Experimental Gerontology, Vol. 33, Nos. 7/8, pp.729–757, 1998

0531-5565/98 $19.00 + .00

ELSEVIER

PII S0531-5565(98)00018-7

ESTROGEN, THE OVARY, AND NEUTOTRANSMITTERS: FACTORS ASSOCIATED WITH AGING

M.M. MILLER,[1,2,3,4] H.P.J. BENNETT,[3,5] R.B. BILLIAR,[1] K.B.J. FRANKLIN[6] and D. JOSHI[1,7]

[1]Department of Obstetrics and Gynecology,
[2]Department of Anatomy,
[3]Division of Experimental Medicine, Department of Medicine,
[4]McGill Centre for Studies on Aging,
[5]Endocrine Laboratories, Department of Medicine,
[6]Department of Psychology, McGill University, Montreal, Quebec, Canada
[7]Present affiliation: B.V. Patel Pharmaceutical Education and Research Development Centre, Taltej, Ahmanabad, India

ABSTRACT—Our studies in the C57BL/6J mouse have been designed to examine the interactions of aging and the ovary, and their mutual effects on neuroendocrine function. In the pituitary, ovarian status and not age determines responsiveness to gonadotropin hormone releasing hormone (GnRH), but estrogen (E_2) is an important mediator in CNS changes, and removal of the ovary (OVX) is deleterious to the neuroendocrine hypothalamus. OVX for just six days in young animals results in synaptic loss between noradrenergic terminals and gonadotropin hormone releasing hormone (GnRH) neurons. Long-term OVX, hypothesized to protect against neuroendocrine aging, fails to guard against any studied age-related changes. Some age-related changes occur as early as midlife. Although neuron number remains constant at middle age, opiatergic neurons undergo significant functional changes by producing opiate antagonist peptides. This change appears to be caused by alterations in the prohormone convertases, which cleave propeptide to peptide. Altered peptides may trigger the loss of reproductive capacity. The midlife shift in opiate peptide production is a component of natural developmental processes that begin in the neonate and continue through old age. In the cholinergic system, E_2 mediates numbers of cholinergic receptors, cholinergic neurons, and cholinergic-modulated memory systems in both young and old animals. Regardless of age, ovarian steroids, if present at physiologic levels, are beneficial to the neuroendocrine CNS, and long-term deprivation from ovarian-produced factors is deleterious in the systems we have examined. Our studies have shown that deprivation from ovarian steroid hormones in the female appears to be a major factor in the health of the CNS and in events associated with aging. *© 1998 Elsevier Science Inc.*

Key Words: aging, estrogen, estrogen receptor, opiates, acetylcholine, norepinephrine, dopamine, female reproductive function, basal forebrain, hypothalamus, menopause, memory, hypothalamic–pituitary-ovarian axis

Correspondence to: M.M. Miller, Department of Obstetrics and Gynecology, Room F3.32, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec H3A 1A1, Canada. Tel: (514) 842-1231 ext. 4745; Fax: (514) 843-1678; E-mail: mmiller@rvhob2.lan.mcgill.ca

INTRODUCTION

AGING IS ACCOMPANIED by alterations in most physiological systems that include structural degeneration, loss of functional capacity, and impaired coordination within and between organ systems. The study of aging has many goals, one being development of strategies to prevent or minimize disability. Examination of this progression requires an understanding of the mechanisms of aging. CNS aging is critical because senescence produces both a primary effect on the brain itself and secondary effects on other organ systems. Brain maturation involves a number of different kinds of changes making it an ideal study system. At the cellular level, changes include alterations in neuron number as well as concomitant shifts in interactions of specific neurotransmitters and neuropeptidergic pathways as well as their receptors. Subcellular changes involve alterations in genetic, synthetic, and metabolic capabilities that lead to accumulation of altered products causing subsequent neuron damage.

Based upon the finding of hypothalamic and preoptic area (POA) estradiol (E_2) concentrating neurons (Stumpf and Sar, 1975), our early studies hypothesized that gonadal steroids, in particular E_2, are important modulators of neurotransmitter function. Forebrain regions were believed to contain a number of neurotransmitters, but their full identities were then unknown. We now understand that E_2 not only affects specific neurons, but also has broad central effects including those on neuronal growth and morphology, electrical activity, neurotransmitter turnover, enzyme regulation, neurosecretion, neurotransmitter uptake, and action on neurotransmitter receptors. E_2 alters behavior, neuroendocrine function, and the CNS affective state (McEwen and Parsons, 1982; McEwen, 1997). E_2 counteracts tardive dyskinesia (Bedard *et al*., 1977), memory loss (Sherwin and Tulandi, 1996), has antidepressant effects in women (Klaiber *et al*., 1979), and modulates emotional lability (Dalton, 1964). E_2 also regulates transcription of a vast array of cellular genes including structural proteins, peptides, neurotransmitters, and their receptors (Sohrabji *et al*., 1995). One one hand, studies in the human had suggested that E_2 has a supportive role in sustaining the LH surge at midlife, while long term deprivation from E_2 is deleterious (Tanaka *et al*., 1982). On other other hand, early studies in animals suggested that long-term exposure to E_2 was injurious (Brawer *et al*., 1983) and that deprivation from E_2 might protect the CNS from age related changes (Felicio *et al*., 1986). Our studies were designed to gain definition of the mechanisms of aging and of the consequences of long-term deprivation from gonadal steroids. We showed strong evidence that removal of the influence of the ovary is deleterious to the neuroendocrine hypothalamus.

To inspect the aging process we have used the well-examined female C57BL/6J mouse whose ovarian cycles are long and irregular immediately after puberty (five weeks). Cycles progressively shorten during the time of greatest fecundity (five to seven months) until reaching regular four to five-day rhythms. At midreproductive life (12–14 months) cycle length can shorten (Finch *et al*., 1984) and again lengthen; ovarian function then begins to decline and the capacity to have normal cycles is ultimately lost. These events parallel human female reproductive cycle changes, but occur within a two year time frame, making the murine reproductive system a good study model (Bronson and Vom Saal, 1979; Finch *et al*., 1980, 1984; Nelson *et al*., 1981, 1992; Gee *et al*., 1983, 1984; Mobbs *et al*., 1984; Felicio *et al*., 1986; Vom Saal and Finch, 1988; Wise *et al*., 1989).

PITUITARY CHANGES ASSOCIATED WITH AGING

The pituitary of the aged animal releases LH appropriately

The E_2-induced LH surge is markedly attentuated in old (24-month) female mice compared with young (6 month) females (Finch *et al*., 1984). We wondered whether this is was due to diminished pituitary capacity to respond to hypothalamic GnRH. We measured the pituitary response in young (five-to six-month) normally cycling and old (24-month) acyclic constant diestrous mice (OVX, seven days). (Figs. 1 and 2). OVX mice were given E_2 capsules producing physiologic levels of steroid to downregulate tonic LH release to baseline levels. Use of an indwelling cannula in freely mobile animals allowed frequent blood sampling and systematic introduction of pharmacologic agents. A GnRH bolus was given after baseline sampling. Significant LH release was obtained in both young and old mice, clearly demonstrating that the pituitary of old female mice is as capable of responding to GnRH as that of young mice. Alteration in pituitary response to GnRH is not a significant factor contributing to age-related alteration in LH secretion (Joshi *et al*., 1993, 1995a).

Ovarian status, and not animal age, determines GnRH responsiveness

The excitatory animo acid *N*-methyl-D,L-aspartic acid (NMA) acts on GnRH neurons resulting in LH release. We tested the hypothalamic NMA response during the aging process indirectly by measuring LH. Short-term OVX (S-OVX) (one week) mice were either prepubertal (five weeks), postpubertal (10 weeks), young (five months), middle-aged (12 months), or old (24 months). Long-term OVX mice (L-OVX) were either young (five months), or old (24 months) and OVX at puberty. Middle-aged mice were OVX at eight months and examined at 12 months (Joshi *et al*., 1995a). While LH levels in OVX (six days) mice were elevated, sustained (six days) physiologic E_2 levels suppressed circulating LH to levels found in intact mice. LH secretion was inhibited by E_2 in both S- and L-OVX mice, regardless of age (Fig. 3 and 4). NMA failed to overcome this inhibition in E_2-primed S-OVX mice. However, NMA did overcome E_2's inhibition of LH secretion in L-OVX mice (Figs. 3 and 4). This occurred regardless of either animal maturity at the time of OVX or animal age. Although the ovaries of old mice failed to produce enough E_2 to result in estrogenized vaginal smears, ovarian modulation of E_2's inhibitory effect on NMA-induced LH secretion was still present. Therefore, the ability of E_2 to inhibit the NMA response is mediated by the length of time between OVX and the initiation of E_2 replacement, and not by animal age. This was the first study of its kind to clearly differentiate ovarian effects from age-related effects in alteration of neuroendocrine function (Joshi *et al*., 1995a).

NEUROENDOCRINE SYSTEM CHANGES ASSOCIATED WITH AGING

An accurate grasp of circumstances surrounding midlife disruption of cyclic gonadotropin function has helped our understanding of its normal function. Reproductive failure occurs long before death in most species. Factors that regulate reproductive aging may be separated from those that involve nonspecific age-related changes (Wise *et al*., 1991). Age-related neuroendocrine alterations play a significant role in the reproductive capacity decline during midlife, which is characteristic of females of most species. Analysis of the "window" at middle age minimizes confounding influences of degenerative disease and focuses on earlier manifestations of aging.

During the estrous cycle of young mice, the hypothalamus and preoptic area (POA) are

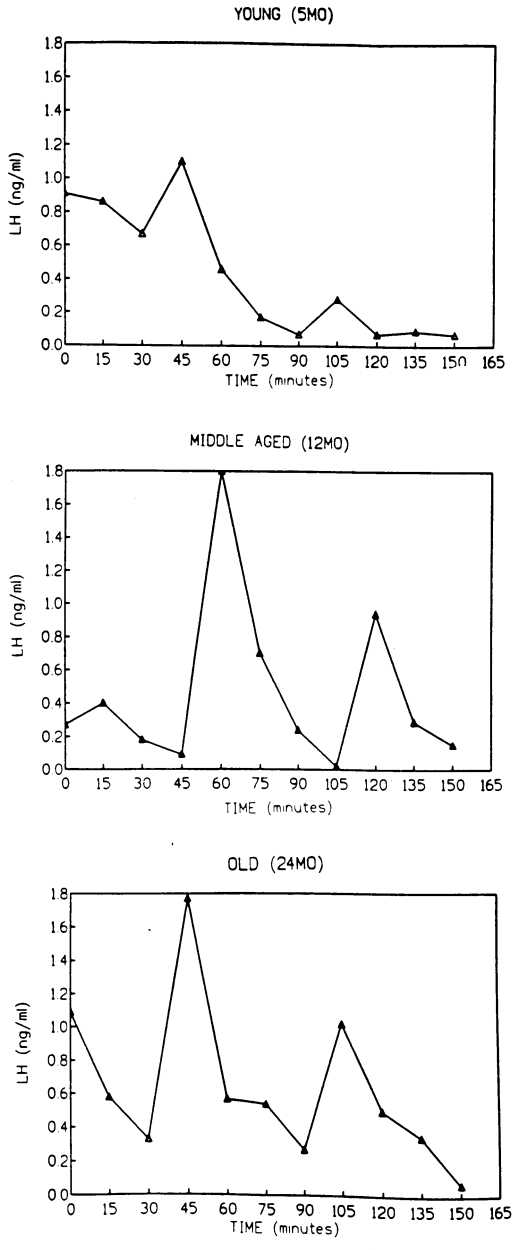


FIG. 1. Representative profile of LH secretion in young, middle aged, and old mice in studies testing the capacity of the pituitary of old animals to synthesize and release LH in response to exogenous stimulation. Animals were OVX for one week (S-OVX), and did not receive exogenous E_2. Blood samples were obtained as described in the text. Reproduced with permission from Joshi, et al. (1995a).

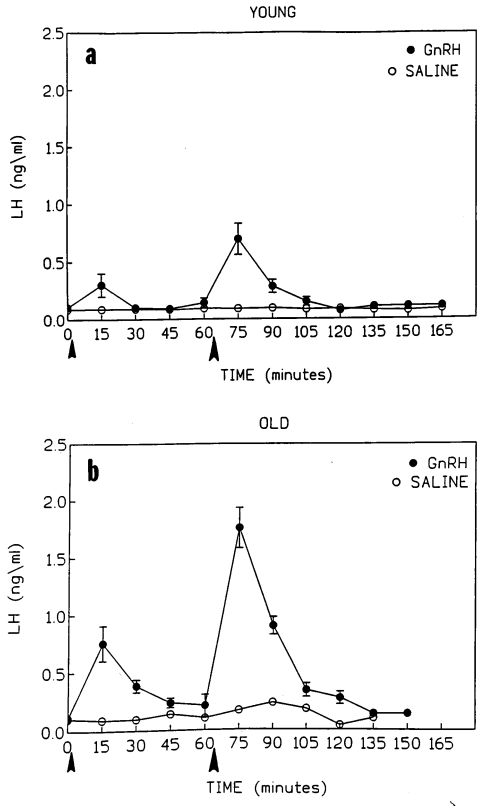


FIG. 2. Effect of GnRH and saline administration on LH secretion in (a) young (5–6 months), cycling and (b) old (24 months), acyclic mice. Animals were OVX and E_2-treated for six days, and serial blood samples were obtained. Animals were challenged with GnRH (5 μg/5 μL saline/kg body weight) immediately after the zero-minute sample followed by 15 μg/15 μL saline/kg body weight one hour after the zero-minute sample or saline 5 μL/kg body weight followed by 15 μL/kg body weight. Each data point is mean ±SE of $n = 6$ for both young and old animals receiving GnRH and $n = 3$ for the saline controls. Reproduced with permission from Joshi *et al*. (1993).

exposed to predictable four- to five-day day peaks and nadirs of circulating E_2 and progesterone (P_4). The POA contains the majority of GnRH [or luteotropin hormone releasing hormone (LHRH)] neurons; axons of POA GnRH neurons project caudally to the GnRH terminals in the hypothalamic arcuate (ARC) nucleus and median eminence (ME) (Hoffman and Wray, 1982; Miller *et al*., 1992a). Negative E_2 feedback effects prevail under the influence of relatively low concentrations of E_2 until this steroid increases to a specific level for a specific duration. Just prior to the LH surge, stimulatory influences increase while inhibitory influences decrease, allowing release from GnRH neuron inhibition, and subsequent release of GnRH into the hypophyseal portal system (Yen, 1986). The consequence is pituitary stimulation of LH and subsequent ovulation.

Ovulatory failure is one of the earliest events of aging, occurring during midlife in many mammalian species (Harmen and Talbert,1987). Contributions of the ovary and brain–pituitary complex to cyclicity cessation are species dependent, but neuroendocrine dysfunction occurs in

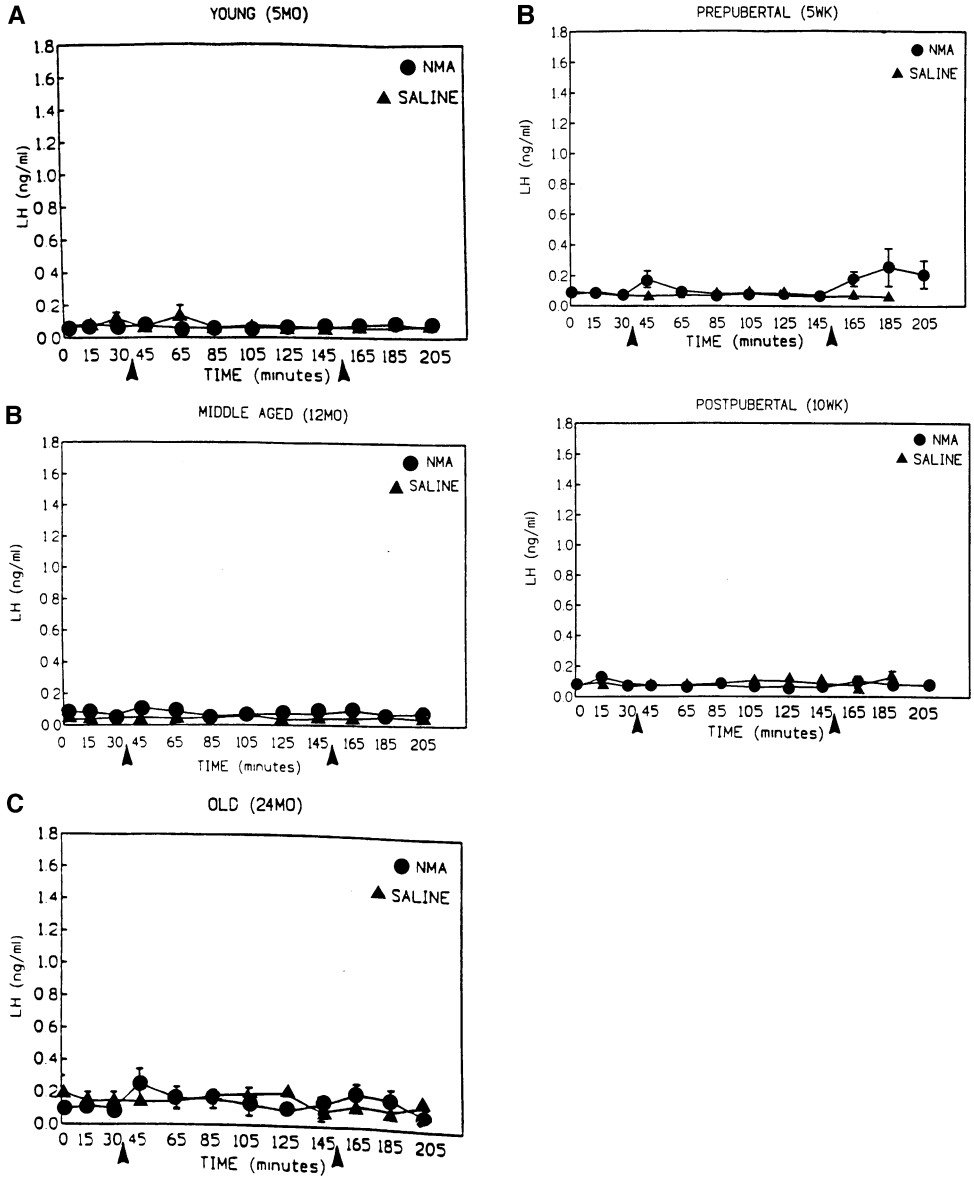


FIG. 3. (A) Effect of *N*-Methyl D,L-aspartic acid (NMA) on LH secretion in young, middle-aged, and old S-OVX mice. Animals were OVX and received E_2 for six days. After three baseline samples, animals were challenged with NMA (20 mg/4 mL saline/kg body weight) or saline 80 min after the last baseline sample. Each data point is a mean ±SE of $n = 6$ for young, middle-aged, and old animals receiving NMA, and $n = 4$ for the saline controls. The number at the top of each figure indicates the age in months at which the NMA challenge was done. Reproduced with permission from Joshi *et al.* (1995a). (B) Comparison of the effect of NMA on LH secretion in mice ovariectomized (S-OVX) before or after puberty. See Fig. 3A for details of ovariectomy-estradiol treatment and the NMA challenge. Each data point is mean ± SE of $n = 8$ for both prepubertal and postpubertal animals receiving NMA, and $n = 4$ for saline controls. Reproduced with permission from Joshi, *et al.* (1995a).

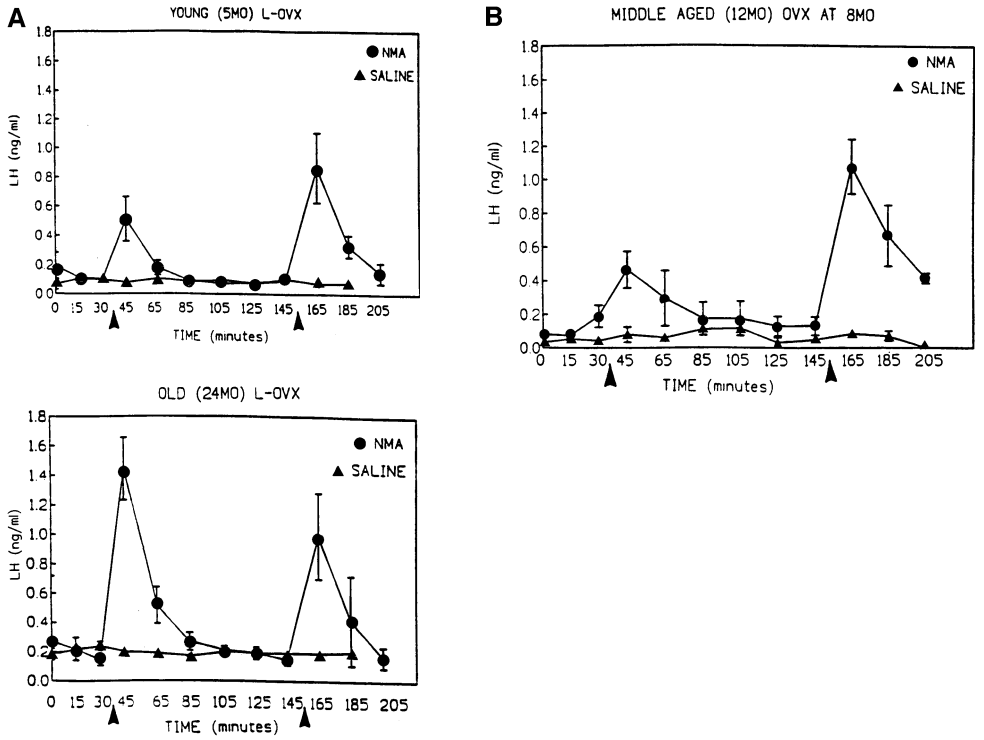


FIG. 4. (A) Effect of NMA on LH secretion in young and old mice ovariectomized (OVX) at puberty (L-OVX). Animals were OVX at seven to eight weeks of age and received E_2 for six days before the NMA test. Each data point is mean ± SE of $n = 6$ for both young and old animals receiving NMA, and $n = 4$ for the saline controls. Please compare these results with those of short-term ovariectomized, estrogen-treated animals in Fig. 3A. Reproduced with permission from Joshi *et al.* (1995a). (B) Effect of NMA on LH secretion in middle aged mice OVX at 8 months and studied at 12 months of age. Each data point is the mean ±SE of $n = 8$ for animals receiving NMA and $n = 4$ for saline controls. Reproduced with permission from Joshi *et al.* (1995a).

all species that have been examined (Nelson and Felicio, 1985). A change in ovarian function occurs in aging female mice, resulting in altered ovarian secretions by middle age (Finch *et al.*, 1980, 1984; Nelson *et al.*, 1981; Felicio *et al.*, 1986; Vom Saal and Finch, 1988). "Altered ovarian secretions" could include changes in the type or pattern of release of ovarian steroids, and/or other ovarian secretions such as inhibin (Jih *et al.*, 1993). At about 18 months in the C57BL/6J mouse a state of reproductive incapacity is achieved, typified by alterations in cycle duration known as persistent diestrous (Nelson and Felicio, 1985). In both rats and mice, changes in CNS function are probably related to ovarian steroid background (Lapolt *et al.*, 1986, 1988; Wise *et al.*, 1991).

Progesterone (P_4) has been hypothesized to protect against age-related reproductive impairments in rat (Lu *et al.*, 1980; La Polt *et al.*, 1986, 1988). However, hormonal correlates to prolonged cycles in mice differ from rats and the possibility of P_4's contribution to extended cycles is weakened: first, because elevated P_4 at midday of day 4 of the cycle of rats is not seen in mice (day 1 = proestrus); and second, because in older mice the midcycle elevation of P_4 is not changed in amplitude (Finch *et al.*, 1980; Nelson *et al.*, 1981). Findings in mice correlate

with observations in perimenopausal women where decreased E_2 levels are associated with irregular cycles.

There are specific endocrine landmarks indicating that the necessary positive feedback response is less efficient at middle age. Ability to produce an LH surge is attenuated, time of surge onset is advanced, and surge amplitude is diminished. With altered cycle length, the hypothalamus is subjected to altered ovarian secretions (Nelson *et al*., 1981; Finch *et al*., 1984; Vom Saal and Finch, 1988; Felicio *et al*., 1986; Lapolt *et al*., 1986). Altered E_2 and P_4 levels and lengthened cycles are, in turn, associated with the diminution in preovulatory elevations of LH (Nelson *et al*., 1992), mobilizing an irretrievable cycle of functional loss.

In mice the LH surge begins to decline by 12 months and is undetectable by 18–20 months (Flurkey *et al*., 1982; Gee *et al*., 1984). Although cycles cease in this strain around 12 months because of even greater decrements in ovarian function, LH secretion is significantly impaired by 16–18 months (Flurkey *et al*., 1982). As impairment in LH surge potential increases, it ultimately cannot support ovulation even in the presence of young ovarian grafts (Felicio *et al*., 1983). Exposure of intact (Mobbs *et al*., 1984, 1985) or OVX mice and rats (Brawer *et al*.,1980) to exogenous E_2 can advance the loss of the LH surge, while long-term withdrawal from ovarian secretions delays the onset of declining LH surge activity (Ascheim, 1964/65; Felicio *et al*., 1983; Nelson and Felicio, 1985). Reduced release of LH can be delayed by L-OVX (Finch *et al*., 1984; Nelson and Felicio, 1985). Older mice OVX at puberty are able to sustain normal cycles for much longer periods of time than are old females with normal cycling histories if they receive an ovary from young cycling donors (Felicio *et al*., 1986). L-OVX failed to protect against any observed age-related changes either in neurons or neuropil (Miller *et al*., 1989). Discerning whether aging, ovarian secretions, the ovary itself, or all of these factors underlie estrous cycle loss has remained problematic because the hypothalamic ultrastructure in L-OVX females remains unstudied.

In the aging rodent, the ovary limits reproductive capacity due to follicle loss. Hypothalamic impairments in the capacity to maintain regular cycles may cause changes in cycle length that precede the loss of cyclicity (Felicio *et al*., 1983; Mobbs *et al*., 1984, 1985; Nelson *et al*., 1992). Middle aged mice undergoing the transition to acyclicity are able to continue to have normal four- to five-day estrous cycles well beyond the expected time of cycle cessation if they are OVX'd and receive donor ovaries from young regularly cycling females. Older mice are less able to do so. This suggests that hypothalamic changes occur during a specific "window of time" marking the transition to acyclicity before ovarian follicles are completely depleted (Felicio *et al*., 1986).

Although cellular mechanisms mediating ovarian hormone influence over neuroendocrine aging are largely unknown, some neuroanatomical correlates have been found. Widespread neuronal loss is not a concomitant of normal aging, but selective alterations in neuron number and synaptic density do occur (Curcio, 1982). For example, a reduction of POA and ARC neurons is found in aged female rats (Hsu and Peng, 1978), but no loss is found in old female hamsters (Hoffman and Finch, 1986). Changes in neuron or synapse number in regions mediating LHRH activity could play a role in reproductive aging. We found surprisingly few alterations in neuroendocrine hypothalamic architecture in aged mice; changes observed were selective and region specific. In both the ARC and POA we found decreased numbers of all types of neurons in old mice. Unlike other species, there was no age-related increase of ARC glia, but the ARC was most susceptible to other effects of aging. It showed a marked increase in blood vessel density, and was the only region with decreased neuron cell surface area (Miller

et al., 1989). The nature and consequences of changes in neural arrangement has not been determined, but morphological changes are consonant with functional loss.

GnRH NEURONS AND AGING

We examined GnRH neuron distribution in young (five to six-month) and old (26–28 month) mice and reported that significant reductions of neurons have occurred in the neuroendocrine hypothalamus at ages after the LH surge has already diminished (Miller *et al*., 1990). In middle-aged mice, GnRH neurons are present in numbers that correspond to those of young mice (Hoffman and Finch, 1986). Therefore, midlife loss of LH surge capacity cannot be accounted for by changes in GnRH neuron number. An increase in synaptic input to GnRH POA perikarya (Witkin, 1989) occurs in aging male rats, where a type of neural plasticity may be at work, but we did not observe such changes in female mice (Miller *et al*., 1990).

OVARIAN FACTORS AND THE FAILURE OF THE NORADRENERGIC NEURON TO ACCOMMODATE CHANGES ASSOCIATED WITH AGING

Many neurotransmitters modulate LH secretion (Barraclough *et al*., 1984); several of these have been implicated in the age-related loss of positive feedback regulation (Wise, 1983; Nelson and Felicio, 1985). Pharmacological and neuroanatomical studies indicate that POA norepinephrine (NE) is important in LH surge induction (Weiland and Wise, 1986; Wise *et al*., 1991). NE terminals are richly distributed in GnRH-containing POA (Miller and Billiar, 1990; Miller and Zhu, 1992, 1995b). In concert with the hypothesis that POA NE nerve terminals stimulate secretion of LH by interaction with GnRH neurons (Kalra and Kalra, 1983; Wise *et al*., 1991), the POA is rich in α-adrenergic (stimulatory) receptors (Weiland and Wise, 1986; Wise *et al*., 1991) and NE terminals (Miller and Zhu, 1992b, 1995). The LH surge is associated with an increase in POA NE turnover and concentration, and noradrenergic (NA) stimulation is increased just prior to ovulation (Wise, 1986). POA NE levels and turnover increase in response to E_2 prior to LH surge induction in OVX steroid-treated animals (Kalra and Kalra, 1983). The LH surge can be blocked by inhibiting dopamine β hydroxylase (DBH), NE's enzyme of synthesis (Alder *et al*., 1983; Kalra and Gallo, 1983), or by decreasing NA transmission via blocking α-adrenergic receptors or ablating POA NA terminals (Alder *et al*., 1983; Kalra and Gallo, 1983). Intracerebroventricularly administered NE will initiate an LH response under appropriate hormonal conditions.

Reproductive aging is associated with reductions in NE concentration and turnover, and α_1-adrenergic receptors (Wise, 1983; Weiland *et al*, 1989; Weiland and Wise, 1990; Wise *et al*., 1991). We studied whether there are direct interactions between the NA system and GnRH neurons that could modulate GnRH release and aging effects. Using electron microscopy, we observed synaptic contacts between POA GnRH dendrites and DBH terminals in young mice with ovaries but there were *no* POA GnRH/DBH interactions in old (24-month) mice. This finding and the possibility that a decremental age-related decline occurs in terminal number may partly explain the age-related loss of LH surges (Miller and Zhu, 1995).

Remarkably, the neuroanatomy of the NA system of young S-OVX mice closely resembles that of old mice. Numbers of DBH terminals and their interactions with GnRH dendrites in young mice OVX for only six days decreased to levels of old mice in persistent diestrous or old L-OVX mice. It follows that NA neurotransmission would also diminish. Thus data emerged that even S-OVX is detrimental to neuronal architecture and that L-OVX fails to protect it. This was an important observation that strengthened our broad conclusion that OVX is generally

deleterious to the neuroendocrine hypothalamus. We concluded that it is the presence of the ovary and not the aging process that accounts for the loss of synaptic interactions in the NA system.

AGE-RELATED CHANGES IN THE OPIATERGIC SYSTEM

Functional interactions between endogenous opiates and the NA system are likely in regulation of LH secretion. For example, both α-adrenergic blockers and inhibitors of NE synthesis prevent the naloxone-induced rise in plasma LH, suggesting that endogenous opiates suppress NE release (Kalra and Simpkins, 1981; Kalra, 1983, 1993; Kalra and Kalra, 1983). Endogenous opiates decrease turnover and presumably release of POA NE (Bicknell, 1985). Activation of hypothalamic adrenergic afferents by electrical ventral tegmental tract stimulation increases plasma LH levels, which can be potentiated by naloxone, an opiate inhibitor (Bicknell, 1985). Simultaneous losses of functional connections to stimulatory and inhibitory influences upon GnRH neurons may partly account for elevated basal LH levels and the decline of LH surge potential seen in aged female mice (Vom Saal and Finch, 1988).

β-Endorphin (β-endo) is an opiate that is implicated as a major regulator of CNS aging changes. It is derived biosynthetically from pro-opiomelanocortin (POMC), is synthesized in a subpopulation of ARC neurons (Bloom *et al*., 1978; Gee *et al*., 1983), and is frequently reduced with age (Barden *et al*., 1981a; Miller *et al*., 1991; Miller, 1994). ARC β-Endo cell bodies project to the POA (Cuello, 1983; Khachaturian *et al*., 1985). Changes in ARC neurons are likely to be reflected in their POA terminal activity fields. β-Endo mediates diverse functions including sexual (Hammer, 1990), eating (Cooper and Kirkman, 1993) and drinking behavior (Gianoulakis and Gupta, 1986), cardiovascular (Field and Kuhn, 1989) thermoregulatory (Adler and Geller, 1993), pain, and stress responses (Fields, 1993). β-Endo has also been implicated in modulation of pituitary hormones including prolactin release (Cella *et al*., 1993). More specifically, age-related impairments in sexual (Larsson, 1962) and eating behavior (Lloyd *et al*., 1993), thermoregulation (Finch *et al*., 1984), hemodynamic (Kiristy-Roy *et al*., 1992), and pain and stress responses (Hoskins *et al*., 1986) have been identified in rodents and may be related to changes in β-endo synthesis and/or secretion. In this regard, β-endo pathways are of interest not only to the neuroendocrinologist, but to those generally interested in aging and the brain.

There is considerable evidence that the opiatergic system is a modulator of gonadotropin secretion. For example, morphine suppresses LH release (Kalra and Gallo, 1983; Barraclough, 1994; Barraclough *et al*., 1984). Naloxone, an opiate antagonist, will prevent this blockade (Gabriel *et al*., 1986), and opiate receptor antagonists will block opiates and opiate agonist effects (Mehmanesh *et al*., 1988). Opiates given intracerebroventricularly will suppress LH release (Barraclough, 1994). Furthermore, POA-implanted naloxone elevates LH (Leadem *et al*., 1985). Opiate fibers (Miller and Zhu, 1992a) and μ opiate receptors, believed to modulate gonadotropin release are found in GnRH-rich regions (Joshi *et al*., 1992, 1995b,c).

β-Endo has tonic inhibitory influences over GnRH and LH secretion throughout the estrous cycle; appropriately timed release from tonic inhibition allows the proestrous GnRH surge to occur (Kalra and Kalra, 1983; Kalra, 1993). Furthermore, β-endo content varies throughout the estrous cycle (Barden *et al*., 1981a; Knuth *et al*., 1983). POA μ opioid receptors in GnRH-rich regions are E_2 modulated (Joshi *et al*., 1993). Although published reports indicate that hypothalamic β-endo is also E_2 modulated, the evidence is inconsistent. β-Endo content and POMC mRNA levels change with the estrous cycle and after E_2 treatment (Karelus and Nelson, 1992). OVX results in increased POMC mRNA levels; E_2 treatment reduces it (Karelus and Nelson,

1992; Weiland *et al*., 1992). However, hypothalamic POMC mRNA levels are lower in rats OVX four weeks than in those OVX two weeks (Wilcox and Roberts, 1985). β-Endo content in (three-week) OVX rats is not different from sham-operated controls (Treiser and Wardlaw, 1992). Regardless of the variable evidence, it is clear that suppression of POMC mRNA by E_2 probably accounts for reduction in β-endo levels after E_2 treatment (Trieser and Wardlaw, 1992). In female C57BL/6J mice a midlife rise in basal LH occurs with lengthened and subsequent ovulatory cycle loss (Gee *et al*., 1983). The opiatergic system is likely to be involved (Kalra and Kalra, 1983; Wise, 1983; Nelson and Felicio, 1985). There are age-related changes in μ opiate receptors (Miller and Zhu, 1992a; Miller, 1994), and endogenous opiates have been linked to the decline in reproductive capacity. Altered opiate tone could affect disinhibition of GnRH secretion (Field and Kuhn, 1988, 1989). Thus, interactions of age, the ovary, and estrogens in endorphin regulation of gonadotropin secretion have been a focus of our investigations.

We examined the impact of aging on ARC opiate neuron number. There was a significant decline (~30%) in arcuate β-endo perikarya in old (24-month) healthy animals, which is likely to have a major impact on opiate neuron function (Miller *et al*., 1991). Opiate terminal fields were also lost. About 20% of rat ARC β-endo neurons (Mobbs *et al*., 1984) and 30% in mice contain estrogen receptor alpha [ER] (Miller *et al*., 1995). Because E_2 modulates POMC mRNA levels, we tested whether age-related changes in β-endo neuron number are selective for those neurons that contain ER. We compared β-endo neuron number in S- and L-OVX mice, among three age groups [5 (young), 12 (middle-aged), or 23–24 months (old)] (Fig. 5A). L-OVX failed to prevent age-related changes in neuron number. There were no significant differences between young and middle aged S-OVX mice for β-endo, ER, or β-endo/ER neuron number. There were significant decreases when older animals were compared to young ones for all three. Age and ovarian status independently affected neuron number. Decreases in β-endo neuron number tended to be greater than those in ER-containing neurons, but subpopulations of β-endo neurons that contain the classical ERα were not affected to any greater degree by age or ovarian status than the β-endo neuron population as a whole. Thus, these observations also failed to support the hypothesis that L-OVX has a protective effect on hypothalamic neurons in older animals.

Tyrosine hydroxylase (TH) is the rate limiting enzyme for the dopamine catecholaminergic pathway (Levitt *et al*., 1965). β-Endo and TH-containing neurons are similarly localized (Miller *et al*., 1995), TH is E_2 regulated (Pasqualini *et al*., 1993), and there is a significant increase in neurotransmitter enzyme activity following OVX (Beattie *et al*., 1972), while acute E_2 treatment of OVX rats results in a decrease in ME TH activity (Pasqualini *et al*., 1993). ARC DA neurons are major regulators of pituitary prolactin release, and DA neurons interact with POMC perikarya (Tong and Pelletier, 1992). In contrast to β-endo, we found no age-related decline in TH neurons, regardless of the presence of ER (Fig. 5B). These data demonstrate a selective effect of aging upon the opiatergic system.

POA OPIATERGIC TERMINALS FAIL WITH AGING

Because GnRH neurons comprise part of the neuronal population that is reduced in older mice (Miller *et al*., 1990), neural pathways affecting GnRH neurons may also be altered with age. β-Endo neurons project to the GnRH neuron-containing POA (Cuello, 1983). While β-endo terminals impinge directly upon GnRH neurons in male rats (Chen *et al*., 1989), the interposition of an interneuron between the GnRH cell and the opiate terminal is probable in mice (Miller, 1994). Because we had observed age-related losses of ARC β-endo neurons, we studied changes

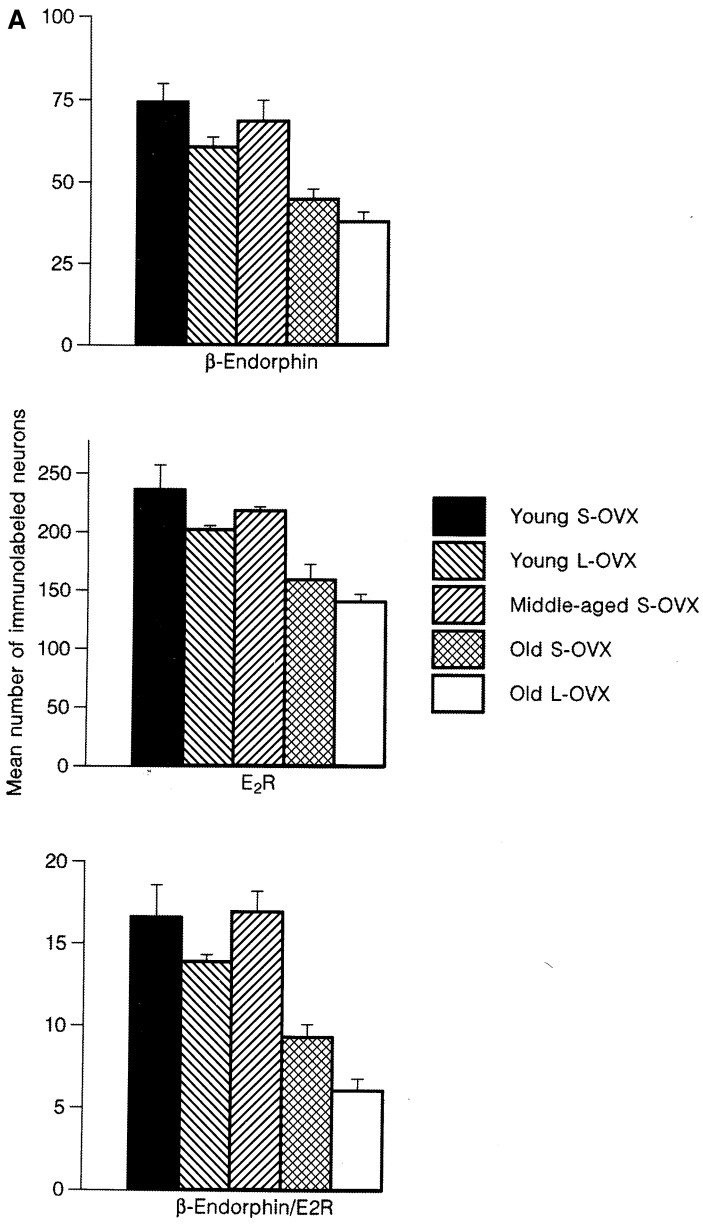


FIG. 5. (A) Histogram demonstrating the mean number of immunolabeled neurons per tissue section (±SE) for β-endorphin (β-endo), estrogen receptor alpha (E_2R) and β-endo/E_2R-containing neurons. Significance levels are for one- and two-way ANOVAs. Reproduced with permission from Miller *et al.* (1995).

in POA terminal fields (Miller and Zhu, 1992a). Overall, the POA neuropil demonstrated a loss in total neuron number in older mice (Miller *et al.*, 1989). Surprisingly, there were few age-related ultrastructural changes in the neuropil. These changes included only a loss of recipient dendrites, and an increase in the number of axons *en passage*. Numbers of opiate-

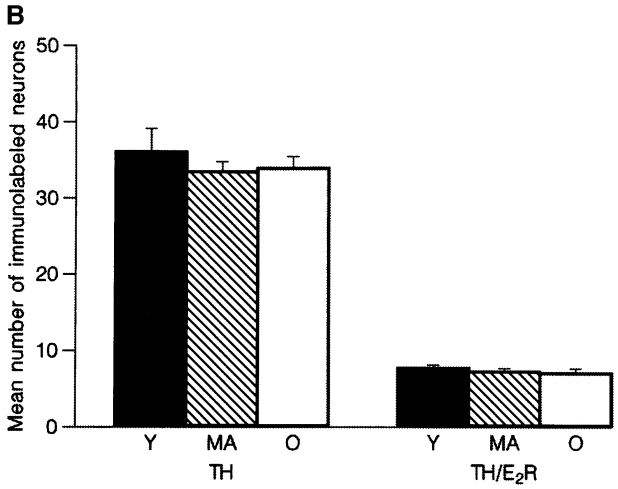


FIG. 5. (B) Mean number of TH, estrogen receptor, and TH/ER immunoreactive neurons per tissue section in the arcuate nucleus of the C57BL/6J mouse. There was no significant difference among the three age groups in the mean number of TH or TH/ER-immunoreactive cell types. There was no significant difference among age groups in the total number of TH-containing neurons/arcuate nucleus. Reproduced with permission from Miller *et al.* (1995).

containing terminals decreased, which was not surprising because their cell bodies of origin had been lost (Miller and Zhu, 1992a). Loss of POA β-endo terminals in old mice could not be accounted for by a general loss of axons of all types because these actually increased with age. This suggests a certain amount of plastisticity of the aging neuroendocrine hypothalamus that did not extend to the opiatergic POA microenvironment.

PROHORMONE CONVERTASES AND THE OPIATERGIC SYSTEM

The opiatergic system is involved in the loss of the LH surge capacity (Kalra and Kalra, 1983; Wise, 1983; Nelson and Felicio, 1985; Wise *et al*., 1991), which begins at middle age. Gonadal steroids may regulate opiate activity by influencing opiate receptor density (Joshi *et al*., 1993; Lloyd *et al*., 1993); regulating opiate neuron gene expression (Barden *et al*., 1981a,b; Bronstein *et al*., 1993; Knuth *et al*., 1983; Karelus and Nelson, 1992); altering opiate concentration in tissues; or modulating terminal opiate release.

Altered function of neuropeptide neurons could be accounted for by altered gene expression and/or changes in prohormone processing. Pro-opiomelanocortin (POMC) is the precursor for the potent endogenous opiate β-endo. We had observed no change in β-endo neuron number at middle age (Miller *et al*., 1995), despite identified changes in levels of peptide and steroid hormones. The fact that others had reported changes in opiate neuron E_2 response at midlife (Nelson *et al*., 1988) lead us to examine opiate neuron function. We wondered whether there are age-related changes in the way β-endo neurons process peptide.

Convertase enzymes, a family of subtilisin/kexin-like proteinases, are responsible for activation of proproteins and prohormones at pairs of basic amino acids (Seidah and Chretian, 1992). These include prohormone convertases (PC) 1, 2, 4, 5, PACE4, and furin. The ubiquitous

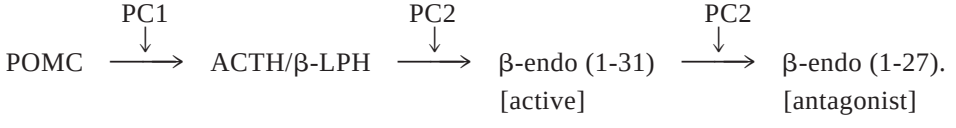
localization of brain furin is consistent with its postulated role as a convertase important in protein processing in the constitutive pathway of secretion (Day *et al*., 1993). Furin mRNA is observed in both neuronal and non-neuronal cells (Day *et al*., 1993; Schafer *et al*., 1993); it can serve as an internal experimental control where levels of mRNA are measured.

Activation of newly synthesized prohormones starts with proteolysis of paired basic amino acids within the protein structure followed by posttranslational modifications (Mains and Eipper, 1979; Eipper and Mains, 1980; Seidah *et al*., 1991; Seidah and Cretian, 1992). PC1 and PC2 were discovered using a PCR-based strategy that took advantage of their high degree of sequence homology with other members of the subtilisin family, especially within sequences critical for serine protease catalysis activity (Smeekens and Steiner, 1990; Nayama *et al*., 1991; Seidah *et al*., 1991). PC1 and PC2 are known to process prohormones and proproteins that negotiate the regulated pathway of secretion (Seidah and Chretain, 1992). Evidence for the prohormone processing function of PC1 and PC2 is based on cell and tissue specific expression and distribution of these neuroendocrine specific proteases (Seidah *et al*., 1991). In the brain, PC1 and PC2, the latter being more widely expressed, have been localized exclusively to neurons (Schafer *et al*., 1993).

Both endocrine and neuroendocrine tissues are subject to a cascade of cleavage events catalyzed by PC1 and PC2. One of the terminal products is β-endo (1-31). In the hypothalamus, this active opiate peptide is subsequently subject to PC2 processing and carboxypeptidase activity to yield essentially inactive forms, β-endo (1–27) and (1–26). Carboxyl terminal cleavage of β-endo (1–31) leads to substantial loss of opiate receptor affinity (Emeson and Eipper, 1986; Akil *et al*. 1991). β-Endo (1–27) and (1–26) retain only 10–30% and ~15%, respectively, of binding affinity relative to the parent molecule (Akil *et al*., 1991). β-Endo (1–27) retains ~30% of β-endo (1–31) potency in displacing [^{3}H] β-endo (1–31) from brain membranes and acts as a competitive antagonist of β-endo (1–31) action (Hammond *et al*., 1984).

The term "processing" defines the steps including β-endo (1–31) biosynthesis and the generation of inactive forms through enzymatic cleavage. Processing of β-endo to either active or inactive forms is likely to be an important regulatory step in mechanisms determining hypothalamic opiatergic activity. Active β-endo (1–31) represents 50% of processed hypothalamic peptide (Emeson and Eipper, 1986). Processing of POMC includes defined and ordered proteolysis, glycosylation, phosphorylation, α-N-acetylation, and carboxyl terminal amidation (Bennett, 1994). End products have been colocalized to hypothalamic secretory granules and are probably secreted together in response to appropriate stimuli. Peptide products are released in a coordinated fashion, and each may interact independently with appropriate target tissues or modulate effects of other POMC derivatives (Eipper and Mains, 1980).

Coexpression experiments have demonstrated the capacity of PC1 and PC2 to process POMC with distinct cleavage specificities (Benjannet *et al*., 1991) including processing of POMC into ACTH and β-lipotropin (β-LPH) by PC1, and subsequently to MSH and β-endo through the action of PC2 (Benjannet *et al*., 1991). Cleavage of β-endo (1–31) to β-endo (1–27) is a PC2-mediated event (Zhou *et al*., 1993). Thus, the processing pathway in which endorphin-related peptides are derived from POMC can be summarized as follows:



Opiatergic properties of β*-endorphin*

Each modified β-endo peptide form has unique pharmacological and/or physiological properties. The (1–27) form displaces β-endo (1–31) from rat brain membranes, is a competitive antagonist to β-endo (1–31) at axon terminals (Hammond *et al*., 1984), inhibits β-endo (1–31) action, and is a likely source of brain opioid system regulation (Hammond *et al*., 1984). Changes in relative concentrations of different β-endo peptides may cause significant changes in opioid information transmitted across synapses (Bronstein *et al*., 1993). β-endo (1–27) alters opiate responses in analgesia reinforcement, Met-enkephalin release, and cardiovascular response. Although antagonist induced upregulation of μ opiate receptors is one possible role for increased opiate sensitivity in animals chronically treated with opiate antagonists, changes in opioid peptide forms also contribute to this effect (Bronstein *et al*., 1993). Shifts in peptide forms may represent a regulatory mechanism of POMC cell response: such shifts may be effected by cleavage of opioid agonist β-endo (1–31) to antagonist forms (Bronstein *et al*., 1993). The ratio of these peptides provides an estimate of relative amounts of opioid agonists and antagonist/nonagonist in particular brain regions and may have significant impact on biological function (Bronstein *et al*., 1993). Decreased levels of hypothalamic β-endo in older animals have been demonstrated within discrete nuclei (Barden *et al*., 1981a).

Physiological shifts in peptide processing of POMC have been defined. With repeated pain stimulation, periaquaductal gray β-endo levels increase and POMC processing is modified in favor of (1–27/1–26) forms (Millan, 1993). In chronically stressed rats, two to three times as much β-endo (1–27)/(1–26) is produced as β-endo (1–31) (Young et al., 1993). A shift toward accumulation of more processed peptides may be due to secretory granule age, longer times giving more time for "slower" cleavage events to take place. Thus, although overall β-endo peptide levels increase, there is a net decrease in opioid activity (Young *et al*., 1993). This effect has been demonstrated in ARC POMC neurons, which are upregulated after chronic naltrexone treatment (Bronstein *et al*., 1993). Reduced activation of opiate receptors at β-endo synapses may also contribute to deficits in opiatergic activity (Young *et al*., 1993). Evidence in support of selective release of different peptide forms at the terminal is meager (Bronstein *et al*., 1993). Regardless of the mechanism, alterations in the relative concentrations of differentially processed β-endo with age would have important functional consequences.

Age-related changes in POMC processing may be reflected in altered physiological function including motor and behavioral responses, temperature regulation, appetite control (Cuello, 1983), and diurnal function (Weiland *et al*., 1992). Increases in the proportion of β-endo (1–26, or 1–27) and acetylated (i.e., inactive) forms, accompanied by a decrease nonacetylated β-endo forms, have been observed in aging male rats (Wilkinson and Dorsa, 1986). We tested whether age or alteration in ovarian secretions are associated with alterations in posttranslational peptide processing in female mice.

INACTIVE OPIATE PEPTIDES IN MIDDLE-AGED MICE

Using high-pressure liquid chromatography we demonstrated that the predominant form of hypothalamic β-endo in young cycling females is β-endo (1–31). Significant age-related alterations in POMC posttranslational peptide processing occur at middle age where the proportion of β-endo (1–31)/(1–27) shifts in favor of the competitive antagonist β-endo (1–27) (Joshi *et al*., 1995b) (Fig. 6). This occurs both in ARC cell bodies and POA terminal fields. In middle-aged females (12 months) undergoing the transition to infertility, numerous inactive opiate peptide forms appear. Because these same altered peptide forms are also present in POA,

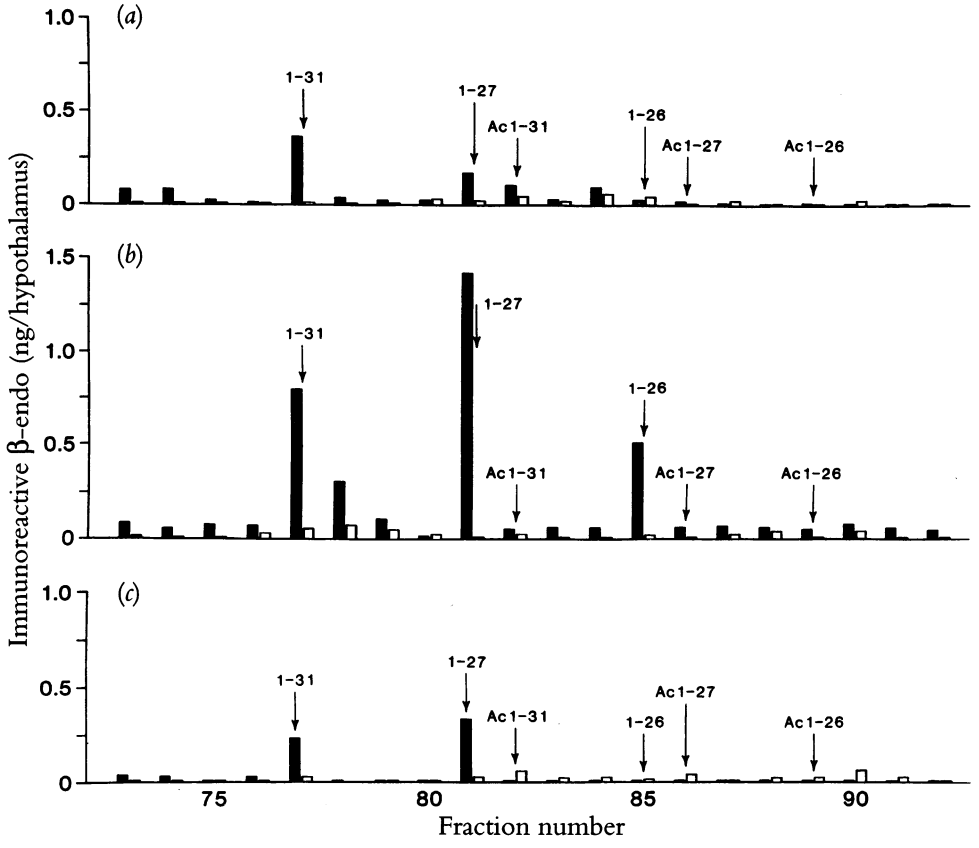


FIG. 6. Reversed-phase HPLC elution profiles of β-endo immunoreactivity in extracts of pooled (n = 3) arcuate nucleus and preoptic area from young (3–4 month), (b) middle-aged (12-13 month), and (c) old (23–24 month) mice. The elution position of the known variants of β-endo were determined separately. Ac 1–31, Ac 1–27, and Ac 1–26 are the N-acetylated derivatives of the 1–31, 1–27, and 1–26 forms of β-endo, respectively. Reproduced with permission from Joshi, *et al.* (1995b).

both neurons and terminals modulating GnRH release are likely to be compromised at middle age (Joshi *et al*., 1995b,c). This change appeared to precede or occur simultaneous with the loss of E_2 responsiveness by POMC neurons (Karelus and Nelson, 1992).

There are significant changes in posttranslational processing of β-endo in favor of opiate antagonist forms in middle aged mice. At midlife, functional changes in β-endo neurons parallel alterations in neuroendocrine function (Joshi *et al*., 1995b,c). By old age (24 months) there are only rare POA opiatergic terminals remaining (Miller and Zhu, 1992a). There can be little or no opiate response. The β-endo neurons are thus likely targets for changes resulting from age-related altered ovarian secretions.

LEVELS OF PC1 AND PC2 IN MIDDLE-AGED MICE

Pituitary POMC mRNA colocalizes with PC1 and PC2 mRNA. In intermediate lobe melanotropes both PC1 and PC2 are coexpressed with POMC mRNA, whereas essentially only PC1

mRNA is coexpressed with POMC in anterior lobe corticotrophs (Day *et al*., 1992). Intermediate lobe PC1 and PC2 mRNA (but not furin) convertase gene expression are coregulated with POMC gene expression (Birch *et al*., 1991; Day *et al*., 1992). In developmental studies of mouse pituitary, the PC1/PC2 expression ratio had a major impact on POMC products. PC2 mRNA expression is highest on postnatal days 1–14, which correlates with the presence of anterior lobe α -MSH–like peptide (Marcinkiewicz *et al*., 1993).

As with the pituitary, the identification of specific combinations of hypothalamic convertase expression suggests that specific enzyme pathways are involved in neuropeptide precursor processing. These specific combinations are responsible for region specific differences in posttranslational processing (Seidah and Chretian, 1992). PC1 acts early in the cleavage of β-LPH to γ-lipotropin and β-endo (1–31). This reaction is enhanced strongly by PC2; it occurs more rapidly under PC2's influence. β-endo (1–31) cleavage is only done by PC2. We hypothesized that hypothalamic PC2 regulation, critical for the step between β-endo (1–31) and β-endo (1–27), may be the means by which the proportion of active (1–31) to antagonist/inactive (1–27, 1–26) forms is modulated in hypothalamic POMC neurons.

Significant increases in PC2, but not PC1, mRNA levels within hypothalamic POMC mRNA neurons correlate with increases in β-endo (1–27) opiate antagonist forms in middle-aged reproductively impaired female mice (Joshi *et al*., 1995b,c). Using Northern blot analyses and single label in situ hybridization histochemistry (ISHH) we measured ARC furin, PC1 and PC2 mRNA in hypothalamic tissues. Paralleling increases in β-endo peptides (1–27), there was a significant elevation in PC2 levels at middle age. This PC2 increase would involve all neuropeptide/PC-containing neurons. Because furin is found in all cell types its age-related loss probably reflects a generalized aging change. Interestingly, there was no decrease in PC1 levels in old animals, suggesting an age-related upregulation of this enzyme as a compensatory event associated with neuron loss. Similarly, there was also no age-related loss of PC2. As predicted, in the middle-aged animal there was a significant increase in PC2, but not PC1 mRNA levels, but no change in POMC mRNA neurons vs. young mice (Fig. 7) (Joshi *et al*., 1995b).

We then used double-label ISHH. Digoxigenin marked POMC mRNA-containing neurons and radioligand tagged PCs in young, middle-aged, or old animals. Furin levels were lower within POMC neurons in older mice, PC1 levels remained constant, but there was a significant increase of PC2 within POMC mRNA containing neurons in middle aged mice. These data confirmed developmentally regulated changes in PC enzyme levels (Day *et al*., 1992), which may serve as an important mechanism by which the ARC can alter biologically active end points, one of which is reproductive capacity.

OVERVIEW OF AGE-RELATED CHANGES IN THE NEUROENDOCRINE HYPOTHALAMUS

We propose the following scenario. In the young animal, POMC neurons process opiate peptide normally. Active opiate is transported to the POA. Under E_2's influence, POMC mRNA levels decrease as the LH surge approaches; less of the active opiate peptide β-endo (1–31) is available due to this downregulation. NA terminals impinge directly upon on POA GnRH neurons (Miller and Zhu, 1992b, 1995). In the presence of E_2, midbrain noradrenalin synthesis increases (Gitler and Barraclough, 1988). Just prior to the LH surge, opiate influences decrease, NA influences increase, and GnRH neurons are stimulated by NE and other neurotransmitters. GnRH peptide in the ARC/ME terminals is released into the hypophyseal portal system.

In middle-aged mice, the LH surge occurs earlier than in young mice. We propose that

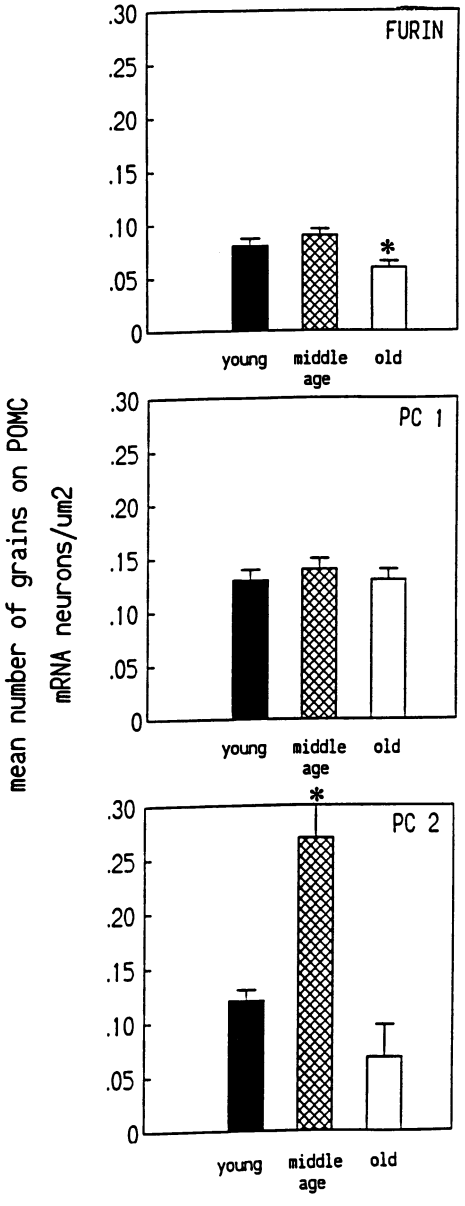


FIG. 7. Effect of age on furin, and the peptide processing enzymes prohormone convertase (PC) 1 and PC 2 mRNA expressed in POMC mRNA containing neurons in the arcuate nucleus of female C57BL/6J mice as measured by double label in situ hybridization histochemistry. Data are expressed as the mean ± SE grain count per unit area (μm^2) of the neurons for each probe in young, middle aged, and old animals. There was a significant decrease in the number of grains per μm^2 surface area of neurons in furin mRNA-containing neurons in old animals ($p \leq 0.05$ by ANOVA). There was no statistical difference among the age groups for PC1. However, for PC2, there was a significanly higher number of grains in the middle-aged group compared to the young (two- to threefold) or old (fourfold) animals, respectively ($p \leq 0.05$, ANOVA). Reproduced with permission from Joshi et al. (1995c).

because there is less of the active opiate, β-endo (1–31), and more of the inactive peptide, β-endo (1–27), in POA terminals, the NA (stimulatory) system can exert its influence earlier. Surge amplitude may be decreased because less time has been allotted for synthesis of appropriate levels of GnRH. Elevated midlife basal LH levels may reflect altered GnRH neuron sensitivity to both stimulatory and inhibitory influences when inactive peptide forms are predominant in the GnRH terminal-rich ARC and POA. Release from opiate inhibition (Joshi *et al*., 1995a,b) along with alterations in the temporal relationships affecting the GnRH neuron (Wise *et al*., 1994) could result in elevated basal LH levels. GnRH neurons could then tonically release incrementally increasing amounts of neuropeptide, which would result in altered pituitary LH stores at the expected time of the LH surge compared to young females. Alterations in E_2 levels and uncoupling of other neurotransmitters (Miller and Zhu, 1992b, 1995; Miller, 1994; Miller *et al*., 1995) may also be important. Although dopamine regulates POMC mRNA levels in the young female (Tong and Pelletier, 1992), there are no changes in TH mRNA levels or in TH neuron number at middle-age (Kohama *et al*., 1992; Miller *et al*., 1995), making alterations in dopamine a less likely candidate for midlife changes in gonadotropin secretion.

Data suggest potential toxic effects of E_2 in the aging animal (Brawer *et al*., 1980, 1983; Schipper *et al*., 1981; McEwen, 1997). Tonic exposure of young animals to high doses of E_2 results in hypothalamic ARC lesions (Brawer *et al*., 1983). Ovarian steroid-dependent ARC cellular degeneration has been demonstrated (Schipper *et al*., 1981; Brawer et al., 1983), the region mediating negative feedback effects of gonadal steroids on gonadotropin secretion (Kalra and Kalra, 1983; Barraclough *et al*., 1984). Chronic exposure of rats to estradiol valerate (EV) significantly increases numbers of ARC microglia and astrocytic granules (Brawer *et al*., 1983). Similar changes have been observed in rats during normal aging (Schipper *et al*., 1981). Because glial proliferation is a characteristic response to neuronal degeneration, these changes may be secondary to deleterious effects of E_2 on neuronal elements. ARC synaptic densities are also altered in adult rats exposed to EV (Garcia-Segura *et al*., 1986). Although selective neurotoxic effects of E_2 on β-endo neurons may be one factor contributing to reproductive senescence (Desjardins *et al*., 1993), we propose that midlife alteration in PC2 mRNA expression is a physiologically relevant event that leads to increased levels of an inactive opiate peptide, and which is linked to the midlife loss of reproductive function. We further propose that the change in PC2 levels is an aging biomarker in the murine model. Ultimately, in old animals a loss of opiatergic and NA terminals occurs, and the likelihood of either neurotransmitter influencing the GnRH neuron is markedly diminished.

OPIATES AND REPRODUCTIVE FUNCTION IN THE HUMAN FEMALE

Human data on β-endo peptide forms or PC levels are unavailable, but alterations in PC-mediated events may change opiate peptide function. Alterations in opiates have been implicated in depression (Zis and Garland, 1991). Cessation of repeated episodes of stress-induced eating is associated with manifestations of a withdrawal syndrome similar to that seen after opiate addiction. This suggests that human obesity may result from autoaddiction to endogenous opiates. Naloxone blockade of opiate receptors reduces food intake in obese humans, those with Prader Willi syndrome, and in traumatic hypothalamic hyperphagia. In anorexia nervosa patients, CSF β-endo levels are elevated and directly correlated with severity of clinical manifestations (Yen, 1986). Patients also show a lack of the naloxone-induced LH responses (Petraglia *et al*., 1993). Central opioids are implicated in stress-related amenorrhea and associated elevated plasma prolactin, ACTH, and LH levels. β-Endo is known to affect release of

these hormones (Yen, 1986). Cyclical changes in peptide processing and endogenously increased hypothalamic β-endo activity may lead to hypogonadotropic amenorrhea in hyperprolactinemic patients (Petraglia *et al*., 1993). In premenstrual syndrome (PMS) administration of naloxone to normal volunteers produces a constellation of PMS-identical symptoms (Reid and Yen, 1983). Abrupt withdrawal or excessive exposure to active opiates triggers psychoneuroendocrine manifestations of PMS (Cohen *et al*., 1981). Midluteal phase opiate level alterations have been linked to binge eating, fatigue, and depression, symptoms that become more severe at midlife (Yen, 1986).

Active opiate peptide and PC levels are likely to be causally linked to pathophysiological responses. Naloxone induces increased frequency and amplitude of pulsatile GnRH secretion in young women but fails to modify gonadotropin secretion in postmenopausal women, who have lost ovarian steroid feedback control (Reid *et al*., 1983). This suggests uncoupling of opiatergic influences on GnRH release after menopause (Yen, 1986). A constellation of symptoms including hot flashes are associated with physiological changes at menopause. Centrally acting β-endo, believed to mediate hypothalamic thermoregulation (Adler and Geller, 1993), has been suggested as an etiological agent of hot flashes. Naloxone treatment reduces flash number (Petraglia *et al*., 1993). Serum β-endo levels increase in association with hot flashes (Jaffe, 1991). Therapeutic modulation of β-endo processing through PC2 enzyme activity could possibly remove or inhibit such changes. Our finding of altered PC levels associated with changes in opiate peptide processing may provide clinically relevant clues to the etiology or treatment of altered of pathological opiate states in humans.

ESTROGEN (E_2) AND THE AGING BRAIN—THE CHOLINERGIC SYSTEM

The importance of E_2 for maintaining the functional integrity of neural systems involved in reproductive behavior is obvious and well accepted. The possibility that E_2 may act to maintain function in forebrain systems not directly related to reproduction is not obvious, and is only beginning to be seriously considered. One neural system in which E_2 seems to promote function is the acetylcholine (ACh) system of the basal forebrain, which is the primary source of cholinergic innervation of the cortex and hippocampal formation (Mesulum *et al*., 1983). These regions of cholinergic innervation are involved in the very general processes of memory, attention, and learning (Fibiger, 1991; Dunnett and Fibiger, 1993; Robbins *et al*., 1997). Loss of ACh associated with aging and neurodegenerative diseases such as Alzheimer's disease (AD) and Down's syndrome contribute to disease-related cognitive decline (Gibbs, 1996).

AD is closely associated with changes in the cholinergic neurotransmitter system (Honjo *et al*., 1989). The loss of memory for everyday events is one of the earliest appearing and most disruptive symptoms of AD. The memory deficit has been linked to the age-related loss of brain cholinergic neurons. To date, the only clinically approved therapies for the dementias associated with aging are cholinergic. Risk factors for AD include reduced cerebral blood flow, alcohol abuse, depression, head trauma, underactivity, being female, old age, sleep disturbance, impaired glucose utilization, Down's syndrome and Parkinson's disease (Crawford, 1996). The greatest risk reduction for patients with AD is among those who smoke (Brenner *et al*., 1993). In patients with an established smoking history, hippocampal nicotinic receptor binding and levels of ChAT are increased *versus* non-smokers.

In animal experiments damage to these cholinergic projections produces major learning and memory deficits (Aigner and Mishkin, 1986; Dekker and McGaugh, 1991). For example, reductions in hippocampal ACh content produced by aging, septal lesions, or the cholinergic

neurotoxin AF64A are associated with working memory impairments (Olton and Marakowska, 1992; McAlonon *et al*., 1995),which are reversed by treatments that restore cholinergic activity (Marakowska *et al*., 1995).

DOES E_2 MODULATE THE CHOLINERGIC SYSTEM?

Recently there has been growing interest in the possibility that E_2 treatment may slow the decline in cognitive function that often occurs with age. Postmenopausal women have a greater risk of showing symptoms of AD than males, and early surgically induced menopause may produce detectable impairment of cognitive function (Sherwin, 1994), which can be reversed with estrogen treatment (Sherwin and Tulandi, 1996). Moreover, women with AD treated with E_2 replacement therapy (ERT) show significant improvement in performance scores for cognitive function (Honjo *et al*., 1989; Fibiger, 1991; Ohkura *et al*., 1995a,b; Henderson *et al*., 1994; Tang *et al*., 1996). Because memory deficits resembling those produced by damage to acetylcholine (ACh) systems are an important feature of age-related cognitive deficits, and because brain ACh production appears to be E_2 modulated (Luine and McEwen, 1983; Gibbs 1996), it is an attractive hypothesis that E_2 might affect cognitive function via the ACh system (Sherwin, 1994). There is a small amount of evidence from animal studies that E_2 and ACh systems interact in memory. E_2 treatment can counteract the memory impairing effects of the cholinergic antimuscarinic drug, scopolamine (Dohanich *et al*., 1994). Even in castrated male rats E_2 can improve spatial working memory (Luine and Rodrigues, 1994) possibly via hippocampal nicotinic cholinergic mechanisms (Packard *et al*., 1996). Thus, both nicotinic and muscarinic cholinergic mechanisms may interact with E_2.

α-Bungarotoxin (α-BTX) is a cholinergic ligand specific to the nicotinic receptor. This ligand binds to specific cholinergic brain areas (Silver and Billiar, 1976; Hunt and Schmidtt, 1978; Miller and Billiar, 1982; Miller *et al*., 1984; Clarke, 1989). We examined the effects of ovariectomy (OVX) on radioligand binding to the hypothalamic suprachiasmatic nucleus. Using intracerebroventricular infusion of ^{125}I α-BTX and autoradiography, OVX caused a marked diminution of nicotinic cholinergic binding sites vs. control animals with ovaries. ERT in OVX females restored binding site number to control levels (Miller and Billiar, 1982; Miller *et al*., 1984). These were the first literature reports demonstrating that gonadal steroids modulate cholinergic receptors.

The idea that age-related memory deficits are specifically due to loss of cholinergic neurons remains unproven, but there is little doubt that cholinergic dysfunction could be deleterious to memory and cognition and preservation of cholinergic function would be beneficial (Fibiger, 1991; Honjo *et al*., 1989; Dunnett and Fibiger, 1993; Robbins *et al*., 1997). Given E_2's supporting effects on the cholinergic system (Luine *et al*., 1980; Luine and McEwen, 1983) and our own early findings (Miller and Billiar, 1882; Miller *et al*., 1984), we investigated the effects of E_2 on memory in young and old mice. There was a significant loss of working memory in old mice, with ovaries, compared to young ones. ERT (30 days) significantly improved working memory performance in both young and old OVX mice. There was no difference in working memory between young mice with ovaries and either young or old mice with ERT (Fig. 8). These data supported our early hypothesis that E_2 is an important factor in sustaining the health of the CNS (Miller *et al*., 1998).

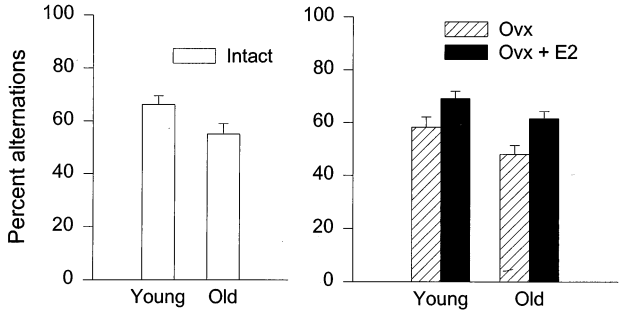


FIG. 8. Effect of age, ovariectomy, and estrogen on working memory in mice. Groups of mice were examined by using the standard "spontaneous alternation" test for working memory. In this test, a mouse is placed at the start of a T-maze with one arm blocked and allowed to explore it until it reached the end of an open arm. It was then removed and after a timed delay placed back at the start with both extensions open. This latter step is repeated nine times. Normal mice prefer to explore the region least recently visited, and tend to enter alternate arms on successive trials. If they do not remember where they were on the previous trial their choices are random. The dependent measure is the proportion of trials on which the mouse alternated. The test can be made more demanding by extending the delay between trials. There was a significant loss of working memory in old mice with ovaries compared to young intact mice ($p < 0.03$). Estrogen replacement therapy (30 days) significantly improved working memory in both young or old OVX mice ($p < 0.05$). There was no significant difference in working memory performance between young mice with ovaries and either young or old OVX mice with estrogen replacement therapy.

SUMMARY

Age-related changes occur in the CNS, but not in a uniform fashion. Neuron loss is not necessarily accompanied by complete functional loss, as indicated by the capacity of the pituitary to respond to a GnRH challenge in the oldest mice. It is clear that other physiological changes also occur, such as those in the estrogen receptor (ER) itself. Continued hypothalamic exposure to ovarian secretions has been a hypothesized cause of neuroendocrine failure (Felicio *et al.*, 1983). After E_2 exposure in the older animal, the steroid is less tightly bound to its receptor, and peak E_2 binding to its receptor is achieved later; receptor loss is accelerated, and its replenishment diminished (Bergman *et al.*, 1992). Age-related changes in the recently discovered ERβ are completely unexplored to date, but like ERα, ERβ does not colocalize with the GnRH neuron (Laflamme *et al.*, 1998) making direct age-related affects of E_2 on GnRH neurons unlikely. It is possible that repetitive steroid receptor occupancy by E_2 may be deleterious to the neuroendocrine system. Cellular clocks or pacemakers may act as signals. This is exemplified by the PC gene, which is upregulated at midlife. These genes may "switch off" neuropeptide function at middle age to protect the female from pregnancy at an age when she may be less likely to successfully carry healthy young; likewise there may be pacemakers or cascades of changes in cell signalling events.

S-OVX is detriminal to E_2-regulated neural architecture, and L-OVX exerts no protective effect on any of the neurotransmitter systems we have examined. In at least some neurotransmitter systems, it is the presence of the ovary and not animal age that dictates the numbers of synaptic connections. If E_2 is replaced following OVX, synapses increase in number in specific E_2-regulated brain areas; this hormone is a significant factor in synaptic remodeling (Nishizuka and Arai, 1981; McEwen, 1997). In our hands, E_2 in physiologic amounts is essential for the

maintenance of specific neuron populations. There are likely to be as yet unidentified age-related genomic effects of E_2. We believe that specific windows of time are components of continuous developmental processes that ultimately become deleterious to the female's reproductive capacity. One of the most important windows appears in the middle-aged female at the transition to loss of reproductive capacity.

Acknowledgments—The authors wish to acknowledge the following funding agencies for grants in aid, which supported the work described in this manuscript: NIH HD19431 and AG07795, Alzheimer Society of Canada, and the Fraser Endowment fund of the Royal Victoria Hospital to M.M.M. MRC MT-6733 to H.P.J.B.; and NSERC and FCAR to K.B.J.F. We also wish to thank Emilia Antecka, Lixia Zhu, Susan Smith, and Kataline Czonka for their excellent technical assistance in generating the data presented in these studies. We would like to express our gratitude to Rene LeSage and Pat Smith for their careful preparation and analyses of peptide and steroid hormones, and Ms. Lorraine Griffin for maintaining a virus and pathogen–free colony of aging mice for 15 years, which encompass these studies.

REFERENCES

ADLER, M.W. AND GELLER, B. Physiological functions of opioids: Temperature regulation. In: Opioids II, Herz, A. (Editors), pp. 205–238, Springer Verlag, Berlin, 1993.

AIGNER, T.G**.** and MISHKIN, M. The effects of physostigmine and scopolamine on recognition memory in monkeys. *Behav. Neural Biol.* **45,** 81–87, 1986.

AKIL, H., YOUNG, E., and WATSON, S.J. Opiate binding properties of naturally occurring N- and C-terminus modified beta-endorphins. *Peptides* **2,** 289–292, 1991.

ASCHEIM, P. Résultats fournis par la greffe hétérochrone des ovaires das l'étude de la régulation hypothalamo–hypophyso–ovarienne de rat sénile. *Gerontologia* **10,** 65, 1964/65.

BARDEN, N., MERAND, Y., ROULEAU, G.M., and DUPONT, A. Changes in the β-endorphin content of discrete hypothalamic nuclei during the estrous cycle of the rat. *Brain Res*. **204,** 41, 1981a.

BARDEN, N., DUPONT, A., FABRIE, F., MERAND, Y., ROULEAU, D., VAUDRY, H., and BOISSIER, J.R. Age-dependent changes in beta-endorpin content of discrete rat brain nuclei. *Brain Res.* **2208,** 209–212, 1981b.

BARRACLOUGH, C.A., WISE PM., and SELMANOFF, M.K. A role for hypothalamic catecholamines in the regulation of gonadotropin secretion. *Recent Prog. in Hormone Res.* **40,** 487–529, 1984.

BARRACLOUGH, C.A. Neurotransmitter regulation of luteinizing hormone-releasing hormone neuronal function. *Acta Biol. Hung*. **45,**189–206, 1994.

BEATTIE, C.W., RODGERS, C.H., and SOYKA, L.F. Influence of ovariectomy and ovarian steroids on hypothalamic tyrosine hyroxylase activity in the rat. *Endocrinology* **91,** 276–279, 1972.

BEDARD, P., LANGELIER, P., and VILLENEUVE, A. Oestrogens and the extrapyramidal system. *Lancet* **2,** 1367–1368, 1977.

BENJANNET, S., RONDEAU, N., DAY, R., CHRETIEN, M., and SEIDAH, N.G. PC1 and PC2 are proprotein convertases capable of cleaving pro-opiomelanocortin at distinct pairs of basic residues. *Proc. Natl. Acad. Sci.* USA **88,** 3564–568, 1991.

BENNETT, H.P.J. Glycosylation, phosphorylation and sulfation of peptide hormones and their precursors. In: *Peptide Biosynthesis and Processing*, Fricker, L.D. (Editor), pp. 111–139, CRC Press, Boston, 1994.

BERGMAN, M.D., SCHACHTER, B.S., KARELUS, K., COMBATSIARIS, E.P. GARCIA T., and NELSON J.F. Up-regulation of the uterine estrogen receptor and its messenger ribonucleic acid during the mouse estrous cycle: The role of estradiol. *Endocrinology* **130,** 1923–1930, 1992.

BICKNELL, R.J. Endogenous opioid peptides and hypothalamic neuroendocrine neurons, *J. Endocrinol.* **107,** 437–446, 1985.

BIRCH, N.P., TRACER, H.L., HAKER, D.J., and LOH,Y.P. Coordinate regulation of mRNA levels of pro-opiomelanocortin and the candidate processing enzymes PC2 and PC3 but not furin in rat pituitary intermediate lobe. *Biochem. Biophys. Res. Commun.* **179,** 1311–1319, 1991.

BLOOM, F., BATTENBERG, E., ROSSIER, J., LING, N., and GUILLEMIN, R.K. Neurons containing beta endorophin in the rat brain exist separately from those containing enkephalin: Immunoctyochemical studies. *Proc. Natl.Acad. Sci.* USA **75,** 1591–1595, 1978.

BRAWER, J.R., SCHIPPER, H., and NAFTOLIN, F. Ovary-dependent degeneration in the hypothalamic arcuate nucleus. *Endocrinology* **107,** 274–279, 1980.

BRAWER, J., SCHIPPER, H., and ROBAIRE, B. Effects of long term androgen and estradiol exposure on the hypothalamus. *Endocrinology* **112,** 194–199, 1983.

BRENNER, D.E., KUKULL, W.A., VAN BELLE, G., BOWEN, J.D., McCORMICK, W.C., TERI, L., and LARSON, E.B. Relationship between cigarette smoking and Alzheimer's disease in a population based case–control study. *Neurology* **43,** 293–300, 1993.

BRONSON, F.H. and VOM SAAL, F.S. Control of the preovulatory release of luteinizing hormone by steroids in the mouse. *Endocrinology* **104,** 1247–1255, 1979.

BRONSTEIN, D.M., DAY, N.C., GUTSTEIN, H.B., TRUJILLO, K.A., and AKIL, H. Pre- and post-translational regulation of β-endorphin biosynthesis in the CNS: Effects of chronic naltrexone treatment. *J. Neurochem.* **60,** 40–49, 1993.

CELLA, S.G., LOCATELLI, V., and MULLER, E.E. Opioid peptides in the regulation of anterior pituitary hormones. In: *Handbook of Experimental Pharmacology*, vol. 104/II. Akil, H. and Simon, E.J. (Editors), 473–495, Springer Verlag, Berlin, 1993.

CHEN, W-P., WITKIN, J.W., and SILVERMAN, A.J. Beta-endorphin and gonadotropin releasing hormone synaptic input to gonadotropin-releasing hormone neurosecretory cells in the male rat. *J. Comp. Neurol*. **286,** 85–95, 1989.

CLARKE, P.B. Mapping of brain nicotinic receptors by autoradiographic techniques and the effect of experimental lesions. *Prog. Brain Res.* **79,** 65–71, 1989.

COHEN, M.R., COHEN, R.M., PICKAR, D., WEINGARTNER, H., MURPHY, D.L., and BUNNEY, W.E. Behavioral effects after high doses naloxone administration to human volunteers. *Lancet* **2,** 1110, 1981.

COOPER, S.J. and KIRKMAN, T.C. Opioid mechanisms in the control of food consumption and taste preferences. In: *Opioids II*, Herz, A, (Editor), pp. 239–262, Springer Verlag, Berlin, 1993.

CRAWFORD, J.G. Alzheimer's disease risk factors as related to cerebral blood flow. *Med. Hypoth.* **46,** 367–377, 1996.

CUELLO, A.C. Central distribution of opioid peptides, *Br. Med. Bul.* **39,** 11–16, 1983.

CURCIO, C.A., BUELL, S.J. and COLEMAN, P.D. Morphology of the central nervous system: not all down hill. In: *Advances in Neurogerontology: The Aging Motor System*, Mortimer, J.A., Pirozzdo, F.J., Maletta, G.I., (Editors), pp. 7–35, Praeger, New York, 1982.

DALTON, K. Heineman Medical Books, The Premenstrual Syndrome, London, 1964.

DAY, R., SCHAFER, M.K-H., WATSON, S.J., CHRETIEN, M., and SEIDAH, N.G. Distribution and regulation of the prohormone convertase PC1 and PC2 in the rat pituitary. *Mol. Endocrinol.* **6,** 485–497, 1992.

DAY, R., SCHAFER, M.K-H., CULLINAN, W.E., WATSON, S.T., CHRETIEN, M., and SEIDAH, N.G. Region specific expression of furin mRNA in the rat brain. *Neurosci. Lett.* **149,** 27–30, 1993.

DEKKER, M.W. and McGAUGH, J.L. The role of interactions between the cholinergic system and other neuromodulatory systems in learning and memory. *Synapse* **7,** 151–168, 1991.

DESJARDINS, G.C., BRAWER, J.R., and BEAUDET, A. Estradiol is selectively neurotoxic to hypothalamic β-endorphin neurons. *Endocrinology* **131,** 86–93, 1993.

DOHANICH, G.P., FADER, A.J., and JAVORSKY, D.J. Estrogen and estrogen-progesterone treatments counteract the effect of scopolamine on reinforced T-maze alternation in female rats. *Behav. Neurosci.* **108,** 988–992, 1994.

DUNNETT, S.B. and FIBIGER, H.C. Role of forebrain cholinergic systems in learning and memory: Relevance to the cognitive deficits of aging and Alzheimer's dementia. *Prog. Brain Res.* **98,** 413–420, 1993.

EIPPER, B.A. and MAINS, R.E. Structure and biosynthesis of proadreno-corticoropin/endorphin and related peptides. *Endocrinol. Rev.* **1,** 1–27, 1980.

EMESON, R.B. and EIPPER, B.A. Characterization of pro-ACTH/endorphin-derived peptides in rat hypothalamus. *J. Neurosci.* **6,** 837–849, 1986.

FELICIO, L.S., NELSON, J.F., GOSDEN, R.G., and FINCH, C.E. 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,** 60676–60680, 1983.

FELICIO, L.S., NELSON, J.F., and FINCH, C.E. Prolongation and cessation of estrone cycles in aging C57BL/6J mice are differentially regulated events. *Biol. Reprod.* **34,** 849–858, 1986.

FIBIGER, H.C. Cholinergic mechanisms in learning, memory, and dementia: A review of recent evidence. *Trends Neurosic.* **14,** 220–223, 1991.

FIELD, E. and KUHN, C.M. Opiate antagonist treatment reinstates estrous cycles in middle-aged persistent-estrous rats. *Biol. Reprod.* **40,** 714–719, 1989.

FIELD, E. and KUHN, C.M. Opioid inhibition of luteinizing hormone release declines with age and acyclicity in female rats. *Endocrinology* **123,** 2626–2631, 1988.

FIELDS, H.L. Brainstem mechanisms of pain modulation: Anatomy and physiology. In: Opioids II, Hertz, A. (Editor), pp. 3–20, Springer Verlag, Berlin, 1993.

FINCH, C.E., FELICIO, L.S., FLURKEY, K., GEE, D.M., MOBBS, C., NELSON, J.F., and OSTERBURG, H.H. Studies on ovarian hypothalamic pituitary interactions during reproductive aging in C57BL/6J mice. *Peptides Suppl.* **1,** 163, 1980.

FINCH, C.E., FELICIO, L.S., MOBBS, C.V., and NELSON, J.F. Ovarian and steroidal influences on neuroendocrine aging processes in female rodents. *Endocr. Rev.* **5,** 467–497, 1984.

FLURKEY, K., GEE, D.M., SINHA, Y.N., and FINCH, C.E. Age effects on luteinizing hormone, progesterone, and prolactin in proestrous and acyclic C57BL/6J mice. *Biol. Reprod.* **26,** 835–846, 1982.

GABRIEL S.M., BERGLUND L.A., and SIMPKINS, J.W. A decline in endogenous opioid influence during the steroid-induced hypersecretion of luteinizing hormone in the rat. *Endocrinology* **118,** 558–561, 1986.

GARCIA-SEGURA, L.M., BAETENS D., and NAFTOLIN, F. Synaptic remodelling in arcuate nucleus after injection of estradiol valerate in adult female rats. *Brain Res.* **366,** 131–136, 1986.

GEE, D.M., FLURKEY, K., and FINCH, C.E. Aging and the regulation of luteinizing hormone in C57BL/6J mice: Impaired elevations after ovariectomy and spontaneous elevations at advanced ages. *Biol. Reprod.* **28,** 598–607, 1983.

GEE, D.M., FLURKEY K., MOBBS C.V., SINHA YN., and FINCH CE. The regulation of luteinizing hormone and prolactin in C57BL/6J mice: effects of estradiol implant size, duration of ovariectomy, and aging. *Endocrinology* **114,** 685–893, 1984.

GEE, C.E., CHEN, C.L.C., ROBERTS, J.L., THOMPSON, R., and WATSON, S.J. Identification of proopiomelanocortin neurons in rat hypothalamus by in situ hybridization cDNA–mRNA hybridization. *Nature* **306,** 374–376, 1983.

GIANOULAKIS C. and GUPTA, A. Inbred strains of mice with variable sensitivity to ethanol exhibit differences in the content and processing of β-endorphin. *Life Sci.* **39,** 2315–2325, 1986.

GIBBS, R.B. Fluctuations in relative levels of choline acetyltransferase mRNA in different regions of the rat basal forebrain across the estrous cycle: Effects of estrogen and progesterone. *J. Neurosci*. **16**: 1049–1055, 1996.

GITLER, M.S. and BARRACLOUGH, C.A., Stimulation of the medullary A1 noradrenergic system augments luteinizing hormone release induced by medial preoptic nucleus stimulation. Evaluation of A1 projections to the hypothalamus and of drugs which affect norepinephrine synthesis and adrenoceptors. *Neuroendocrinology* **48,** 351–359, 1988.

HAMMER, R.P. μ-Opiate receptor binding in the medial preoptic area is cyclical and sexually dimorphic. *Brain Res.* **515,** 187–192, 1990.

HAMMOND, R.G., NICOLAS, P., and LI, C.H. β-Endorphin (1–27) is an antagonist of β-endorphin analgesia. *Proc. Natl. Acad. Sci. USA* **81,** 1389–1390, 1984.

HARMEN S.M. and TALBERT B.G. Reproductive Aging. In: *Handbook of the Biology of Aging*, 2nd ed, Finch, C.E. and Schneider, E.L. (Editors), pp. 457–510, Van Nostrand, New York, 1987.

HENDERSON, V.W., PAGANINI-HILL, A., EMANUEL, C.K., DUNN M.E., and BUCKWALTER, J.G. Estrogen replacement therapy in older women: Comparison between Alzheimer's Disease and nondemented control subjects. *Arch. Neurol*. **51,** 896–900, 1994.

HOFFMAN, G.E., WRAY S., and GOLDSTEIN, M. Relationship of catecholamines and LHRH: Light microscopic study, *Brain. Res. Bull.* **9,** 417–430, 1982.

HOFFMAN, G.E. and FINCH C.E. LHRH neurons in the female C57BL/6J mouse brain during reproductive aging: No loss up to middle age. *Neurobiol. Aging* **7,** 45–48, 1986.

HONJO, H., OGINO, Y., NAITOH, K., URABE, M., KITAWAKI, J., YASUA, J., YAMAMOTO, T., ISHOHARA, S., OKADO, H., YONEZAKWA, T., HAYAHI, K., and NAMBARA, T. In vivo effect of estrone sulfate on the central nervous system-senile dementia (Alzheimer's type). *J. Steroid Biochem.* **34,** 521–525, 1989.

HOSKINS, B., BURTON, C.K., and HO, I.K. Differences in morphine induced antinociception and locomotor activity in mature adult and aged mice. *Pharmacol. Biochem. Behav.* **25,** 599–605, 1986.

HSU, H.K. and PENG, M.T. Hypothalamic neuron number of old female rats. *Gerontology* **24,** 434–440, 1978.

HUNT, S. and SCHMIDT, J. Some observations on the binding patterns of alpha-bungarotoxin in the central nervous system of the rat. *Brain Res.* **157,** 213–232, 1978.

JAFFE, R.B. The menopause and perimenopausal period. In: *Reproductive Endocrinology*, Yen, S.S.C. and Jaffe, R.B. (Editors), pp. 393–394, W.B. Saunders, Philadelphia, 1991.

JIH, M.H., LU, J.K.H., and WAN, T-C. Inhibin subunit gene expression and distribution in the ovaries of immature, young adult, middle aged, and old female rats. *Endocrinology* **132,** 319–326, 1993.

JOSHI, D., BILLIAR, R.B., and MILLER, M.M. Modulation of hypothalamic mu-opioid receptor density by estrogen: A quantitative autoradiographic study of the female C57BL/6J mouse. *Brain Res. Bull.* **30,** 629–634, 1992.

JOSHI, D., LEKHTMAN, I., BILLIAR, R.B., and MILLER, M.M. Gonadotropin hormone releasing hormone induced responses in young and old female C57BL/6J mice. *Proc. Soc. Exp. Biol. Med*. **204,** 191–194, 1993.

JOSHI, D., BILLIAR, R.B., and MILLER, M.M., Luteinizing hormone response to *N*-Methyl D,L-aspartic acid in the presence of physiological estradiol concentrations: Influence of age and the ovary in the female C57BL/6J mouse. *Proc. Soc. Natl Acad. Exp. Biol. Med.* **209,** 237–244, 1995a.

JOSHI, D., BENNETT, H.P.J., JAMES, S., TOUSIGNANT, P., and MILLER, M.M. Hypothalamic processing of β-endorphin is altered at middle age. *J. Endocrino*l. **144,** 405–415,1995b.

JOSHI, D., MILLER, M.M., SEIDAH, N.G., and DAY, R. Age-related alterations in the expression of prohormone convertase messenger ribonucleic acid (mRNA) levels in hypothalamic proopiomelanocortin mRNA neurons in the female C57BL/6J mouse. *Endocrinology* **136,** 2721–2729, 1995c.

KALRA, S.P, and SIMPKINS, J.W. Evidence for noradrenergic mediation of opioid effects on luteinizing hormone secretion. *Endocrinology* **109,** 776–782, 1981.

KALRA, S.P. Opiate peptides: Inhibitory neuronal systems in regulation of gonadotropin secretion. In: The Role of Peptides and Proteins in Control of Reproduction, McCann, S.M. and Dhindsa, D.S. (Editors), pp. 63–87, Elsevier, New York, 1983a.

KALRA, S.P. and KALRA, P.S. Neuronal regulation of luteinizing hormone secretion in the rat. *Endocr. Rev.* **4,** 311–356, 1983.

KALRA, S.P. and GALLO, RV. Effects of intraventricular administration of catecholamines on luteinizing hormone release in morphine-treated rats. *Endocrinology.* **113,** 23–28, 1983.

KALRA, S.P. Mandatory neuropeptide-steroid signalling for the preovulatory luteinizing hormone-releasing hormone discharge. *Endocr. Rev.* **14,** 507–538, 1993.

KARELUS, K. and NELSON, J.F. Aging impairs estrogenic suppression of hypothalamic proopiomelanocortin messenger ribonucleic acid in mouse. *Neuroendocinology.* **55,** 627–633, 1992.

KHACHATURIAN, H., LEWIS, M.E. SCHAFER M.K-H., and WATSON, S.J. Anatomy of CNS opioid systems. *Trends Neurosci.* **8,** 111–119, 1985.

KIRISTY-ROY, J.A., HALTER, J.B., SMITH, M.J., and TERRY, L.C. Selective impairment of neuroendocrine and hemodynamic responses to a mu-opioid peptide in aged rats. *J.Gerontol.* **47,** 889–897, 1992.

KLAIBER, E.L., BOVERMAN, D.M., VOGEL, W., and KOBAYASHI, Y. Estrogen therapy for several persistent depression in women. *Arch. Gen. Psychiatry* **36,** 550–554, 1979.

KNUTH, U.A., SIKAND, G.S., CASANUEVA, F.F., HAVLICEK, V., and FRIESEN, H.G. Changes in beta-endorphin content in discrete areas of the hypothalamus throughout proestrous and diestrous of the rat. *Life Sci.* **33,** 1443–1450, 1983.

KOHAMA, S.G., BROWN, S.A., FINCH, C.E., and McNEILL, T.H. Chronic estradiol administration did not cause loss of hypothalamic or TIDA neurons in young or middle aged C57BL/6J mice. *Brain Res.* **574,** 341–344, 1992.

LaFLAMME, N., NAPPI, R.E., DROLET, G., LABRIE, C., and RIVEST, S. Expression and neuropeptidergic characterization of estrogen receptors (ERα and ERβ) throughout the rat brain: Anatomical evidence of distinct roles of each subtype. *J. Neurobiol.* (in press) 1998.

LaPOLT, P.S., MATT, D.W., JUDD, H.L., and LU, J.K.H. The relation of ovarian steroid levels in young female rats to subsequent estrous cyclicity and reproductive function during aging. *Biol. Reprod.* **35,** 1131–1139, 1986.

LaPOLT, P.S., YU, S.M., and LU, J.K. Early treatment of young female rats with progesterone delays the aging-associated reproductive decline: A counteraction by estradiol. *Biol. Reprod.* **38,** 987–995, 1988.

LARSSON, K. Effect of age on the sexual behavior of the male rat. *Gerontologia* **6,** 133–138, 1962.

LEADEM, C.A. and KALRA, S.P. Effects of endogenous opioid peptides and opiates on luteinizing hormone and prolactin in ovariectomized rats. *Neuroendocrinology* **41,** 342–352, 1985.

LEVITT, M., SPECTOR, S., SJOEERDSMA, A., and UDENFRIEND, S. Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea pig heart. *J. Pharmacol. Exp. Ther.* **48,** 1–8, 1965.

LLOYD, J.M., WEILAND, N.G., and WISE, P.M. Age-related changes in opiate receptor densities in discrete hypothalamic nuclei of ovariectomized (OXV) and estradiol (E_2)-treated rats. *Mol. Cell Neurosci.* **4,** 173–180, 1993.

LU, J.K.H. DAMASSA, D.A., GILMAN, D.P., JUDD, H.L., and SAWYER, C.H. Differential patterns of gonadotropin responses to ovarian steroid and the LH-releasing hormone between constant-estrous and pseudopregnant states in aging rats. *Biol. Reprod.* **23,** 345–351, 1980.

LUINE, V., PARK, D., JOH, T., REIS, D., AND McEWEN, B., Immunochemical demonstration of increased choline acetyltransferase concentration in rat preoptic area after estradiol administration. *Brain Res.* **191,** 273–277, 1980.

LUINE, V.N. and McEWEN, B.S. Sex differences in cholinergic enzymes of diagonal band nuclei in the rat preoptic area. *Neuroendocrinology* **36,** 475–482, 1983

LUINE, V. and RODRIGUEZ, M. Effects of estradiol on radial arm maze performance of young and aged rats. *Behav. Neural. Biol.* **62,** 230–236, 1994.

McALONAN, G.M., DAWSON, G.R., WILKINSON, L.O., ROBBINS, T.W., and EVERITT, B.J. The effects of

AMPA-induced lesions of the medial septum and vertical limb nucleus of the diagonal band of Broca on spatial delayed non-matching to sample and spatial learning in the water maze. *Eur. J. Neurosci*. **7,** 1034–1049, 1995.

MCEWEN, B.S. and PARSONS, B. Gonadal steroid action on the brain: Neurochemistry and neuropharmacology. *Annu. Rev. Pharmacol. Toxicol*. **22,** 555–598, 1982.

MCEWEN, B.S. The brain is an important target of adrenal steroid actions. A comparison of synthetic and natural steroids. *Ann. NY Acad. Sci.* **823,** 201–213, 1997.

MAINS, R.E. and EIPPER, B.A. Synthesis and secretion of corticotropins, melanotropins, and endorphins by rat intermediate pituitary cells. *J. Biol. Chem.* **254,** 7885–7894, 1979.

MARKOWSKA, A.L., OLTON, D.S., and GIVENS, B. Cholinergic manipulations in the medial septal area: Age-related effects on working memory and hippocampal electrophysiology. *J. Neurosci.* **15,** 2063–2073, 1995.

MARCINKIEWICZ, M., DAY, R., SEIDAH, N.G., and CHRETIEN, M. Ontogeny of the prohormone convertases PC1 and PC2 in the mouse hypophysis and their colocalization with corticotropin and alpha-melanotropin. *Proc. Natl. Acad. Sci. USA* **90,** 4922–4926, 1993.

MEHMANESH, H., ALMEIDA, O.F., NIKOLARAKIS, K.E., and HERZ, A. Hypothalamic LH-RH release after acute and chronic treatment with morphine studied in a combined in vivo/in vitro model. *Brain Res.*. **451,** 69–76, 1988

MESULUM, M-M., MUFSON, E.J., WAINER, B.H., and LEVEY, A.I. Central cholinergic pathways in the rat: An overview based on an alternative nomenclature (Chj1–Ch6). *Neuroscience* **10,** 1185–1201, 1983.

MILLAN, M.J. Multiple opioid systems and chronic pain. In: *Handbook of Experimental Pharmacology,* vol. 104/II, Akil, H. and Simon, E.J. (Editors), pp. 2127–162, Springer Verlag, New York, 1993.

MILLER, M.M. and BILLIAR, R.B. Effects of ovariectomy on the binding of (^{125}I)alpha-bungarotoxin (2.2 and 3.3) to the suprachiasmatic nucleus of the hypothalamus: An in vivo autoradiographic analysis. *Brain Res.* **247,** 355–364, 1982.

MILLER, M.M., SILVER, J., and BILLIAR, R.B. Effects of gonadal steroids on the in vivo binding of (^{125}I)alpha-bungarotoxin to the suprachiasmatic nucleus, *Brain Res.* **290,** 67–75, 1984.

MILLER, M.M., GOULD B. E., and NELSON J.F. Aging and long-term ovariectomy alter the cytoarchitecture of the hypothalamic–preoptic area of the C57BL/6J mouse *Neurobiol of Aging* **10,** 683–690, 1989.

MILLER, M.M., JOSHI, D., BILLIAR, R.B., and NELSON, J.F. Loss of LH-RH neurons in the rostral forebrain of old female C57BL/6J mice. *Neurobiol. Aging* **11,** 217–221, 1990.

MILLER, M.M. and BILLIAR, R.B. Methods for enhancing immunolabeling of beta-endorphin-containing neuronal terminals in rat and mouse hypothalamus. *Neuroendocrinol. Lett*. **12,** 25–30, 1990.

MILLER, M.M., JOSHI, D. BILLIAR, R.B., and NELSON, J.F. Loss during aging of beta-endorphinergic neurons in the hypothalamus of female C57BL/6J mice. *Neurobiol. Aging* **12,** 239–244 1991.

MILLER, M.M. and ZHU, L. Aging changes the beta-endorphin neuronal system in the preoptic area of the C57BL/6J mouse: An ultrastructural analysis. *Neurobiol. Aging* **13,** 773–781, 1992a.

MILLER, M.M. and ZHU, L. Age-related changes in dopamine beta hydroxylase immunoreactivity in the rostral forebrain of female C57BL/6J mice. *Neuroendocrinol. Lett.* **14,** 127–132, 1992b.

MILLER, M.M. Morphometric and immunocytochemical analysis of age-related changes in neuropeptidergic neurons. In: *Neuroprotocols: A Companion to Methods in Neurosciences*, vol. 4, Conn, M. (Editor), 188–203, Academic Press, New York, 1994.

MILLER, M.M., TOUSIGNANT, P., YANG, U. PEDVIS, S., and BILLIAR, R.B. Effects of age and long-term ovariectomy on the estrogen-receptor containing subpopulations of β-endorphin immunoreactive neurons in the arcuate nucleus of female C57BL/6J mice. *Neuroendocrinology* **61,** 542–551, 1995.

MILLER, M.M. and ZHU, L. Ovariectomy and age alter gonadotropin hormone releasing hormone–noradrenergic interactions. *Neurobiol. Aging* **16**: 613–625, 1995.

MILLER, M.M., HYDER, S.M., ASSAYAG, R., PANARELLA, S.R, TOUSIGNANT, P., and FRANKLIN, K.B.J. Estrogen modulates memory and the cholinergic phenotype in the basal forebrain. *Neuroscience* (in press).

MOBBS, C.V., GEE, D.M., and FINCH, C.E. Reproductive senescence in female C57BL/6J mice: Ovarian impairments and neuroendocrine impairments that are partially reversible and delayable by ovariectomy. *Endocrinology* **115,** 1653–1662, 1984.

MOBBS, C.V., CHEYNEY, D., SINHA, Y.N., and FINCH, C.E. Age-correlated and ovary-dependent changes in relationships between plasma estradiol and luteinizing hormone, prolactin, and growth hormone in female C57BL/6J mice. *Endocrinology* **116,** 813–820, 1985.

NAKAYAMA, K., HOSAKA, M., HATSUZAWA, K., and MURAKAMI, K. Cloning and functional expression of a novel endoprotease involved in prohormone processing at dibasic sites. *J. Biochem.* **109,** 803–806, 1991.

NELSON, J.F., FELICIO, L.S., OSTERBURG, H.H., and FINCH, C.E Altered profiles of estradiol and progesterone

associated with prolonged estrous cycles and persistent vaginal cornification in aging C57BL/6J mice. *Biol. Repro.* **24,** 784–794, 1981.

NELSON, J.F. and FELICIO L.S. Reproductive aging in the female: An etiological perspective updated. *Rev. Biol. Res. Aging* **2,** 251, 1985.

NELSON, J.F., BENDER, M., and SCHACHTER, B.S. Age-related changes in proopiomelanocortin messenger ribonucleic acid levels in hypothalamus and pituitary of female C57BL/6J mice *Endocrinology* **123,** 340–344, 1988.

NELSON, J.F., FELICIO, L.S., OSTERBURG, H.H., and FINCH, C.E. Differential contributions of ovarian and extraovarian factors to age related reductions in plasma estradiol and progesterone during the estrous cycle of C57BL/6J mice. *Endocrinology* **130,** 805–810, 1992.

NISHIZUKA M. and ARAI, Y. Organizational action of estrogen on synaptic pattern in the amygdala: implications for sexual differentiation of the brain. *Brain Res*. **213,** 422–426, 1981.

OHKURA, T., ISSE, K., AKAZAWA, K., HAMAMOTO, M., YAOI, Y., and HAGINO, N. Long term estrogen replacement therapy in female patients with dementia of Alzheimer's type: 7 case reports. *Dementia* **6,** 99–107, 1995a.

OHKURA, T., ISSE, K., AKAZAWA, K., HAMAMOTO, M., YAOI, Y., and HAGINO, N. Evaluation of estrogen treatment in female patients with dementia of Alzheimer's type. *Endocr. J.* **41,** 361–371, 1995b.

OLTON, D.S. and MARKOWSKA, A.L. The ageing septohippocampal system: Its role in age-related memory impairments. In: *Neuropsychology of Memory*, Squire, L.R. and Butters, N. (Editors), Guilford, New York, 1992.

PACKARD, M.G., KOHLMAIER, J.R., and ALEXANDER, G.M. Posttraining intrahippocampal estradiol injections enhance spatial memory in male rats: Interaction with cholinergic systems. *Behav. Neurosci.*. **110,** 626–632, 1996.

PASQUALINI, C., GUIBERT, B., and LEVIEL, V. Short-term inhibitory effect of estradiol on tyrosine hyrdoxylase activity in tuberonfundibular dopaminergic neurons in vitro. *J. Neurochem.* **60,** 1707–1713, 1993.

PETRAGLIA, F., COMITINIE, G., and GENAZZANI, A.R. β-Endorphin in human reproduction, In: *Handbook of Experimental Pharmacology*, vol. 104/II, Akil, H. and Simon, E.J. (Editors), pp. 763–780, Springer Verlag, Berlin, 1993.

REID, R.L., QUIGLEY, M.E., and YEN, S.C.C. The disappearance of opioidergic regulation of gonadotropin secretion in postmenopausal women. *J. Clin. Endocrinol. Metab.* **57,** 1107–1110, 1983.

REID, R.L. and YEN, S.C.C. The premenstrual syndrome. *Clin. Obstet. Gynecol.* **26,** 710–718, 1983.

ROBBINS, T.W., MCALONAN, G., MUIR, J.L., and EVERITT, B.J. Cognitive enhancers in theory and practice: Studies of the cholinergic hypothesis of cognitive deficits in Alzheimer's disease. *Behav. Brain Res.* **83,** 15–23, 1997.

SCHAFER, M.K.H., DAY, R., CULLINAN, W.E., CHRETIAN, M., SEIDAH, N.G. and WATSON, S.J. Gene expression of prohormone and proprotein convertase in the rat CNS: A comparative in situ hybridization analysis. *J. Neurosci.* **13,** 1258–1279, 1993.

SCHIPPER, H., BRAWER, J.R., NELSON, J.F., FELICIO, L.S., and FINCH, C.E. Role of the gonads in the histologic aging of the hypothalamic arcuate nucleus. *Biol. Reprod*. **25,** 413–419, 1981.

SEIDAH, N.G., MARCINKIEWICZ, M., BENJANNET, S., GASPER, L., BEAUDIAN, G., MATTEI, M.G., LAZURE, C., MBIKAZ, M., and CHRETIEN, M. Cloning and primary sequence of a mouse candidate prohormone convertase PC1 and homologous to PC2, furin and kexl: Distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. *Mol. Endocrinol*. **5,** 111–122, 1991.

SEIDAH, N.G. and CHRETIEN, M. Prohormone and proprotein convertases of the subtilisin family: Recent developments and future perspective trends. *Endocrinol. Metab.* **3,** 133–140, 1992.

SHERWIN, B.B. Estrogenic effects on memory in women. *Ann N.Y. Acad. Sci*. **743,** 213–230, 1994.

SHERWIN, B.B. and TULANDI, T. Add-back estrogen reverses cognitive deficits induced by a gonadotropin-releasing hormone agonist in women with leiomyomata uteri. *J. Clin. Endocrinol. Metab.* **81,** 2545–2549, 1996.

SILVER, J. and BILLIAR, R.B. An autoradiographic analysis of [^{3}H]alpha-bungarotoxin distribution in the rat brain after intraventricular injection, *J. Cell. Biol.* **71,** 956–963, 1976.

SMEEKENS, S.P. and STEINER, D.F. Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. *J. Biol. Chem*. **265,** 2997–3000, 1990.

SOHRABJI, F., MIRANDA, R.C., and TORAN-ALLERAND, D. Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. USA* **92,** 11110–11114, 1995.

STUMPF, W.E. and SAR, M. Autoradiographic techniques for localizing steroid hormones. *Methods Enzymol*. **36,** 135–156, 1975.

TANAKA, Y., KATAYAMA, K., and TOJO, S. Failure of positive feedback in hypothalamo–pituitary-system in aged women and its recovery with estrogen treatment. *Acta Obstet. Gynaecol. Jpn*. **34,** 1907–1915, 1982.

TANG, M.X., JACOBS, D., STERN, Y., MARDER, K., SCHOFIELD, P., GURLAND, B., ANDREWS, H., and

MAYEUX, R. Effects of oestrogen during menopause and age at onset of Alzheimer's Disease. *Lancet* **348,** 429–432, 1996.

TONG, Y. and PELLETIER, G. Role of dopamine in the regulation of proopiomelanocortin (POMC) mRNA levels in the arcuate nucleus and pituitary gland of the female rat as studied by in situ hybridiazation. *Brain. Res. Mol. Brain Res.* **15,** 27–32, 1992.

TREISER, S.L. and WARDLAW, S.L. Estradiol regulation of proopiomelanocortin gene expression and peptide content in the hypothalamus. *Neuroendocrinology* **55,** 167–173, 1992.

VOM SAAL, F.S. and FINCH, C.E. Reproductive Senescence: Phenomena and Mechanisms in Mammals and Selected Vertebrates, In: *The Physiology of Reproduction.* vol. 2, Knobil, E., Neill, J.D. (Editors), p. 2351, Raven Press, New York, 1988.

WEILAND, N.G., SCARBROUGH, K., and WISE, P.M. Aging abolishes the estradiol-induced suppression and diurnal rhythm of proopiomelanocortin gene expression in the arcuate nucleus. *Endocrinology* **131,** 2959–2964, 1992.

WEILAND, N.G. and WISE, P.M. Aging progressively decreases the densities and alters the diurnal rhythm of $\alpha 1$ adrenergic receptors in selected hypothalamic regions. *Endocrinology* **126,** 2392–2397, 1990.

WEILAND, N.G., COHEN, J.R., and WISE, P.M. Age associated alteration in catecholaminergic concentrations, neuronal activity, and receptor densities in female rats. *Neurobiol. Aging* **10,** 323–329, 1989.

WEILAND, N.G., and WISE, P.M. Effects of age on $beta_1$ and $beta_2$ adrenergic receptors in the brain assessed by quantitative autoradiography. *Brain Res.* **398,** 305–312, 1986.

WILCOX, J.N. and ROBERTS, J. Estrogen decreases rat hypothalamic proopiomelanocortin messenger ribonucleic acid levels. *Endocrinology* **117,** 2392–2396, 1985.

WILKINSON, C.W. and DORSA, D.M. The effects of aging on molecular forms of β- and γ-endorphins in rat hypothalamus. *Neuroendocrinology* **43,** 124–131, 1986.

WISE, P.M. Aging of the female reproductive system. In: *Review of Biological Research in Aging*, vol. I, Rothstein, M. (Editor), pp. 195–222, Alan R. Liss, New York, 1983.

WISE, PM. Effects of hyperprolactinemia on estrous cyclicity, serum luteinizing hormone, prolactin, estradiol, and progesterone concentrations, and catecholamine activity in microdissected brain areas, *Endocrinology* **118,** 1237–1246, 1986.

WISE, P.M., SCARBROUGH, K., LARSON, G.H., LLOYD, J.M., WEILAND, N.G., and CHIU, S. Neuroendocrine influences on aging of the female reproductive system. *Front. Neuroendocrinol.* **12,** 323, 1991.

WISE, P.M., WEILAND, N.G., SCARBROUGH, K., SORTINO, M.A., COHEN, I,R., and LARSON, GH., Changing hypothalamopituitary function: Its role in aging of the female eproductive system. *Horm. Res*. **31,** 39–44, 1989.

WISE, P.M., WEILAND, N.G., SCARBROUGH, K., and LLOYD, J.M. Changing diurnal and pulsatile rhythms during aging. *Neurobiol. Aging* **15,** 503–507, 1994.

WITKIN, J.W. Morphology of luteinizing hormone-releasing hormone neurons as a function of age and hormonal condition in the male rat. *Neuroendocrinology* **49,** 344–348, 1989.

YEN, S.S.C. Neuroendocrine control of hypophyseal function. In: *Reproductive Endocrinology, Physiology, Pathophysiology, and Clinical Management*, pp. 33–74, W.B. Saunders, Philadelphia, 1986.

YOUNG, E., BRONSTEIN, D., and AKIL, H. Proopiomelanocortin biosynthesis, processing, and secretion: Functional implication, In: *Handbook of Experimental Pharmacology*, vol. 1041I, Herb, A. (Editor), Springer Verlag, New York, 1993.

ZHOU, A., BLOOMQUIST, B.T., and MAINS, R.E. The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during proopiomelanocortin biosynthetic processing. *J. Biol. Chem.* **268,** 1763–1769, 1993.

ZIS, A.P. and GARLAND, J.E. Opioid peptides and depression: The neuroendocrine approach. *Bailleres Clin. Endocrinol.* **5,** 97–117, 1991.