Regulation of Arcuate Nucleus Synaptology by Estrogen[a]

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ESTROGEN AND THE DEVELOPMENT OF THE HYPOTHALAMUS

Our laboratory has long been interested in the control of gonadotrophins by sex steroids. It has become clear that estrogen plays a long term and critical role in gonadotrophin secretion by regulating the synaptology of the GNRH delivery system. Our present understanding of this neuroendocrine constellation is that circulating unbound estrogen and estrogen formed in the brain from aromatization of testosterone determine the final number of neurons in the rat hypothalamus and influence neuronal differentiation, process formation and synaptogenesis.[1-3] The regulation of synaptogenesis appears to be completed by the time of sexual maturation in the rat[1,4] and may contribute to the achievement of adult reproductive function.

Owing to the presence in the circulation of an avid specific estrogen binder (α-fetoprotein) during the developmental period in rats, only a minor portion of the circulating estrogen is unbound and available to influence the earliest developmental events.[3] In the presence of the additional estrogen in the brain arising from the *in situ* conversion of androgen secreted by the male testis,[3] the developmental program is elaborated to that of the male, where no estrogen-induced gonadotrophin surge is possible in adult life (sexual differentiation of the brain). In females, the absence of increased estrogen during the perinatal period, neuronal maturation, process formation, and synaptogenesis lead to the formation of a network capable of an estrogen-induced augmentation in GNRH secretion (positive feedback).[5]

The role of the sex chromosomes in development appears largely to determine the gonadal sex. Thereafter, it is testosterone from the testis that differentiates the indeterminate fetal GNRH delivery system to the male pheno-

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type. In the absence of this stimulus, but in the presence of a permissive level of unbound circulating estrogen,[1] the GNRH-delivery system develops a feminine neural and glial complement with a synaptology capable of an estrogen-induced gonadotrophin surge. In the presence of a testis, the developmental program is elaborated to block positive feedback. Thus, while the presence of intrahypothalamic aromatase or estrogen synthetase is critical during the perinatal period, it is the presence of a second perinatal androgen surge in the male that drives the loss of positive feedback.

In summary, in males during brain differentiation in the rat, estrogen made in the brain from circulating testosterone from the testis organizes the GNRH-delivery system to permit only tonic gonadotrophin secretion; positive feedback is not possible. In the female, a neural network capable of an estrogen-induced augmentation in GNRH secretion is formed in the presence of permissive levels of estrogen. We have studied the cellular mechanisms underlying this sexual differentiation of the brain.

ESTROGEN AND ARCUATE NUCLEUS POST-SYNAPTIC MEMBRANE ORGANIZATION

Following the work of Matsumoto and Arai,[6] who showed that estrogen regulates post-natal synaptogenesis in the arcuate nucleus, and our own work which validates these studies, attention focused on a better understanding of the mechanism by which estrogen acts. Since post-synaptic membranes are a major determinant of which synapses a neuron will form, we turned to freeze-fracture techniques to study the post-synaptic membrane organization of the developing rat arcuate nucleus. Freeze-fracture permits an evaluation of the components of membranes, including intramembranous domains of receptors, structural proteins and channels. By knowing the geometry of the freeze-fracture replica, it is possible to determine the size of particles and divide them into small (<10 nM) and large (>10 nM) intramembranous particles. We observed that the female rat's arcuate nucleus has an excess of intramembranous protein particles over males from birth into adult life[7] (FIG. 1). The excess intramembranous particles in females are primarily those of less than 10 nM diameter. Males have a slight excess of the large particles. Thus, by the day of birth there is already a recognizable sex difference in neuronal post-synaptic membrane organization in the arcuate nucleus, which could play a role in differing synaptic networks being formed in males and females.

To assess the effect of locally formed estrogen neuronal membrane organization, we treated 5-day-old female rats with testosterone proprionate (TP).[8] On day 10, perfusion-fixed tissue was obtained and post-synaptic membrane organization studied. Arcuate nucleus neuronal membranes from TP-treated females were indistinguishable from those of control males in protein particle content. Since the dose of TP given also blocks the development of the estrogen-induced gonadotrophin surge and produces postpubertal constant vaginal estrus, we hypothesize that the sexual dimorphism in postsynaptic intra-

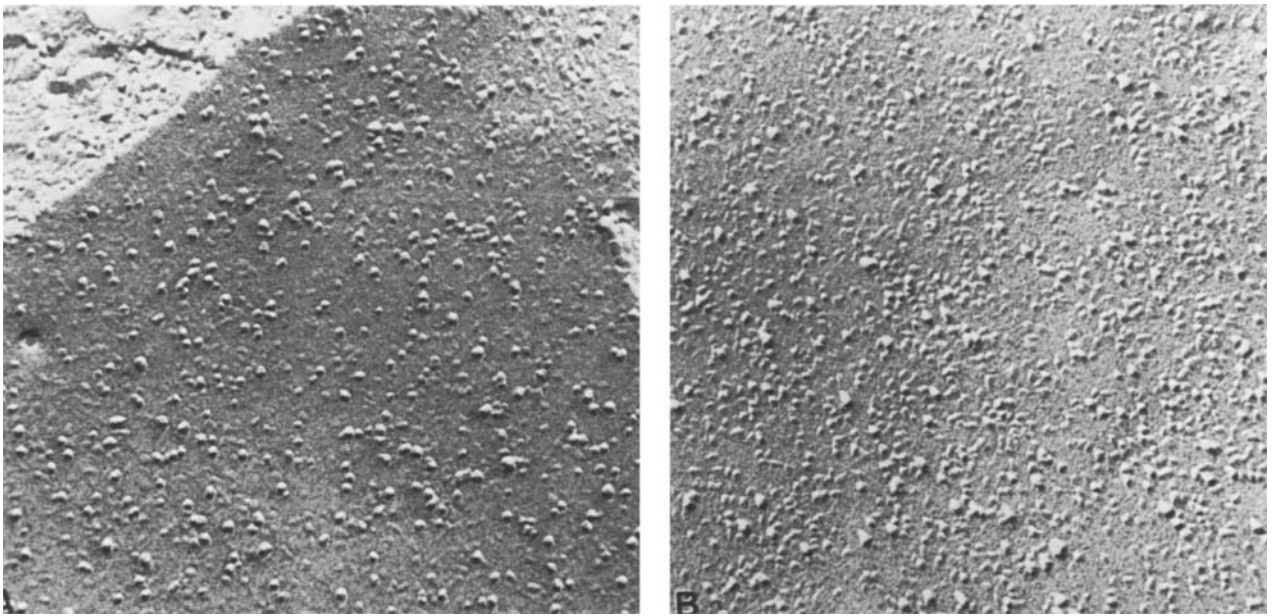

FIGURE 1. Freeze-fracture replicas of neuronal membrane protoplasmic face demonstrating neuronal intramembranous protein particle density for male **(A)** and female **(B)** arcuate nucleus. Note the higher particle density in female as compared to male neuronal membranes. (Reproduced from Dev. Brain Res. **19:147**, with permission.)

membranous proteins is related to the development of sexually dimorphic neuronal circuits.

During these and other studies, an increased number of endo-exocytotic images or pits was seen in the post synaptic membranes of male rats. Further, when we studied the number of pits in TP-treated females, we found increases in the number of endo-exocytotic images.[9] From these data, we hypothesized that endo-exocytotic pits play are responsible for the decrease in intramembranous proteins found in males and TP-treated females.

We have also conducted freeze-fracture studies of arcuate nucleus neuronal membranes from estrogen-treated adult female rats.[10] The following sequence of events was observed upon estrogen treatment. There was a rapid lateral movement of intramembranous protein particles which resulted in clustering of the particles. This was followed by the appearance of endo-exocytotic images often with dense aggregation of particles in the pits. This was followed by a decrease in the average number of particles in post-synaptic membranes. We proposed that the clustering of intramembranous protein particles followed by preferential endocytosis of the high density membrane resulted in a decrease in the number of small intramembranous protein particles. To test this hypothesis, slices of unfixed arcuate nucleus were incubated in artificial cerebral spinal fluid (ACSF) and estradiol added to the bath.[11] The slices were fixed at varying intervals following the addition of estradiol. It was found that there was an increase in the number of pits in the post-synaptic membranes which *plateued* at 1 minute.

To test whether endocytosis was occurring, the experiments were repeated with ACSF-containing horseradish perioxidase (HRP). HRP itself has no effect on endo-exocytotic rates but is internalized along with extracellular fluid. We found that the increase of intracellular HRP paralleled the rapid rise in the number of pits observed with estrogen treatment. This indicated that estrogen caused a sequence of events including endocytosis, which likely leads to the depletion of small intramembranous protein particles.

The effect of estrogen on the number of endo-exocytotic images in freeze-fracture replicas from arcuate nucleus post synaptic membranes is stereospecific to 17β estradiol.[9] The estrogen-induced increase in the number of pits is dose-related and is reversed by the receptor antagonist tamoxifen. Tamoxifen has no effect on its own in this model. Dihydrotestosterone (DHT), a ring A-reduced non-aromatizable androgen has no effect, even at high doses. TP has a considerably smaller effect when compared to estradiol, which could be due to its metabolism into estradiol by the incubating hypothalamic slices. It should also be noted that the effect of estradiol on neuronal membranes has been observed in cultured rat cortical neurons[12] and in neuronal membranes from primate, hypothalamus.[13]

In a preliminary attempt to determine the identity of the protein particles that are affected by estrogen, the technique of fracture-labeling was employed.[14] Tissue was prepared as for freeze-fracture; however, the slices were initially incubated with sucrose to enlarge the intercellular space and concanavelin A (a lectin that binds to specific oligosaccarides on glycoproteins) was added. Unbound concanavalin A was washed out and HRP-coated colloidal

gold was added to label the bound concanavalin A with an electron-dense marker. It was found that the concanavalin A bound over the glycocalyceal face of the membrane corresponded very closely to the small intramembranous protein particles. Furthermore, TP/estrogen treatment of 5-day-old female rats resulted on day 10 in a picture of concanavalin A binding identical to that seen in control male rats. Thus, the fracture-labeling technique has identified glycoproteins which are likely to be the extracellular domains of the small intramembranous particles that are lost during the action of estrogen.

ESTROGEN-INDUCED SYNAPTIC PLASTICITY DURING THE OVARIAN CYCLE

The above described effects of estrogen on arcuate nucleus synaptology during development result in modulation of synaptic circuitry. Therefore, we questioned whether changes in the circulating levels of estrogen which occur during the normal estrous cycle could affect the number of synapses in the GNRH-delivery system, particularly in the arcuate nucleus. Freeze-fracture for evaluation of post-synaptic membranes and transmission electron microscopy for axosomatic synapse counting were performed[8] on the arcuate nucleus of cycling female rats at each day of the estrous cycle. The preovulatory rise in circulating estradiol was mirrored by a fall in intramembranous protein particles which reached a nadir by the morning of proestrus, coincident with the plasma estrogen peak. The number of axosomatic synapses at that time was

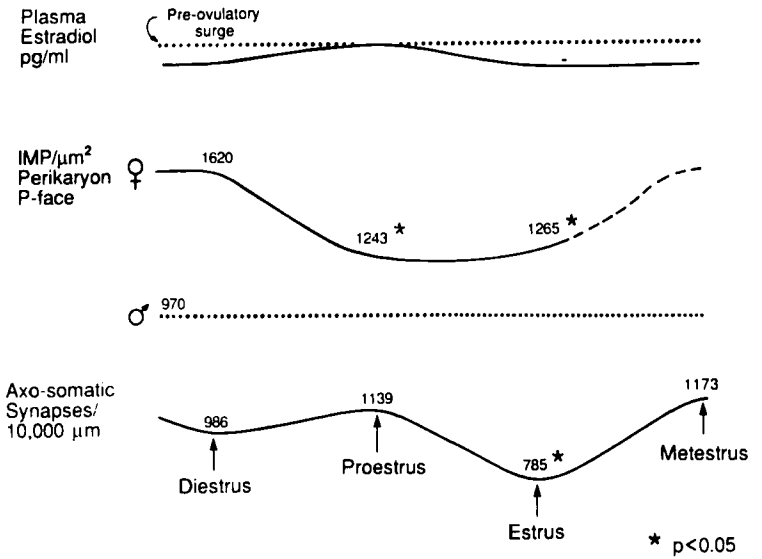

FIGURE 2. Phased synaptic remodeling in the rat arcuate nucleus. For explanation see text.

unchanged from levels seen during diestrus-metestrus, but by the afternoon of proestrus 32% of the axosomatic synapses in the arcuate nucleus were lost. The number of synapses remained low until the synapses recovered by the morning of metestrus (FIG. 2).

We investigated whether these physiologic changes in arcuate nucleus synaptology actually are induced by estrogen. We blocked the estrogen-induced gonadotrophin surge using an anti-estrogen antibody. This also blocked the loss of axosomatic synapses in the arcuate nucleus. The effect was reversed by diethylstilbestrol, a non-competing, nonsteroidal estrogen (unpublished). We concluded that the physiologic changes in arcuate nucleus axosomatic synapses are due to the preovulatory estrogen surge from the ovary.

ESTROGEN AND AGING OF THE FEMALE REPRODUCTIVE SYSTEM

The normal aging female rat begins to show failure of the estrogen-induced gonadotrophin surge around 11 months of age. This failure of ovulation at proestrus results in a second day of peak estrogen levels and five- rather than four-day cycles. By 15 months most female rats have lost the capacity for an estrogen-induced gonadotrophin surge and are thereafter acyclic.[15] The loss of cyclicity in the aging female rat is termed "senescent constant estrus," reflecting the characteristic cornified cells shed from the vaginal mucosa in these animals which have many unruptured ovarian follicles. In cycling adult female rats, the preovulatory estrogen surge is accompanied by synaptic remodeling. We questioned whether repeated estrogen-induced remodeling could eventuate in the loss of the female phenotype in neuronal intramembranous protein particle content and the synaptic networks that support the estrogen-induced gonadotrophin surge, *i.e.* the hypothalamic component of reproductive aging.

We found that premature failure of the reproductive system can occur in rats exposed to pharmacologic doses of estrogen. One injection of estradiol valerate (EV), 1.0 mg/kg body weight, leads to three times the usual preovulatory estradiol levels for a period of 2–3 weeks. Within 6 weeks, administration of this pharmacologic dose to adult female rats results in constant estrus and loss of positive feedback.[16] Freeze-fracture study of arcuate nucleus neuronal membranes from these animals[17] revealed a decline in small intramembranous protein particles to levels seen in normal males. The decline in intramembranous protein particles was accompanied by a loss of axosomatic synapses which was observed by three weeks after injection. In observations made at 3, 8, 16 and 32 weeks following injection, the lowest number of synapses was seen at eight weeks and the density of arcuate nucleus synapses recovered to pretreatment levels by 32 weeks. By 4–6 weeks the rats had entered constant estrus and therefore we may assume that the synaptology of the GNRH-delivery system no longer supported positive feedback.

Despite the fact that these are pharmacologic studies, it is interesting to note that the axodendritic synapses followed the same changes as the axoso-

matic synapses; however, axon-spine synapses of the arcuate nucleus did not change. Moreover, despite the dramatic effect on intramembranous proteins and the very high levels of estrogen involved, the percentage of axosomatic synapses which were lost during this 32 week study was no different from the number that are lost every 4 days during the normal rat estrous cycle (approximately 30–50%). This led us to hypothesize that the action of estrogen is on specific synapses rather than simply a dose-related effect on the total number of arcuate nucleus synapses. To test this hypothesis we have used immunocytochemistry for identification of specific neurotransmitter networks affected by estrogen (for review see ref. 16). These techniques have been developed in our laboratory and others over the past two decades.

GnRH-containing neurons do not have estrogen receptors and therefore appear to be only indirectly affected by estrogen. Another population of estrogen-sensitive cells may therefore serve as intermediaries between estrogen input and the activity of GnRH neurons. Combined immunocytochemical and tracing experiments have revealed that γ-amino-butyric-acid (GABA) and catecholamine (CA)-containing neurons in the arcuate nucleus form connections with pro-opiomelanocortin-β-endorphin (POMC-B-END) containing neurons.[18,19] These β-endorphin cells project onto GnRH-immunoreactive neurons in the preoptic area. We therefore studied the effect of estrogen on the GABA and CA neurotransmitter systems (Lewis and Naftolin unpublished study). Our findings were that there was a fall of axo-somatic synapses at 8 weeks, which included a loss of GABA and CA connections in both estradiol-treated, intact, and ovariectomized females. These findings are partly consistent with the recent reports by Parducz *et al.*[20] who showed that administration of unconjugated estradiol resulted in a rapid loss of GABA synapses in the arcuate nucleus. While the number of GABA synapses returned to control levels by 32 weeks post-estradiol valerate (EV) treatment in intact animals the number of GABAergic presynaptic boutons increased 31% above control values. CAergic synapses increased 70% of their pretreatment values in intact females and to control levels in ovariectomized females. Thus the effect of EV is on both the GABAergic and catecholaminergic synapses in the arcuate nucleus.

Because of the persistently reduced numbers of neuronal intramembranous protein particles in the EV-treated constant estrus rats, we conducted freeze-fracture studies of groups of young and old, female and male rats.[19] We found that the arcuate nucleus neuronal intramembranous protein particle content of control male rats did not change with aging. However, senescent constant estrus and diestrus rats of 15 and 18 months of age respectively, had neuronal membranes with a particle content indistinguishable from males. This finding supports our hypothesis that there may be a specific neuronal membrane organization that determines the performance of arcuate nucleus GNRH delivery system (FIG. 3). We have tested this hypothesis at selected points in development and aging. Our findings support the idea that during the recurrent rises of estradiol which trigger the preovulatory gonadotrophin surge there is a subtle cumulative reorganization of the synaptology of the arcuate nucleus which eventuates in the loss of the estrogen-induced gonadotrophin

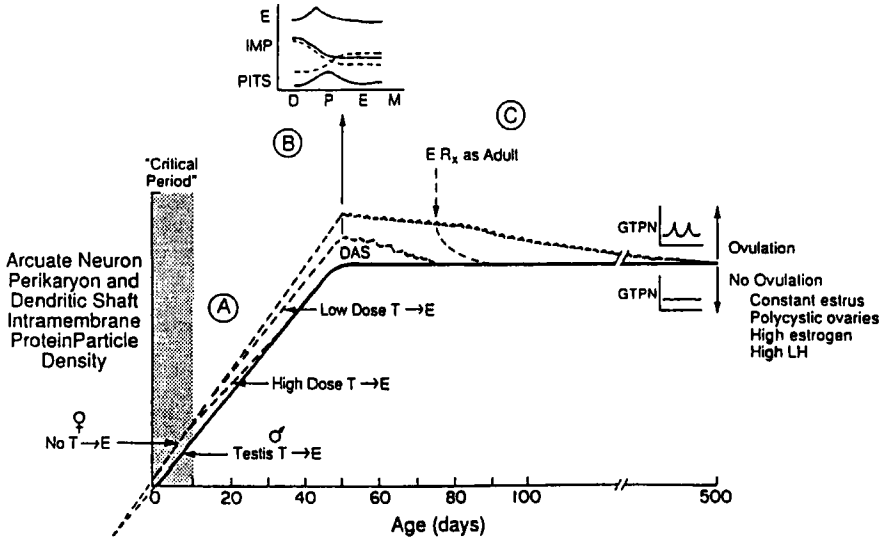

FIGURE 3. The Critical Neuronal Membrane Organization Hypothesis: A general scheme depicting the development of arcuate neuron intramembranous particle density throughout the life of the rat. The effects of pharmacologically administered, as well as endogenously produced, sex steroids on arcuate neuron intramembranous particle density are summarized by the solid and dashed lines. In the absence of locally produced central nervous system estrogen, the female develops cyclic gonadotropin release *(dashed line)*. Androgen or estrogen administered to the immature female, or estrogen administered to the adult female alters arcuate neuron intramembrane protein particle density and the mode of gonadotropin release so that both resemble the male. The aging female's arcuate nucleus neuronal intramembrane particle density as well as her pattern of gonadotropin release come to resemble that of the male: senescent constant estrus. Thus, the solid line demarcates the hypothetical limit of intramembranous protein particle density that is necessary to support a cyclic LHRH-delivery system. (Reproduced from Biol. Repro. **42:24**, 1990, with permission.)

surge.[20] This formulation needs further direct observation on arcuate nuclei of aged rats for confirmation. But in any case, we have enlarged our view of the effects of estrogen to extend past development into adult reproductive life and aging.

SUMMARY

Estrogen modulates the synaptology of the hypothalamic arcuate nucleus during sexual differentiation of the rat brain in both males and females. In males, testosterone of gonadal origin is converted to estrogen in the brain by an enzyme, aromatase, which is also present in females. The exposure of

the male's hypothalamus to relatively high levels of estrogen (following a perinatal testosterone surge) leads to the development of a pattern of synaptogenesis which does not support an estrogen-induced gonadotrophin surge in the adult. In female rats, hypothalamic development occurs with permissively low levels of estrogen, enabling a midcycle estrogen-induced gonadotrophin surge and ovulation in adulthood.

During adult reproductive life in female rats, circulating estrogen modulates the synaptology of the arcuate nucleus. The most physiological example of this is the 30–50% loss of axosomatic synapses following the preovulatory estrogen surge on diestrus-proestrus.

Studies on post-synaptic membranes of the arcuate nucleus reveal sex differences in membrane organization and protein content which are estrogen-dependent. Estrogen apparently stimulates endocytosis of areas of post-synaptic membrane that are dense with small intramembranous protein particles, resulting in a reduction in the number of small intramembranous particles. This also appears to be the physiologic mechanism of neuronal changes in females during the estrus cycle.

Repeated exposure to preovulatory levels of estrogen may lead to an age-related decline in reproductive capacity in female rats. Aging females lose the estrogen-induced gonadotrophin surge responsible for ovulation. This loss of function may result from a cumulative estrogen effect during the repeated ovarian cycles which results in a reorganization of the synaptology on which regulates the estrogen-induced gonadotrophin surge. The membrane organization of the senescent constant estrus aged female appears indistinguishable from the males.[1]

The hypothalamic circuits modulated by estrogen have yet to be delineated. However, recent research has shown that GABA, the monoamines, and several neuropeptides are participants in the estrogen-sensitive network which regulates GNRH secretion. In this regard, present work shows estrogen-induced changes in GABA and dopamine synapses in the arcuate nucleus.

REFERENCES

1. ARAI, Y. & A. MATSUMOTO. 1978. Synapse formation of the hypothalamic arcuate nucleus during post-natal development in the female rat and its modification by neonatal estrogen treatment. Psychoneuroendocrinology **3:** 31–45.
2. GORSKI, R. A. 1984. Sexual differentiation of brain structure in rodents. *In* Sexual Differentiation: Basic and Clinical Aspects. M. Serio, M. Motto, M. Zannsisi, L. Martini, Eds.: 56–77. New York: Raven Press.
3. NAFTOLIN, F., K. RYAN, I. DAVIES, V. REDDY, F. FLORES, Z. PETRO, M. KUHN, R. WHITE, Y. TAKAOKA & L. WOLIN. 1975. The formation of estrogens by central nervous system tissue. Rec. Prog. Horm. Res. **31:** 295–319.
4. PEREZ, J., S. LUQUIN, F. NAFTOLIN & L. M. GARCIA-SEGURA. 1993. The role of estradiol and progesterone in phased synaptic remodeling of the rat arcuate nucleus. Brain Res. **608:**38–44.

5. BROWN-GRANT, K., A. MUNCK, F. NAFTOLIN & M. R. SHERWOOD. 1971. The effects of the administration of testosterone propionate alone or with phenobarbitone and of testosterone metabolites of neonatal female rats. Horm. Behav. **2:** 173–182.
6. MATSUMOTO, A. & Y. ARAI. 1980. Sexual dimorphism in "wiring pattern" in the hypothalamic arcuate nucleus and its modification by neonatal hormone environment. Brain Res. **190:** 238–242.
7. GARCIA-SEGURA, L. M., D. BAETENS & F. NAFTOLIN. 1985. Sex differences and maturational changes in arcuate nucleus neuronal plasma membrane organization. Dev. Brain Res. **19:** 146–149.
8. GARCIA-SEGURA, L. M., P. HERNANDEZ, G. OLMOS, P. A. TRANQUE & F. NAFTOLIN. 1988. Neuronal membrane remodelling during the oestrus cycle: A freeze-fracture study in the arcuate nucleus of the rat hypothalamus. J. Neurocytol. **17:** 377–383.
9. GARCIA-SEGURA, L. M., J. PEREZ, P. A. TRANQUE, G. OLMOS & F. NAFTOLIN. 1989. Sexual differentiation of the neuronal plasma membrane: Neonatal levels of sex steroids modulate the number of exo-endocytotic images in the developing rat arcuate neurons. Neurosci. Lett. **91:** 19–23.
10. OLMOS, G., P. AGUILERA, P. TRANQUE, F. NAFTOLIN & L. M. GARCIA-SEGURA. 1987. Estrogen-induced synaptic remodelling in adult rat brain is accompanied by the reorganization of neuronal membranes. Brain Res. **425:** 57–64.
11. GARCIA-SEGURA, L. M., G. OLMOS, P. TRANQUE & F. NAFTOLIN. 1987. Rapid effects of gonadal steroids upon hypothalamic neuronal membrane ultrastructure. J. Ster. Biochem. **27:** 615–623.
12. GARCIA-SEGURA, L. M., G. OLMOS, R. J. ROBBINS, P. HERNANDEZ, J. H. MEYER & F. NAFTOLIN. 1989. Estradiol induces rapid remodelling of plasma membranes in developing rat cerebrocortical neurons in culture. Brain Res. **498:** 339–343.
13. NAFTOLIN, F., J. PEREZ, C. S. LERANTH, D. E. REDMOND & L. M. GARCIA-SEGURA. 1990. African green monkeys have sexually dimorphic and estrogen-sensitive hypothalamic neuronal membranes. Br. Res. Bull. **25:** 575–579.
14. GARCIA-SEGURA, L. M., J. PEREZ, P. A. TRANQUE, G. OLMOS & F. NAFTOLIN. 1989. Sex differences in plasma membrane concanavalin A binding in the rat arcuate neurons. Br. Res. Bull. **22:** 651–655.
15. WISE, P. M., N. G. WEILAND, K. SCARBOROUGH, G. H. LARSON & J. M. LLOYD. 1990. Effects of aging on reproductive neuroendocrine function. *In* Ovarian Secretions and Cardiovascular and Neurological Function. F. Naftolin, Ed. New York: Raven Press.
16. BRAWER, J. R., H. SCHIPPER & F. NAFTOLIN. 1980. Ovary-dependent degeneration in the hypothalamic arcuate nucleus. Endocrinology **107:** 274–279.
17. LERANTH, C., N. J. MACLUSKY & F. NAFTOLIN. 1986. Interconnections between neurotransmitter- and neuropeptide-containing neurons involved in gonadotropin release in the rat. *In* Neural and Endocrine Peptides and Receptors. M. Moody, Ed.: 177–193. New York: Plenum Press.
18. HORVATH, T., F. NAFTOLIN & C. LERANTH. 1993. Luteinizing hormone releasing hormone and gamma-amino-butyric acid neurons in the medial preoptic area are synpatic targets of dopamine axons originating in anterior periventricular areas. J. Neuroendocrinol. **5:** 71–79.

19. HORVATH, T., F. NAFTOLIN & C. LERANTH. 1992. GABAergic and catecholaminergic innervation of the mediobasal hypothalamic β-endorphin cells projecting to the medial preoptic area. Neuroscience **51:** 391–399.
20. PARDUCZ, A., J. PEREZ & L. M. GARCIA-SEGURA. 1993. Estradiol induces plasticity of GABAergic synapses in the hypothalamus. Neuroscience **53:** 395–401.