Neuroendocrine Modulation and Repercussions of Female Reproductive Aging

PHYLLIS M. WISE, MATTHEW J. SMITH, DENA B. DUBAL, MELINDA E. WILSON, SHANE W. RAU, ADRIENNE B. CASHION, MARTINA BÖTTLER, AND KATHERINE L. ROSEWELL

Department of Physiology, University of Kentucky College of Medicine, Lexington, Kentucky 40536-0298

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 ‘menopause.’ 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 postmeno-
pausal 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
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 (Scarborough,

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.
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

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.]
disruption of the catecholaminergic input to GnRH neurons halts estrous cyclic-
ity 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 senes-
cence. 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 signi
f
fi
ca
c
ificant 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 neuropep-
tides 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.
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

![Graph showing VIP mRNA levels/cell in young and middle-aged ovariotomized, estradiol-treated rats.](image)
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.]
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 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
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.

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.]
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
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.

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.
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, ERα mRNA is dramatically upregulated and estradiol pretreatment prevents injury-induced downregulation of ERβ 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 ERα 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 ERα 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 ERα is a critical functional link in estradiol-mediated neuroprotection, we performed parallel studies in ERα knockout mice (Dubal et al., 2001b). We found that deletion of ERα completely abolishes the protective actions of estradiol in all regions of the brain, whereas estradiol’s ability to protect against

![Graph](image-url)  

FIG. 9. Estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) gene expression are differentially modulated after MCAO. (Left panel) ERα 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 ERβ mRNA in the ipsilateral cortex. In the absence of estradiol, ERβ 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 ERβ (Figure 10). These results firmly establish ERα 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 17α-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 ERβKO mice but not in ERαKO mice. (Left panel) Estradiol significantly decreased infarct volume in wild-type compared with oil (vehicle)-treated controls. In contrast, in ERαKO mice, estradiol did not exert any protective effect. (Right panel) Estradiol significantly decreased infarct volume in wild-type and ERβKO mice. Brain sections were stained with hematoxylin 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-α, not -β, is a critical link in estradiol-mediated protection against brain injury. Proc Natl Acad Sci USA 98:1952–1957. Copyright National Academy of Sciences.]
These findings complement those of Gollapudi and Oblinger (1999a,b), who showed that PC12 cells transfected with full-length rat ERα 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.
REFERENCES

Ahmed, Hissong SA 1999 Gender and risk of autoimmune diseases: possible role of estrogenic compounds. Envir Health Perspect Supp 107:681


Clifton DK, Sawyer CH 1979 LH release and ovulation in the rat following depletion of hypothalamic norepinephrine: chronic vs. acute effects. Neuroendocrinology 28:442–449


Compston JE 2001 Sex steroids and bone. Physiol Rev 81:419–447


DePaolo LV 1987 Age-associated increases in serum follicle-stimulating hormone levels on estrus are accompanied by a reduction in the ovarian secretion of inhibin. Exptl Aging Res 13:3–7


Erkkola R 1996 Female menopause, hormone replacement therapy, and cognitive processes. Maturitas 23:S27–S30

Everett JW, Sawyer CH 1953 Estimated duration of the spontaneous activation which causes release of ovulating hormone from the rat hypophysis. Endocrinology 52:83–92

Everett JW, Sawyer CH, Markee JE 1949 A neurogenic timing factor in control of the ovulatory discharge of luteinizing hormone in the cyclic rat. Endocrinology 44:234–250

Eyigor O, Jennes L 1996 Identification of glutamate receptor subtype mRNAs in gonadotropin-releasing hormone neurons in rat brain. Endocrine 4:133–139


Felicio LS, Nelson JF, Gosden RG, Finch CE 1983 Restoration of ovulatory cycles by young ovarian grafts in aging mice; potentiation by long-term ovariectomy decreases with age. Proc Natl Acad Sci USA 80:6076–6080


Klein NA, Illegworth PJ, Groome NP, McNeilly AS, Baftaglia DE, Soules MR 1996 Decreased inhibin B secretion is associated with the monotropic FSH rise in older, ovulatory women: a study of serum and follicular fluid levels of dimeric inhibin A and B in spontaneous menstrual cycles. J Clin Endocrinol Metab 81:2742–2745
Lindsay R 1996 The menopause and osteoporosis. Obstet Gynecol 87:16S–19S
Nilsen J, Mor G, Naftolin F 2000 Estrogen-regulated developmental neuronal apoptosis is determined by estrogen receptor subtype and the fas/fas ligand system. J Neurobiol 43:64–78
Rubin BS, Jimenez-Linan M 1999 LHRH receptor mRNA levels and gonadotropin subunit mRNA levels are reduced in the pituitaries of middle-aged rats on the day of a steroid-induced LH surge. Soc Neurosci Abstr 25:1960
Sawyer CH, Everett JW, Markee JE 1949 A neural factor in the mechanism by which estrogen induces the release of luteinizing hormone in the rat. Endocrinology 44:218–233
Scarbrough K, Wise PM 1990 Age-related changes in the pulsatile pattern of LH release precede the transition to estrous acyclicity and depend upon estrous cycle history. Endocrinology 126:884–890
Smith WA, Cooper RL, Conn PM 1982 Altered pituitary responsiveness to gonadotropin-releasing hormone in middle-aged rats with 4-day estrous cycles. Endocrinology 111:1843–1848
van der Beek EM, Wiegant VM, van der Donk HA, van den Hurk R, Buijs RM 1993 Lesions of the suprachiasmatic nucleus indicate the presence of a direct vasoactive intestinal polypeptide-containing projection to gonadotropin-releasing hormone neurons in the female rat. J Neuroendocrinol 5:137–144

van der Beek EM, Swarts HJ, Wiegant VM 1999 Central administration of antiserum to vasoactive intestinal peptide delays and reduces luteinizing hormone and prolactin surges in estrogen-treated rats. Neuroendocrinology 69:227–237


Weiland NG, Wise PM 1990 Aging progressively decreases the densities and alters the diurnal rhythm of alpha-1-adrenergic receptors in selected hypothalamic regions. Endocrinology 126:2392–2397


Wise PM 1982b Norepinephrine and dopamine activity in microdissected brain areas of the middle-aged and young rat on proestrus. Biol Reprod 27:562–574

Wise PM 1984 Estradiol-induced daily luteinizing hormone and prolactin surges in young and middle-aged rats: correlations with age-related changes in pituitary responsiveness and catecholamine turnover rates in microdissected brain areas. Endocrinology 115:801–809


