Neuroendocrine control of reproductive aging: roles of GnRH neurons

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Abstract

The process of reproductive senescence in many female mammals, including humans, is characterized by a gradual transition from regular reproductive cycles to irregular cycles to eventual acyclicity, and ultimately a loss of fertility. In the present review, the role of the hypothalamic gonadotropin-releasing hormone (GnRH) neurons is considered in this context. GnRH neurons provide the primary driving force upon the other levels of the reproductive axis. With respect to aging, GnRH cells undergo changes in biosynthesis, processing and release of the GnRH decapeptide. GnRH neurons also exhibit morphologic and ultrastructural alterations that appear to underlie these biosynthetic properties. Thus, functional and morphologic changes in the GnRH neurosecretory system may play causal roles in the transition to acyclicity. In addition, GnRH neurons are regulated by numerous inputs from neurotransmitters, neuromodulators and glia. The relationship among GnRH cells and their inputs at the cell body (thereby affecting GnRH biosynthesis) and the neuroterminal (thereby affecting GnRH neurosecretion) is crucial to the function of the GnRH system, with age-related changes in these relationships contributing to the reproductive senescent process. Therefore, the aging hypothalamus is characterized by changes intrinsic to the GnRH cell, as well as its regulatory inputs, which summate to contribute to a loss of reproductive competence in aging females.

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Overview of reproductive senescence in female mammals

Aging of the female reproductive system involves all three levels of the reproductive axis, comprising the brain, pituitary and ovary. Most research on the mechanisms of menopause in women has focused on the role of declining ovarian follicular reserves. Although this is unquestionably important, it is becoming increasingly clear from human, nonhuman primate, and rodent models that the hypothalamus and pituitary also play critical roles. Gonadotropin-releasing hormone (GnRH) cells of the hypothalamus are the primary regulatory factor of the reproductive axis (Gore 2002a), driving puberty and ovulation. This primacy of the GnRH system makes it reasonable to postulate that they are also associated with senescent changes in reproductive function, and there is increasing support for such a role. In fact, in the hypothalamus and functionally associated regions, such as the preoptic area (POA), alterations in the GnRH neurosecretory system occur prior to ovarian failure. Nevertheless, the exact mechanisms by which GnRH cells change in terms of their intrinsic properties (e.g. biosynthesis, transport and release), extrinsic regulatory factors

(e.g. inputs from central neurotransmitters, neurotrophic factors and steroid feedback) and morphologic changes are only beginning to be understood. In addition, age associated changes in anterior pituitary gonadotrope function, controlling luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion are also implicated in reproductive senescence (Hall & Gill 2001). At the level of the ovary, numbers and viability of follicles; ovulation; and secretion of estrogens, progestins, inhibin and activin change with aging (Burger *et al.* 2002). Thus, no single level of the hypothalamic-pituitary-ovarian axis can account for the entire process of reproductive senescence. Here, we will address the role of the hypothalamic GnRH neurosecretory system in the loss of reproductive competence in female mammals.

Evidence for a role of the hypothalamus in reproductive aging

Rodents

The depletion of ovarian oocytes is not the primary cause of reproductive senescence in rodents. This was addressed

in an early study in which young, reproductively competent rats were ovariectomized, and then given transplanted ovaries from senescent rats. These young recipient animals show follicular development and ovulation from the aged ovaries, demonstrating that the ovary is not the limiting factor in reproductive failure (Krohn 1955). Moreover, although rats become spontaneously acyclic with aging, their ovaries may resume ovulation in response to various external stimuli, and contain functional follicles even in acyclic rats (Huang & Meites 1975). Such evidence that rodents become acyclic in the presence of a functional ovary indicates a nonovarian, putative hypothalamic or pituitary signal that plays a causal role in reproductive failure.

Further support for a role of the hypothalamus is provided by studies utilizing electrolytic lesions of the medial POA in young rats. These animals show irregular estrous cycles as in older rats (Clemens & Bennet 1977). Electrochemical stimulation of the POA of aged acyclic rats results in enhanced LH secretion (Wuttke & Meites 1973). This latter finding indicates that the pituitary continues to be responsive to GnRH release (which is elicited by the POA stimulation) and suggests that altered GnRH output, not pituitary responsiveness, is responsible for age-related acyclicity.

Primates

There are clear species differences in reproductive aging processes. As discussed above, the hypothalamus probably plays the primary role in reproductive senescence in rodents. However, the relative roles of the hypothalamus, pituitary and ovary in both human and nonhuman primates are less clear, although all three of these levels appear to contribute, at least to some extent, to the reproductive aging process. A study in female rhesus monkeys shows that pulsatile GnRH release (specifically mean GnRH concentrations and to a lesser extent GnRH pulse amplitude) becomes elevated very late in life (28–31 years) (Gore *et al.* 2004), concomitant with the perimenopause in this species. Studies in women have measured serum gonadotropin (LH or FSH) or glycoprotein free α -subunit (FAS), the latter as a reflection of hypothalamic GnRH release (Gill *et al.* 2002) to determine age-related changes. One study compared pulsatile gonadotropin release (frequency and amplitude) in postmenopausal women relative to premenopausal levels during the early follicular stage and showed that the postmenopausal women had increased pulse amplitude, but no change in pulse frequency, compared with the premenopausal cohort (Rossmanith 1995). However, because the premenopausal women were sampled during the early follicular stage, when pulsatile gonadotropin release is relatively low, it is difficult to generalize about increased gonadotropin release between pre- and postmenopausal women. More insight is provided by studies comparing early and late postmenopausal women. The study by Rossmanith (1995) shows that, as aging

progresses, gonadotropin levels decrease, as do gonadotropin pulse frequency and amplitude. Another group compared early postmenopausal women (45–55 years) with late postmenopausal women (70–80 years) and demonstrated significantly lower LH and FSH concentrations in older than younger postmenopausal women (Gill *et al.* 2002). A second report from this group shows that FAS pulse frequency is significantly decreased, LH pulse frequency is modestly decreased and both FAS and LH pulse amplitude are lower in the aged group (Hall *et al.* 2000).

It could be argued that because the perimenopause is characterized by decreased circulating estradiol concentrations relative to the premenopausal period (Gore *et al.* 2004), the increased GnRH/gonadotropin secretion seen in monkeys (and possibly in early postmenopausal women) (Rossmanith 1995) is simply due to a diminution of estradiol negative feedback. However, this is probably not entirely the case, suggesting a hypothalamic and/or pituitary level of regulation. Again, studies comparing early and late postmenopausal women have shed light on this phenomenon. Estradiol levels are not significantly different between early and late postmenopausal women (Hall *et al.* 2000), yet serum LH, FSH and FAS levels are significantly lower in these late postmenopausal women (Hall *et al.* 2000, Gill *et al.* 2002), and gonadotropin negative feedback regulation by exogenous steroids is maintained (or even increased) in late postmenopausal women (Gill *et al.* 2002). A recent paper on the SWAN study (Study of Women's Health Across the Nation) (Weiss *et al.* 2004) shows that perimenopausal women at about 50 years of age who exhibit cyclic changes in urinary estrone conjugates (a reflection of estrogens) across a menstrual cycle can be subdivided into two groups: those with LH profiles consistent with a preovulatory LH surge, and those with no LH response. Although this finding is not entirely consistent with earlier work, it supports a hypothalamic and/or pituitary loss of sensitivity to estrogen feedback in women, and not a change in ovarian output per se, as being a primary initiator of the menopausal process.

To follow is a summary of the literature on the role of the hypothalamic GnRH neurosecretory system in reproductive aging. Our focus will be GnRH functional, morphologic and anatomic properties that correlate with, or may cause, reproductive failure. We will also discuss briefly some of the regulatory factors controlling GnRH neurons that themselves undergo age-related changes, the end result of which is the process of reproductive senescence.

GnRH gene activation and aging

The synthesis of the GnRH decapeptide, like other secreted peptides, begins with the transcription of the GnRH gene to an RNA primary transcript in the nucleus. After processing, including 5' capping, polyadenylation and intron splicing, the mature GnRH mRNA is translocated to the cytoplasm. Relative to most genes, the GnRH mRNA precursors in the nucleus are relatively abundant *in vivo*

(Gore 2002b). This led us to hypothesize that there is a large pool of GnRH mRNA precursors, enabling most of the regulation of GnRH biosynthesis to occur primarily by a post-transcriptional mechanism such as mRNA stability (Gore 2002b). Once the mRNA is produced, translation of GnRH occurs in the endoplasmic reticulum and Golgi apparatus, and ultimately via budding of secretory vesicles, which contain the GnRH precursor peptide. Processing of the GnRH precursor peptide continues in the vesicles, which contain the enzymes involved in proGnRH peptide splicing. Thus, within vesicles, both the 10-amino-acid GnRH and its 56-amino-acid GnRH-associated peptide (GAP) can be detected, and both are released from nerve terminals in the median eminence (Rangaraju *et al.* 1991, Wetsel & Srinivasan 2002). The former exerts its hypophysiotropic actions at the pituitary, and the latter has functions that are only partially understood.

GnRH gene expression in ovarian-intact models

GnRH mRNA levels are measured as an index of biosynthetic capacity. In ovarian-intact Sprague-Dawley female rats, used either on proestrus or diestrus (the two extremes of the estrous cycle), our group used the RNase protection assay to show that overall GnRH mRNA levels are significantly higher in middle-aged (12–14 months) and old (25–26 months) than young (4–5 months) rats (Gore *et al.* 2000a) (Table 1). In these same animals, GnRH primary transcript levels, an index of gene transcription,

are significantly lower in old than young or middle-aged rats (Gore *et al.* 2000a). This finding suggests that the age-associated increase in GnRH mRNA occurs independently of *de novo* gene transcription; in fact, the increase in mRNA occurs despite a decrease in gene transcription. Another study in Sprague-Dawley rats shows a small (20%) age-related decrease from 2 to 18 months of age, but that study did not take reproductive cyclicity into consideration (Li *et al.* 1997b). Age differences may explain the differing results between these two rat studies. It is also important to point out that ovarian-intact rats, in contrast to humans, may still possess functional ovaries that are capable of responding to external stimuli with ovulation even at very advanced ages (Huang & Meites 1975). Rance's laboratory used *in situ* hybridization to show that GnRH mRNA levels are significantly higher in postmenopausal than in premenopausal women, the latter of varying menstrual cycle status (Table 1) (Rance & Uswandi 1996). The similarity of Rance's finding in women to our finding in rats (Gore *et al.* 2000a), that is, age-associated increases in GnRH mRNA levels, suggests that, whereas the ovarian environment is very different, the hypothalamic level may be conserved across mammalian species.

GnRH gene expression in ovariectomy models

Using the RNase protection assay, we found two different results for GnRH mRNA. In one study, levels remained unchanged with age in both ovariectomized (OVX) and

Table 1 GnRH gene expression and activation in aging females.

Species	Treatment	Method	Result	Reference
<i>A. Intact models: overall changes with aging</i>				
Human	Intact	ISH	GnRH mRNA is significantly higher in postmenopausal (54–86 years) than premenopausal (21–41 years) women	(Rance & Uswandi 1996)
S-D rat	Intact	RPA	GnRH mRNA is higher in MA (acyclic or on proestrus or diestrus I) and old (acyclic) than young rats (proestrus or diestrus I); GnRH primary transcript is lower in these same old than young or MA rats	(Gore <i>et al.</i> 2000a)
S-D rat	Intact	ISH	GnRH mRNA levels decrease 20% from 2 to 18 months or age (cycling status not specified)	(Li <i>et al.</i> 1997a)
<i>B. OVX models: overall changes with aging</i>				
S-D rat	OVX	RPA	No change in GnRH mRNA with age (young, MA or old)	(Gore <i>et al.</i> 2002)
S-D rat	OVX	RPA	GnRH mRNA decreases from young to MA	(Miller & Gore 2001)
<i>C. Intact models: proestrous GnRH/LH surge</i>				
S-D rat	Intact	RPA	Young rats have a proestrous increase in GnRH mRNA. MA rats lack this increase	(Gore <i>et al.</i> 2000a)
<i>D. OVX models: steroid-induced GnRH/LH surge</i>				
S-D rat	OVX	ISH	The number of GnRH mRNA-positive cells peaks at 1800 h during the surge in young, but not MA, rats	(Rubin <i>et al.</i> 1997)
<i>E. Intact models: Fos coexpression in GnRH neurons during the GnRH/LH surge</i>				
S-D rat	Intact	IHC	Fos coexpression in GnRH neurons is lower in MA than young rats during the proestrous gonadotropin surge	(Lloyd <i>et al.</i> 1994, Rubin <i>et al.</i> 1994, Le <i>et al.</i> 2001)
<i>F. OVX models: Fos coexpression in GnRH neurons during the steroid-induced GnRH/LH surge</i>				
S-D rat	OVX	IHC	Fos coexpression in GnRH neurons is lower during the steroid-induced LH surge in MA than young rats	(Rubin <i>et al.</i> 1994, Le <i>et al.</i> 2001)

IHC: immunocytochemistry; ISH: *in situ* hybridization; MA: middle-aged; OVX: ovariectomized; RPA: RNase protection assay; S-D: Sprague-Dawley.

OVX plus estradiol-replaced rats (Gore *et al.* 2002), while in a second study we detected a significant age-related decrease (Miller & Gore 2001). Although it is difficult to reconcile these differing results from our own laboratory, our recent preliminary data using real-time PCR to measure GnRH mRNA in aging rats also support a small but significant age-related decline in middle-aged compared with young OVX rats, independent of hormone replacement status (D M Walker & A C Gore, unpublished observation). Thus, unlike intact female rats, which show an age-related *increase* in GnRH mRNA levels, OVX rats usually undergo an overall, small, age-associated *decrease* (Table 1). This highlights the importance of using the appropriate animal model for studies on reproductive aging, as the presence or absence of the ovary can affect the outcome.

GnRH gene activation during the preovulatory GnRH/LH surge of intact rats

One of the hallmarks of reproductive aging in rats is the attenuation of the preovulatory GnRH/LH surge that is responsible for ovulation (Lloyd *et al.* 1994). Therefore, this is an important endpoint at which to study GnRH gene expression. To address this, we euthanized young and middle-aged proestrous rats at 1000 and 1500 h. These time points were chosen as we previously showed in young female rats that GnRH mRNA and primary transcript levels are elevated at 1500 compared with 1000 h on proestrus, the day of the surge (Gore & Roberts 1995). This latter finding was recently confirmed in young rats by another laboratory using real-time PCR (Schirman-Hildesheim *et al.* 2005). In our study comparing young and middle-aged intact rats during the proestrous gonadotropin surge, we showed that GnRH mRNA levels are higher at 1500 than 1000 h. A trend for the GnRH primary transcript to be increased at 1500 compared with 1000 h was also seen in young rats. By contrast, middle-aged rats lack an increase in GnRH mRNA or primary transcript on proestrus (Gore *et al.* 2000a) (Table 1). This lack of drive of the GnRH system of middle-aged rats may explain the delay and attenuation of the preovulatory GnRH/LH surge.

The immediate early gene Fos has been used for many years as a marker of transcriptional activation (reviewed in Hoffman & Lyo 2002). In GnRH cells, Fos coexpression is upregulated within GnRH neurons of young rats during the preovulatory GnRH/LH surge (Lloyd *et al.* 1994). This implies an activation of GnRH gene expression at this time, and is consistent with our report, discussed above, that GnRH gene transcription is elevated shortly before the surge in young female rats (Gore & Roberts 1995). In proestrous middle-aged rats whose preovulatory surge is attenuated, Fos coexpression in GnRH neurons is substantially decreased compared with young proestrous rats (Lloyd *et al.* 1994, Le *et al.* 2001) (Table 1). Again consistent with this, we reported that GnRH primary transcript RNA levels in middle-aged rats do not increase on the afternoon of the GnRH/LH surge, implying a loss of drive

of GnRH gene activation in middle-aged rats, even those which are still exhibiting a surge and can ovulate. Thus, this change precedes the transition to acyclicity and may play a causal role in this process.

GnRH gene activation during the steroid-induced GnRH/LH surge of OVX rats

In OVX rats, GnRH gene expression has been quantified by *in situ* hybridization during the steroid-induced surge in young and middle-aged rats (Rubin *et al.* 1997). The authors show an increased number of GnRH mRNA-expressing neurons at 1800 h (near the peak of the surge) in young, but not middle-aged, animals. Le *et al.* (2001) studied OVX young and middle-aged rats during the estradiol plus progesterone-induced surge, and show that Fos coexpression in GnRH neurons is significantly lower in middle-aged than young rats.

Taken together, these studies support the importance of measuring age-related changes in GnRH gene expression, as they relate to the functional outcome of reproductive failure. Studies performed during the preovulatory or steroid-induced GnRH/LH surge have been particularly informative in this regard, as they consistently support a diminution of the activation of surge-associated GnRH gene activity by middle age, prior to reproductive failure (Table 1).

GnRH release and aging

After synthesis, GnRH is packaged into secretory granules and transported to the median eminence, where the peptide is released from GnRH terminals into the perivascular space of the portal capillary system (King *et al.* 1982). In adult rodents, GnRH is released from the median eminence at a frequency of about one pulse every 30 min. A slightly slower frequency of release (approximately 50–60 min intervals) is observed in primates (Terasawa 2001, Gore *et al.* 2004). The pulsatile pattern of GnRH (or LH) release is superimposed upon longer cycles of release, such as menstrual (primates) or estrous (rat, mouse, sheep) cycles. During aging, all of the parameters of pulsatile release (basal and mean concentrations, and pulse frequency) and the preovulatory surge are altered (as described below and summarized in Table 2). As all of these biologic rhythms are critical for normal reproductive function (Levine 1997, Terasawa 2001), age-related changes in these patterns may underlie the transition to acyclicity. To follow is a summary of *in vivo* and *in vitro* studies on GnRH or LH release in aging females.

***In vivo* studies of pulsatile LH release in OVX rats**

Using young (3–4 months), middle-aged (8–10 months) and old (18–22 months) rats that were OVX for 4 weeks, Wise *et al.* (1988) showed that the amplitude, frequency and mean concentration of serum pulsatile LH decrease

Table 2 GnRH/LH release in aging females (*in vivo* studies).

Species	Age	Treatment	Result	Reference
<i>A. Pulsatile GnRH/LH release in intact females</i>				
Rhesus monkey	Y (~5 yr) O (~26 yr)	Intact	Pulsatile LH release (mean and basal levels) increases with age; no change in pulse frequency	(Woller <i>et al.</i> 2002)
Rhesus monkey	Y (4–13 yr) O (27–31 yr)	Intact	Pulsatile GnRH release (measured by push-pull perfusion) increases (mean levels). There are also large ‘bursts’ of GnRH release in old monkeys; no difference in pulse frequency	(Gore <i>et al.</i> 2004)
Japanese macaque	Y (7–15 yr) O (26 yr)	Intact	Serum LH is higher in perimenopausal (O) than Y monkeys; old monkeys continue to exhibit positive feedback to estradiol treatment	(Nozaki <i>et al.</i> 1995)
Women	Early postmenopausal (45–55 yr) Late postmenopausal (70–80 yr)	Mostly intact	FAS pulse frequency, and LH and FAS pulse amplitude are lower in late than early postmenopausal women	(Hall <i>et al.</i> 2000)
Women	Same as Hall <i>et al.</i> 2000		LH and FSH concentrations are lower in late than early postmenopausal women	(Gill <i>et al.</i> 2002)
<i>B. Pulsatile GnRH/LH release in OVX females</i>				
S-D rat	Y (3–4 mo) MA (8–10 mo) O (18–22 mo)	OVX 4 weeks	Pulsatile LH release (mean levels, frequency, amplitude) decreases progressively during aging	(Wise <i>et al.</i> 1988)
S-D rat	Y (~3 mo) MA (~11 mo)	OVX 4 weeks	Pulsatile LH release (mean levels, frequency) are lower in MA than Y rats, particularly in MA rats that were irregularly cycling or in PE prior to OVX	(Scarbrough & Wise 1990)
Wistar rat	Y (3 mo) O (26 mo)	OVX 4 weeks	Pulsatile LH and hypothalamic multiunit activity decrease with aging	(Sano & Kimura 2000)
<i>C. Preovulatory GnRH/LH surge in intact females</i>				
S-D rat	Y (~4 mo) MA (~11 mo)	Intact	The preovulatory gonadotropin surge is delayed and attenuated with aging	(Rubin <i>et al.</i> 1994, Lloyd <i>et al.</i> 1994, Wise 1982 <i>b</i> , Weiss <i>et al.</i> 2004)
Women	Perimenopausal (~49 yr)	Intact	LH surges are detected in only about half of women with cyclic changes in estrogens, suggesting loss of hypothalamic/pituitary sensitivity to estrogen feedback	
<i>D. Steroid-induced GnRH/LH surge in OVX females</i>				
S-D rat	Y (3–4 mo) MA (~11 mo)	OVX 4 weeks, treat with E2 + P	Pulsatile GnRH release (measured by push-pull perfusion) decreases during the LH surge with aging (GnRH mean levels and pulse frequency are decreased; overall GnRH output is decreased in MA compared to Y rats)	(Rubin & Bridges 1989)
S-D rat	Y (~4 mo) MA (~11 mo)	OVX, treat with E2 + P	The steroid-induced gonadotropin surge is delayed and attenuated in MA rats	(Rubin <i>et al.</i> 1994, Le <i>et al.</i> 2001)

E2: estradiol; FAS: gonadotrophin free α -subunit; L-E: Long-Evans; MA: middle-aged; mo: month; O: old; OVX: ovariectomized; P: progesterone; PE: persistent estrus; S-D: Sprague-Dawley; Y: young; yr: year.

with aging. Effects of reproductive history (regular vs irregular estrous cycles, or acyclicity) prior to OVX were studied by Scarborough and Wise (1990). They reported that LH concentrations, pulse amplitude and pulse frequency are lower in middle-aged than young OVX rats, and that rats with irregular estrous cycles or that were acyclic prior to OVX have even lower pulsatile LH release than do regularly cycling, young or middle-aged rats (Scarborough & Wise 1990).

In vivo studies of the preovulatory GnRH/LH surge in intact rats

LH release has also been measured during the preovulatory GnRH/LH surge, which is temporally delayed and attenuated in middle-aged rats (Lloyd *et al.* 1994, Rubin *et al.* 1994, Le *et al.* 2001) (Table 2), despite the fact that these animals can still have regular reproductive cycles,

ovulate, and become pregnant. Nevertheless, this diminution of preovulatory GnRH/LH release, which is correlated temporally with diminution in the proestrous increase in GnRH gene expression (Gore *et al.* 2000a), seems to presage the transition to acyclicity. Indeed, both positive and negative feedback regulation of GnRH/LH release become compromised with aging in rats (Huang *et al.* 1976). Although the mechanisms for the surge attenuation and the diminished response to estradiol feedback are not well understood, they probably involve changes in the capacity of GnRH neurons to synthesize and release the decapeptide, as well as to respond to neural and glial inputs and steroid hormone sensitivity.

In vivo studies of the steroid-induced GnRH/LH surge in OVX rats

Direct measurements of GnRH release by push-pull perfusion in aging female rats show a decrease in pulsatile

GnRH release during a steroid-induced LH surge in middle-aged compared with young OVX rats (Rubin & Bridges 1989). Specifically, mean GnRH levels and pulse frequency (but not pulse amplitude) are diminished, and mean GnRH output during the surge is lower in middle-aged rats (Table 2). Sano and Kimura (2000) measured multiunit activity (MUA) in the median eminence of young (3 months) and old (26 months) OVX rats, together with serial serum LH measurements, as a reflection of GnRH activity. They showed that both MUA and LH frequency decrease, and LH pulse amplitude is smaller, in the aged rats. These findings of decreased GnRH release and hypothalamic MUA are consistent with the age-related changes in LH release in rats discussed above.

In vivo studies in primates

GnRH and LH release have been measured in aging rhesus monkeys undergoing menopause by Woller *et al.* (2002) and Gore *et al.* (2004) (Table 2). Both of these studies demonstrate the maintenance of robust GnRH and LH pulses in aged animals, with overall elevated pulse amplitudes but no change in pulse frequency compared with young monkeys sampled during the early follicular phase. Our study measuring GnRH release in monkeys also reports large GnRH bursts in some of the old animals (Gore *et al.* 2004), and we speculated that these incidents may be related to hot flashes. Japanese macaques show elevated serum LH as they age, and continue to exhibit positive feedback responses to estradiol treatment (Nozaki *et al.* 1995). These results contrast markedly with those in rats discussed above that show a decrease in pulsatile GnRH and LH release and a loss of positive feedback to estradiol with age. However, rats and monkeys differ in the age at which they undergo reproductive senescence. Rats experience this transition in midlife, whereas nonhuman primates, such as rhesus monkeys, undergo this process much later in life. Some light may be shed on this issue by studies measuring serum gonadotropins or glycoprotein free α -subunit (FAS) in women as an index of GnRH release (Table 2). Although these studies differ in the age of experimental subjects, overall they show increases in gonadotropin concentrations from the pre- to the perimenopausal or early postmenopausal period (Rossmanith 1995, Gill *et al.* 2002), but subsequent decreases in gonadotropin or FAS release from the early to the late postmenopausal period, including lower hormone concentrations and decreased pulse frequency (Ushiroyama *et al.* 1999, Hall *et al.* 2000). Thus, the chronologic age relative to reproductive failure is an important parameter in understanding species differences.

In vitro release of GnRH

Studies of GnRH release from explanted hypothalamic or median eminence dissections have provided some information on how these cells change their function during

aging. These experiments have the advantage of facilitating direct measurements of GnRH, although they also have some caveats, as described below. In intact (proestrous), OVX, or OVX plus estradiol-treated female rats (the latter during the LH surge), Rubin (1992) reported that basal GnRH release is higher from isolated hypothalami of middle-aged than young rats, although potassium-stimulated GnRH release is equivalent between the two ages. A different result was reported by Hwang *et al.* (1990), who demonstrated that basal GnRH release from explants of young (~4 months) and old (21–24 months) OVX rats is comparable, whereas potassium-stimulated GnRH release is greater in the young rats. Differences between these two reports may be due to differences in ages of animals, as the Hwang study used much older rats than the Rubin study. Another group showed no difference in basal GnRH release, but a decrease in stimulated GnRH release caused by glutamate agonists in middle-aged proestrous compared with young proestrous rats (Zuo *et al.* 1996). Although some of these inconsistent results may be attributable to differences in the ages at which rats were studied, others may also result from properties of explants, which lack a functionally intact GnRH network. Thus, if explants are cut or prepared differently between laboratories, this may result in differential GnRH neural inputs that might influence basal and/or stimulated GnRH release. Therefore, while results in explants have provided some valuable information, the more difficult *in vivo* studies are more relevant to age-related changes in GnRH release.

Morphologic changes in GnRH neurons with age

According to the evidence above, the nature of GnRH neuronal activity changes with age. This change may be related to alterations in numbers of GnRH cells and/or their morphology, reflecting the manufacture, transport and release of GnRH.

The question of whether aging affects GnRH cell numbers has been pursued at the light-microscopic level. As summarized in Table 3, the results vary, possibly in relation to the different treatments, ovarian status and species. Nevertheless, most studies report either no age-related change or a small difference in cell numbers with aging. An increase in GnRH cell numbers in old (constant estrous) compared with young (proestrous) rats was reported by one group (Merchenthaler *et al.* 1980). Several other investigations report no change in GnRH cell numbers, distribution or morphology with aging in female rats (Rubin *et al.* 1984, Miller & Gore 2002), mice (Hoffman & Finch 1986) or rhesus macaques (Witkin 1986). Still other studies have reported an age-associated decrease in GnRH cell numbers in mice and rats (Miller *et al.* 1990, Funabashi & Kimura 1995), but the detected decreases were modest (<20%). Therefore, the majority of studies suggest that the total number of GnRH neurons may not be a critical parameter in the determination of reproductive senescence.

Table 3 Changes in GnRH cell number and morphology during aging in female mammals.

Species	Age and treatment	Methods	Results	Reference
<i>A. GnRH cell number</i>				
Rat (W-R) (Intact)	Y (2–4 mo, proestrous) O (16–20 mo, PE)	LM: IHC	Old rats have more GnRH cells than young rats in medial POA and septum	(Merchenthaler <i>et al.</i> 1980)
Rat (S-D) (Intact)	Y (4–5 mo) MA (9–10 mo)	LM: IHC	No difference in GnRH cell # between Y and MA rats	(Miller & Gore 2002)
Rat (W-I) (OVX)	Y (2 mo) O (26 mo)	LM: IHC	Significant decrease in GnRH cell # with aging, particularly in rostral POA	(Funabashi & Kimura 1995)
Mouse (OVX)	Y (6 mo) O (26–28 mo)	LM: IHC	GnRH cell # decreases 18% in old mice	(Miller <i>et al.</i> 1990)
Mouse (OVX)	Y (5–6 mo) MA (15–16 mo)	LM: IHC	No change in GnRH cell # or distribution in MA mice	(Hoffman & Finch 1986)
<i>B. GnRH distribution, morphology and ultrastructure of cell bodies</i>				
Rat (S-D) (Intact)	Y (3–4 mo) MA (10–15 mo, PE)	LM: IHC	Distribution and morphology of GnRH neurons is similar in young and MA rats	(Rubin <i>et al.</i> 1984)
Rat (S-D) (OVX or intact)	Y (4 mo) O (19–20 mo)	EM: IHC	GnRH perikarya of old rats show decreased RER and Golgi; OVX has opposite effects in Y and O rats	(Romero <i>et al.</i> 1994)
Rat (S-D) (Intact)	Y (2–3 mo) MA (8–9 mo)	LM: IHC	The temporal and spatial pattern of GnRH neuronal distribution is more restricted in MA than Y rats during the LH surge	(Rubin <i>et al.</i> 1995)
Rhesus monkey (Intact)	2 to 22 yrs	LM: IHC	No change in GnRH cell #, distribution or morphology with age	(Witkin 1986)
<i>C. GnRH neuroterminals in the median eminence (ME)</i>				
Rat (S-D) (Intact)	Y (2–3 mo) MA (9–10 mo)	LM: IHC in ME	During the preovulatory LH surge, GnRH immunoreactivity in ME decreases more in Y than MA rats	(Rubin & King 1995)
Rat (L-E) (Intact)	Y (2 mo) O (22 mo)	LM: IHC in ME	The #, area and immunoreactivity of GnRH axons decreases with aging	(Bestetti <i>et al.</i> 1991)

IHC: immunohistochemistry; L-E: Long-Evans; LM: light microscopy; MA: middle-aged; ME: median eminence; O: old; OVX: ovariectomized; PE: persistent estrus; POA: preoptic area; S-D: Sprague-Dawley; W-I: Wistar-Imamichi; W-R: Wistar-R-Amsterdam; Y: young; yr: year; #: number.

The distribution of GnRH neurons, in general, also does not change with aging in rats (Rubin *et al.* 1984) and monkeys (Witkin 1986). A more careful analysis of the pattern of distribution of GnRH neurons during the preovulatory GnRH/LH surge shows that there are more subtle differences between young and middle-aged rats (Rubin *et al.* 1995) (Table 3). The medial-lateral distribution of GnRH neurons and their coexpression of Fos are more restricted in middle-aged rats. This latter study highlights the importance of careful temporal analyses of the GnRH system during the transition to acyclicity.

In the POA, the relationship between GnRH perikarya and astrocytes may change with aging. Cashion *et al.* (2003) evaluated the number of astrocytes that are apposed to GnRH neurons in young and middle-aged cycling rats across different times on proestrus and estrus. They showed that glial apposition to GnRH neurons undergoes circadian fluctuations on proestrus (but not estrus) in young, but not middle-aged, rats, and suggest that this loss of astrocytic plasticity may contribute to the delayed and attenuated LH surge in middle-aged rats.

GnRH neuronal ultrastructure undergoes age-related alterations, as shown by electron-microscopic studies and described in Table 3. By pre-embedding immunohistochemistry, GnRH-positive cell bodies are identified as containing a large round or ovoid nucleus, well-devel-

oped rough endoplasmic reticulum (RER), Golgi complexes, lysosomes, scattered dense core vesicles characteristic of peptidergic vesicles (100 nm in diameter), and small clear vesicles characteristic of monoaminergic neurotransmitters (30–40 nm) (Jennes *et al.* 1985). A large proportion of the surface area of GnRH perikarya appears to be covered by a thin glial sheath, which separates the plasma membrane from other axon processes (Jennes *et al.* 1985, Witkin *et al.* 1991). Reports on the effects of age on the ultrastructure of GnRH neurons have focused on synaptic inputs, the volume fraction of RER and the Golgi apparatus (Table 3). In intact rats, RER and Golgi volume fractions are significantly lower in GnRH cells of old, acyclic rats (18–20 months) than in those of young, cycling rats (4 months, used on diestrus). In similarly aged rats that have been OVX for 4 weeks, the percentage of Golgi fraction is lower in aged than young rats, but the percentage of RER is much higher in the old rats. These results suggest that aging is associated with changes in protein synthesis of GnRH cells with aging in an ovarian status-dependent manner (Romero *et al.* 1994). In addition, these studies on GnRH ultrastructural changes, showing decreases in protein biosynthetic machinery, are consistent with some of the changes observed in GnRH gene expression and release discussed above.

GnRH neuronal processes and terminals change with aging

Relatively few studies have investigated the question of age-associated changes at the level of GnRH neuronal processes and terminals. Nevertheless, it is important to perform studies at this level of analysis, and to contrast results with effects of age on the perikarya. At the cell body, the age-related changes discussed above in synaptic input or intracellular organelles would probably affect GnRH biosynthesis. By contrast, at the GnRH nerve terminal, as discussed below, age-related alterations would directly affect GnRH neurosecretion. Moreover, neuronal or nonneuronal elements regulating GnRH nerve terminals in the median eminence may change with aging to result in altered GnRH neurosecretion.

GnRH processes project caudally through the hypothalamus, with their terminals ultimately projecting to the external zone of the median eminence. At the light-microscopic level, GnRH axon swellings (Herring bodies), often referred to as puncta or varicosities, can be easily identified by immunocytochemistry. At the electron-microscopic level, these structures are seen to contain clusters of large dense-core and small vesicles in GnRH processes and, rarely, to form synaptic contacts with other neurons. Besides having a storage function, the axon swellings may be a site of neuronal interaction (Jennes *et al.* 1985, Tweedle *et al.* 1989). A few studies have investigated changes in GnRH terminal morphology in the context of aging (Table 3). There is reduced GnRH fiber staining in the median eminence of old male rats (Hoffman & Sladek 1980). In intact female rats, GnRH immunoreactivity is decreased in the median eminence of young compared with middle-aged female rats during the preovulatory GnRH/LH surge (Rubin & King 1995), suggesting that greater GnRH release occurred from the terminals of the young rats (Table 3). Using light microscopy, Bestetti *et al.* found that the number, area and immunoreactivity of GnRH axons in the median eminence are decreased in old compared with young rats (Bestetti *et al.* 1991).

Electron-microscopic analyses of terminal regions of young rats show that the distance between GnRH terminals and the basal lamina, the ensheathment of GnRH terminals by tanycytes, and the proximity of GnRH terminals to portal capillaries are affected by sex, developmental age and hormonal status (King & Rubin 1994). Considerable regulation of GnRH terminals may result from ensheathment by tanycytes (specialized ependymal cells lining the third ventricle) (Flament-Durand & Brion 1985), which could play a role in allowing GnRH terminals access to the portal vasculature or to other neuronal processes. Tanycytes are believed to be involved in the transport and release of hormones, including estrogen and GnRH, in the median eminence (Akmayev & Fidelina 1981). It has also been proposed that tanycytes may play a role in the remodeling of the aged basal hypothalamus in male and female rats through phagocytotic actions on

degenerating neurons (Brawer & Walsh 1982, Zoli *et al.* 1995). Light- and electron-microscopic studies show that tanycytes undergo morphologic modifications with age in both male and female rats (Brawer & Walsh 1982). Variations of estrogen and neurotransmitter levels in the median eminence during reproductive cycles have been shown to affect the morphologic, synthetic and transport activity of tanycytes (Flament-Durand & Brion 1985, Zoli *et al.* 1995). Therefore, the relationship between GnRH terminals and tanycytes is a level of regulation that appears to be affected during aging, and has potential consequences for GnRH release into the portal capillary circulation.

GnRH is present in secretory granules within axon profiles and nerve terminals of the median eminence (Goldsmith & Ganong 1975, Yin *et al.* 2002). The size of the granule is smaller in the portal plexus (40–70 nm) than in the GnRH process itself (90–120 nm) in adult male guinea pigs (Silverman & Desnoyers 1976). Recently, immunogold labeling has been applied to study the GnRH network in rat (Liposits *et al.* 1995, Prevot *et al.* 1998) and rhesus monkey (Durrant & Plant 1999). With cryofixation, low temperature embedding and quantitative immunogold labeling techniques at the electron-microscopic level, it is possible to probe the subcellular localization of a neurotransmitter or protein within a very small compartment, linking function with morphology. We have been applying this technique to understand the ultrastructural regulation of GnRH terminals by estrogen (Yin *et al.* 2002) and are currently extending our analyses to aging rats.

Inputs to the GnRH system change with age

The regulation of pulsatile GnRH/LH release and the preovulatory surge involves multiple neuronal circuits (Gore 2002a, 2002b). Although the number of neurotransmitters and neuromodulators is too large to cover in the present review, we have summarized information on a few of the major neurotransmitters commonly believed to regulate GnRH function and for which there is evidence of age-related alterations, the outcome of which is a diminution of reproductive competency.

Dopamine

In the aging hypothalamus of male and female rats, dopamine (DA) secretion into the portal vasculature declines (Gudelsky & Porter 1981, Reymond & Porter 1981). The release of DA from tuberoinfundibular dopaminergic (TIDA) neurons into hypophysial portal blood vessels is sexually dimorphic, and related to the stimulatory action of estrogen (Gudelsky & Porter 1981). The importance of DA in reproductive function has been shown by the ability of dopaminergic agonists to restore estrous cycles in acyclic rats, either young (with lesions in medial POA) or aged (Clemens *et al.* 1977). In male rats, DA fluorescence intensity decreases in the median eminence of old compared

with young rats, but increases with age in the dopaminergic perikarya of the arcuate nucleus (Hoffman & Sladek 1980). Morphologic evidence shows a rapid and dramatic decrease in the total number of neurovascular contacts in the median eminence after DA infusion into the lateral ventricle (Hokfelt 1973). DA and cAMP regulated phosphoprotein (DARPP-32, a marker for cells containing DA receptors) show strong immunoreactivity in tanycytes surrounding GnRH neuroterminals in the median eminence of male rats and monkeys (Meister *et al.* 1988). These results suggest that DA may control GnRH release in the median eminence by modifying the degree of tanycytic enclosure on GnRH terminals (Meister *et al.* 1988).

Norepinephrine

Norepinephrine, acting primarily through the α -adrenergic receptor, stimulates GnRH release, and this noradrenergic influence declines with aging (Temel *et al.* 2002). Using young and middle-aged OVX rats during the steroid-induced gonadotropin surge, Wise (1982a) showed that norepinephrine concentrations in several hypothalamic regions are elevated on the afternoon of the surge in young rats, but that this is attenuated in middle-aged rats. There is a potential anatomic basis for this decreased responsiveness with aging, as a study reported direct appositions between GnRH and noradrenergic cells in young (5 months), but not old (24 months), female OVX mice (Miller & Zhu 1995). Clonidine, an α -adrenergic agonist, can restore the pulsatile pattern of LH secretion in middle-aged acyclic rats (Estes & Simpkins 1982). Therefore, changes with aging in the anatomic and functional relationships between noradrenergic and GnRH neurons may contribute to the decline in reproductive function.

Glutamate

Abundant evidence indicates that the excitatory neurotransmitter glutamate is extremely important in the regulation of GnRH function. GnRH cells express glutamate receptors (both N-methyl-D-aspartate (NMDA) and non-NMDA receptors) at their cell bodies and nerve terminals (Gore *et al.* 1996, Kawakami *et al.* 1998, Miller & Gore 2002). In POA of intact (young, middle-aged and old) rats, mRNA levels of NMDA receptor subunits change with aging (Gore *et al.* 2000b). Overall levels of the NMDA receptor 2b (NR2b) subunit decrease, not with chronological age but rather with reproductive status. Thus, young and middle-aged rats that are regularly cycling (used either on proestrus or diestrus I) have higher NR2b mRNA levels in the POA than do middle-aged acyclic or old acyclic rats. In addition, the coexpression of NR2b specifically on GnRH perikarya *increases* in intact middle-aged (either proestrous or persistent estrous) compared with young, proestrous rats, suggesting an alteration in the NMDA receptor stoichiometry with aging, but largely independent of reproductive cycling status (Miller & Gore 2002).

This probably has a functional outcome, as the ability of NMDA agonists to stimulate GnRH gene expression in young proestrous rats is abolished in middle-aged proestrous or persistent estrous rats (Gore *et al.* 2000b). Additionally, the stimulatory effect of NMDA on GnRH release is diminished in old (18 months) compared with young (3 months) OVX rats (Arias *et al.* 1996). Finally, new evidence shows that GnRH neurons of male rats coexpress vesicular glutamate transporter-2 (VGLUT2), the presence of which indicates a glutamatergic phenotype (Hrabovszky *et al.* 2004). This suggests that GnRH neurons themselves may synthesize and potentially release glutamate. To date, this has been reported only for male rats; it will be interesting to extend this finding to females, and to determine whether this coexpression changes with age.

Vasoactive intestinal polypeptide

The suprachiasmatic nucleus (SCN), the circadian pacemaker in the brain, makes projections to GnRH neurons that are critical for circadian (daily) cycles of GnRH release (Krajnak *et al.* 1998). A strong candidate for the regulation of GnRH neurons by the SCN is vasoactive intestinal polypeptide (VIP). VIP neurons project directly to GnRH neurons, where they make direct contacts (Van der Beek *et al.* 1997) in a sexually dimorphic manner, and there are more contacts in female than male rats (Horvath *et al.* 1998). VIP neurons undergo developmental and age-related changes in their circadian rhythms, and in their inputs to GnRH perikarya (Krajnak *et al.* 1998, Kriegsfeld *et al.* 2002). VIP mRNA levels exhibit a 24 h rhythm in young, but not middle-aged, female rats, which is associated with declines in cellular VIP mRNA and VIP neuron number in SCN (Krajnak *et al.* 1998). It has been suggested that the effects of VIP on GnRH neuron are mediated through cAMP. A study of cAMP level in SCN and POA (the location of GnRH perikarya) demonstrates a diurnal rhythm in young, but not middle-aged, female rats (Gerhold *et al.* 2005). When the VIP rhythm is suppressed, this produces an aging-like cAMP rhythm, and in turn leads to inhibition of the GnRH activation (Gerhold *et al.* 2005).

Insulin-like growth factor-I

Insulin-like growth factor-I (IGF-I) is a neurotropic factor in the brain, where it is involved in neuronal development. IGF-I receptors (tyrosine kinase receptors) are widely distributed in the brain, including the hypothalamus and POA (Daftary & Gore 2005). Recently, we showed that IGF-I immunoreactivity is coexpressed in GnRH cell bodies of intact male and female mice (female mice were randomly cycling), and that IGF-I immunoreactivity is abundantly expressed in regions enriched with GnRH nerve processes and terminals (Daftary & Gore 2003, 2005). IGF-I mRNA levels are substantially lower in both the POA (the location of GnRH perikarya) and medial basal hypothalamus-median eminence (the site of

GnRH processes and terminals) of middle-aged than young OVX rats (either with or without estradiol replacement) (Miller & Gore 2001). The finding that IGF-I treatment does not affect GnRH gene expression in rats of either age suggests that it may act on other levels of the GnRH circuit rather than gene expression (Miller & Gore 2001).

Summary and future directions

Reproductive function declines with aging in female mammals, and the contribution of the hypothalamic GnRH system to this process is becoming better understood. Studies on how the GnRH neurosecretory system changes with aging have demonstrated alterations in GnRH gene expression, the pattern of pulsatile and preovulatory GnRH release, and the morphology and ultrastructure of the GnRH cell body and neuroterminal. Neurotransmitters and neuromodulators also show important regulatory effects on the GnRH circuit, and these, too, can undergo age-related alterations. The use of advanced molecular and structural biology techniques may provide us with improved resolution and a broader perspective on the study of GnRH neural activity in the future. Although this review has limited itself to changes within GnRH cells and their endogenous regulatory factors in the brain, it is important to consider the influence of the environment on the biosynthesis of GnRH. Interactions among GnRH neurons, environmental factors and genes will be fruitful areas for further studies on the mechanisms of reproductive aging.

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