The Role of RFamide-Related Peptide-3 in Age-Related Reproductive Decline in Female Rats

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Reproductive senescence, the point in time when females cease to show estrous cyclicity, is associated with endocrine changes in the hypothalamus, pituitary, and gonads. However, the mechanisms triggering this transition are not well understood. To gain a better understanding of the top-down control of the transition from reproductive competence to a state of reproductive senescence, we investigated middle-aged female rats exhibiting varying degrees of reproductive decline, including individuals with normal cycles, irregular cycles, and complete cessation of cycles. We identified hormonal changes in the brain that manifest before ovarian cycles exhibit any deterioration. We found that females exhibit an increase in RFamide-related peptide-3 (RFRP3) mRNA expression in the hypothalamus in middle age prior to changes in estrous cycle length. This increase is transient and followed by subsequent decreases in kisspeptin (*KiSS1*) and gonadotropin-releasing hormone (*GnRH*) mRNA expression. Expression of *RFRP3* and its receptor also increased locally in the ovaries with advancing age. While it is well known that aging is associated with decreased GnRH release and downstream disruption of the hypothalamic–pituitary–gonadal (HPG) axis, herein, we provide evidence that reproductive senescence is likely triggered by alterations in a network of regulatory neuropeptides upstream of the GnRH system.

Keywords: GnIH, RFRP3, GnIH/RFRP3, aging, reproductive senescence

INTRODUCTION

Female reproduction is a complex process that requires precise neurochemical timing. From puberty through senescence, a complex interplay among central neuroendocrine circuits, peripheral hormonal systems, and internal and external cues is required for successful procreation. The hormonal regulation that is involved in successful reproductive functioning is well studied. However, less is known about the neural and hormonal mechanisms initiating the degradation of the reproductive axis as females approach reproductive senescence.

Reproductive senescence in females is defined as the point in an organism's life when it ceases to show regular cyclic ovarian activity, exiting from an active reproductive stage to an inactive reproductive life, akin to menopause in humans. In humans, menopause is characterized as three stages – premenopausal, when females still maintain regular menstrual cycles; perimenopausal,

when cycles become increasingly irregular; and postmenopausal, defined as 1 year post amenorrhea (1) – and is associated with specific endocrine changes in both the brain and the ovaries (2–4). Rats represent a useful, non-human model for the study of menopause as they experience reproductive decline analogous to that of humans. With advancing age, middle-aged rats transition from regular 4- to 5-day estrous cycles to irregular cycle lengths of over 6 days, followed by a complete cessation of cyclicity, characterized as either a state of persistent estrus or diestrus. This final transition occurs in female rats between 12 and 15 months of age (5), appropriately corresponding to about 45–50 years old in women (4). The hypothalamic–pituitary–gonadal (HPG) axis is highly conserved across mammals, and the hypothalamic and ovarian changes in the rodent resemble menopausal changes in humans (6).

In female rodents, ovulation requires the precisely timed release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. In rats, the transition to reproductive senescence is associated with reductions in GnRH neuron activity (7, 8) and secretion (9, 10) on the day of the preovulatory luteinizing hormone (LH) surge, resulting in an attenuation of LH and ovulation (10, 11). These changes are likely due to a modification in the network of upstream hypothalamic mediators of GnRH function. In particular, two neuropeptides identified in the past 15 years have been shown to regulate GnRH in opposing ways. Kisspeptin (the product of the *KiSS1* gene) found in the anteroventral periventricular (AVPV) and arcuate nuclei stimulates GnRH release, whereas RFamide-related peptide-3 (RFRP3) found in the dorsal medial hypothalamus of rodents acts to inhibit GnRH release (12–14). Kisspeptin mRNA levels decrease with aging (15), along with a decrease in the number of immunoreactive kisspeptin neurons in the AVPV in middle age (16). In contrast, the role of RFRP3 in female reproductive aging is yet to be discovered.

In this study, we examined *RFRP3* expression in regularly cycling young (3 months old), regularly cycling middle aged (8 months old), irregularly cycling (10 months old), and acyclic (12 months old) Long–Evans female rats. To explore the possibility that alterations in neurochemical systems upstream of GnRH neurons initiate the transition to reproductive senescence, we measured *RFRP3*; its receptors *GPR147*, *KiSS1*, and *GnRH*; and pituitary gonadotropin mRNA expression throughout aging. We found that *RFRP3* expression increased in middle-aged animals along with reductions in *KiSS1* and *GnRH* expression with advancing age. Expression of *RFRP3* and *GPR147* also increased with age in the ovaries. These data reveal an association between alterations in the RFRP3 and kisspeptin systems with age-related reproductive decline both centrally and peripherally.

MATERIALS AND METHODS

Experimental Subjects

Adult female Long–Evans rats were housed in trios and exposed to a 12/12-h light–dark cycle. Lights came on at 0700 hours and *ad libitum* food and water were available. For all studies, rats were acclimated to their housing conditions for a week, and then vaginal smears were obtained daily to determine cyclicity for 30 days before the studies commenced. All tissue was collected between the hours of 0900 and 1200 hours. In the irregularly cycling group, only females that showed irregular cycles for at least 15 of the 30 days (cycles above 6 days in length) were used in the 10-month-old group ($n = 21$ of a total of 30 animals measured). For the acyclic group, only 12-month-old animals that exhibited persistent estrus or diestrus for at least 15 of the 30 days were used ($n = 12$ of a total of 18 animals measured). "Young" control animals were measured in the diestrus phase of the estrous cycle. All animal care and procedures were approved by the University of California Berkeley Animal Care and Use Committee.

Real-time Reverse Transcriptase PCR

Rats were lightly anesthetized with isoflurane and rapidly decapitated before bilateral hypothalami (dissected from bregma −2.5 to bregma −3.5), pituitary, and whole ovaries were dissected on ice in RNA-later (Thermo Fisher Scientific, Santa Clara, CA, USA) and flash-frozen in liquid nitrogen. For all studies, total RNA was extracted using Trizol extraction methods and purified with DNase (DNA-free, Ambion). cDNA was synthesized following the manufacturer's instructions for iScript cDNA synthesis kit (BioRad), and then RT-PCR was run using the manufacturer's instructions for SsoAdvanced SYBR supermix (BioRad). Samples were run in a BioRad CFX96 real-time PCR system. After the PCR was complete, specificity of each primer pair was confirmed using melt curve analysis, and samples run on a 2% ethidium bromide agarose gel with a 50-bp DNA ladder (Invitrogen) to verify the generation of a single product of correct size. Rat primers were designed using the NCBI Primer BLAST software, which verifies specificity. The primer efficiencies were determined by standard curve, and C_t values were determined using PCR miner (17) and normalized to the ribosomal reference gene, ribosomal protein L16P (RPLP). There were no significant differences in RPLP values across any groups. Fold change was evaluated using the delta delta C_T method as outlined in Pfaffl 2001 (18).

Primer sequences are given below.

Primer	Forward	Reverse	Temp	Product size
RPLP	ATCTACTCCG CCCTCATCCT	GCAGATGAGG CTTCCAATGT	55	159
RFRP3	CCAAAGGTTT GGGAGAACAA	GGGTCATGGC ATAGAGCAAT	55	110
GPR147	GGTCAGAACG GGAGTGATGT	AGGAAGATG AGCACGTAGGC	55	119
LHβ	GCAAAAGCCA GGTCAGGGATAG	AGGCCCAC ACCACACTTGG	55	92
FSHβ	TTCAGCTTTCCC CAGGAGAGATAG	ATCTTATGGTCT CGTACACCAGCT	55	305
Cga	CTATCAGTGTATG GGCTGTTG	CTTGTGGTAG TAACAAGTGC	55	199
KISS1	TGGCACCTGTG GTGAACCCTG	ATCAGGCGAC TGCGGGTGGCA	61.4	202
GnRH	GCAGATCCCTA AGAGGTGAA	CCGCTGTTGT TCTGTTGACT	55	201

Statistical Analysis

Differences in gene expression examined *via* RT-PCR were analyzed by a one-way ANOVA followed by Tukey's multiple comparison test for *post hoc* analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Statistics were performed using the Prism software.

RESULTS

Hypothalamic Neuropeptide and Receptor Expression Level in the Hypothalamus across the Reproductive Span

To characterize changes in the reproductive axis during the transition from reproductive competence to reproductive senescence in female rats, naturally aging rats that exhibited different stages of reproductive decline were utilized. Expression profiles in four groups were examined: a 3-month regularly cycling group labeled "young," an 8-month regularly cycling group referred to as "middle-aged cycling," a 10-month group of irregularly cycling rats referred to as "irregularly cycling," and a 12-month group of acyclic animals referred to as "acyclic."

Hypothalamic *RFRP3* mRNA levels were significantly higher in middle-aged and acyclic females compared with young cycling females (relative fold change, 8 months old, 2.15 ± 0.23, 12 months old, 1.97 ± 0.25, **Figure 1A**). *RFRP3* was significantly lower in irregularly cycling females (10 months old, 0.24 ± 0.04,

Figure 1A) compared with all other groups. Expression of the RFRP3 receptor, *GPR147*, was significantly higher in middle-aged animals regularly cycling females, relative to young controls (relative fold change, 1.5 ± 0.09, **Figure 1B**).

Kisspeptin is a potent activator of GnRH, and others have found that *KiSS1* decreases with aging (16). In our study, we also found a significant decrease in expression of *KiSS1* in all groups compared with young controls (relative fold change, 8 months old, $0.002 \pm 1.9 \times 10^{-3}$, 10 months old, $0.001 \pm 1.8 \times 10^{-4}$, and 12 months old, $0.002 \pm 4.7 \times 10^{-4}$, **Figure 1C**). We also see a downstream decrease in *GnRH* mRNA levels, as the acyclic animals exhibit a significant downregulation of GnRH compared with both the young controls and regularly cycling middle-aged animals (0.45 ± 0.05, **Figure 1D**).

Pituitary Expression Level in the Pituitary across the Reproductive Span

Next, we examined gonadotropin changes in the pituitary and found no differences in expression of the RFRP3 receptor, *GPR147* across the four groups (**Figure 2A**). However, the expression level of the LH subunit β (LHβ) in the pituitary was decreased in the middle-aged regularly cycling group (relative fold-change, 0.57 ± 0.22, **Figure 2B**) and irregularly cycling animals (0.73 ± 0.31, **Figure 2B**) relative to young controls. In acyclic rats, LHβ mRNA expression was not significantly different
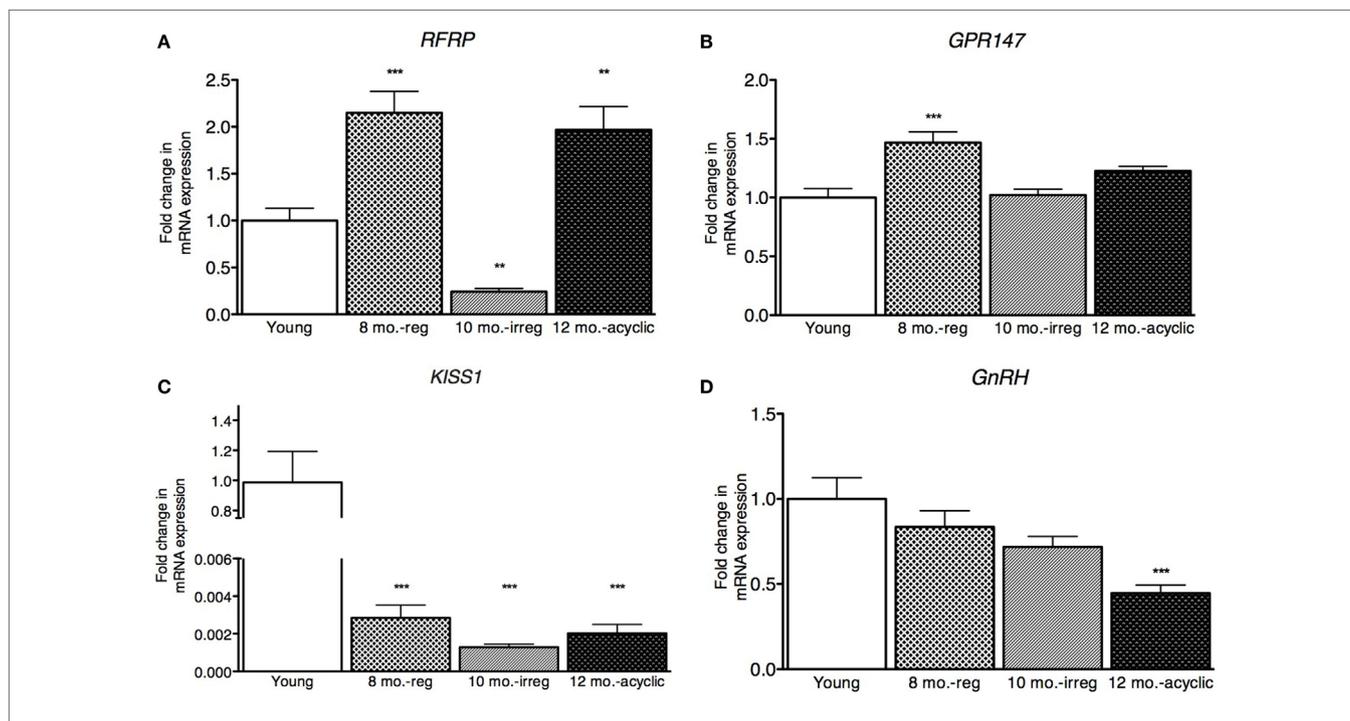

FIGURE 1 | Hypothalamic peptide mRNA expression in female rats. **(A–D)** Gene expression changes in the hypothalamus at different age ranges through middle age. Young = 3-month regularly cycling females, 8-month-old reg. = 8-month-old females with regular estrous cycles, 10-month-old irreg. = 10-month-old females exhibiting irregular cycles, i.e., over 6–7 days rather than 4–5, and 12-month-old acyclic = 12-month-old rats that exhibit persistent estrous or diestrous over a period of more than 14 days. mRNA levels of all (mean ± SEM, $N = 18$/group for young and 8-month-old reg., $N = 21$ for 10-month-old irreg., and $N = 12$ for 12-month-old acyclic) were determined using qRT-PCR relative to the ribosomal reference gene RPLP. Estrous cycle staging was determined by inspection of daily vaginal smears (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
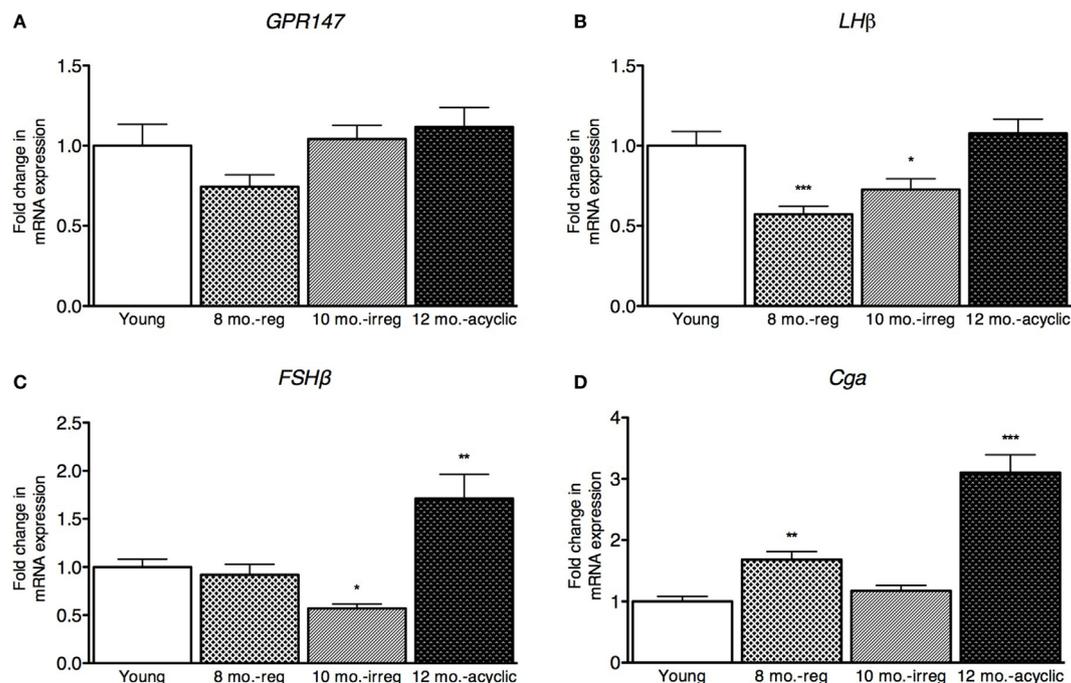
FIGURE 2 | Pituitary mRNA expression in female rats. (A–D) Gene expression changes in the pituitary at different age ranges through middle age. Young = 3-month regularly cycling females, 8-month-old reg. = 8-month-old females with regular estrous cycles, 10-month-old irreg. = 10-month-old females exhibiting irregular cycles, i.e., over 6–7 days rather than 4–5, and 12-month-old acyclic = 12-month-old rats that exhibit persistent estrous or diestrous over a period of more than 14 days. mRNA levels of all (mean ± SEM, $N = 18$/group for young and 8-month-old reg., $N = 21$ for 10-month-old irreg., and $N = 12$ for 12-month-old acyclic) were determined using qRT-PCR relative to the ribosomal reference gene RPLP. Estrous cycle staging was determined by inspection of daily vaginal smears (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

from young controls (1.1 ± 0.31). FSHβ expression was decreased in irregularly cycling females (0.57 ± 0.21, **Figure 2C**) relative to young controls, but increased in acyclic females, compared with the young controls (1.71 ± 0.88). Synthesis of LH and FSH requires a common glycoprotein alpha subunit (Cga), in addition to a specific beta subunit. Cga expression was increased in the middle-aged group (1.68 ± 0.55, **Figure 2D**) relative to young and 10-month-old irregularly cycling females and the acyclic group (3.00 ± 1.00) relative to all other groups.

RFRP3 and GPR147 Expression Increase Dramatically in the Aging Ovary

Next, we explored how *RFRP3* and *GPR147* changed in the ovaries during reproductive decline. We found that *RFRP3* was higher in the ovaries in the acyclic group (2.5 ± 0.29, **Figure 3A**) compared with young and middle-aged cycling females. *GPR147* was upregulated in both middle-aged and irregularly cycling females compared with young animals (8 months old, 2.9 ± 0.34 and 10 months old, 2.44 ± 0.21, **Figure 3B**).

DISCUSSION

The present findings suggest that the inhibitory neuropeptide, RFRP3, may play a role in the initiation of reproductive decline

in female rats, prior to change in cyclicity. Specifically, we found that hypothalamic RFRP3 expression increases significantly in middle age, concomitant with an increase in the RFRP3 receptor, *GPR147*, in the hypothalamus and gonads. These findings imply a transient increase in RFRP3 signaling around 8 months of age, prior to the onset of cycle irregularity. This transient increase in inhibitory signaling possibly acts as an initiating signal to inhibit regular cycling, leading to subsequent reproductive decline. This initial inhibitory signaling at 8 months is followed by a decrease in *KiSS1* in the hypothalamus. RFRP3 receptor mRNA has been shown to colocalize with KiSS1 neurons (19), suggesting that increased RFRP3 expression in middle age prior to reproductive cessation might act to signal *KiSS1*-expressing cells to further reproductive decline. Though transient, this effect may be sufficient to trigger a downstream decrease in HPG axis activity, as we see decreases in *LH*β and *FSH*β subunits in early aging. Since RFRP3 mRNA levels were measured in RNA extracted from whole hypothalamic in this study, it cannot provide nucleus-specific localization. However, RFRP3 expression was only documented in the DMH (12, 20). The data presented in this study provide a timeline of changes in expression of the major regulators, providing correlative evidence for the role played by RFRP3. Future studies using RFRP shRNA or overexpression viral vector or pharmacological inhibition of the RFRP3 receptor GPR147 would be required in order to prove the mechanistic role
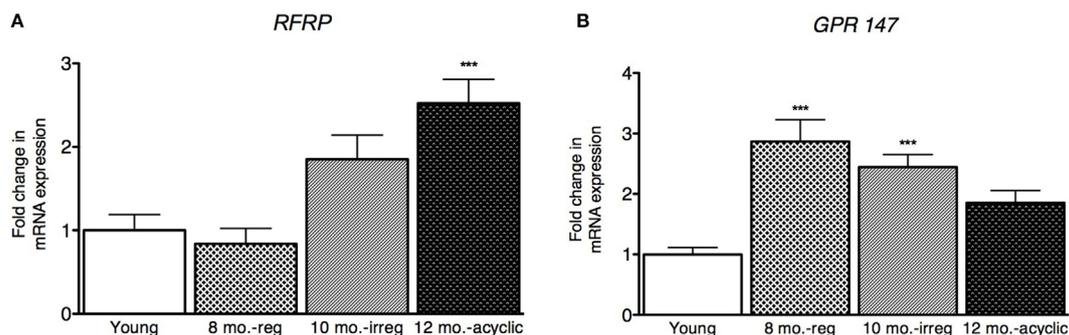
FIGURE 3 | Ovarian hormone mRNA levels in female rats. (A,B) Gene expression changes in the ovary at different age ranges through middle age. Young = 3-month regularly cycling females, 8-month-old reg. = 8-month-old females with regular estrous cycles, 10-month-old irreg. = 10-month-old females exhibiting irregular cycles, i.e., over 6–7 days rather than 4–5, and 12-month-old acyclic = 12-month-old rats that exhibit persistent estrous or diestrous over a period of more than 14 days. mRNA levels of all (mean ± SEM, N = 18/group for young and 8-month-old reg., N = 21 for 10-month-old irreg., and N = 12 for 12-month-old acyclic) were determined using qRT-PCR relative to the ribosomal reference gene RPLP. Estrous cycle staging was determined by inspection of daily vaginal smears (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

of RFRP3 in the timing of senescence. For example, a recently published inducible RFRP3 shRNA lentiviral vector (21) could be used to test the effect of blocking the transient increase in RFRP3 on onset of senescence. Alternatively, use of a GPR147 antagonist would provide information as to the role of RFRP3 in reproductive senescence, and the compound GJ14 might be a useful tool in this respect (22).

Previous studies have shown that LH serum concentrations decrease with aging, whereas FSH serum concentrations increase (6). This decrease in LH is likely triggered by a decrease in GnRH stimulation from the hypothalamus. It is hypothesized that an increase in FSH is seen in rodents, in contrast to what is observed in women, because follicles continue to develop throughout aging. However, these follicles are acyclic as they do not produce corpus lutea. The increase in $LH\beta$, $FSH\beta$, and Cga mRNA levels in acyclic, aged females has been seen in previous studies (23, 24) and is hypothesized to represent a decrease in the ability to transcribe gonadotropin proteins successfully, or potentially a decrease in the ability to release the gonadotropins. Here, we show that, similar to our hypothalamic findings, early decreases in LH and FSH transcription precede cycle cessation and increase thereafter, with this latter decrease likely representing the loss of negative feedback control.

Perhaps the most striking finding in this study is the increase in both *RFRP3* and *GPR147* in the ovaries as aging progresses. The increase in RFRP3 is maintained through at least 12 months of age female become acyclic. What is particularly interesting is the different kinetics of RFRP3 regulation in the hypothalamus and the gonads. RFRP3 increases transiently in the hypothalamus in middle-aged animals while still cycling normally, is suppressed in irregularly cycling females, and is upregulated again when cycling ceases. In contrast, RFRP3 expression in the ovaries increases after cycle irregularity begins and is maintained at least until cycles cease. This differential expression dynamics can potentially reflect hypothalamic RFRP signaling inducing subsequent downstream suppression of the axis. The correlative

evidence described in this study would require further experimental manipulations to discover the mechanistic hierarchy. For instance, is the increase of ovarian RFRP3 induced by the hypothalamic RFRP3 increase, or occurs independently? There is evidence for local RFRP3 production in the ovary that might be independently regulated, separate from signals from the hypothalamus, providing support for this latter possibility (25–27). Decreased gonadal steroid production throughout the stages of reproductive decline (6) is thought to result from decreased preovulatory gonadotropin release. Our data suggest that this decrease in gonadal steroids may be due to local inhibition *via* RFRP3 in the ovaries.

Successful reproduction is dependent on the coordination of many endocrinological events in the brain and the periphery. In the aging rat, HPG axis functionality decreases, manifesting as decreases in GnRH synthesis and release, decreases in LH, and a decrease in gonadal steroids. However, the regulatory cues that initiate the transition from function to decline are largely yet unknown. We have described here the dynamic regulation of key neuropepetides in the HPG axis along this transition. Importantly, a transient increase in the expression of RFRP3 and its receptor precedes irregular cycling, and later cycling cessation, adding a new piece to the puzzle of reproductive decline.

AUTHOR CONTRIBUTIONS

AG, GB, LK, and DK designed experiments and wrote the manuscript. AG and SM performed experiments. AG analyzed data.

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