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Neuroendocrine Mechanisms for Reproductive Senescence in the Female Rat

Gonadotropin-Releasing Hormone Neurons

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Reproductive aging in female rats is characterized by profound alterations in the neuroendocrine axis. The preovulatory luteinizing hormone (LH) surge is attenuated, and preovulatory expression of the immediate early gene *fos* in gonadotropin-releasing hormone (GnRH) neurons is substantially reduced in middle-aged compared with young rats. We tested the hypothesis that alterations in GnRH gene expression may be correlated with the attenuation of the LH surge and may be a possible mechanism involved in neuroendocrine senescent changes. Sprague-Dawley rats ages 4 to 5 mo (young), 12–14 mo (middle-aged), or 25 to 26 mo (old) were killed at 10:00 AM or 3:00 PM on proestrus, the day of the LH surge, or diestrus I in cycling rats, and on persistent estrus or persistent diestrus in acyclic rats. RNase protection assays of GnRH mRNA and GnRH primary transcript were performed. GnRH mRNA levels increased significantly with age, whereas GnRH primary transcript levels, an index of GnRH gene transcription, decreased in old compared to young and middle-aged rats. This latter result suggests that an age-related change in GnRH mRNA levels occurs independently of a change in gene transcription, indicating a potential posttranscriptional mechanism. On proestrus, GnRH mRNA levels increased significantly from 10:00 AM to 3:00 PM in young rats. This was in contrast to proestrous middle-aged rats, in which this afternoon increase in GnRH mRNA levels was not observed. Thus, the normal afternoon increase in GnRH mRNA levels on proestrus is disrupted by middle age and may represent a substrate for the attenuation of the preovulatory GnRH/LH surge that occurs in rats of this age, prior to reproductive failure.

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Introduction

Although female rodents do not experience menopause, they undergo a transition from regular ovulatory cycles to irregular cycles to acyclicity *(1,2). T*he mechanisms for these changes are largely unknown, and the relative contributions of the different levels of the reproductive axis (brain [hypothalamus], pituitary, or gonad) have not been completely elucidated. In female rats, it is likely that reproductive senescence is regulated to a large extent by neuroendocrine, as opposed to ovarian events, based on the following observations:

1. The ovary of an aged, acyclic rat is capable of ovulating in response to various stimuli, and contains functional follicles *(3,4).*
2. Pulsatile gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) release has been reported to change during aging *(5–8).*
3. The preovulatory GnRH/LH surge is significantly attenuated by middle age, prior to reproductive failure *(9–12).*
4. The expression of the immediate early gene *c-fos*, a marker of gene activation, is significantly lower in GnRH neurons during the preovulatory GnRH/LH surge in middle-aged compared with young rats *(13–15).*
5. Temporally, these neuroendocrine changes precede ovarian changes *(2).*

GnRH neurons in the preoptic area-anterior hypothalamus (POA-AH) of rodents are the key cells regulating reproductive function. Changes in GnRH release and gene expression play a critical role in the control of the timing of reproductive development and puberty, as well as in the maintenance of adult ovarian cycles *(16–20).* However, a direct role for GnRH neurons in reproductive senescence remains to be elucidated. Changes in the numbers of GnRH neurons with aging are probably not responsible for neuroendocrine senescence, because whereas some laborato-

Table 1
LH Levels (ng/mL) in Intact Rats[a]

Age group	Cycle stage	LH (10:00 AM)	LH (3:00 PM)
Young (4 to 5 mo)	Proestrus	0.34 ± 0.04	0.29 ± 0.06
	Diestrus I	0.52 ± 0.10	0.27 ± 0.06
Middle-aged (12 to 14 mo)	Proestrus	0.55 ± 0.13	0.59 ± 0.14
	Diestrus I	0.50 ± 0.10	0.35 ± 0.03
	Persistent estrus	0.58 ± 0.17[b]	0.69 ± 0.15[b]
	Persistent diestrus	0.45 ± 0.04	0.28 ± 0.07
Old (25 to 26 mo)	Persistent estrus	0.81 ± 0.22[b]	0.89 ± 0.31[b]
	Persistent diestrus	0.45 ± 0.09	0.45 ± 0.20

[a]Data are expressed as mean ± SEM.
[b]$p < 0.05$ vs persistent diestrous rats.

ries have reported small decreases in the number of GnRH neurons with aging *(21,22)*, others have reported no such change *(8,23,24)*. Studies on changes in GnRH release and gene expression with aging are similarly controversial, with some reporting increases *(25)*, others decreases *(26–28)*, and still others no change *(5–8,29,30)* in these parameters.

In the present study, we tested the hypothesis that alterations in GnRH neurons, specifically GnRH gene expression, occur during reproductive senescence, concomitantly with the attenuation of the preovulatory GnRH/LH surge in middle-aged rats. We measured changes in GnRH mRNA levels, and GnRH primary transcript levels, an index of GnRH gene transcription (*31*), in intact female rats at young, middle-age, and old age, and of differing reproductive cycling statuses.

Results

Serum LH Levels in Aging Intact Rats

Serum LH levels were measured by radioimmunoassay (RIA) and were found to be low, but within the detectable range of the RIA, in intact female Sprague-Dawley rats at all ages and reproductive statuses (Table 1). An overall effect of age on serum LH was found ($p < 0.05$), with higher LH levels in old than young rats. In cycling rats (proestrus and diestrus I), no significant effects of age, cycle stage, or time of day were observed. In acyclic rats (persistent estrus and persistent diestrus), a significant effect of cycle stage was detected ($p < 0.005$), with significantly higher levels in persistent estrous rats than in persistent diestrous rats. No effects of time of day or age were found in persistent estrous or persistent diestrous rats, and no significant interaction among these variables was found. Comparisons of the effects of reproductive status on LH levels in middle-aged rats demonstrated no significant effects. These results confirm that proestrous animals were sacrificed before the preovulatory LH surge, as we have reported previously *(32)*.

Serum Estradiol Levels in Aging Intact Rats

Table 2 gives serum estradiol levels as measured by RIA for intact female Sprague-Dawley rats. A significant effect of age on serum estradiol levels was observed with significantly higher levels in old than in middle-aged or young rats ($p < 0.05$). In cycling (proestrus and diestrus I) rats, no significant effect of age but a significant effect of cycling status on serum estradiol levels was observed ($p < 0.005$). Post-hoc analysis indicated that estradiol levels were significantly higher in proestrous than in diestrous I rats ($p < 0.01$). There was no effect of time of day on estradiol levels in these cycling animals ($p = 0.16$), nor any interaction of time of day with cycling status or age. In acyclic rats (persistent estrus and persistent diestrus), although there was no effect of age or cycling status on estradiol levels, a significant effect of time of day was observed ($p < 0.05$), owing to significantly lower estradiol levels at 3:00 PM than at 10:00 AM in old rats ($p < 0.05$). In middle-aged rats, no effect of cycling stage or time of day was observed.

GnRH Cytoplasmic mRNA Levels

Figure 1 is an autoradiogram showing GnRH and cyclophilin mRNA in representative POA-AH dissections of individual rats. Results on GnRH mRNA levels were identical whether expressed as absolute amounts of GnRH mRNA, or when normalized to cyclophilin. Cyclophilin mRNA levels did not vary by age, time of day, or reproductive status. Because the same amount of RNA was run in each lane (20 μg), we chose to express our results as femtograms of GnRH mRNA, in order to facilitate comparisons with GnRH primary transcript (below). A significant effect of age on GnRH mRNA levels was found, with higher levels in middle-aged and old compared with young rats (Table 3; $p < 0.05$). In cycling (proestrus and diestrus I) rats, a significant effect of age was seen ($p < 0.05$), with significantly higher levels in middle-aged than young cycling rats (Fig. 2). No effect of cycling stage on GnRH mRNA was seen ($p = 0.53$), nor an interaction of age with cycling stage ($p = 0.49$). For time of day, no overall effect was observed; however, an interaction of time of day with age and cycling stage was observed ($p < 0.05$). Post-hoc analysis demonstrated that young proestrous rats had higher GnRH mRNA levels at 3:00 PM than at 10:00 AM ($p < 0.05$).

Table 2
Estradiol Levels (pg/mL) in Intact Rats[a]

Age group	Cycle stage	Estradiol (10:00 AM)	Estradiol (3:00 PM)
Young (4 to 5 mo)	Proestrus	18 ± 7[b]	17 ± 4[b]
	Diestrus I	8 ± 0	8 ± 1
Middle-aged (12 to 14 mo)	Proestrus	14 ± 6[b]	26 ± 6[b]
	Diestrus I	7 ± 1	13 ± 2
	Persistent estrus	21 ± 5	21 ± 4
	Persistent diestrus	18 ± 2	15 ± 5
Old (25 to 26 mo)	Persistent estrus	28 ± 3	21 ± 4
	Persistent diestrus	30 ± 3	15 ± 3

[a]Data are expressed as mean ± SEM.
[b]$p < 0.01$ vs diestrus I.

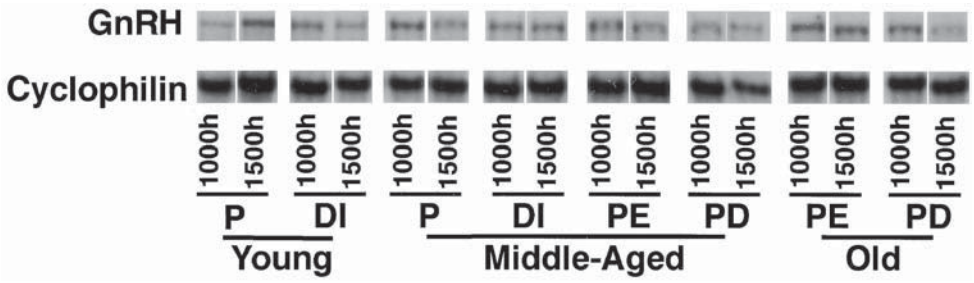

Fig. 1. Composite autoradiogram showing representative examples of GnRH mRNA (**top**) and cyclophilin mRNA (**bottom**) in cytoplasmic POA-AH RNA samples from individual rats at 10:00 AM and 3:00 PM on different days of the estrous cycle and in acyclic rats. Each lane contains RNA from a single animal. P, proestrus; DI, diestrus I; PE, persistent estrus; PD, persistent diestrus.

Table 3
GnRH mRNA and Primary Transcript Levels (fg) in Intact Rats[a]

Age group	GnRH mRNA	GnRH primary transcript
Young (4 to 5 mo)	176 ± 10	28 ± 3
Middle-aged (12 to 14 mo)	210 ± 10[b]	30 ± 2
Old (25 to 26 mo)	239 ± 16[b]	21 ± 1[c]

[a]Data are expressed as mean ± SEM.
[b]$p < 0.05$ vs corresponding young groups.
[c]$p < 0.05$ vs corresponding young and middle-aged groups.

Additionally, middle-aged proestrous rats had higher GnRH mRNA levels than young proestrous rats at 10:00 AM. This is probably responsible for the significant effect of age on GnRH mRNA levels in cycling rats. For acyclic rats (persistent estrus and persistent diestrus), no significant effect of age was observed ($p = 0.09$). However, a significant effect of cycle stage ($p < 0.05$) was determined, with higher levels in persistent estrous than persistent diestrous rats ($p < 0.05$). No effect of time of day was found ($p = 0.52$), although there was a significant interaction of time of day with age and cycling stage ($p < 0.05$), with post-hoc analysis demonstrating that GnRH mRNA levels were significantly lower at 3:00 PM compared to 10:00 AM in old persistent diestrous rats ($p < 0.05$). In middle-aged rats, no effects of cycle stage ($p = 0.39$) or time of day ($p = 0.69$) or an interaction of these variables ($p = 0.38$) were observed (Fig. 2).

GnRH Nuclear Primary Transcript Levels

Figure 3 is an autoradiogram showing GnRH primary transcript in individual POA-AH samples. A significant effect of age was seen, with old rats having significantly lower GnRH primary transcript levels than young and middle-aged rats ($p < 0.05$; Table 3). For cycling (proestrus and diestrus I) rats, no significant differences in GnRH primary transcript were seen (Fig. 4). For acyclic rats (persistent estrus and persistent diestrus), a significant effect of age ($p < 0.05$), but no effect of cycle stage ($p = 0.32$), or an interaction of cycle stage with age ($p = 0.15$), was seen (Fig. 4). Additionally, no effect of time of day, nor any

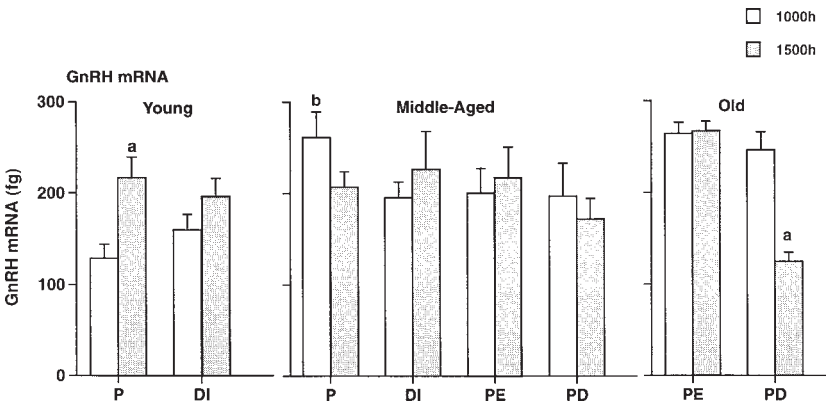

Fig. 2. Changes in GnRH mRNA levels (fg/20 μg) in young, middle-aged, and old female rats of differing cycling statuses and times of day. GnRH mRNA levels were significantly elevated at 3:00 PM compared with 10:00 AM in young proestrous rats, and lower at 3:00 PM compared with 10:00 AM in old persistent diestrous rats. Middle-aged proestrous rats had higher GnRH mRNA levels than young proestrous rats at 10:00 AM and middle-aged diestrous I rats at 10:00 AM. a, $p < 0.05$ vs 10:00 AM. b, $p < 0.05$ vs corresponding young rats. P, proestrus; DI, diestrus I; PE, persistent estrus; PD, persistent diestrus.,

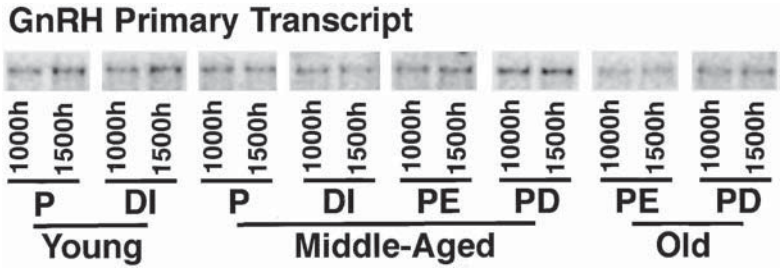

Fig. 3. Composite autoradiogram showing representative examples of GnRH primary transcript in nuclear POA-AH RNA isolated from individual rats killed at 10:00 AM or 3:00 PM on different days of the estrous cycle, or in acyclic rats. Each lane contains RNA from a single animal. P, proestrus; DI, diestrus I; PE, persistent estrus; PD, persistent diestrus.

interaction of time with any other parameter, was seen. For middle-aged rats, no effect of cycling status ($p = 0.23$), time of day ($p = 0.85$), or an interaction of these parameters ($p = 0.17$) was found.

Discussion

The results of the present study indicate that GnRH gene expression changes during reproductive senescence in intact female Sprague-Dawley rats. Overall, GnRH mRNA levels increase significantly with aging, with higher levels in middle-aged and old than in young rats. With respect to proestrus, the day of the preovulatory LH surge, the increase in GnRH mRNA levels seen from 10:00 AM to 3:00 PM in young rats does not occur in middle-aged rats. This latter change suggests that there is a decreased drive on GnRH neurons during reproductive aging that may be related to, or even responsible for, the transition to acyclicity *(13,15,33)*. We also examined the mechanism for the changes in GnRH mRNA levels and determined that activation of GnRH gene transcription is unlikely to be responsible for these changes, because GnRH primary transcript levels do not reflect GnRH mRNA levels. Instead, we postulate that a post transcriptional mechanism may underlie these age-related changes in GnRH mRNA levels.

Our observation that GnRH mRNA levels increase with aging in female Sprague-Dawley rats is consistent with one made in humans in which GnRH mRNA levels, as determined by *in situ* hybridization (ISH), were reported to be lower in premenopausal (ages 21–41) compared with postmenopausal (ages 54–86) women (*25*). In the human, it is speculated that an increase in GnRH gene expression may be a result of the removal of ovarian negative feedback in postmenopausal women. However, humans and rats differ in that aging rats do not experience follicular atresia, and estradiol levels do not decrease with aging in the rat. Indeed, in the present study, we observed an overall increase in circulating estradiol levels with aging (Table 2). Thus, it is possible that rather than absolute estradiol levels exerting an effect on GnRH gene expression, it is the cyclic fluctuations in estradiol that occur during the cycle that may have an impact on overall GnRH mRNA levels. Future experiments are necessary to test this hypothesis, e.g., by inducing cycles in aging rats (which can be accomplished

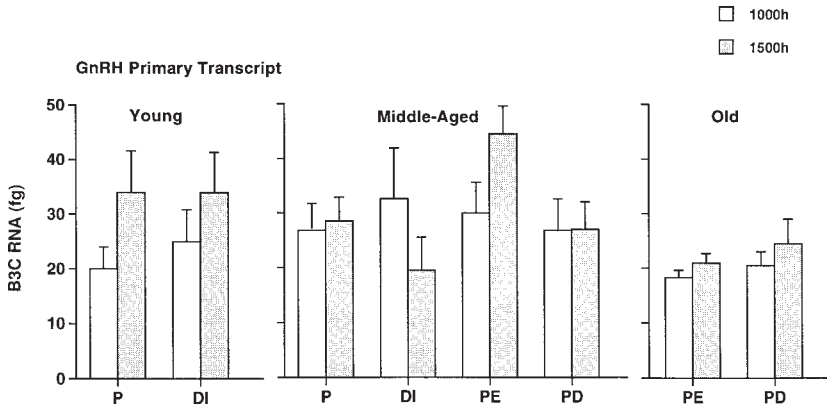

Fig. 4. GnRH primary transcript levels at differing cycling statuses and times of day.

by neurotransmitter or behavioral manipulations) and comparing them to acyclic rats of the same age. Moreover, animals in the present study were examined only on proestrus and diestrus I, and the age-related increase in GnRH mRNA occurred only in proestrous rats. We do not know how GnRH mRNA may change with aging on the other days of the estrous cycle, namely, estrus and diestrus II. It is possible that the overall increase in GnRH mRNA from the young to the middle-aged group, which is owing primarily to the increase on proestrus, would be altered by the inclusion of these additional groups.

The present finding of an increase in GnRH mRNA levels in rats, using the RNase protection assay, differs from a report demonstrating a significant decrease, as determined by ISH (*28*), in overall GnRH mRNA levels in ovariectomized, steroid-primed Sprague-Dawley rats. Another group reported decreased GnRH mRNA levels between old (18 mo) and young (~2 mo) Sprague-Dawley rats killed on the morning of diestrus I (*34*), also as determined by ISH. Differences among studies may be owing to differing ovarian or hormonal statuses, or to different techniques. An example of the effects of differing hormonal status is demonstrated by a report documenting age-related decreases in GnRH mRNA levels in intact but not castrate male F344 rats (*26*). Preliminary data from our laboratory indicate that GnRH mRNA levels do not differ among young, middle-aged, and old ovariectomized rats (*35*), in contrast to the present study done in intact rats of similar ages. With respect to differences in techniques, our study provides data on changes in GnRH mRNA levels in virtually the entire GnRH neurosecretory population, because our dissection contains most of these cells. By contrast, ISH hybridization studies provide important data regarding the localization of changes in GnRH gene expression in specific subpopulations of these cells (*28*). Thus, it is quite possible that certain subsets of GnRH neurons may undergo age-related decreases, or no changes, in GnRH gene expression, whereas the total population of GnRH neurons may exhibit an increase in gene expression.

Although hypothalamic GnRH content was not measured in the present study, it has been measured by other laboratories in the context of aging. This area is also controversial. One laboratory did not observe any differences in GnRH content in the medial basal and anterior hypothalamus between young (3 to 4 mo) and old (18–20 mo) ovariectomized female Wistar rats *(29)*. Another group found a decrease in GnRH peptide concentrations in the median eminence of middle-aged compared with young rats at the beginning of the LH surge on proestrus *(36)*. However, another laboratory reported slightly or significantly higher GnRH peptide levels, depending on the endocrine condition, in middle-aged (9–16 mo) compared with young (3 to 4 mo) rats, in the anterior hypothalamus or medial basal hypothalamus, but not the POA-AH *(30,37)*. Nevertheless, the observation that GnRH peptide levels can be elevated in the anterior hypothalamus and median eminence in middle-aged rats corresponds to our present observation of increased GnRH mRNA levels in middle-aged compared with young rats.

In the present study, we also investigated the effects of time of day on GnRH gene expression by measuring levels of GnRH mRNA at a morning (10:00 AM) and an afternoon (3:00 PM) time point. These time points were chosen based on previous observations of our laboratory that GnRH mRNA levels are significantly elevated at 3:00 PM on proestrous compared to earlier (11:00 AM) and later (6:00 AM) time points (*32*), and a similar finding was made by another group (*38*). Unfortunately, the large number of groups of animals for comparisons prevented us from looking at additional time points, which may have been enlightening. We observed a significant increase in GnRH mRNA levels from 10:00 AM to 3:00 PM in young proestrous rats, which was not present in middle-aged proestrous rats. These data are consistent with those of Rubin et al. *(28)*, who

demonstrated alterations in GnRH mRNA levels with time of day in young but not middle-aged ovariectomized rats, similar to our finding in intact rats. Our laboratory and others have also reported afternoon increases in GnRH gene expression in young female rats on proestrus ([*32,38–41*]; reviewed in refs. *42* and *43*). The abolition of this rhythm in middle-aged rats may be related to the attenuated preovulatory LH surge *(13,15,33)*, as well as the diminution of Fos expression in GnRH neurons in middle-aged rats *(13–15)*.

A significant effect of time of day was also observed in old persistent diestrous rats, which, in contrast to young proestrous rats, exhibited an afternoon decrease in GnRH mRNA levels. We do not know the physiologic significance of this morning-afternoon difference; however, it is interesting that this same group of rats also exhibited an afternoon decrease in estradiol levels, which was not seen in any other age group. It is unknown whether there is a relationship between the parallel afternoon decrease in GnRH mRNA and in serum estradiol levels in old persistent diestrous rats, and this merits future investigation.

The mechanism for the regulation of GnRH mRNA levels in vivo appears to be primarily posttranscriptional (reviewed in ref. *42*), as indicated by a lack of change in GnRH primary transcript levels. There is currently no direct evidence that GnRH primary transcript levels are reflective of GnRH gene transcription. Nevertheless, studies in the immortalized GT1-7 cells comparing changes in GnRH primary transcript to GnRH gene transcription as measured by nuclear run-on assays indicate virtually identical regulation by factors such as phorbol ester or calcium secretagogues *(31,44–46)*. Because nuclear run-on assays are extremely difficult to perform in animal tissue, we have extrapolated from the data on the GT1 cells that GnRH primary transcript levels are an accurate reflection of GnRH gene transcription. Under many circumstances, GnRH mRNA levels change independently of changes in GnRH primary transcript levels *(32,45,47–49)*. We have also explored whether the processing of GnRH primary transcript to the mature GnRH mRNA is affected by those factors that regulate GnRH mRNA levels, and this does not appear to be the case *(47)*. We have reported in the GT1-7 cell line that GnRH poly (A) tail length, an indicator of mRNA stability *(50,51)*, is altered by various secretagogues *(45,52)*. Preliminary data suggest that GnRH poly (A) tail length in the rat changes under circumstances in which GnRH mRNA levels, but not GnRH primary transcript levels, change (*53*). Taken together, these studies provide evidence for GnRH primary transcript levels being a reflection of GnRH gene transcription, and a possible posttranscriptional mechanism for the alteration of GnRH mRNA levels, i.e., poly (A) tail length.

GnRH primary transcript levels were quantified in the same aging rats in which GnRH mRNA levels were measured. We found that GnRH primary transcript levels decrease with aging, with lower levels in old than in young or middle-aged rats. This is in contrast to the increase in GnRH mRNA levels that occurs during aging in the same rats of the present study. Thus, the alterations in GnRH mRNA levels, occurring independently of a change in GnRH gene transcription, are most likely due to a change in GnRH mRNA stability. Such a posttranscriptional mechanism has also been postulated to occur during early development and puberty in rats and mice *(49,54,55)* and in response to *N*-methyl-D-aspartate (NMDA) receptor activation *(47)*. In a previous study, we observed that GnRH primary transcript levels increased significantly between 11:00 AM and 3:00 PM *(32)*, and a similar result was found by another laboratory using ISH *(40)*. Nevertheless, in the present study, although a trend toward an increase in GnRH primary transcript levels from 10:00 AM to 3:00 PM was observed, it did not attain significance. This may be because overall no effects of reproductive status nor time of day on GnRH primary transcript levels were observed, possibly owing to large variabilities among groups, and, thus, small but consistent differences within groups could not be detected statistically.

Regarding effects of aging on posttranscriptional regulation of mRNA, it has been reported by Colman et al. *(56)* that total poly (A) mRNA stability and complexity does not change with aging in brains of Sprague-Dawley and Fischer 344 male rats (*56*). They conclude that age-related changes in gene expression are not expressed throughout the brain, and this latter result suggests that alterations in mRNA stability, as seen for the GnRH system, may be restricted to specific populations of neurons or nonneuronal cells in the brain. Other genes, such as vasopressin and prolactin, are also regulated at a posttranscriptional level *(57,58)*, and proenkephalin can be regulated at a posttranslational level *(59)*. Again, these latter results suggest that an increase in GnRH mRNA stability with aging may be specific to these neurons. Another article reported that poly (A) mRNA size decreases with aging in quail oviduct and other non-neuronal tissues *(60)*. However, it remains to be proven whether GnRH mRNA in aging rats undergoes any changes in poly (A) tail size.

Changes in neuroendocrine function during aging could be owing to changes intrinsic to GnRH neurons such as alterations in GnRH gene expression and/or release, as well as to changing inputs to GnRH neurons from other neurotransmitters, neuropeptides, growth factors, and steroid hormones. Alterations in neuropeptide Y (NPY) gene expression in the medial basal hypothalamus (MBH) have been reported in middle-aged compared with young Sprague-Dawley rats, and, indeed, the diurnal rhythm of NPY mRNA is abolished in middle-aged rats on proestrus *(33)*. Because NPY is believed to play an important role in the regulation of GnRH neurons *(61–63)*, this change in NPY neurons may be involved in the change in GnRH

mRNA levels and release that occurs during reproductive aging. Additionally, age-related alterations in catecholamine turnover *(10)* and changes in morphologic interactions between GnRH and catecholaminergic neurons *(64)* have been reported. Similarly, several laboratories have been investigating the role of the NMDA receptor and have seen age-related decreases in the responsiveness of GnRH neurons to NMDA receptor activation *(5,7,65,66)*. We are actively pursuing the role of changing NMDA receptor input to GnRH neurons in aging rats *(67)*.

Inputs from the suprachiasmatic nucleus (SCN), the circadian pacemaker, to GnRH neurons may underlie the rhythmic changes in GnRH mRNA levels observed in the present study. Thus, the attenuation of the afternoon increase in GnRH mRNA that is observed in young but not middle-aged proestrous rats may be due to decrements in SCN function. Indeed, it is well established that the SCN undergoes age-related decreases in rhythmic function (reviewed in ref. *68*). SCN neurons have been reported to make direct synaptic contacts on GnRH neurons *(69,70)*, and this is a possible anatomical substrate for this diurnal change in GnRH neuronal function. Alterations in GnRH mRNA levels, Fos expression in GnRH neurons (*13–15*), and the preovulatory LH surge (*9–12*) in middle-aged rats on proestrus are consistent with a diminution of circadian drive from the SCN.

In summary, the present study has demonstrated that GnRH mRNA levels increase during reproductive aging in ovarian-intact female rats. GnRH gene transcription decreases at this time, suggesting a possible posttranscriptional mechanism for the increase in GnRH mRNA levels. On proestrus, young animals experience an increase in GnRH mRNA levels shortly before the preovulatory LH surge, which is not seen in middle-aged animals on proestrus. This age-related change in GnRH gene expression is consistent with other alterations that occur in middle-aged rats such as a diminished preovulatory LH surge and decreased fos expression in GnRH neurons *(9,11–15,71)*. Taken together, these findings suggest a change in the drive from the GnRH system that precedes the transition to acyclicity.

Materials and Methods

Animals

Female Sprague-Dawley rats were purchased at 3 to 4 mo (young), 10–12 mo (middle-aged), or 24 mo (old) of age from Harlan Sprague-Dawley (Indianapolis, IN). Rats were housed two per cage in a room with a controlled temperature and light cycle (12 h light: 12 h dark, lights on at 7:00 AM), and provided food and water ad libitum. Six to 8 animals were used for each group. Animals were monitored for estrous cyclicity or acyclicity by daily vaginal smear for at least 3 wk before experimentation, and this monitoring was continued throughout the duration of experimentation. Young rats with regular 4-d and middle-aged rats with regular 4- or 5-d estrous cycles were used in cycling groups. Approximately half the cycling middle-aged animals exhibited 4- or 5-d cycles, and there was no difference in GnRH gene expression between these groups. All young and middle-aged rats were virgins, and some old rats were virgins and others retired breeders. However, no differences in GnRH gene expression were observed based on previous breeding status, and, thus, old rats were pooled for analyses.

Animals were killed rapidly by decapitation on proestrus, diestrus I, persistent estrus, or persistent diestrus at 10:00 AM or 3:00 PM. These time points were chosen based on previous observations in our laboratory of a significant increase in GnRH mRNA from 11:00 AM to 3:00 PM in young proestrous rats (*32*). The brain was removed, and the POA-AH was dissected using a stainless steel brain slicer (model RBM-4000, Activational Systems, Warren, MI), snap-frozen on dry ice, and stored at –70° C until use. The POA-AH was dissected as described previously *(49,72)* and as follows: The caudal border was made by a coronal cut just posterior to the entry point of the optic chiasm. The rostral border was exactly 4 mm anterior, made by a coronal cut at the posterior third of the olfactory tubercle. This coronal section (4 mm thick) was laid rostral side up on a chilled glass plate. An isosceles triangle–shaped cut was made with the apex of the triangle just under the midline of the corpus callosum, and the two legs of the triangle passing through the anterior commissure. Trunk bloods were collected, allowed to clot, centrifuged, and serum stored at –70° C.

RNA Extraction and RNase Protection Assay

RNA from frozen POA-AH dissections was extracted exactly as described previously using a double-detergent lysis buffer system *(47,73)*. A similar amount of RNA was recovered from POA-AH dissections of animals of different ages, with 37.9 $\pm$ 2.2 μg, 33.7 $\pm$ 1.5 μg, and 31.5 $\pm$ 3.8 μg of RNA in young, middle-aged, and old POA-AH dissections, respectively (no significant difference among groups). Cytoplasmic and nuclear RNA from individual POA-AH dissections were suspended in 20 μL of hybridization solution (0.1 *M* EDTA, pH 8.0, and 4 *M* guanidine thiocyanate; final pH 7.5) for RNase protection assay. The following DNA clones were used to make riboprobes:

1. GnRH complementary DNA (cGnRH), 362 bp in length, spanning the *Hind*III site in exon 1 to the *Bam*HI site in exon 4, and subcloned into a pBS(+) vector (Stratagene, La Jolla, CA) to measure GnRH mRNA in the cytoplasm *(72)*;
2. A proGnRH (B3C) genomic fragment spanning 506 bp of the intron B-exon 3-intron C junction and subcloned in the *Eco*RI and *Hind*III sites of a pBS(+) vector to measure GnRH primary transcript in the nucleus *(47)*, an index of GnRH gene transcription *(31)*;

3. Cyclophilin (1B15) measured using a 111-bp cDNA clone, spanning from the *Pst*I and *Xmn*I restriction sites and subcloned in a Bluescript KS(+) vector *(72)*.

Solution hybridization/RNase protection assay was performed as described previously *(47,73)*. Briefly, GnRH cDNA and B3C probes were labeled with [α-^{32}P]UTP to high specific activity (~1,300,000 cpm/ng) and cyclophilin to low specific activity (~60,000 cpm/ng). The probes were brought to a final volume of 5 μL and added to samples and standards for a final volume of 25 μL (20 μL of RNA and 5 μL of probe). Twenty micrograms of each cytoplasmic sample were incubated with GnRH and cyclophilin probes in the same tubes, and all cytoplasmic samples were assayed in a single assay. Measurements of GnRH primary transcript were accomplished in a single assay, utilizing the entire nuclear fraction of an individual POA-AH dissection dissolved in 20 μL of hybridization buffer, to which 5 μL of probe was added. For standard curves, probes were mixed with increasing known amounts of reference RNAs. Samples and standards were allowed to hybridize for 16–18 h at 30° C; the remainder of the assay was conducted exactly as described previously *(47,73)*. Gels were exposed to X-ray film for 18–48 h to produce an autoradiogram, and to a phosphor imaging screen (Molecular Dynamics, Sunnyvale, CA) for 18 h for quantitation. The amount of radioactivity in each sample was determined by comparison with the amount of reference RNA in standard curves as calculated by regression analysis.

RIA of LH

LH levels in serum samples were determined in a single RIA in duplicate samples by double-antibody RIA (*74*) using the rat LH RP-3 standard from the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) kindly provided by Dr. A. Parlow. The intraassay coefficient of variation was 6.9%.

RIA of Estradiol

Estradiol levels in serum samples were determined by RIA of duplicate samples using the DSL ultrasensitive estradiol RIA kit (DSL-4800) according to the instructions. Assay sensitivity was 5 pg/mL, interassay variation was 4.6% and intraassay variation ranged from 1 to 3%.

Statistical Analyses

Differences in GnRH mRNA, GnRH primary transcript RNA, LH, and estradiol levels were analyzed. In all cases, an *F*-test on equality of variances was performed for the relevant variable. If this assumption was met, then the statistic was performed. First, an overall effect of age on each parameter was determined by one-way analysis of variance (ANOVA). Then, comparisons were made between animals of similar cycling status, first using two-way ANOVA. Thus, effects were compared in young and middle-aged cycling animals (proestrus or diestrus I), or in acyclic middle-aged and old animals (persistent estrus or persistent diestrus). Additionally, comparisons were made between middle-aged cycling and acyclic groups in order to examine the effects of reproductive status in animals of a similar age. In the case of GnRH primary transcript, significant differences in variance were determined, and, therefore, paired data were analyzed using a *t*-test for independent samples with unequal variances. Post-hoc comparisons were performed using Fisher's Protected Least Significant Difference analysis. Significance was set at $p < 0.05$.

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