

Gonadotropin-Releasing Hormone Messenger Ribonucleic Acid Levels Are Unaltered with Changes in the Gonadal Hormone Milieu of the Adult Male Rat*

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ABSTRACT. Testicular function is regulated by the negative feedback effect of sex hormones acting at the brain and pituitary to inhibit the secretion of LH and FSH. An important component of this feedback axis is presumed to involve regulation of secretion and possibly synthesis of GnRH by the brain. We tested the hypothesis that the castration-induced increase in gonadotropin secretion is subserved, at least in part, by increased synthesis of GnRH. Using *in situ* hybridization and an oligonucleotide probe to pro-GnRH messenger RNA (GnRH mRNA), we compared the level of cellular GnRH mRNA and the relative number of GnRH mRNA-containing neurons between intact and 21-day castrate adult male rats. To derive estimates of the number of GnRH cells and the cellular GnRH mRNA content, coronal sections from each animal were anatomically matched between intact and castrate groups. All identifiable cells within these sections were counted and analyzed with the aid of a computerized image analysis system, by an observer unaware of the animal's experimental group and were assigned an anatomical location for reference.

In an initial experiment, we observed no difference in cellular GnRH mRNA signal level between intact ($n = 4$) and castrate ($n = 5$) animals (129 ± 8 vs. 139 ± 5 grains per cell); however, we did find a statistical difference between the intact and castrated groups in the relative number of GnRH mRNA-containing cells (intact: 212 ± 15 vs. castrate: 320 ± 18). To confirm this observation, we repeated the experiment by again comparing the number of GnRH mRNA-positive cells between intact ($n = 4$) and castrate ($n = 4$) rats. In this second experiment, we found no difference in the number of identifiable GnRH mRNA-containing cells between intact and castrate animals (272 ± 14

vs. 274 ± 36 , respectively); this was the case for the total cell count as well as when the data were analyzed by anatomical region. To clarify the conflicting results on cell counts of Exps 1 and 2, we repeated the experiment a third time, again comparing both the number of GnRH mRNA-containing cells and the cellular content of GnRH mRNA. In this experiment, we observed that neither cell number nor content of GnRH mRNA differed between the intact and castrate groups. Again, this was the case for total cell count, as well as when the data were analyzed by anatomical region. In a fourth experiment, instead of the oligonucleotide probe we had used in the previous 3 experiments, we developed a cloned complementary RNA probe to the prepro-GnRH message and applied this with *in situ* hybridization to address the same question. In this experiment, as in 2 of the previous experiments, we observed no difference in either GnRH mRNA-positive cell number (intact: 136 ± 6 vs. castrate: 126 ± 6) or cellular GnRH mRNA signal level (intact: 169 ± 7 vs. castrate: 158 ± 7 grains per cell) between the intact and castrate groups. Moreover, in this, as in the previous 2 experiments when the data were analyzed by anatomical region, no difference in either cell number or message content was detected between groups. These results confirmed the findings of Exps 1 and 3 with respect to cellular GnRH mRNA content and corroborate the findings of Exps 2 and 3 for cell count. We conclude that neither the number of GnRH mRNA-positive cells nor the cellular GnRH mRNA content is altered after long term castration in the adult male rat, suggesting that feedback control of gonadotropin secretion does not depend upon compensatory changes in GnRH biosynthesis. (*Endocrinology* 127: 523-532, 1990)

GONADAL function is coordinated by reciprocal interactions between pituitary and gonadal hormones (1), a concept that was fully developed by Moore and Price in the early 1930s (2, 3). At nearly the same time, Hohlweg and Junkmann (4) postulated that gonadal hormones act on the sex center of the brain as one

aspect of the control system (4). Together, these classical studies provide the empirical basis for the current view that sex hormones, such as testosterone, act in a negative feedback fashion within the brain-pituitary axis to regulate the secretion of LH and FSH (5, 6). However, the anatomical substrate and cellular mechanisms whereby gonadal hormones effect this process remain unresolved.

An important component of this feedback axis is presumed to involve the negative feedback regulation of GnRH secretion and possibly synthesis by relatively few neurons in the basal forebrain and hypothalamus. This assumption rests on evidence that is consistent with the

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argument that gonadal hormones inhibit GnRH secretion and that the castration-induced rise in gonadotropin secretion is due, in part, to increased GnRH secretion (7-9). After castration of the adult male rat, the content of GnRH within the mediobasal hypothalamus declines (10, 11). This decline in hypothalamic GnRH content after castration has been interpreted to reflect a drain in the releasable store of GnRH due to an increase in secretory demand. Indeed, several reports testify to the fact that GnRH secretion does increase after castration in the rat and ram (12, 13). However, this belief has recently become the subject of controversy, based on contradictory evidence [(14) see Ref. 15 for review].

To examine this issue from another vantage point, we argued that if there were an increase in secretory demand placed on GnRH neurons after castration, GnRH synthesis would likely be increased as well. We tested the hypothesis that upon removal of gonadal inhibition there would be a commensurate increase in the biosynthetic capacity of GnRH neurons. Using mRNA levels as an index of biosynthetic capacity, we measured levels of pro-GnRH mRNA (GnRH mRNA) in individual cells of the forebrain and hypothalamus by *in situ* hybridization and compared levels observed between intact and castrate adult male rats. We report that neither the cellular levels of GnRH mRNA nor number of GnRH cells expressing the message changes with long term castration in the male rat.

Materials and Methods

Animals

Adult male Sprague-Dawley rats were purchased (Tyler Laboratories, Bellevue, WA) and housed under light-controlled conditions (12-h light, 12-h darkness), with lights on at 0700 h. Animals were provided rat chow and tap water *ad libitum*.

Experimental design

Exp 1. In the first experiment we sought to determine whether gonadal hormones influence the level of GnRH mRNA in individual neurons or the number of GnRH message-positive neurons throughout the basal forebrain and rostral hypothalamus of adult male rats. Animals were either castrated ($n = 5$) or sham-castrated ($n = 4$) under ether anesthesia and killed 21 days later. Using an oligonucleotide probe and *in situ* hybridization, we counted the number of GnRH mRNA-positive neurons, measured the relative cellular GnRH mRNA content in individual neurons, and compared these values between the intact and castrated groups.

Exp 2. In this experiment, we sought to confirm the findings of Exp 1. Adult male rats were either castrated ($n = 4$) or sham-castrated ($n = 4$) and killed 21 days after surgery. As we had seen a difference between the number of GnRH message-positive neurons and not in the cellular level of GnRH mRNA,

we only compared the number of GnRH message-positive neurons between intact and castrated groups in this experiment.

Exp 3. To clarify the conflicting results of Exps 1 and 2 with respect to GnRH cell counts, we again repeated the experiment and obtained estimates of cellular GnRH mRNA as well as cell counts. Adult male rats were either castrated ($n = 3$) or sham-operated ($n = 3$) and killed 21 days after surgery. We counted the number of GnRH message-positive neurons, measured their relative cellular GnRH mRNA content, and compared these values between the castrated and intact groups.

Exp 4. Here, we performed the identical experiment we had for Exp 3, but instead of the oligonucleotide probe we had used in the previous 3 experiments, we conducted the *in situ* hybridization with a complementary RNA (cRNA) probe to rat GnRH mRNA. Again, adult male rats were either castrated ($n = 5$) or sham-operated ($n = 5$) and killed 21 days after surgery. We counted the number of GnRH message-positive neurons, measured their relative cellular GnRH mRNA content, and compared these values between the castrated and intact groups.

Tissue preparation

Between 0800 and 1100 h, animals were asphyxiated with carbon dioxide and decapitated within 30 sec. The brains were quickly removed, frozen on dry ice, and stored at -80°C . Trunk blood was collected and the serum fractions stored at -20°C before being assayed for testosterone and LH. Coronal brain sections ($20\ \mu\text{m}$) were cut in a cryostat with consecutive sections thaw-mounted onto 3 sets of poly-L-lysine-coated slides, thereby placing every third slice into a given set. Sections were collected from the point at which the corpus callosum joins at the midline, through the preoptic area, and into the anterior hypothalamus. Slides were stored in airtight boxes at -80°C until hybridization histochemistry was conducted. One set of slides from each animal was then processed for GnRH mRNA with either an oligonucleotide (Exps 1-3) or a cRNA probe (Exp 4).

GnRH oligonucleotide probe synthesis

For Exps 1-3, we used a 48-base oligodeoxynucleotide probe that was complementary to the entire GnRH coding region, the 9 intervening nucleotides between the coding regions for GnRH and GnRH-associated-peptide (GAP), and the first 9 bases of the GAP coding region of rat pro-GnRH mRNA (16). This probe was 3' end-labeled with [^{35}S]dATP [New England Nuclear (NEN), Wilmington, DE], purified, and reconstituted in TED (10 mM Tris HCl, 1.0 mM EDTA, and 10 mM dithiothreitol). This stock solution of GnRH oligoprobe was diluted in hybridization buffer to a concentration of approximately $0.42\ \mu\text{g}/[\text{ml} \cdot \text{Kilobase (kb)}]$. For a detailed description of the synthesis, purification, and radiolabeling of this probe see Ref. 17.

GnRH cRNA probe synthesis

A plasmid, pSP65, containing an insert complementary to rat GnRH mRNA, was generously provided by Tony Mason [Genentech, South San Francisco, CA] (16). The 5' terminus

contained a *SalI* site while the 3' end consisted of a *BamHI* site. This 462 base pair (bp) probe was complementary to 170 bp of 5' untranslated message, the entire 276 bp of open reading frame, and the first 16 bases of 3' untranslated message. Radioactive cRNA was synthesized *in vitro* as previously described (18). By linearizing the plasmid with *SalI* and transcribing cRNA with SP6 polymerase, we produced an antisense probe that was complementary to GnRH mRNA; this probe was used in Exp 4. Conversely, when the plasmid was linearized with *BamHI* and cRNA transcribed with T7 polymerase, a sense probe that was identical, not complementary, to GnRH mRNA was produced and used as a specificity control. The transcription reactions contained a total of 50 μmol α -thio-UTP, of which 7 μmol were ^{35}S -labeled (NEN) and 43 μmol were unlabeled α -thio-UTP. The final specific activity was 3.22×10^8 dpm/ μg . The probe was hydrolyzed to an average length of 150 bases and diluted in cRNA hybridization buffer.

GnRH oligonucleotide in situ hybridization protocol

The oligonucleotide hybridization protocol was carried out as previously described (17). In brief, tissue sections were taken out of the freezer, dried, and postfixed in 4% paraformaldehyde (pH 7.4) for 5 min. The slides were then washed in phosphate buffer and treated with 0.25% acetic anhydride. Next, the tissue was rinsed in $2 \times \text{SSC}$ ($1 \times \text{SSC} = 150 \text{ mM NaCl}$ and 15 mM Na citrate) and delipidated. The slides were then dried; GnRH oligonucleotide probe was applied to each slide; and a coverslip was placed over the tissue sections. The slides were incubated in moist chambers at 37 C for approximately 20 h. After incubation, the slides received several washes in $1 \times \text{SSC}$, including washes at 48 C. The slides were then rinsed in distilled H_2O and dried.

GnRH cRNA in situ hybridization protocol

Tissue sections were dried, fixed in 4% paraformaldehyde, and washed in 0.1 M phosphate buffer. Next, the slides were treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The slides were then rinsed in $2 \times \text{SSC}$ and delipidated; 60 μl GnRH riboprobe were placed on a slide; and a coverslip was placed over each slide. Tissue sections were incubated with the cRNA probe for approximately 20 h in moist chambers at 50 C. After incubation the coverslips were removed and the slides were washed in $4 \times \text{SSC}$ containing 4.2 mM DTT. After a series of washes in SSC that increased in stringency, RNase treatment, and a wash at 60 C, the slides were passed through a series of alcohols and air-dried (for additional details see Ref. 18).

Autoradiography and histological staining

After *in situ* hybridization histochemistry, the slides were dipped into photographic emulsion (Eastman Kodak, Rochester, NY), allowed to expose at 4 C until developed to visualize autoradiographic silver grains, and then counterstained with cresyl violet (17). The tissue treated with oligonucleotide probe was allowed to expose at 4 C for 2 to 3 weeks, while the cRNA-treated tissue was exposed for 4 days.

Anatomical matching of brain sections

Using a rat brain atlas as a guide (19), we anatomically matched coronal brain sections across animals from both experimental groups. These sections were taken from the anterior septal region (plate 12; see Ref. 19 for plate identification), through the diagonal band of Broca (plate 15), and the preoptic area (plate 19), with the most caudal slice at the level of the suprachiasmatic nucleus and the rostral aspect of the lateral hypothalamus (plate 23). (see Fig. 7 for locus of the coronal sections with respect to Bregma.)

Quantification of the number and GnRH mRNA content of cells

Tissue sections were matched and then analyzed by an operator unaware of the animal's experimental group. For deriving estimates of cell counts in Exps 1–3, 20 sections from each animal were matched between all animals in the experiment, and all cells were counted in each section; in Exp 4, 10 sections from each animal were matched between all animals, and all cells were counted in each section. GnRH mRNA-containing cells were identified by scanning the tissue with low power, transmitted darkfield optics to locate clusters of silver grains. All grain clusters that could be identified by the operator as being above background were viewed under highpower, brightfield optics. If the grain cluster was verified as being associated with a cresyl violet-stained nucleus, this cluster was then considered to be a GnRH mRNA-containing neuron.

To obtain estimates of cellular GnRH mRNA content, 10 sections from each animal were matched between all animals, and all cells within each of these sections were analyzed. The signal levels in individual cells were measured by an automated computerized image-processing system (20), except that the microscope used in the current studies was an ACM Zeiss Photomicroscope (Zeiss, New York, NY). As above, silver grain clusters were verified as being associated with a cresyl violet-stained nucleus under brightfield optics, and individual grains were then counted with a $40\times$ epiillumination darkfield objective.

Probe validation and saturation analysis

GnRH oligonucleotide probe. A detailed description of the experiments performed for validation and specificity of the GnRH oligonucleotide probe has been previously published (17). The experiments described here were performed with a tissue-saturating probe concentration of $0.42 \mu\text{g}/(\text{ml} \cdot \text{kb})$.

GnRH cRNA probe. Determination of the concentration of GnRH cRNA probe required to fully saturate the endogenous GnRH mRNA in intact male rats was determined by generating a saturation curve (Fig. 1). The endogenous GnRH mRNA was saturated by a probe concentration of approximately $0.15 \mu\text{g}/(\text{ml} \cdot \text{kb})$. Experiment 4 was performed at a concentration of $0.21 \mu\text{g}/(\text{ml} \cdot \text{kb})$. It was determined that the probe concentration used in Exp 4 also saturated the endogenous GnRH mRNA in castrated male rats (data not shown).

To determine the specificity of the antisense probe (*i.e.* complementary to the endogenous GnRH message), a sense ^{35}S -labeled GnRH cRNA probe was applied to tissue sections

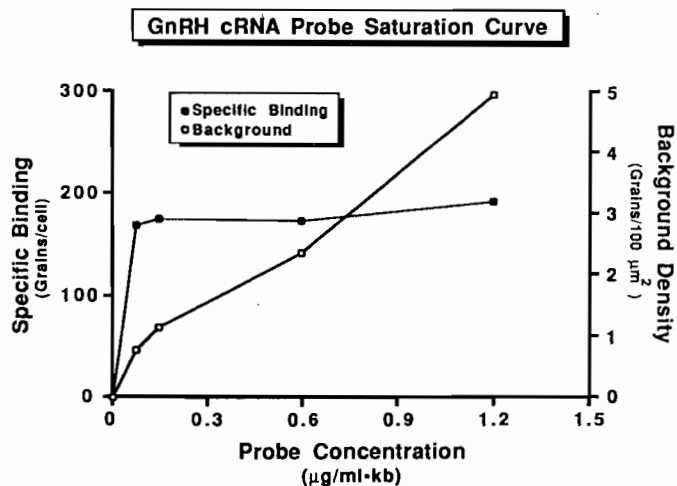


FIG. 1. Saturation curve for the prepro-GnRH cRNA probe. The tissue is saturated at a concentration of approximately 0.15 $\mu\text{g}/(\text{ml} \cdot \text{kb})$, and Exp 4 was performed with a probe concentration of 0.21 $\mu\text{g}/(\text{ml} \cdot \text{kb})$.

containing the diagonal band of Broca and the medial preoptic area; this probe had the same sequence as that of GnRH mRNA, instead of its complementary sequence. There were no silver grain clusters in the sections that received sense GnRH cRNA probe.

Testosterone assay

Serum samples were analyzed for testosterone by a double antibody RIA with reagents provided by the World Health Organization. The minimum sensitivity was 0.10 ng/ml. The interassay and intrassay coefficients of variation were 9.8 and 4.8%, respectively. The first antibody cross-reacts with the following steroids: 5 α -dihydrotestosterone (1.3%); 4 δ -androstenedione (3.5%); 5 α -androstane diol (1.3%); androstane 3 α -diol (0.7%); androstane 3 β -diol (1.8%).

LH assay

Reagents for this assay were provided by the National Institutes of Health. The reference standard was NIDDK-rLH-RP-3, the first antibody was NIDDK-anti-rLH-S-10, and the LH antigen was NIDDK-rLH-I-7. The maximum detectability was 40 ng/ml. The intraassay and interassay coefficients of variation were 5.5% and 8.1%, respectively.

Statistical analysis

For purposes of statistical analysis, the *n* for all experiments refers to the number of experimental animals. For cellular GnRH mRNA content determinations, the mean grains per cell from individual animals and the mean grains per cell from discrete anatomical areas in these individuals were used to calculate the mean \pm SEM for each group. Likewise, the mean number of GnRH mRNA-positive cells from individual animals and the mean number of cells from discrete anatomical areas in these individuals were used to calculate the mean \pm SEM for each group. In each experiment, the difference between intact and castrated rats in grains per cell for all of the cells measured

in an animal, and for the total number of GnRH message-positive neurons, was assessed by Student *t* test. To test for differences in GnRH message content and the number of GnRH message-positive neurons in specific anatomical regions, only areas in which there were at least 10 cells per animal per region measured in all animals were evaluated. Variations across anatomical areas were assessed by two-way (group *vs.* area) analysis of variance with repeated measures. The rejection level for statistical tests was set at $\alpha = 0.05$.

Power calculations were performed on the data to determine our ability to detect differences between groups (21). Given an *n* of 4 animals in each group and a pooled cell count SD of 29 cells, we had a 90% chance of detecting a true difference between the two groups as small as 27%. With respect to cellular GnRH mRNA content as reflected by grains per cell, assuming a pooled standard deviation of 11 grains per cell, we had a 90% chance of detecting a difference as small as 19%.

Results

Exp 1

We observed no difference between groups in cellular levels of GnRH mRNA signal (intact: 129 \pm 8 *vs.* castrate; 139 \pm 5 grains per cell; Fig. 2B); however, the number of GnRH mRNA-containing neurons was significantly higher in castrated compared with intact animals (intact: 212 \pm 15 *vs.* castrate: 320 \pm 18, $p < 0.01$; Fig. 2A). The increase in the number of GnRH message-positive neurons was greatest in the area of the organum vasculosum of the lamina terminalis, while no difference in cellular mRNA content between areas was observed (data not shown).

Exp 2

After counting the number of GnRH mRNA-positive cells in this second experiment, we observed that intact and castrated animals had nearly identical numbers of GnRH mRNA-containing cells (intact: 272 \pm 14 *vs.* castrate: 274 \pm 36 neurons). There was no difference in the number of GnRH neurons between anatomical regions (data not shown).

Exp 3

Dark-field photomicrographs of representative cells from this experiment are shown in Fig. 3. We detected no difference between groups in either the number of GnRH mRNA-positive cells (intact: 252 \pm 11 *vs.* castrate: 219 \pm 18 neurons; Fig. 4A) or in the cellular level of GnRH mRNA signal (intact: 105 \pm 2 *vs.* castrate: 105 \pm 3 grains per cell; Fig. 4B). There was no difference in the number of GnRH neurons or the cellular content of GnRH mRNA across anatomical areas (data not shown).

Experiment 1

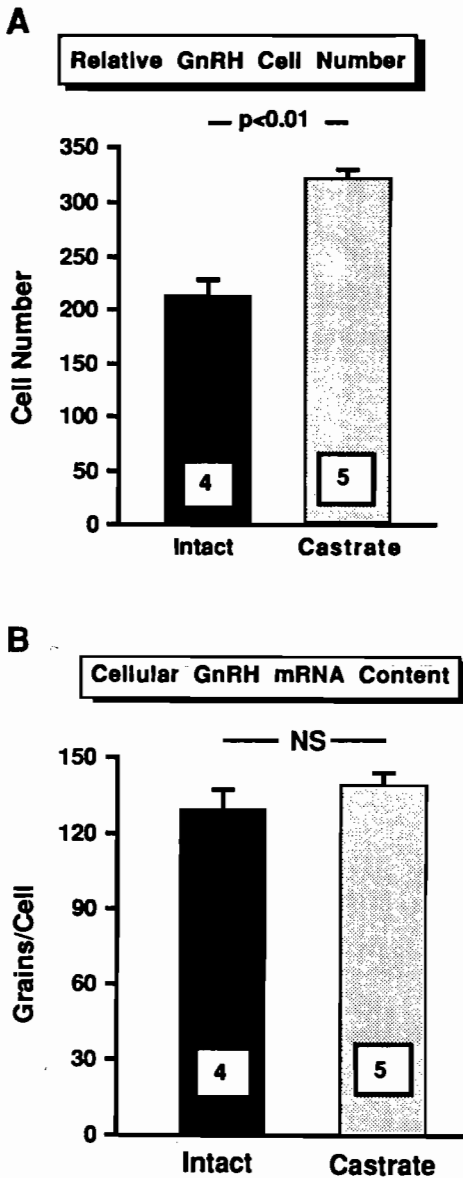


FIG. 2. A, A comparison between intact and 21-day castrate male rats in the relative number of GnRH mRNA-positive neurons (mean \pm SEM) throughout the forebrain and rostral hypothalamus. Twenty sections per animal were analyzed, and the number of rats studied in each group is identified at the base of each column. B, A comparison between intact and 21-day castrate male rats in GnRH mRNA signal levels (grains per cell \pm SEM) in labeled cells throughout the forebrain and rostral hypothalamus. Ten sections per animal were analyzed, and the number of rats examined is given at the base of each column.

Exp 4

In this experiment, performed with the cRNA probe for GnRH mRNA instead of the oligonucleotide probe, we detected no difference between groups in the relative number of GnRH mRNA-positive cells (intact: 136 ± 6 vs. castrate: 126 ± 6 ; Fig 5A) or cellular GnRH mRNA

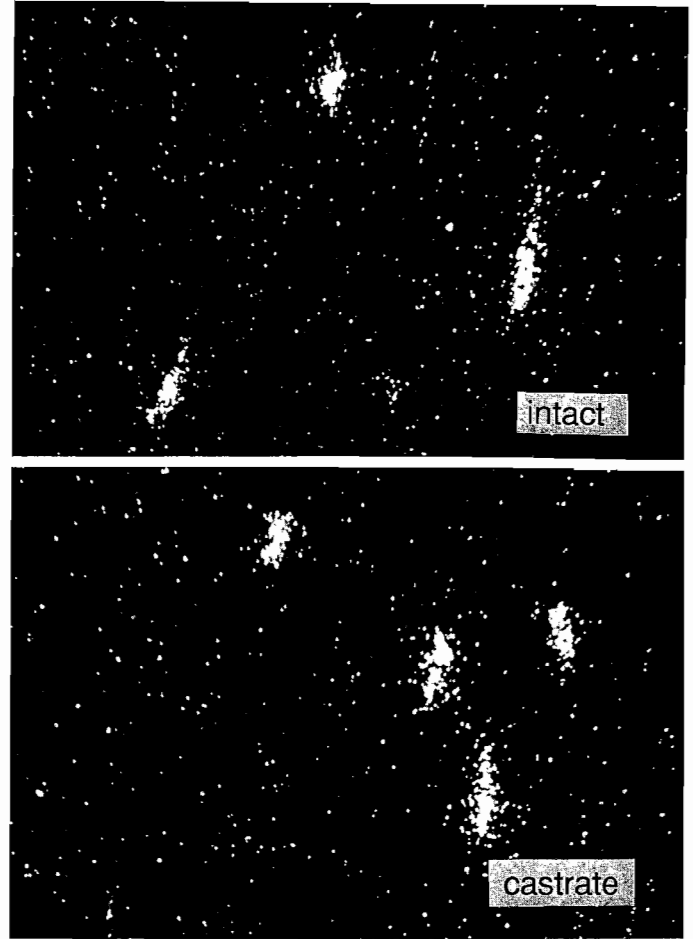


FIG. 3. Dark-field photomicrographs of the medial preoptic area showing cells labeled with a probe for GnRH mRNA in sections obtained from intact (upper panel) and 21-day castrate (lower panel) adult male rats.

signal level (intact: 169 ± 7 vs. castrate: 158 ± 7 ; Fig. 5B). When 4 discrete anatomical regions containing GnRH mRNA-positive neurons [medial septum (MS); vertical limb of the diagonal band of Broca (DBBV); medial preoptic area (MPOA); lateral preoptic area (LPOA)] were analyzed for differences in GnRH cell number, we observed no difference between groups and no interaction between experimental group and area [analysis of variance (ANOVA); Fig. 6A]. There were significant differences across areas in the number of GnRH cells, with the DBBV containing greater numbers of GnRH mRNA-positive neurons. With respect to cellular GnRH mRNA signal level, we observed no difference between groups across anatomical areas and no interaction between experimental group and anatomical region (ANOVA; Fig. 6B).

To visualize the approximate distribution of GnRH mRNA-containing neurons for both the intact and castrate groups, the average number of GnRH mRNA-containing neurons per sample section was plotted across

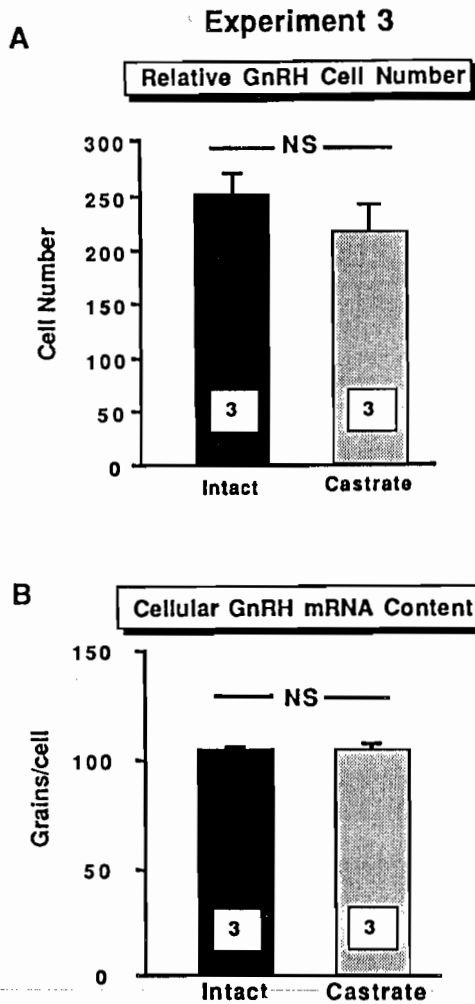


FIG. 4. A, A comparison between intact and 21-day castrate male rats in the relative number of GnRH mRNA-positive neurons (mean \pm SEM) throughout the forebrain and rostral hypothalamus. The number of rats examined is given at the base of each column, and 20 sections per animal were analyzed. B, A comparison between intact and 21-day castrate male rats in GnRH mRNA signal levels (grains per cell \pm SEM) in labeled cells throughout the forebrain and rostral hypothalamus. Ten sections per animal were analyzed, and the number of rats is given at the base of each column.

the rostral-caudal distribution of the diencephalon (Fig. 7). Most of the GnRH mRNA-positive neurons were found at the level of the organum vasculosum of the lamina terminalis and the rostral portion of the third ventricle (near Bregma), with the density of neurons tapering off both rostrally and caudally from this point. Inspection of the graph suggests no obvious difference in the distribution of GnRH mRNA-containing cells in the rostral-caudal plane between the groups.

Hormone analysis

For Exps 2-4, the results presented in Table 1 verify that intact animals had high circulating levels of testos-

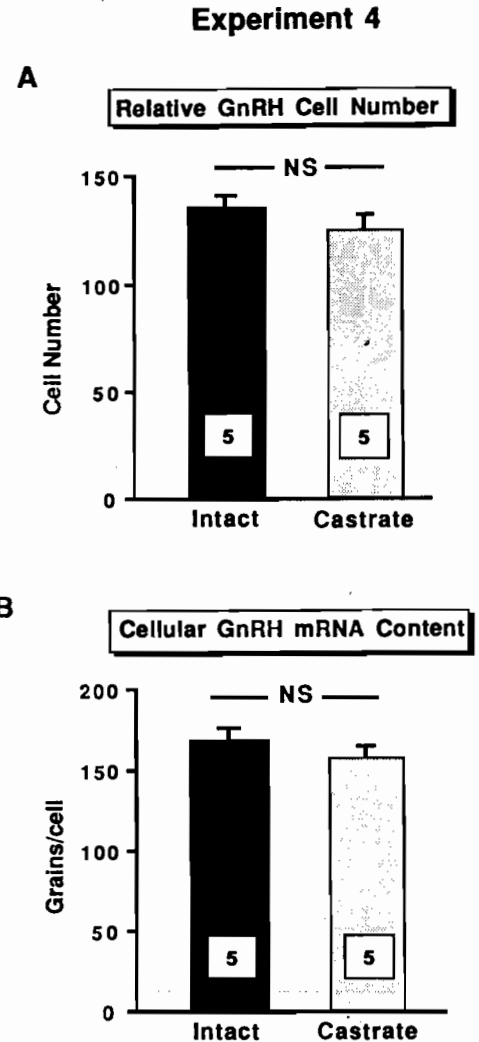


FIG. 5. A, A comparison between intact and 21-day castrate male rats in the relative number of GnRH mRNA-positive neurons (mean \pm SEM) counted throughout the forebrain and rostral hypothalamus. In this case, ten sections per animal were analyzed, and the number of rats examined is given at the base of each column. B, A comparison between intact and 21-day castrate male rats in GnRH mRNA signal levels (grains per cell \pm SEM) in labeled cells analyzed throughout the forebrain and rostral hypothalamus. Ten sections per animal were analyzed, and the number of rats examined is given at the base of each column.

terone and low levels of LH, typical of normal adult male rats, whereas castrated animals had low testosterone and high LH levels, as would be expected. (Blood samples were unavailable for analysis from animals used in Exp 1.)

Discussion

We report that neither cell number nor GnRH mRNA content is altered as a function of long term castration in the adult male rat. While an initial study had suggested a possible difference in cell number between intact

Experiment 4

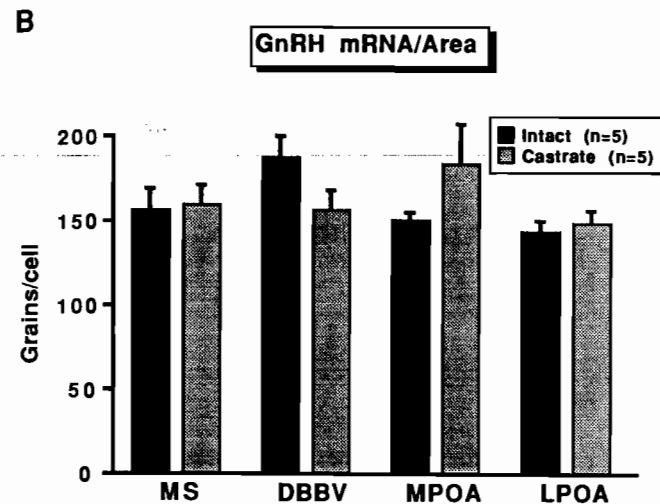
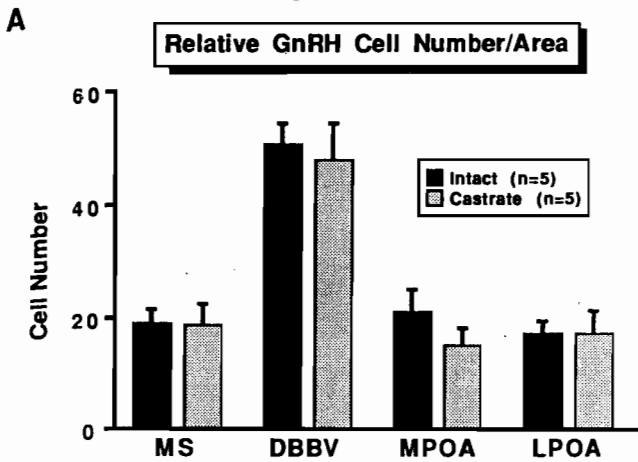


FIG. 6. A, A comparison between intact and 21-day castrate male rats in the relative number of GnRH mRNA-positive neurons (mean \pm SEM) from four discrete anatomical regions, including the MS, the DBBV, the MPOA, and the LPOA. No difference was found between groups, and no interaction between group and anatomical area was detected (two-way ANOVA). B, A comparison between intact and 21-day castrate male rats in GnRH mRNA signal levels (grains per cell \pm SEM) in labeled cells from four anatomical regions (see Fig. 6A legend for identification of regions). No difference was found between groups or anatomical areas, and no interaction between group and area was detected (two-way ANOVA).

and castrated animals, this observation could not be confirmed in three subsequent experiments. We can offer no logical explanation for the outcome of the first experiment and conclude that the number of neurons capable of expressing the GnRH gene is unchanged as a function of the gonadal hormone milieu. These findings suggest that the population of neurons expressing the GnRH gene is stable in the face of castration and that the compensatory gonadotropin response to castration does not involve a recruitment of previously nonexpressing

Rostral-Caudal Distribution of GnRH Neurons

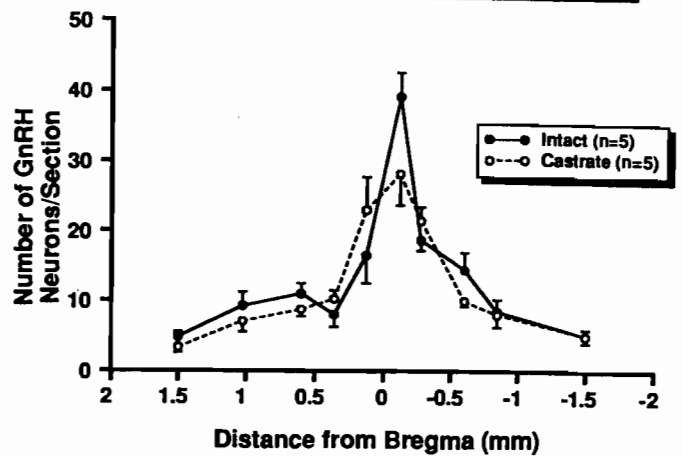


FIG. 7. Rostral to caudal distribution of the number of GnRH neurons from both intact and castrate rats analyzed in Exp 4.

TABLE 1. Plasma levels of testosterone and LH in both intact and castrated male rats from Exps 2-4

	Testosterone (ng/ml)	LH (ng/ml)
Exp 2		
Intact (n = 4)	3.83 \pm 0.44	4.34 \pm 0.88
Castrate (n = 4)	0.21 \pm 0.07	>40.0
Exp 3		
Intact (n = 3)	1.50 \pm 0.04	10.0 \pm 4.4
Castrate (n = 3)	<0.10	>40.0
Exp 4		
Intact (n = 5)	2.27 \pm 0.51	7.4 \pm 2.9
Castrate (n = 5)	<0.10	>40.0

Values are means \pm SEM.

GnRH cells into the preexisting population. This conclusion corroborates the results of an earlier study by Rothfeld and his co-workers (22), and the current literature is entirely consistent on this point, at least as far as the number GnRH mRNA-expressing cells is concerned. However, this is not the case with cellular GnRH mRNA content.

On the one hand, several groups have reported that castration in the male rat is associated with an apparent decrease in GnRH mRNA (22, 23). On the other hand, Toranzo and co-workers (24) have reported an increase in GnRH message after castration and go on to demonstrate that this increase is prevented by replacement with either estradiol or dihydrotestosterone. Now, our study testifies to a third result: no change in cellular GnRH mRNA content with castration. In our case, given the experimental n and variance of analytical technique, we determined, by power calculation, that with our protocol we could have identified a 27% difference in cell count and a 19% difference in GnRH message content 90% of the time with $\alpha = 0.05$. There is a possibility that

cellular GnRH message levels do change after castration within a small subset of a larger, heterogeneous population of GnRH neurons. If this were the case, it might prove difficult to detect even a large change in GnRH message content within this subpopulation of cells.

Another caveat is the possibility that immediately after castration, GnRH gene expression is transiently increased but that by 21 days a new level of activity is achieved, not reflected by steady state levels of GnRH mRNA. We compared intact and 21-day castrated rats, while others (22–24) studied 14-day castrates. The possibility that GnRH secretion increases immediately (within days) after castration is supported by the work of Badger and his colleagues (25). They investigated the time course of both LH release and pituitary sensitivity to GnRH after castration. While circulating LH levels are markedly elevated within 24 h after castration, pituitary sensitivity to GnRH is not increased until 7 days after castration (25). These data suggest that the early postcastration rise in LH is due, at least in part, to increased GnRH production and secretion. However, once the pituitary becomes supersensitive to GnRH, less GnRH could be required to produce the high serum LH levels observed in long term (e.g. 21-day) castrates. Hence, there could be an initial increase in the production and secretion of GnRH, including elevated GnRH mRNA levels, but GnRH mRNA content may return to intact levels sometime after 2 weeks.

Some technical shortcomings in the previous studies could also account for the discrepancies between our results and those of the earlier reports. First, in the study performed by Rothfeld *et al.* (22), silver grains were counted by hand, relatively few cells were analyzed, and grain densities over individual cells were very low (~24–30 grains per cell). Since the variance related to grains appearing over a cell is roughly proportional to the square root of the total number of counts (*i.e.* grain counts approximate a Poisson distribution), the coefficient of variation associated with grain counting when there are 25 grains per cell is more than twice as large as it is with 150 grains per cell. Second, it is unclear from the earlier accounts whether saturating concentrations of the GnRH probe were applied to fully hybridize and saturate endogenous GnRH mRNA (22, 24). If this were not the case and the probe concentrations applied were at the steepest part of the saturation curve, subtle differences in the handling of tissue between intact and castrate animals could result in large changes in grain counts and the derived message estimates, despite there being no actual differences between the groups in cellular message content. In the experiments of Park *et al.* (23) the hybridization assay used for their experiments may lack sufficient sensitivity to have a valid application to resolving the question posed, since in their report GnRH

mRNA was apparently undetectable in castrated animals. The question of how much GnRH mRNA may be present in the castrated state aside, there certainly is a detectable level of GnRH mRNA in the castrated male rat.

Discrepancies in results concerning the effects of gonadal hormones on the GnRH system are not restricted to mRNA studies but also apply to the measurement of other aspects of this system. For example, reports of GnRH content in the hypothalamus testify to a decrease (10, 11, 26, 27), an increase (28), and no change (29) after gonadectomy. Several groups have counted the number of GnRH neurons identified by immunocytochemistry after castration, and their findings include an increase in GnRH cell number (30), no change (27, 31), as well as a decrease in the number of GnRH neurons (32). *In vivo* measurements of GnRH release in unanesthetized male rats have yielded results suggesting a decrease in overall release when measured in the mediobasal hypothalamus (14), but an increase in release when measured in the anterior pituitary (33). These equivocal findings make it difficult to derive a satisfactory conclusion about the role of the GnRH system in the feedback control of testicular function. Part of the problem may reside in the fact that we are steeped in the historical view that after gonadectomy, unrestrained GnRH neurons release increased quantities of the neurohormone into the hypophysial-portal vasculature to subserve a compensatory increase in gonadotropin secretion. The absolute necessity of GnRH for the support of normal gonadotropin secretion is unquestioned (34, 35); however, based on the present results, it would seem unlikely that the 10-fold increase in LH secretion after castration can be attributed to a dramatic alteration in the synthesis of the pro-GnRH molecule (6, 36).

If not by increased GnRH synthesis and secretion, how then can increased LH secretion after gonadectomy be explained? Several factors are undoubtedly involved. First, castration leads to alterations in the pattern of pulsatile LH (and presumably GnRH) release (6), and this is accompanied by increased pituitary sensitivity to GnRH, perhaps through an increase in GnRH receptor number (37–40). Therefore, one could expect increased LH release after the removal of the gonad, even if there were no change in the total amount of GnRH released. Second, even without a change in the rate of synthesis of the proGnRH molecule, its posttranslational processing or its rate of degradation could be altered by the presence of gonadal hormones, effectively changing the amount of mature GnRH available for release. Indeed, recent evidence testifies to this fact. It has been demonstrated that after orchidectomy there are alterations in the GAP/GnRH ratio (41). Roselli *et al.* (27) have also shown that castration brings about an increase in the

pro-GnRH/GnRH ratio. Together, these observations focus attention on events downstream of synthesis as sites for regulation of the GnRH neuron by steroid hormones.

We detected no overall difference between groups in either mRNA levels or in the number of GnRH-positive neurons, and when we analyzed GnRH neurons with respect to their anatomical location, no difference was found between intact and castrated rats. The relative number and distribution of GnRH neurons that we detected via *in situ* hybridization are similar to those observed with immunocytochemistry (32). For lack of a better system of partitioning groups of GnRH neurons we placed them in classical anatomical regions such as the MS, DBBV, MPOA, and LPOA. The GnRH system has not been shown to consist of different groups of neurons on a functional basis, but it is known that at least 70%, if not all, GnRH neurons throughout the forebrain and hypothalamus project to the median eminence (42). However, there may be two or more populations of GnRH neurons, including those that regulate anterior pituitary function and others that mediate behavioral or unknown actions of GnRH (42). Hence, there may be a subset of GnRH neurons wherein mRNA levels are affected by gonadal hormones, but the identification of these neurons will require more specific criteria to isolate them from the general population of GnRH cells.

The effect of gonadal steroid feedback onto GnRH mRNA has been studied in the female rat and, as in the male, the results are conflicting. A positive feedback effect of estradiol on GnRH mRNA abundance has been demonstrated (43), whereas another group (44) observed that estradiol decreased GnRH mRNA content. Further evidence for a negative feedback effect of ovarian steroids has been shown by Toranzo *et al.* (24) in that ovariectomy produces an increase in GnRH mRNA, and this augmentation of GnRH message can be prevented by replacement with either estradiol or dihydrotestosterone, implicating a role for both androgens and estrogens in the control of GnRH mRNA in the female. Overall, the steroid feedback control of GnRH mRNA in the female rat remains unresolved; studies of gonadal feedback in the female are confounded by estradiol having both positive and negative feedback effects on the hypothalamo-pituitary axis, and previous studies have not properly addressed this problem.

In summary, we have shown that neither the number of cells capable of expressing the GnRH gene nor the level of that expression change as a function of long term castration in the adult male rat. These results suggest that the compensatory rise in gonadotropin secretion after castration is not subserved by alterations in GnRH synthesis, but instead is mediated by alterations in the patterning of GnRH secretion, which can occur inde-

pendently of a measurable increment in steady-state messenger RNA levels.

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