a GnRH response for this receptor in the perifusion model supports an intrinsic pituitary defect. Additional studies in an OVX model with addition of a GnRH antagonist may be useful to further isolate the site of dysfunction.

In conclusion, normal female reproductive function depends on the precise quantitative and temporal stimulation of follicular development by pituitary gonadotropins. Our data suggest that, in addition to alterations in ovarian and hypothalamic physiology, aging is associated with deficits in anterior pituitary gene expression. It is interesting to note that expression of a number of the factors examined did not differ between Y and MA females, suggesting that the observed deficits were not due to a global decline in anterior pituitary function. Each gene studied in this report encodes a protein with a well-defined role in the control of gonadotropin biosynthesis and secretion, which is ultimately critical for ovarian function. Therefore, abnormalities in basal or cyclic expression of these genes might negatively impact estrous cyclicity and fertility. For example, *Nr5a1* stimulates expression of the gonadotropin subunit genes, and therefore, the blunted *Nr5a1* mRNA levels observed in middle-aged animals correlates well with the observed loss of gonadotropin expression in these animals.

In addition to changes in pituitary gene expression in middle-aged females soon after estrous cycles ceased, it is important to note that altered gene expression and abnormal responses to GnRH were observed in pituitary cells harvested from middle-aged females prior to the development of age-related alterations in estrous cyclicity. This temporal relationship strongly implies that pituitary dysfunction, in concert with hypothalamic and ovarian deficits, plays a role in the age-associated reproductive decline in females.

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