# Neuroendocrine Modulation and Repercussions of Female Reproductive Aging

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ABSTRACT

The menopause marks the end of a woman's reproductive life. During the postmenopausal period, plasma estrogen concentrations decrease dramatically and remain low for the rest of her life, unless she chooses to take hormone replacement therapy. During the past 20 years, we have learned that changes in the central nervous system are associated with and may influence the timing of the menopause in women. Recently, it has become clear that estrogens act on more than just the hypothalamus, pituitary, ovary, and other reproductive organs. In fact, they play roles in a wide variety of nonreproductive functions. With the increasing life span of humans from approximately 50 to 80 years and the relatively fixed age of the menopause, a larger number of women will spend over one third of their lives in the postmenopausal state. It is not surprising that interest has increased in factors that govern the timing of the menopause and the repercussions of the lack of estrogen on multiple aspects of women's health. We have used animal models to better understand the complex interactions between the ovary and the brain that lead to the menopause and the repercussions of the hypoestrogenic state. Our results show that when rats reach middle age, the patterns and synchrony of multiple neurochemical events that are critical to the preovulatory gonadotropin-releasing hormone (GnRH) surge undergo subtle changes. The precision of rhythmic pattern of neurotransmitter dynamics depends on the presence of estradiol. Responsiveness to this hormone decreases in middle-aged rats. The lack of precision in the coordination in the output of neural signals leads to a delay and attenuation of the luteinizing hormone surge, which lead to irregular estrous cyclicity and, ultimately, to the cessation of reproductive cycles. We also have examined the impact of the lack of estrogen on the vulnerability of the brain to injury. Our work establishes that the absence of estradiol increases the extent of cell death after stroke-like injury and that treatment with low physiological levels of estradiol are profoundly neuroprotective. We have begun to explore the cellular and molecular mechanisms that underlie this novel nonreproductive action of estrogens. In summary, our studies show that age-related changes in the ability of estradiol to coordinate the neuroendocrine events that lead to regular preovulatory GnRH surges contribute to the onset of irregular estrous cycles and eventually to acyclicity. Furthermore, we have shown that the lack of estradiol increases the vulnerability of the brain to injury and neurodegeneration.

## I. Introduction

Women undergo the menopause at approximately 51 years of age. The timing of this dramatic physiological change has remained essentially constant

since records have been kept. The menopause occurs at the time of the exhaustion of the ovarian follicular reserve. Since the ovarian follicles are not only the source of germ cells but also are the primary source of estrogens, plasma estrogen concentrations drop precipitously during the postmenopausal years and remain low for the remainder of a woman's life, unless she chooses to take hormone replacement therapy (HRT). Thus, the end of the reproductive life has far-reaching implications for women because they become permanently hypoestrogenic at this time. In recent years, we have come to appreciate that estrogens are not only reproductive hormones but also pleiotropic hormones that play roles in a wide variety of nonreproductive functions as disparate as bone and mineral metabolism (Lindsay, 1996; Manolagas, 2000; Compston, 2001), memory and cognition (Fillit, 1994; Erkkola, 1996; Sherwin, 1996,1999), cardiovascular function (Wild, 1996; Matthews *et al.*, 2000; Mendelsohn, 2000; Stevenson, 2000; Losordo and Isner, 2001), and the immune system (Jansson and Holmdahl, 1998; Ahmed and Hissong, 1999). With the substantial increase in the average life span of humans from approximately 50 to 80 years that occurred during the last century, and the relatively fixed age of the menopause, the number of women who will spend over one third of their lives in the postmenopausal state has increased dramatically. It is not surprising, then, that an increasing number of clinical and basic science researchers have focused their efforts on understanding reproductive aging, since it has become increasingly important to understand the factors that drive the menopausal transition. A number of excellent reviews that discuss the role of the brain in female reproductive aging have appeared during the past 10 years (Lu *et al.*, 1994; Wise *et al.*, 1997; Rubin, 2000; Lapolt and Lu, 2001). This review focuses primarily on our work; however, we have benefited immensely from the studies of our colleagues and will cite them in appropriate places. It should be noted that a better understanding of the mechanisms regulating female reproductive aging will be important to gerontologists because the female reproductive system undergoes senescence relatively early during the aging process, in the absence of pathological changes that often confound gerontological studies. Therefore, we hope that concepts derived from our understanding of the aging reproductive system may shed light on the process of the biology of aging of other systems (Kirkwood, 1998).

## II. The Central Nervous System Plays an Important Role in Female Reproductive Aging

Our studies have focused on the influence of changes in the central nervous system (CNS) on female reproductive aging and the repercussions of hypoestrogenicity on the brain. For many years, it was accepted that reproductive aging – and, in particular, the menopause in women – resulted simply from the depletion of the postmitotic pool of ovarian follicles that is set down during embryonic

development (vom Saal *et al.*, 1994). It was thought that CNS changes that accompany the menopause were merely a consequence of declining ovarian function. More recently, investigators have realized that the brain plays an important role in the sequence of events leading to reproductive senescence. It appears that the temporal patterns of neural signals undergo subtle but important changes during middle age in both women and animal models, before the cessation of reproductive cycles, and that these changes may accelerate the loss of follicles leading to the menopause.

These conclusions are based predominantly upon studies performed in rodent models. Therefore, one must ask whether experimental results using animal models of reproductive senescence will improve our understanding of the human menopause. This remains controversial, with advocates on both sides of this lively discussion. On the one hand, since rodents do not undergo a real menstrual cycle, by definition, they do not undergo a true 'meno-pause.' Arguments that rodents are not good models of the human menopause are based on two important observations. First, in postmenopausal women, plasma gonadotropin levels are high; whereas in aged acyclic, repeatedly pseudopregnant rats, they remain relatively normal, despite substantial decreases in estradiol in both species (for a review, see Wise, 2000). This suggests that decreased estrogen secretion is paramount to the postmenopausal state and hypothalamic influences may not be as critical in the human female. In contrast, hypothalamic influences are paramount to the postreproductive state in rodents: ovaries of young rodents transplanted into old hosts fail to cycle normally, whereas ovaries of old rodents transplanted into young hosts respond to neuroendocrine signals and reproductive cyclicity is restored. However, the regularity of the cycle is not totally restored (Peng and Huang, 1972; Felicio *et al.*, 1983). Second, it has been suggested that the temporal dynamics of loss of the ovarian follicular reserve are fundamentally different in women, compared to rodents. However, we would contend that thorough study and careful analysis of the rate of loss of primordial follicles have not been performed in rodents the way they have been in humans (Faddy *et al.*, 1983; Richardson *et al.*, 1987).

Despite these differences between older postmenopausal women and older acyclic rats, striking parallels exists between middle-aged female rats and pre- and perimenopausal women. First, the sentinel event that heralds impending reproductive decline in both humans and rats is a rise in follicle-stimulating hormone (FSH) concentrations (DePaolo, 1987; Klein *et al.*, 1996). In humans, the change is prominent during the periovulatory phase of the menstrual cycle. In a similar manner, middle-aged rats exhibit elevated FSH levels during estrous afternoon. Second, the pattern of luteinizing hormone (LH) secretion changes in both perimenopausal women and middle-aged rats as they enter the transition to acyclicity. Controversy exists as to the changes in pulsatile LH release in pre- and perimenopausal women. A report by Matt *et al.* (1998) shows that, in regularly

cycling, middle-aged women, the duration of LH pulses increases and the frequency of pulses decreases. To our knowledge, the work of Matt and colleagues is the only study performed in regularly cycling, premenopausal women. None of the other studies in humans have controlled for changing cycle length during the perimenopausal period when they monitored LH pulses. This may underlie the discrepancy among studies in humans. These results mirror the changes that we observed in middle-aged, regularly cycling rats (Scarbrough and Wise, 1990). Third, menstrual cycle length in women and estrous cycle length in rats become highly variable (Sherman *et al.*, 1976; Fitzgerald *et al.*, 1994). Cycles of increased and decreased length have been reported in women between the ages of 37–45 years, as they enter the perimenopausal transition. Likewise, rats exhibit highly variable estrous cycles, with prolonged periods of estrus or diestrus between each preovulatory LH surge. Fourth, although the postmenopausal period is characterized by extremely low levels of estradiol, several studies (Klein *et al.*, 1996; Santoro *et al.*, 1996) have shown that estradiol concentrations do not decrease during the pre- and perimenopausal period but, in fact, remain normal or are elevated. These new findings are strikingly similar to what has been observed in middle-aged rats as they enter the transition to irregular cyclicity (Butcher and Page, 1981; Lu, 1983). Finally, the ability of estradiol to induce LH surges is attenuated in both perimenopausal women and middle-aged rats. Van Look and colleagues (1977) showed that estradiol was able to induce LH surges of attenuated amplitude in only a small portion of the women studied. This parallels precisely the changes that we observed in middle-aged rats (Wise, 1984). For all these reasons, we believe that rodents serve as excellent models in which to examine the factors that initiate the process of reproductive aging during middle age. We assume that information gained from these species can be extrapolated to humans and will allow us to uncover and explore concepts that can be generalized to human reproductive aging.

## III. Changes in the Pattern of Gonadotropin Secretion Reflect Changes in Hypothalamic GnRH Input

We (Wise, 1982a) and other investigators (van der Schoot, 1976; Lu, 1983; Nass *et al.*, 1984) established that one of the earliest changes that occurs during middle age is in the secretion pattern of the preovulatory LH and FSH surge. In rats that had not exhibited any change in estrous cycle length and still maintained normal, regular, 4-day estrous cycles, we observed a consistent delay in the onset of the LH surge and attenuation in peak concentrations (Figure 1). These changes occur at an age when we could not detect any changes in the responsiveness of the pituitary gland to GnRH. Therefore, we asked: do GnRH neurons change during the middle-aged period, leading to alterations in the ability to drive LH secretion? Methods to directly quantitate changes in the secretion pattern of

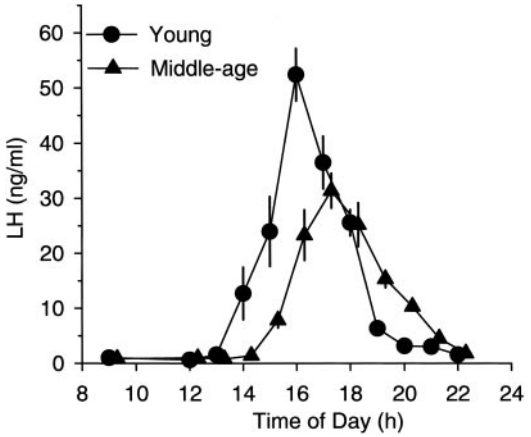


FIG. 1. The luteinizing hormone (LH) surge is blunted and delayed in middle-aged, compared to young, rats. Young and middle-aged regularly cycling rats were sequentially bled from right atrial cannulae during the day of proestrus. Plasma was radioimmunoassayed for LH. The first significant increase in LH was delayed by 1 hour and attenuated significantly in middle-aged rats.

GnRH have been problematic, since the hormone is not detectable in peripheral blood, GnRH neurons are few in number, and are distributed diffusely in the anterior preoptic region of the hypothalamus. Instead, investigators have used immunocytochemical methods to determine the number of GnRH neurons and the percentage that are activated. Expression of immediate early genes (e.g., Fos) by individual neurons has been used as a marker of increased neuronal activity (Hoffman *et al.*, 1993). Therefore, we used this method to test whether alterations in the timing and amplitude of the proestrous LH surge involve alterations in the activation of GnRH neurons. In these studies, we have never detected any age-related change in the number of GnRH neurons (Lloyd *et al.*, 1994; Krajnak *et al.*, 2001). However, the number of GnRH neurons that express Fos during the proestrous LH surge decreased dramatically in middle-aged, regularly cycling rats (Figure 2). These results have been confirmed and extended using three-dimensional reconstructions of the forebrain populations of GnRH neurons in young and middle-aged rats (Rubin *et al.*, 1995). Our studies strongly suggest that the pattern of afferent input to GnRH neurons changes during the early stages of reproductive aging. It should be noted that additional changes in GnRH activity have been reported by investigators who have utilized rats that have reached more advanced stages of reproductive senescence, particularly during times of increased demand on the population of GnRH neurons. These changes include decreased *in vivo* output of GnRH in conjunction with the steroid-induced LH surge using push-pull perfusion (Rubin and Bridges, 1989) and decreased LH pulse frequency and amplitude in ovariectomized rats (Scarbrough

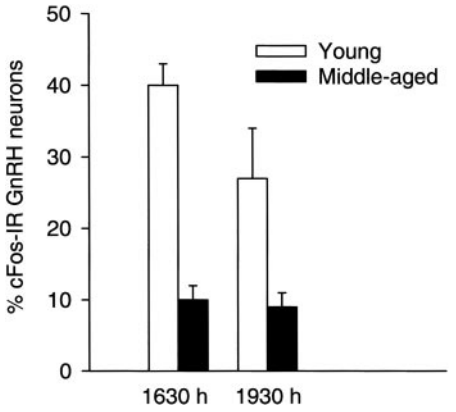


FIG. 2. Percent of gonadotropin-releasing hormone (GnRH) neurons that express Fos during the proestrous LH surge decreases with age. Young and middle-aged rats regularly cycling rats were perfused with paraformaldehyde and their brains sectioned for dual immunocytochemical localization of GnRH and Fos. Age significantly decreased the level of activation of GnRH neurons. [Reprinted with permission from Lloyd JM, Hoffman GE, Wise PM 1994 Decline in immediate early gene expression in gonadotropin-releasing hormone neurons during proestrus in regularly cycling, middle-aged rats. Endocrinology 134:1800–1805. Copyright The Endocrine Society.]

and Wise, 1990). In addition, investigators have observed a decline in pituitary GnRH receptor mRNA (Rubin and Jimenez-Linan, 1999) and binding (Marchetti and Cioni, 1988), which are regulated by the pattern of pulsatile GnRH secretion. These age-related receptor changes are likely to lead to decreased responsiveness to GnRH (Smith *et al.*, 1982; Hogg *et al.*, 1992; Brito *et al.*, 1994).

## IV. Changes in the Temporal Pattern and Synchrony of Neurotransmitter Input May Alter the Pattern of GnRH Secretion

The pattern of preovulatory GnRH secretion is determined by afferent input from multiple neurotransmitters and neuropeptides. Over the past 50 years, researchers have come to recognize the complexity of the signaling system that ultimately leads to the GnRH surge. We continue to add to the list of neurotransmitters and neuropeptides that modulate the pattern of GnRH secretion. The hierarchy of modulators is still unclear. Indeed, it is not clear whether a single neurotransmitter is essential or whether there is plasticity in the repertoire of neurotransmitters that can participate or substitute for one another in the generation of the GnRH surge. Data favor the latter possibility: there is remarkable redundancy and plasticity in the ensemble of factors that influences GnRH secretion. Together, they insure the maintenance or reappearance of LH surges when one of the players is disrupted. Thus, Clifton and Sawyer (1979) found that

disruption of the catecholaminergic input to GnRH neurons halts estrous cyclicity but only temporarily, suggesting that, when necessary, other inputs are able to replace the important role that catecholamines normally play in GnRH secretion. It is clear that the synchrony, timing, and interplay among the multiple neural signals are required to insure that the proper timing and amplitude of preovulatory LH surges is maintained.

In an elegant and seminal series of studies beginning over 50 years ago, Everett and Sawyer (Everett *et al.*, 1949; Sawyer *et al.*, 1949; Everett and Sawyer, 1950,1953) showed that if the neural signals that regulate the LH surge are delayed by even 2 hours, the surge is delayed by an entire day, occurring at the proper time 24 hours later. Thus, these studies implicated that a circadian pacemaker regulates the precise timing of the events leading to the LH surge and the exact timing of the surge itself. Thus, the daily rhythmicity in the activity of neural events serves as a foundation for the orderly timing of the GnRH surge and hence the preovulatory release of LH. Over the past several years, we have examined whether the diurnal rhythm in various aspects of neural activity is altered in middle-aged rats as they begin the transition to reproductive senescence. We measured the diurnal rhythm of monoamine turnover rates (Wise, 1982b; Cohen and Wise, 1988), neurotransmitter receptor densities (Weiland and Wise, 1990), and neuropeptide mRNAs (Weiland *et al.*, 1992; Cai and Wise, 1996; Krajnak *et al.*, 1998; M.J. Smith, A.B. Cashion, L. Jennes and P.M. Wise, unpublished observations). The clear theme that emerges is that virtually all of the rhythms are dampened or altered when female rats are middle aged and begin the transition to irregular estrous cycles. Often, the attenuation in rhythmicity was progressive and changes were more exaggerated as animals aged. The change in rhythmicity of any single neurotransmitter must be considered subtle, since the overall average often did not change. Investigators who measure these endpoints at only one time of day would be unlikely to detect a significant age-related change. Yet, together, disruption of the synchrony and coordination of multiple neural signals that govern the precise timing of GnRH secretion may ultimately lead to profound changes in the ability of rats to maintain regular reproductive cyclicity.

Until very recently, we thought that deterioration in the integrity of the circadian biological clock, which is located in the suprachiasmatic nuclei (SCN) in mammals, may underlie the desynchronization of multiple rhythms (Wise *et al.*, 1988,1997). However, our newest findings suggest that we must modify our thinking. We tested our hypothesis by measuring the rhythm of key neuropeptides in the SCN. We reasoned that if aging involves a change in the integrity of the SCN itself and all its essential elements (i.e., inputs, oscillators, outputs), the pattern of expression of all critical neuropeptides of the SCN would be affected. On the other hand, if only some of the components of the clock are affected with age, we might observe differential effects on these neuropeptides. We measured

the rhythm of gene expression of two key functionally critical neuropeptides heavily expressed in the SCN: vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP) (Krajnak *et al.*, 1998). VIP neurons are located primarily in the ventrolateral aspect of the SCN, where they receive direct retinal input (Ibata *et al.*, 1989). The rhythmic expression of VIP mRNA and protein depends upon exposure to the light/dark cycle (Albers *et al.*, 1990). Therefore, this neuropeptide is likely to convey time-of-day information to efferent targets in different regions of the brain. In contrast, AVP neurons are predominantly located in the dorsomedial portion of the SCN. The 24-hour rhythm in its gene and peptide expression is endogenous and does not depend upon the light/dark cycle for its existence. The AVP rhythm, therefore, serves as a marker of the integrity of the SCN (Gillette and Reppert, 1987). Both VIP and AVP neurons relay circadian information to several regions of the brain by sending efferent projections to diverse regions of the brain, including the rostral preoptic area, where they may influence GnRH neurons or other neurotransmitters that regulate GnRH secretion (Harney *et al.*, 1996; van der Beek *et al.*, 1999; Smith *et al.*, 2000; Krajnak *et al.*, 2001). Our results were clear – and surprising. As expected, VIP and AVP mRNA levels exhibited a 24-hour rhythm in young females. Furthermore, the rhythm in VIP mRNA disappeared by the time animals were middle aged (Figure 3). In marked contrast, the AVP mRNA rhythm was totally unaffected with age: the rhythm and overall level of mRNA were the same in

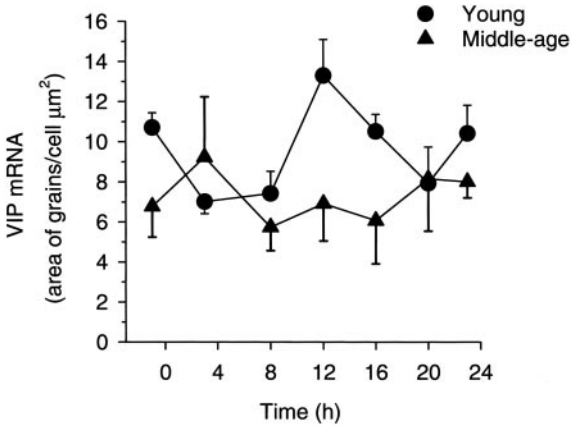


FIG. 3. VIP mRNA levels/cell in young and middle-aged ovariectomized, estradiol-treated rats as measured by *in situ* hybridization exhibits age-related changes in rhythmicity. Young, middle-aged, and old rats were killed at 7 times of day over a 24-hour period. Young rats exhibited a diurnal rhythm in gene expression. The rhythm was no longer detectable in middle-aged or old rats. [Reprinted from Krajnak K, Kashon ML, Rosewell KL, Wise PM 1998 Aging alters the rhythmic expression of vasoactive intestinal polypeptide mRNA, but not arginine vasopressin mRNA in the suprachiasmatic nuclei of female rats. J Neurosci 18:4767–4774.]

young, middle-aged, and old rats, both in terms of the amount of mRNA/cell (Figure 4) and the number of cells expressing AVP mRNA. This was the first time that we had observed the preservation of a neural rhythm in middle-aged rats. Based on these results, we concluded that the integrity of the entire biological clock does not deteriorate in a unified manner; instead, age differentially influences various components of the SCN.

The disappearance of the rhythm in VIP expression in the SCN is particularly intriguing, since this neuropeptide may play a uniquely important role in conveying time-of-day information directly to GnRH neurons. We hypothesized that the decrease in VIP levels in the SCN and the disappearance of its rhythm may lead to the delay in the timing of the LH surge and the attenuation in its amplitude. To test this possibility, we suppressed the level and rhythm of VIP by administering antisense oligonucleotides to VIP directed at the SCN of ovariectomized, estradiol-treated rats and assessed the effect on the LH surge (Harney *et al.*, 1996). Peak LH concentrations during the surge were delayed and attenuated in antisense-treated animals, compared to random, oligo-treated control rats, in a manner that was strikingly similar to that observed previously in middle-aged rats (Figure 5). Similar results have been obtained using administration of VIP antisera intracerebroventricularly to ovariectomized, estradiol-treated rats (van der Beek *et al.*, 1999). More recently, we determined whether aging alters the innervation of GnRH neurons by VIP and/or the ability of VIP to activate GnRH

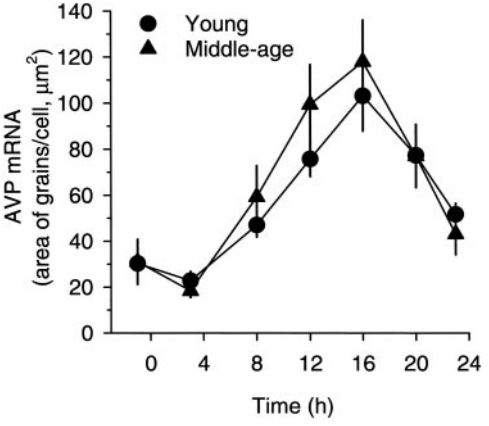


FIG. 4. Arginine vasopressin (AVP) mRNA levels/cell in young and middle-aged ovariectomized, estradiol-treated rats as measured by *in situ* hybridization exhibits age-related changes in rhythmicity. Brain sections from the same young, middle-aged, and old rats were used to measure AVP and vasoactive intestinal peptide (VIP) (see Figure 3). The diurnal rhythm was identical in all age groups. [Reprinted from Krajnak K, Kashon ML, Rosewell KL, Wise PM 1998 Aging alters the rhythmic expression of vasoactive intestinal polypeptide mRNA, but not arginine vasopressin mRNA in the suprachiasmatic nuclei of female rats. J Neurosci 18:4767–4774.]

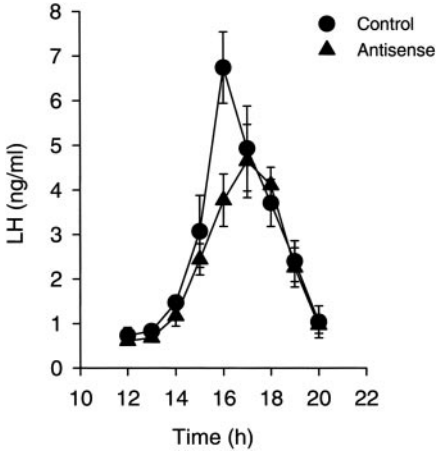


FIG. 5. The steroid-induced LH is blunted and delayed in rats that were treated with antisense oligos to VIP or control scrambled oligos directly at the suprachiasmatic nucleus (SCN). Ovariectomized, estradiol-treated young rats were administered antisense or scrambled oligos and sequentially bled. The steroid-induced surge of LH exhibited changes that are remarkably like those observed during aging. [Reprinted with permission from Harney JP, Scarbrough K, Rosewell KL, Wise PM 1996 *In vivo* antisense antagonism of vasoactive intestinal peptide in the suprachiasmatic nucleus causes aging-like changes in the estradiol-induced LH and prolactin surge. Endocrinology 137:3696–3701. Copyright The Endocrine Society.]

neurons by examining the effects of aging on the number of GnRH neurons apposed by VIP fibers and the number of GnRH neurons that receive VIP input that express Fos. Using triple-label immunocytochemistry for GnRH, VIP, and Fos in young and and middle-aged females, we quantified the number of GnRH neurons, GnRH neurons apposed by VIP fibers, and the number of GnRH neurons that express Fos that are apposed by VIP fibers. Our results clearly demonstrate that aging does not alter the number of GnRH neurons that receive VIP innervation. However, the number of GnRH neurons that receive VIP innervation and co-express Fos decreases significantly (Krajnak *et al.*, 2001) (Figure 6). Immunocytochemical methods and light microscopy did not allow us to evaluate whether VIP acted directly upon GnRH neurons or through another neurotransmitter. However, several lines of evidence suggest that the communication between VIP and GnRH neurons is direct. 1) Using triple-label immunofluorescence to simultaneously localize GnRH, VIP, and $VIP_2$ receptor protein, we showed that about 40% of all GnRH neurons analyzed contain $VIP_2$ receptor immunoreactivity and that VIP-containing processes were seen in close apposition to a significant number of $VIP_2$ receptor-positive GnRH neurons (Smith *et al.*, 2000). 2) Horvath and colleagues demonstrated synaptic contacts between VIP- and GnRH-containing neurons (Horvath *et al.*, 1998). 3) Lesions of the

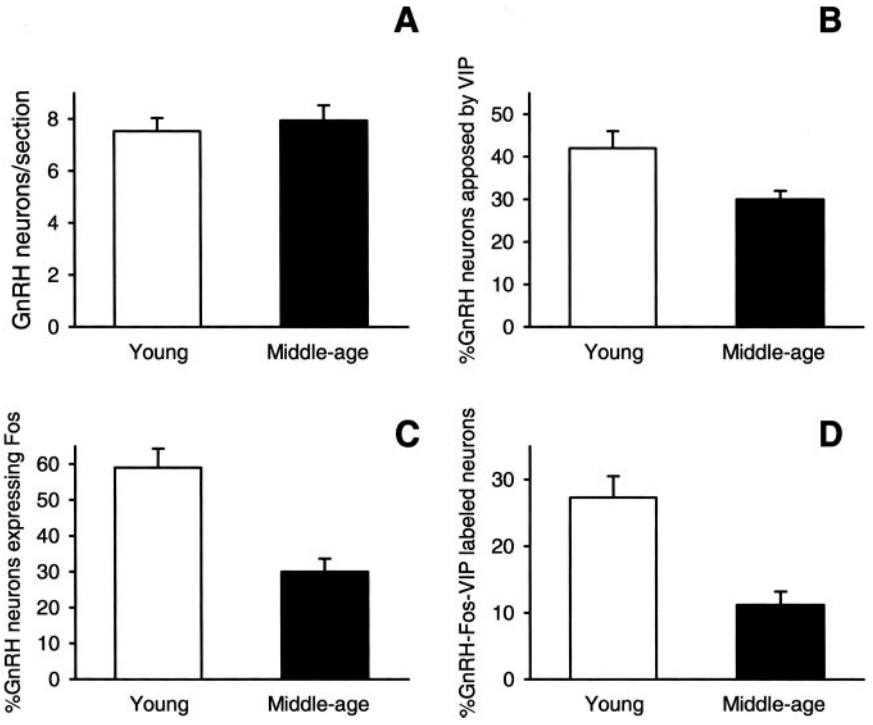


FIG. 6. (A) Number of GnRH-immunopositive neurons per section; (B) percent of GnRH and VIP-immunopositive neurons; (C) percent of GnRH and Fos-immunopositive neurons; and (D) percent of GnRH, Fos, and VIP immunoreactive neurons in the preoptic area of young and middle-aged females during the peak of a steroid-induced LH surge exhibit age-related changes. Aging is associated with no change in the number of GnRH immunopositive neurons or the percent of GnRH and VIP immunopositive neurons. However, percent of activated GnRH neurons and the percent of activated GnRH that were closely apposed to VIP neurons decreased with age. [Reprinted from Krajnak K, Rosewell KL, Wise PM 2001 Fos-induction in gonadotropin-releasing hormone neurons receiving vasoactive intestinal polypeptide innervation is reduced in middle-aged female rats. Biol Reprod 64:1160–1164.]

SCN indicate the presence of direct, VIP-containing projections to GnRH neurons (van der Beek *et al.*, 1993). Together, these findings provide further support for a direct, VIP-containing pathway from the SCN to GnRH neurons and indicate that VIP can communicate directly with GnRH neurons. Furthermore, it appears that the age-related delay in the timing of the LH surge is not due to a change in VIP innervation of GnRH neurons but instead may result from a decreased sensitivity of GnRH neurons to VIP input.

## V. Aging Influences the Balance Between Stimulatory and Inhibitory Neural Inputs to GnRH Neurons

The ultimate pattern of GnRH release is governed by the orchestration of stimulatory and inhibitory inputs. Most work has focused on stimulatory side of the balance sheet, since, clearly, this is critical to the GnRH surge. Most recently, investigators have focused attention on the possible role of decreased glutamate input to GnRH neurons in aging rats (Zuo *et al.*, 1996; Gore *et al.*, 2000a,b), since glutamate is a neurotransmitter that exerts important direct stimulatory effects on GnRH neurons (Brann, 1995; Eyigor and Jennes, 1996). However, we are beginning to appreciate more deeply that the amplitude and timing of the preovulatory LH surge depends upon a decrease in inhibitory tone (Akabori and Barraclough, 1986a,b; Smith and Gallo, 1997). Opioid peptides and gamma aminobutyric acid (GABA) are critical inhibitory neurotransmitters that normally restrain GnRH secretion during the estrous cycle. A decrease in their activity normally occurs on proestrous afternoon, permitting stimulatory factors to maximally influence GnRH neurons. Researchers have found that, unless the inhibitory inputs to GnRH neurons are suppressed, the effects of norepinephrine and other stimulatory factors do not result in LH surges of normal amplitude or timing (for a review, see Kalra and Kalra, 1984). In addition, pharmacological blockade of the inhibitory tone early on proestrus results in a premature LH surge. We currently are examining the diurnal rhythm of preprodynorphin mRNA levels in young and middle-aged proestrous rats to determine whether the rhythm of this neuropeptide is altered and whether its activity increases in middle-aged rats and may contribute to the diminished LH surge (M.J. Smith, A.B. Cashion, L. Jennes, and P.M. Wise, unpublished observations). Preliminary data (Figure 7) demonstrate that, in young proestrous rats, preprodynorphin mRNA levels decrease prior to the LH surge, decreasing inhibitory tone and possibly allowing stimulatory factors to be maximally effective. In middle-aged rats, the diurnal rhythm is no longer detectable and overall mRNA levels are higher, compared to young controls. Thus, it is possible that the inhibitory tone that dynorphin communicates to GnRH neurons is amplified in middle-aged rats and does not subside to allow stimulatory factors to act. This could contribute to the attenuated and delayed preovulatory surge observed in middle-aged animals. We currently are examining whether GABAergic tone also may increase with age. This is another important inhibitory neuropeptide that communicates directly with GnRH neurons (Petersen *et al.*, 1993) and exerts an important inhibitory tone on GnRH secretion. The role of this inhibitory neurotransmitter may be particularly important, since estradiol's actions on GABA may regulate cyclic morphological changes in astrocytes (Tranque *et al.*, 1987; Parducz *et al.*, 1993; Mong *et al.*, 1999). These changes in the stellation of astrocytes on proestrus may then affect ensheathment of neurons, including possibly GnRH

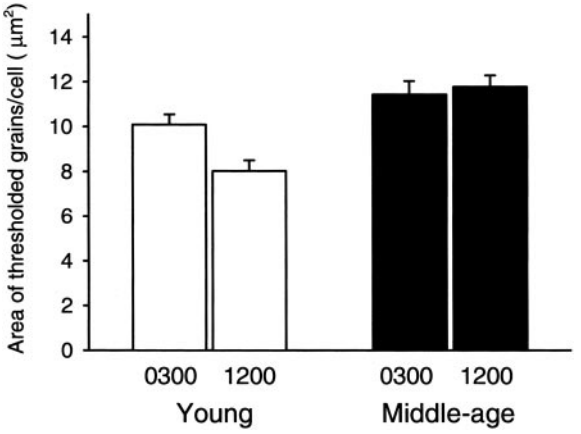


FIG. 7. Preprodynorphin mRNA levels are higher and lack rhythmicity in middle-aged compared to young rats on proestrus. Young and middle-aged rats were killed at 0300 and 1200h on proestrus. Brains were prepared for *in situ* hybridization. Preprodynorphin mRNA was analyzed in the anteroventral periventricular nucleus (AVPV). Overall levels of gene expression are elevated and the rhythm is no longer detectable by the time rats reach middle age.

neurons (Cashion *et al.*, 2001), leading to changes in the ability of neurotransmitters to communicate with each other to coordinate the GnRH surge.

## VI. Estradiol Is a Neuroprotective Factor

Recently, many researchers have focused their attention on the nonreproductive protective actions of estrogen. Aging and the menopause involve the gradual depletion of the ovarian follicular reserve and, with it, a decrease in plasma levels of estradiol. Since women will be spending a considerable portion of their lives in a hypoestrogenic state, the potential that women will be more vulnerable to neurodegenerative diseases and injury, due to the lack of estrogen, becomes even more important to understand. Numerous recent reviews provide excellent documentation of the many experimental and clinical circumstances in which estrogens provide profound protection against neuronal cell death (Green and Simpkins, 2000; Hurn and Macrae, 2000; Roof and Hall, 2000; Garcia Segura *et al.*, 2001; Wise *et al.*, 2001a,b). We will focus on our studies in this emerging area of interest.

We reported that low physiological levels of estradiol replacement dramatically decrease the degree of brain injury and cell death in an animal model of cerebrovascular stroke (Dubal *et al.*, 1998). When we occlude the middle cerebral artery (MCAO) and permanently decrease blood flow to approximately 50% of normal, both the cerebral cortex and striatum undergo cell death.

Ovariectomized rats are particularly vulnerable to injury and exhibit progressive infarction that evolves over a 24-hour period. Replacement with estradiol, to levels that mimic those which normally occur during the estrous cycle, results in profound protection of the cortex but not the striatum. Interestingly, middle-aged rats were equally responsive to the protective actions of estradiol (Dubal and Wise, 2000) (Figure 8). Our findings that estradiol replacement exerts equivalent neuroprotection in young and middle-aged female rats were unexpected because responsiveness of the hypothalamus to estradiol, as measured by a variety of endpoints – such as estradiol-induced activation of GnRH neurons that leads to LH surges (Wise, 1982b,1984; Lloyd *et al.*, 1994), organization of diurnal rhythmicity in the hypothalamic neurotransmitter activity (Wise, 1982b; Cohen and Wise, 1988) or gene expression (Weiland *et al.*, 1992; Krajnak *et al.*, 1998), and stimulation of progesterone receptor binding (Wise *et al.*, 1984) – diminishes with age. Therefore, we had hypothesized that estradiol would be less able to protect the brains of older animals against ischemic brain injury.

We have begun to investigate the cellular and molecular mechanisms that mediate the protective actions of estradiol. Several of our observations lead us to believe that low physiological levels of estradiol act through estrogen receptor alpha (ER$\alpha$)-dependent mechanisms, leading to changes in gene expression that favor cell survival and suppress apoptotic cell death. First, we found that estradiol slows the progression, rate, and extent of cell death in the brain. Hormone treatment does not influence the extent of cell death that occurs immediately after stroke injury. Instead, its effects are confined to protecting against delayed cell death that occurs during the later phases of injury. Thus, it appears that estradiol protects specifically against apoptotic cell death (Dubal *et*

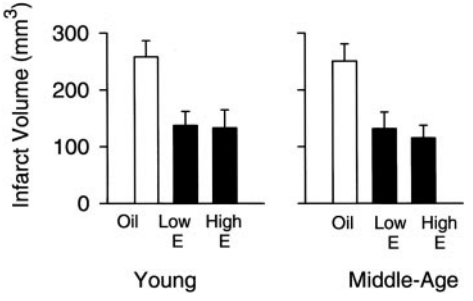


FIG. 8. Estradiol protects against middle cerebral artery occlusion (MCAO) in young and middle-aged rats. Low and high physiological levels of estradiol decreased total injury as measured by staining of brain sections with 2% triphenyltetrazolium chloride and measurement of infarct size using a computer-assisted imaging system and NIH Image. [Reprinted with permission from Dubal DB, Wise PM 2000 Neuroprotective effects of estradiol in middle-aged female rats. Endocrinology 142:43–48. Copyright The Endocrine Society.]

*al.*, 2001a; Rau *et al.*, 2001) but does not protect against immediate necrotic cell death. Second, we (Dubal *et al.*, 1999a) reported that, within 24 hours of MCAO, ER$\alpha$ mRNA is dramatically upregulated and estradiol pretreatment prevents injury-induced downregulation of ER$\beta$ in the cerebral cortex (Figure 9). These data suggest that brain injury may influence responsiveness of the injured cerebral cortex to estradiol and induce differential actions that are mediated by each receptor subtype (Nilsen *et al.*, 2000; Patrone *et al.*, 2000). It is important to note that ER$\alpha$ is not usually detectable in the cerebral cortex of the adult rat and is only transiently expressed in this brain region during neonatal development when the cortex undergoes dramatic neurogenesis, neuritogenesis, and differentiation. It is intriguing to speculate that the dramatic reappearance of ER$\alpha$ in the cerebral cortex may allow a recapitulation of the developmental actions of estradiol in promoting neurogenesis and re-differentiation of the cortex. Several studies support the concept that, following stroke injury, specific features of brain function revert to those seen during early stages of development, with the process of recovery recapitulating ontogeny (reviewed in Cramer and Chopp, 2000). To test whether ER$\alpha$ is a critical functional link in estradiol-mediated neuroprotection, we performed parallel studies in ER$\alpha$ knockout mice (Dubal *et al.*, 2001b). We found that deletion of ER$\alpha$ completely abolishes the protective actions of estradiol in all regions of the brain, whereas estradiol's ability to protect against

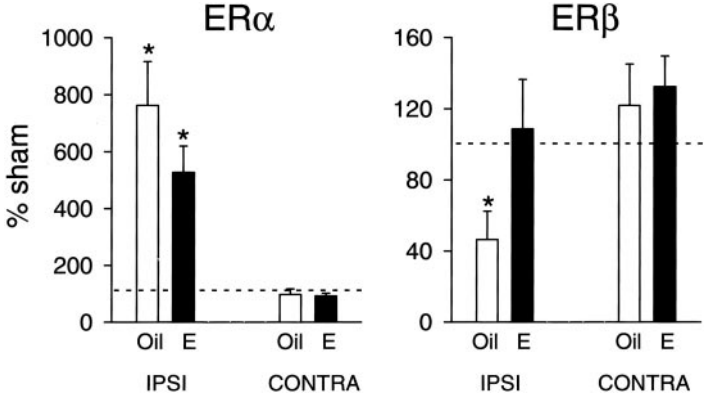


FIG. 9. Estrogen receptor alpha (ER$\alpha$) and estrogen receptor beta (ER$\beta$) gene expression are differentially modulated after MCAO. (Left panel) ER$\alpha$ mRNA was dramatically upregulated in the ipsilateral cortex of oil (vehicle)- and estradiol-treated rats, compared to the contralateral cortex and compared to constitutive levels in both oil- and estradiol-treated sham controls. (Right panel) Estradiol treatment prevented the injury-induced downregulation of ER$\beta$ mRNA in the ipsilateral cortex. In the absence of estradiol, ER$\beta$ gene expression declined significantly after injury below sham control constitutive levels. [Reprinted from Dubal DB, Shughrue PJ, Wilson ME, Merchenthaler I, Wise PM 1999 Estradiol modulates Bcl-2 in cerebral ischemia: a potential role for estrogen receptors. J Neurosci 19:6385–6393.]

brain injury is totally preserved in the absence of ER$\beta$ (Figure 10). These results firmly establish ER$\alpha$ as the critical mechanistic link that mediates the neuroprotective effects of physiological levels of estradiol. Finally, we have found that estradiol treatment alters the expression of multiple genes that have been implicated in the balance between cell survival and cell death, including bcl-2 (Dubal *et al.*, 1999a), c-fos (Rau *et al.*, 2000), galanin (Dubal *et al.*, 1999b), and activin (Böttner *et al.*, 2001). Whether any or all of these genes are critical functional mediators of estradiol's protective actions is not clear at present. Much more work must be done to understand the roles of each of these factors in protecting the brain against injury and cell death.

We have used *in vitro* methods to assess the protective actions of estradiol because this methodology provides invaluable tools that complement *in vivo* approaches. Multiple manipulations can be performed *in vitro* that are not technically or financially feasible using *in vivo* models. In particular, organotypic explants provide a powerful way to manipulate cellular environments *in vitro*, while maintaining interneurons, spatial relationships, local synaptic connections, and interactions with the local glial environment. Using explant cultures of the neonatal cerebral cortex, we (Wilson *et al.*, 2000) have shown that low concentrations of estradiol protect against cell death. Our studies strongly suggest that ERs are critical, since the protection was not observed using 17$\alpha$-estradiol and was blocked by co-incubation with ICI 182,780, an ER antagonist (Figure 11).

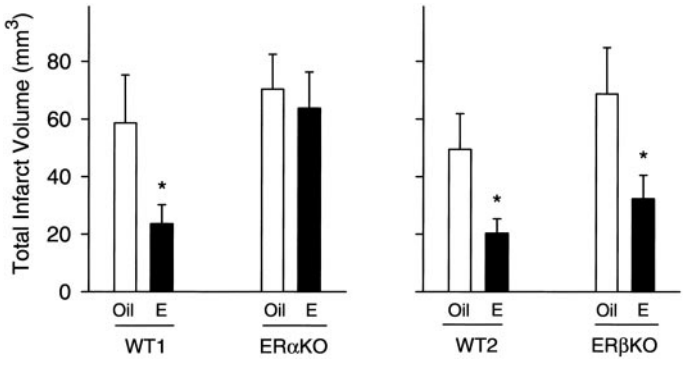


FIG. 10. Estradiol protects against MCAO in wild-type mice of both genetic backgrounds and in ER$\beta$KO mice but not in ER$\alpha$KO mice. (Left panel) Estradiol significantly decreased infarct volume in wild-type compared with oil (vehicle)-treated controls. In contrast, in ER$\alpha$KO mice, estradiol did not exert any protective effect. (Right panel) Estradiol significantly decreased infarct volume in wild-type and ER$\beta$KO mice. Brain sections were stained with hematoylin and eosin and the volume of the infarct was quantified with a computer-assisted imaging system using NIH Image. [Reprinted from Dubal DB, Zhu B, Yu B, Rau SW, Shughrue PJ, Merchenthaler I, Kindy MS, Wise PM 2001 Estrogen receptor-$\alpha$, not -$\beta$, is a critical link in estradiol-mediated protection against brain injury. Proc Natl Acad Sci USA 98:1952–1957. Copyright National Academy of Sciences.]

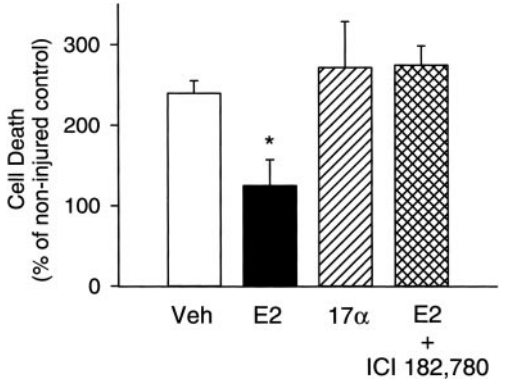


FIG. 11. Estradiol protects against ischemic injury-induced cell death in explant cultures of the cerebral cortex. Cell death, as measured by lactose dehydrogenase (LDH) release, is significantly decreased when explants were cultured in the presence of 17$\beta$-estradiol. However, 17$\alpha$-estradiol failed to protect and ICI182,780, an ER antagonist, blocked the protective actions of 17$\beta$-estradiol. [Reprinted from Wilson ME, Dubal DB, Wise PM 2000 Estradiol protects against injury-induced cell death in cortical explant cultures: a role for estrogen receptors. Brain Res 873:235–242.]

These findings complement those of Gollapudi and Oblinger (1999a,b), who showed that PC12 cells transfected with full-length rat ER$\alpha$ respond to the protective effects of estradiol, whereas cells transfected with vector DNA alone are not protected by estradiol. Other investigators have found that pharmacological levels of estradiol protect, even in the absence of the ER (for reviews, see Green and Simpkins, 2000; Hurn and Macrae, 2000; Roof and Hall, 2000; Garcia Segura *et al.*, 2001; Wise *et al.*, 2001a,b). The cellular and molecular mechanisms that underlie these receptor-independent protective actions are likely to result from estrogen's antioxidant, scavenging, immune-suppressing, and vascular actions. Together, these studies emphasize the breadth of the repertoire of mechanisms that estrogens use to protect against injury and cell death.

## VII. Summary

In summary, our understanding of the role of the brain in reproductive aging – and, conversely, the impact of reproductive aging on the brain – has increased dramatically during the past 20 years. Subtle changes in hypothalamic function and the ability of estradiol to influence the secretion of GnRH begin early during the aging process, ultimately leading to reproductive acyclicity. We are increasingly aware that the permanent hypoestrogenic state has major repercussions on multiple organs and physiological systems. Much attention is focused on the possible mechanisms through which estrogens may act as protective factors.

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