NEUROKININ B AND THE HYPOTHALAMIC REGULATION OF REPRODUCTION

by

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DEDICATION

Aquí dejo huella de todo lo que no hice

Un lustro de caminos explorados

De pertenecer a medias,

Espía escurridizo

Que malgasta el tiempo de su invisibilidad

Escucho voces rojas

Almas que quedaron en la línea

De verguenza y ausencia

Almas cazadas por los acoplados

Entrada a contratiempo

Corazónes de barro, ojos pequeños

De un espíritu incansable

Cambiaron esta vida, la llenaron de vivencias,

Pasos de duende en tierras de gigantes

Con mano fuerte sostenemos el azadón

Sangre, sol y canción

Todo revienta en las heridas,

Las pocas nubes de esta tierra

Atardeceres malva, solitud azulada

Ciencia y conciencia

Azar y metáfora.

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ABSTRACT

The morphology and gene expression of neurokinin B (NKB) neurons is altered in the human infundibular (arcuate) nucleus in association with the ovarian failure of menopause. Also, gonadotropin releasing-hormone (GnRH) mRNA is elevated and proopiomelanocortin (POMC) mRNA decreased. To determine if loss of ovarian steroids could produce comparable changes in gene expression in primates, we measured the effects of ovariectomy on NKB and GnRH in young cynomolgus monkeys. We also measured POMC gene expression, serum leptin and body weight to examine the consequences of ovariectomy on energy balance. Neurokinin B neurons in the infundibular nucleus of ovariectomized monkeys were larger, more numerous and displayed increased levels of NKB mRNA than the intact controls. Ovariectomy increased the number of neurons expressing GnRH gene transcripts. In contrast, the energy balance parameters were unchanged by ovariectomy. This study provides strong support for the hypothesis that ovarian failure contributes to the mophological changes and increased NKB and GnRH gene expression observed in postmenopausal women.

We hypothesized that hypothalamic NKB neurons participate in the hypothalamic circuitry regulating LH. We determined if intracerebral infusion of a NK_3 receptor agonist alters serum LH in the ovariectomized estrogen-treated rat. A significant inhibition of serum LH was observed after senktide injection, accompanied by changes in Fos expression in medial preoptic area, arcuate, paraventricular and supraoptic nuclei.

This study provides evidence that stimulation of the NK_3 receptor may inhibit LH secretion via activation of hypothalamic neurons.

To futher investigate the role of NKB in gonadotropin regulation, we infused an antisense oligodeoxynucleotide targeted to the NKB gene in gonadectomized rats. In support of our hypothesis, the downregulation of NKB decreased serum LH by 25%. To analize the participation of the NKB receptor, NK_3, we targeted an antisense to the receptor. Rats injected with the NK_3 antisense exhibited no change in serum LH. Furthermore, injection of SB-222200, a NK_3 antagonist, did not modify serum LH. These data suggest that NKB may regulate gonadotropin secretion through more than one receptor. Taken together, these studies provide some of the first detailed information on the relationship between NKB neurons, and the reproductive axis.

CHAPTER ONE

INTRODUCTION

Mammalian reproduction is a dynamic and finely orchestrated process that can be observed throughout the life of the organism. The process includes the formation of a sexually dimorphic brain during development, differentiation of the gonads and the external sex organs (puberty), the formation of the mature gametes, the union of the female and male gametes, the embryo survival and growth in the uterus, and finally, the senescence of the reproductive phase. In addition to the physiological events, reproduction includes the complex behaviors that are necessary for copulation and parenthood (Card 1999).

The principal components of the reproductive axis are the GnRH cells in the hypothalamus, the gonadotrophs in the pituitary gland and the gonads. This axis influences and is influenced by several other systems as energy the balance system, limbic system, and sleep (Steiger 2003,Williams *et al* 2001,Young and Korszun 2002). The integrity and coordination of the reproductive axis are necessary for proper reproductive function. The reproductive system is the focus of extensive research in diverse areas of investigation including physiological and behavioral studies.

The hypothalamus, pituitary gland and gonads

The hypothalamus is a collection of bilateral and symmetrical nuclei at the base of the brain, forming the walls and the floor of the third ventricle. The hypothalamus, with complex projections and inputs, performs different integrative processes that are essential for survival of the organism and reproduction of the species (Card 1999). In the coronal plane, the hypothalamus is divided into preoptic, anterior, tuberal and mammillary regions (posterior). Sagittally, is divided into medial and lateral regions. Table I lists the major nuclei of the subdivisions of the hypothalamus.

The hypothalamus projects to essentially every major subdivision of the central nervous system (CNS). Transfer of information is primarily via synapses; however, the hypothalamus also influences, via the pituitary gland, peripheral organs such as adrenals, thyroid, mammary glands, uterus, kidneys, skeletal muscle, bone and gonads. In several nuclei of the hypothalamus there are specialized neurons that function as neurosecretory cells. These cells have the morphology of conventional neurons, and yet, are capable of releasing neurohormones in response to neuronal impulses. These neurohormones can be released not only as conventional neurotransmitters (into the synaptic cleft) but also into blood vessels in the hypothalamus. The axons of neurosecretory cells converge in the median eminence, the lower part of the infundibulum. The neurohormones can acces the portal vasculature (Page 1988), were they travel to target cells in the pituitary gland.

Some examples of neurohormones include the corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) from the paraventricular nucleus, growth hormone-releasing hormone (GRH) from the arcuate nucleus and gonadotropin hormone-releasing hormone (GnRH) from preoptic area and medial basal hypothalamus. The only direct neural connection between the hypothalamus and the pituitary gland is the tuberohypophysial tract. Magnocellular neurons in the supraoptic and paraventricular nuclei send axons that terminate in the posterior lobe of the pituitary gland. These neurons secrete vasopressin, which is involved in water homeostasis and oxytocin, which causes milk letdown in lactating females.

The pituitary gland (also called the hypophysis) lies in the sella turcica of the sphenoid bone, beneath the hypothalamus and the optic chiasm. The pituitary gland is formed from tissues having different embryological origins. The anterior pituitary (also called the adenohypophysis) develops from an embryological structure called Rathke's pouch. This structure pinches off from the roof of the mouth and migrates to what will become the anterior pituitary. The posterior pituitary (also called the neurohypophysis) is an outgrowth of the base of the brain. The cellular types that exist in the anterior pituitary are somatotrophs, lactotrophs, corticotrophs, thyrotrophs and gonadotrophs. The hormones secreted by the pituitary cells are growth hormone (GH), prolactin (PRL), adrenocorticotropin hormone (ACTH), thyroid-stimulating hormone (TSH), and the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). With the exception of LH and FSH, which are secreted from a common cell type, each of the

anterior pituitary hormones is contained in a particular cell type. In contrast, the neurohypophysis contains only neuronal axonal endings, glial-like cells called pituicytes and blood vessels. Magnocellular neurosecretory cells from the hypothalamus innervate the posterior pituitary and secrete oxytocin and vasopressin adjacent to fenestrated capillaries in this structure. The hormones then penetrate the blood vessels to follow the general circulation and interact with their target organs. The precise secretion of the pituitary hormones is a result of the sum of positive and negative signals from the hypothalamus and feedback from systemic signals (Card 1999).

The gonads are sexually dimorphic glands with two main functions: the production of hormones and the production of gametes (sperm and eggs). The steroid hormones that are produced in the gonads have a variety of functions which include the development of the brain, gamete formation and sexual behavior (Erickson 2000). Hormones from the anterior pituitary regulate gonadal hormones and reciprocally, steroids modulate hypothalamic and pituitary function.

The male gonads, the testes, contain seminiferous tubules that convolute in an ovoid shape. Spermatozoa (sperm cells) are produced and develop within these tubules. Two linings form the seminiferous tubules, the outer wall is made up of connective tissue and contractile fibroblasts, and the inner sheet is composed of the Sertoli cells or support cells and the spermatogenic cells. The surrounding tissue of the seminiferous tubes contains interstitial Leydig cells, which are endocrine cells that synthesize the male sex

hormone testosterone. Sertoli cells, in response to FSH from the anterior pituitary, synthesize and secrete androgen binding protein. Androgen binding protein binds testosterone and this complex is released into the lumen of the seminiferous tubule. The elevated level of testosterone is thought to enhance spermatogenesis.

The female gonads, the ovaries, have three functional subunits: follicles, which each contain a developing egg or oocyte; corpora lutea, which are the remains of the follicle after the egg is released and the stroma, which is supporting tissue. The follicles consist of an immature egg, or oocyte, surrounded by a layer of epithelial cells called the granulosa cells. Granulosa cells secrete two peptide hormones called activin and inhibin, which are important in the regulation of hormone secretion from the hypothalamus and pituitary gland. There are two other cell types surrounding the follicle, the theca interna and theca externa. The theca interna cells secrete androgens, which are converted to estrogen by the aromatase activity in granulosa cells. Estrogens are the primary sex hormones in females. At birth, the ovary is populated with primordial follicles, a pool of non-growing follicles from which all dominant preovulatory follicles are selected (Erickson 2000).

The menstrual cycle

The reproductive capacity of women is determined by the cyclicity of ovarian function, which begins at puberty with the first cycle (or menarche) and ends at the menopause with the permanent cessation of menses. The age of menarche is genetically

determined and may be correlated with acquiring a critical body weight. The adult human ovary is a mass of follicles, luteal tissue, blood vessels, nerves and connective tissue. The continuous and progressive changes in the follicles and corpora lutea is what gives rise to the cyclical changes in the menstrual cycle. Normal ovarian function requires the orchestrated activity of the hypothalamus, the pituitary, the ovary and the endometrium (Knobil and Hotchkiss 1988). This axis requires a fine tuned signaling: gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile manner from the preoptic and arcuate nucleus of the hypothalamus into the hypophyseal portal system. GnRH is transported to the anterior pituitary gland where it stimulates the expression and secretion of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH). The gonadotropins are secreted into the systemic circulation and target the ovaries where they stimulate follicle growth, ovulation and the secretion of steroidal hormones and other peptides. The ovarian steroids provide both positive and negative feedback signals to the hypothalamus (Silberstein and Merriam 2000) and pituitary, and in turn regulate their function (Figure 1.1).

The menstrual cycle is divided into the following phases: menses, follicular phase, ovulation and luteal phase. The beginning of the menstrual cycle is considered the first day of the menses and the cycle ends the day prior to the next menses. Steroid hormones are low at the start because of the absence of follicles large enough to produce them. This absence of ovarian steroids causes an increasing secretion of the gonadotropin hormone FSH. The recruitment of 6 to 10 follicles begins with elevated

plasma FSH in the early follicular phase. One or two dominant follicles mature covered by two layers of steroidogenic tissue, granulosa cells and theca cells. The remaining recruited follicles undergo spontaneous degeneration or atresia. The maturating follicles secret estradiol and inhibin which exert a negative feedback on pituitary FSH secretion. The negative feedback also inhibits LH secretion, which remains low during the follicular phase. During the early follicular phase the concentration of estradiol remains relatively low. The growing follicles, however, secrete estradiol in proportion to their size, thus, estradiol rises slowly at first and then more rapidly as the follicular phase progresses. When the estradiol concentration rises and remains above a critical point for at least 36 hours, the negative feedback process is reverted to positive feedback (Karsch 1987). In this late follicular phase, gonadotropin secretion, mainly LH, reaches a peak known as the preovulatory gonadotropin surge. This LH surge provokes rupture of the follicle and the release of the oocyte (ovulation). Ovulation carries away the oocyte and the cumulus of granulosa cells; the remaining theca and granulosa cells differentiate into an endocrine structure known as the corpus luteum. The corpus luteum is active for about two weeks (luteal phase) and secretes progesterone, some estrogen and the inhibins that inhibit the estrogen–induced gonadotropin surge. During the luteal phase, LH and FSH concentrations remain low. The function of estrogen and progesterone in this phase is to finish preparing the uterus for pregnancy. If implantation of the fertilized ovum does not occur, then the corpus luteum degrades and involutes into a nodule of dense connective tissue termed the corpus albicans. As the corpus luteum regresses, steroid hormone concentration declines. At the end of the cycle, luteolysis occurs and the lining of the

uterus, which developed during the whole cycle, is shed (menses). In the absence of steroid hormones, pituitary gonadotrophs are released from negative feedback control and LH and FSH slowly begin to increase. The increase in FSH stimulates a new group of follicles to develop, consequently the next cycle begins.

Gonadotropin hormone-releasing hormone

The decapeptide gonadotropin hormone-releasing hormone (GnRH) is the molecule in the brain that is, in essence, the conductor of reproduction. The absence of GnRH results in absolute impairment of reproduction. GnRH neurons are one of the few neuronal cell populations that originate outside the brain: they migrate from the olfactory placode into the ventral medial forebrain during fetal development (Schwanzel-Fukuda and Pfaff 1989). Rather than forming a specific nucleus, GnRH neurons are widely scattered throughout of the hypothalamus, including the diagonal band of Broca, the bed nucleus of the stria terminalis, dorsal septum, lateral and medial preoptic area, and mediobasal hypothalamus (Silverman *et al* 1979). In addition to hypothalamus, GnRH neurons are also found in other brain areas (Rance *et al* 1994). In rodents, most GnRH neurons are found in the rostral medial preoptic area and only a few caudally (Merchenthaler *et al* 1984).

At least three subtypes of GnRH neurons have been described in the human based on morphology and gene expression (Rance *et al* 1994). Type I neurons are small with heavy GnRH mRNA expression, oval to fusiform in shape and are located in mediobasal

hypothalamus and medial preoptic area. Type II neurons are small with light GnRH mRNA expression, round to oval in shape and are scattered in the dorsal preoptic area, the septal area, the amygdala and the substantia innominata. Type III neurons are large with intermediate mRNA expression, round to oval and are located in the putamen and the magnocellular basal forebrain complex. From these three types, type I increases to 50% the mRNA expression in postmenopausal women when compared to premenopausal women (Rance and Uswandi 1994). In the monkey, gene expression decreases in type I neurons after hormone replacement therapy (Krajewski *et al* 2003). Although the functional relevance of the three subtypes is not fully understood, in reproductive research the focus has been on the type I cell, because of its location, projections, responsiveness to steroid hormones and similarity among different animal species.

Axons arising from GnRH neurons establish contact with fenestrated capillaries in the median eminence, which form the hypophyseal portal system. Through this vascular route, GnRH travels to the anterior pituitary where it binds to specific receptors on gonadotrophs to stimulate secretion of the gonadotropins, LH and FSH. Luteinizing hormone is secreted in two markedly different patterns: one is a pulsatile secretion and the other is the pre-ovulatory surge. These patterns are controlled by the GnRH pulse generator sytem (Dyer and Robinson 1989,Knobil 1990) and the GnRH surge generator system respectively (Kalra 1993).

Neurotransmitters/Neurochemicals as intermediaries of steroid effect

Steroid hormones regulate GnRH secretion, but they also affect many other neurons that in turn regulate GnRH synthesis and secretion (Figure 1.1). The focus of many studies, therefore, has been the steroid-responsive neurons that directly or indirectly affect GnRH or LH. Neurotransmitter/neuromodulators that have direct effect on GnRH neurons are galanin (Maiter *et al* 1990), norepinephrine (NE) (Terasawa *et al* 1988), neuropeptide Y (NPY) (Woller *et al* 1992), and gamma-aminobutyric acid (GABA) (Leranth *et al* 1985). Galanin is colocalized with GnRH (Merchenthaler *et al* 1990), augments LH responses to GnRH in the presence of steroid hormones and stimulates GnRH release (Lopez and Negro-Vilar 1990). Norepinephrine is secreted from the median eminence in pulses. The pulses occur in synchrony with GnRH pulses (Terasawa *et al* 1988), and disruption of the NE system suppresses pulsatile LH secretion.

The medial preoptic area of the hypothalamus is innervated by NPY neurons originating from two different locations, one in the arcuate nucleus and one in the brainstem. Neurons in the arcuate nucleus that express NPY are modulated by steroids (Sahu *et al* 1992) and some coexpress estrogen receptors (Kalra 1993). In rodents, there is morphological evidence that NPY neurons regulate GnRH (Tsuruo *et al* 1990). In the rhesus monkey, NPY stimulates GnRH release from median eminence and this release is potentiated by estrogen (Woller and Terasawa 1992). This effect is also seen in the rat (Crowley *et al* 1987). Intraventricular injection of NPY in ovariectomized rats results in

an inhibitory effect on LH release. However, when administered to the ovariectomized steroid-primed rat, NPY has a stimulatory effect on LH release (Kalra and Crowley 1984,Sahu *et al* 1987).

The role of GABA in the GnRH network has been substantiated by several studies. GABA neurons synapse on GnRH neurons (Leranth *et al* 1985), and GnRH neurons express $GABA_A$ receptors (Petersen *et al* 1993). The effect of GABA is modulated by steroids, GABA neurons in the preoptic area express estrogen receptor alpha and GABA release is influenced by the presence of steroids (Herbison 1997). GABA neurons are also located in the arcuate nucleus, where they modulate local networks of peptides involved in reproduction such as NPY, opioids and galanin (Horvath *et al* 1992,Lasaga *et al* 1988,Nishihara and Kimura 1987).

The majority of the regulatory peptides in the reproductive axis are synthesized in the hypothalamus and function mainly in the modulation of GnRH. There is also evidence that some of these regulatory peptides have a physiological effect on pituitary gonadotrophs. There are several pathways by which a peptide can alter gonadotropin secretion. Peptides can directly target pituitary cells, synergize the effect of GnRH, or stimulate the secretion of paracrine factors in the pituitary that affect gonadotrophs. Anterior pituitary cells contain receptors for hypothalamic peptides such as NPY, substance P, opioids, oxytocin (Evans 1999). Neuropeptide Y synergizes with GnRH in pituitary cells (Crowley *et al* 1987). Substance P is secreted from the hypothalamus

(Ronnekleiv *et al* 1984) and regulates LH secretion, but also it is secreted from pituitary cells (Kerdelhue *et al* 1985) and may exert a paracrine influence on gonadotrophs.

Proopiomelanocortin

Among several different physiological functions, endogenous opioids participate in gonadotropin secretion and GnRH regulation (Genazzani and Petraglia 1989,Kalra 1993,Rasmussen *et al* 1983,Ropert *et al* 1981). An opioid precursor, proopiomelanocortin (POMC), is synthesized in the hypothalamic infundibular nucleus. One of the cleavage products of POMC, β-endorphin, fluctuates during the menstrual cycle in the nonhuman primate (Ferin *et al* 1984). Also in the monkey, pulsatile LH is inhibited by β-endorphin, and this effect is reversed by estrogen replacement (Wardlaw *et al* 1982). Opioid antagonists increase GnRH secretion into the hypophyseal portal blood of monkeys (Pau *et al* 1996). Furthermore, POMC gene expression is decreased in the infundibular nucleus of postmenopausal women and is inversely proportional to age (Abel and Rance 1997,Abel and Rance 1999). POMC neurons innervate medial preoptic neurons (Simerly *et al* 1986) and synapse on GnRH neurons (Thind and Goldsmith 1988). Opioid receptors are modified by ovarian steroids, their availability is increased during estrous and diestrous compared to proestrous. In addition to the participation of POMC in reproduction, infundibular POMC neurons are a critical component in energy homeostasis and appetite control (Mountjoy and Wong 1997,Schwartz *et al* 1997). The functional basis of the role of POMC in appetite is mostly due to the POMC-derived

peptides and their interaction with melanocortin receptor 3 and 4 (MC3R and MC4R) (Cone *et al* 2001,Huszar *et al* 1997).

Tachykinins

The mammalian tachykinins form a family of small peptides that share a common C-terminal sequence (Phe-X-Gly-Leu-MetNH2) (Maggio 1988). The tachykinin family includes substance P (SP), neurokinin A (NKA), neurokinin B (NKB), and neuropeptide γ (NPγ). Substance P was the first tachykinin described by P. Von Euler in 1931 and the term tachykinin was chosen because of its rapid action (contractile) on smooth muscle. Tachykinins are derived from two genes encoding two precursors, preprotachykinin A (PPTA or PPT1) and preprotachykinin B (PPTB or PPT2). PPTA codes for SP, NPγ and NKA; PPTB codes only for NKB. Alternate splicing of the primary transcript PPTA produces three different mRNAs: α, β, γ. When α-PPTA is expressed, only SP can be synthesized, when β-PPTA is expressed, SP, NPK, and NKA can be synthesized and when the third transcript, γ-PPTA is expressed, SP, NKA and NPγ can be synthesized. The effects of tachykinins are mediated by three different neurokinin (NK) receptors that display pharmacological differences and different binding affinities for the tachykinins. NK1 has higher affinity for SP>NKA>NKB; NK2 for NKA>NKB>SP; NK3 for NKB>>NKA>SP (Helke *et al* 1990). In the central nervous system mainly NK1 and NK3 are present (Tsuchida *et al* 1990).

The broad distribution of tachykinins in the nervous system suggests an important role for tachykinins as neurotransmitters/neuromodulators. Tachykinins are present in brain areas involved in the central control of several peripheral autonomic functions including blood pressure, micturition, respiration and gastrointestinal motility. Tachykinins are also involved in emotion related functions such as anxiety, aggression and pain, as well as higher functions such as learning and memory.

Tachykinins are found in hypothalamic sites implicated in the control of reproduction, and sexual and appetite behavior (Merchenthaler *et al* 1992,Severini *et al* 2002). Substance P/NKA neurons are particularly abundant in the dorsomedial, ventromedial and premammillary nuclei. Lower densities of SP/NKA neurons are shown in the preoptic, dorsolateral, lateral, posterior, suprachiasmatic, retrochiasmatic, septohippocampal and supramamillary nuclei. The anterior pituitary gland contains PPTA but not PPTB mRNA. Neurokinin B neurons are found in moderate amounts in the medial preoptic area, anterior and lateral hypothalamic areas, the arcuate and paraventricular nuclei (Merchenthaler *et al* 1992).

Tachykinins appear to play an important role in reproduction. Neuropeptide K effectively suppresses LH release in a dose-dependent manner in ovariectomized rats at 30 minutes, and prevents the LH surge in ovariectomized estrogen-primed rats (Sahu and Kalra 1992). These data suggest an inhibitory role for NPK on LH secretion in female rats. In contrast, NPK stimulates LH release in intact males (Kalra 1992). The influence

of testosterone on the action of NPK on gonadotropin secretion remains to be determined. Neuropeptide γ only suppresses LH release at high concentrations in ovariectomized rats (Sahu and Kalra 1992). In intact male rats however, intracerebral injection of NPγ elicits a dose-related increase in plasma LH. When administered to gonadectomized male rat, NPγ suppresses LH release (Kalra *et al* 1992). Neurokinin A does not affect the regulation of LH in any hormonal environment in the female rat (Sahu and Kalra 1992), but increases LH plasma concentration when administered to intact males and furthermore, decreases plasma LH when administered to orchidectomized rats (Kalra *et al* 1992). The action of NKA on gonadotropin secretion appears to be dependent on sex and hormonal environment.

The effect of SP on gonadotropin secretion has been widely studied in rodents. In 1979, Vijayan et al. showed that SP has a stimulatory effect on LH release in ovariectomized rats (Vijayan and McCann 1979). The same effect was shown by Arisawa et al in 1990 (Arisawa *et al* 1990). Furthermore, intracerebral injection of a SP antiserum or antagonist in gonadectomized rats significantly suppressed LH (Dees *et al* 1985). In 1991, Tsuruo et al, showed innervation of GnRH neurons by SP fiber terminals (Tsuruo *et al* 1991). These studies revealed a physiological excitatory role of SP in the control of LH release in female rats. In intact male rats, SP was ineffective on LH regulation, but its administration in the gonadectomized male rat suppressed LH release (Kalra *et al* 1992). In addition to regulation of gonadotropin secretion by SP, there is evidence that gonadal steroids regulate the expression of SP and its receptor. Ovarian

steroids regulate SP receptor mRNA in vivo (Brown *et al* 1990) and in vitro in a time and dose-related manner (Villablanca and Hanley 1997). SP gene expression is regulated by steroid hormones in the ventromedial nucleus of the hypothalamus and could modulate lordosis behavior in the rat (Priest *et al* 1995). Moreover, SP content in the rat median eminence varies over the estrous cycle (Parnet *et al* 1990) and cells undergo ultrastructural transformations associated with stages of the estrous cycle (Tsuruo *et al* 1984).

In summary, the effect of tachykinins on gonadotropin secretion differs depending on the sex and/or gonadal steroid environment. This differential effect is not generalized for all the tachykinins. Neuropeptide K regulates LH secretion in both sexes, whereas NPγ does so only at high concentrations in females. The dependence of some peptides on gonadal steroids to regulate gonadotropin secretion is also diverse among tachykinins, NKA does not affect LH secretion in females but has opposing effects in the male when the animal is castrated. In contrast, NPK is inhibitory in the intact and ovariectomized female rat. Gonadal steroids play an important role in tachykinin function, but a general effect can not be described.

Neurokinin B

From the studies relating tachykinins with the reproductive axis, none has demonstrated a role for NKB. Despite this fact, there are several reasons to believe NKB plays an important role in reproduction. In the arcuate nucleus of postmenopausal

women, NKB neurons undergo hypertrophy and increase NKB mRNA expression. These hypertrophied neurons also express estrogen receptor-α, suggesting they are targets for steroid action (Rance *et al* 1990,Rance and Young 1991). Arcuate neurons in rats present increased NKB gene transcripts after ovariectomy, a phenomenon similar to that in postmenopausal women. In addition, NKB expression changes in different estrous cycle phases, and changes with an induced constant estrous cycle (Rance and Bruce 1994). Continuos estrogen decreases NKB expression to five fold less in the arcuate nucleus of the ovariectomized rat (Akesson *et al* 1991). The same effect occurs in male rats with different gonadal steroid environments (Danzer *et al* 1999).

At this point, it is possible to hypothesize that ovarian steroids in both sexes regulate NKB neurons in the arcuate nucleus, and that the hypertrophy and increase gene expression observed in postmenopausal women could be due to ovarian failure. Subsequent experiments have further supported these hypotheses. Estrogen treatment of ovariectomized cynomolgus monkey reduces hypothalamic NKB gene expression (Abel *et al* 1999). In the ovine brain, NKB expressing cells in the arcuate nucleus are responsive to steroids (Pillon *et al* 2003), are sexually dimorphic and project to the rostral preoptic area where GnRH neurons are located (Goubillon *et al* 2000a). Ovarian steroid treatment in the rat uterus decreases NK3 receptors (Crane *et al* 2002,Pinto *et al* 1999). Therefore, there is a possibility that gonadal steroids modulate the NKB receptor, NK3 in the central nervous system. In addition, the hypothalamus has the highest concentration of NKB in central nervous system. Also, NKB immunoreactive nerve terminals are

present in the external zone of the median eminence (Merchenthaler *et al* 1992), a neurosecretory site for pituitary regulation.

Human menopause and reproductive aging

The end of the reproductive phase in the human is menopause, the cessation of menses. Ovarian failure and a decline in estrogen secretion to castrate levels characterize menopause. The process preceding the menopause is complex and long. The probability of becoming pregnant decreases at the mean age of 37, but menstrual cycles are still regular. At about the age of 45, menstrual cycles become irregular and a perimenopausal state becomes apparent. Menopause occurs at the mean age of 51 when almost no follicles are left. The mechanisms of reproductive aging are still a subject of active investigation. There are two different hypotheses to explain the origin of reproductive senescence. The ovarian hypothesis states that the decreasing quantity and quality of the follicle pool dictate the decreasing fertility after the age of 30. The neuro-endocrine hypothesis states that aging of the hypothalamus is the main initiator of a cascade of events leading to menopause. Evidence supporting both hypotheses exists in the literature. A gradual decrease in the ovarian follicular pool is observed even at a young age, which underlies the associated decline in fertility and endocrine changes observed as menopause approaches (Richardson *et al* 1987).

The early perimenopausal state is characterized by a substantial fall in inhibin B and a tendency for FSH to increase. The role of inhibin B is to suppress FSH, therefore it

is suggested that the falling of inhibin B is the important factor in allowing the rise in FSH observed in older, regularly cycling women (Klein *et al* 1996). In a study by Welt et al., two groups of women were compared, one young group of ages 20-34 and a group of perimenopausal women of ages 35-46. The perimenopausal group had lower serum inhibin B across the entire luteal phase, whereas changes in inhibin A where not clear and changes in progesterone were not present (Welt *et al* 1999). Estrogen in the perimenopausal state increased in both the luteal and follicular phases (Santoro *et al* 1996). A remark here is that if perimenopausal ovaries retain the ability to produce adequate patterns of estrogen secretion, then the hormonal changes of the perimenopause are not only due to a deficiency in ovarian function.

A possible sequence of events could be as follows: the decline in the follicular pool in the ovary leads to a fall in inhibin B and a subsequent rise in FSH. At this point, it is still possible to maintain ovulatory function, secretion of estrogen and inhibin A by the dominant follicle. The stimulating influence of FSH could accelerate depletion of the residual follicles in the perimenopausal period (Nelson *et al* 1995). The number of follicle declines to a point where menstrual cycles become irregular, inhibin B decreases even more, leading to a more marked increase in FSH. Around the time of menopause, the number of follicles is almost exhausted and ovulatory function stops, decreasing inhibin A and ovarian steroids to castrate levels (Burger *et al* 1998).

In the early perimenopausal state, the remarkable changes are the decrease of inhibin B and increase in FSH, while LH remains unchanged. In the postmenopausal state, the loss of ovarian steroids removes the negative feedback effect and promotes the increasing secretion of luteinizing hormone from the anterior pituitary gland. The postmenopausal state is then characterized by hypoestrogenism and hypergonadotropism (Ebbiary *et al* 1994).

Estrogen modulates many functions in the human body, and decreased levels of estrogen in menopause affects the quality of life in women. The estrogen receptor (ER) is located in numerous cells throughout the body, making many organs and tissues a substrate for estrogen action. When ovarian steroid depletion occurs, several physiological alterations occur. The uterus decreases in volume and the endometrium becomes atrophic. Atrophic vaginitis as well as vaginal dryness may occur. During the period preceding termination of the reproductive age, women experience episodic sensations of heat known as hot flushes. The experience of a hot flush is most often described by women as sensations of heat, sweating, flushing, chills, clamminess and anxiety. Sweating is most often reported in the face, head, neck, and chest. The menopausal flushes disappear after estrogen replacement. Estrogen withdrawal also plays an important role in the development of bone disease and reduction in total bone calcium (Gallagher *et al* 1987,Grainge *et al* 2001). Normal estrogen action includes direct stimulation of chondrocyte activity through a receptor-mediated effect.

After the onset of menopause, there is a reduction in the cortical thickness and the tensile strength of bones as a consequence of a loss of mineral content (Thomas *et al* 2001).

The hippocampus, a brain structure that is involved in learning and memory, also expresses the estrogen receptor. After estrogen withdrawal, there is a dynamic remodeling of dendritic morphology in the hippocampus (Gould *et al* 1990). In addition to cognition, estrogen may influence mood in women. Women in menopause or surgical menopause have higher depression scores than premenopausal women or menopausal women receiving hormone replacement therapy (HRT). A deficit in the neurotransmitter serotonin has been implicated in depression, and estrogen is involved in the regulation of serotonin (Pecins-Thompson *et al* 1996).

Some of the symptoms associated with menopausal women as hot flushes are easily explained by the lack of ovarian steroids. Young women experience hot flushes after oophorectomy which disappear soon after estrogen replacement (Chakravarti *et al* 1977). To explain other symptoms solely by the absence of estrogen becomes difficult with the aging process also present. One possibility is that the aging process causes some of the symptoms, which are potentiated in the absence of estrogen. The etiology of alterations in energy balance in older women is still on debate. Some studies have found an association between body weight and age (Davies *et al* 2001,Lamberts *et al* 1997,Ley *et al* 1992). Other studies however, have shown no link between body mass index and menopausal status (Lindquist 1982) or hormonal replacement (Haffner *et al* 1997).

Neuropeptide gene expression

The postmenopausal state is accompanied by remarkable changes in the human hypothalamus. The medial basal hypothalamus of postmenopausal women contains more GnRH mRNA than the hypothalamus of premenopausal women (Rance and Uswandi 1996). The increase in GnRH gene expression occurs only in the type I neurons and may reflect a direct effect of steroid hormone depletion. In support of this hypothesis, GnRH gene expression increases in the medial basal hypothalamus of gonadectomized rhesus monkeys (El Majdoubi *et al* 2000). The increase in GnRH content is reversed with steroid hormone replacement. In ovariectomized monkeys, hormone replacement therapy decreases GnRH gene expression in the type I neurons within the medial basal hypothalamus (Krajewski *et al* 2003). Furthermore, using a push-pull perfusion cannula implanted in the median eminence of monkeys, Chongthammakun et al. demonstrated a suppressive effect of estrogen on GnRH secretion (Chongthammakun and Terasawa 1993). Human studies are limited because of the invasive techniques needed to determine GnRH secretion, but GnRH secretion is increased in postmenopausal women and decreased after hormone replacement therapy (Gill *et al* 2002). Although this study used indirect measurements of GnRH, it is a good indication that in older women the increase in GnRH is in part due to the ovarian failure of menopause. The increase in gonadotropin secretion in the postmenopausal state may, in part, result from an increase in GnRH.

Another remarkable change is the neuronal hypertrophy that occurs in the infundibular (arcuate) nucleus of the hypothalamus after menopause. The hypertrophied neurons manifest morphological features of activated neurons such as enlarged nuclei and nucleoli, and increased Nissl substance (rough endoplasmic reticulum) (Sheehan and Kovács 1966). Quantitative studies have shown that the mean profile area of neurons in the infundibular nucleus increased 30% in postmenopausal women (Rance *et al* 1990). Rance et al., using oligonucleotide probes to reveal the neuropeptide/neurotransmitter content of the hypertrophied neurons (Rance and Young, 1991), found that the hypertrophied neurons express neurokinin B (NKB), substance P (SP) and estrogen receptor (ER). The changes in neuronal size are also accompanied by an increase in NKB gene expression. The location of the NKB neurons and the fact that they express estrogen receptor alpha suggested a role for these neurons in the reproductive axis. Animal models have been useful in elucidating this hypothesis. Gonadectomy increases NKB gene expression and the number of cells expressing NKB in the arcuate nucleus of female and males rats (Danzer *et al* 1999,Rance and Bruce 1994). The increase in the NKB gene expression in the rat arcuate nucleus is prevented by steroid replacement (Danzer *et al* 1999). In addition, hormone replacement therapy in the ovariectomized monkey also reduces NKB gene expression in the infundibular nucleus (Abel *et al* 1999).

The number of neurons expressing proopiomelanocortin (POMC) mRNA, the precursor of β-endorphin, decreases in the infundibular nucleus of postmenopausal women (Abel and Rance 1997). The reduced number of neurons is inversely

proportional to age (Abel and Rance 1999). POMC neurons are an inhibitory signal for GnRH gene expression, and the decrease in menopause could contribute to the elevation of GnRH and gonadotropin hypersecretion. Long term hormone replacement therapy (HRT) had no effect on POMC gene expression in ovariectomized cynomolgus monkeys (Abel *et al* 1999). This data suggested that the decrease in POMC gene expression in older women is secondary to factors other than estrogen withdrawal. POMC products are involved in many regulatory functions in the hypothalamus and a change in POMC neuronal function could have a considerable impact on the physiology of postmenopausal women.

In summary, the hypothalamic changes occurring in menopause include: (1) Increased GnRH gene expression in the type I neurons within the medial basal hypothalamus. (2) Hypertrophy of neurons containing NKB, substance P and estrogen receptor within the hypothalamic infundibular nucleus accompanied by increased tachykinin gene expression. (3) Decreased numbers of neurons expressing POMC mRNA within the infundibular nucleus.

In general, the changes in hypothalamic neuropeptide content are not due to cell death since there is no change in the total number of infundibular neurons of postmenopausal women when compared to premenopausal women (Abel and Rance 2000). The etiology of hypothalamic changes that have been observed in postmenopausal women has been difficult to analyze. The reason for this difficulty is the

presence of more than one variable that could affect the hypothalamus: ovarian failure and age. The fact that invasive research is necessary to explore this topic also hinders progress. Therefore, we have directed our attention to the usefulness of animal models.

Animal models of menopause

Nonhuman primate

Research in the physiology of menopause is a difficult task. The solution to conduct this type of research is to find an animal model where invasive techniques can be used in controlled experimental procedures. There is considerable debate whether there is an animal model that can be used to mimic human menopause. The most accepted model is the nonhuman primate since there are many similarities in endocrine and metabolic profiles, cognitive changes, and cardiovascular symptomology during pre and postmenopausal stages. The progression from regular to irregular cycles as reproductive aging advances and the cessation of menses occur in both the nonhuman primates and humans. Also, there is a decline in fertility in both species as cyclicity becomes irregular and aging progresses. Both humans and monkeys have hot flushes in the perimenopausal and postmenopausal stage (Dierschke 1985). Bone density has been associated with ovarian failure of human menopause, as well as with aging. In the monkey, there are bone changes also related to ovarian steroid withdrawal (Jerome *et al* 1997).

The most important differences between nonhuman primates and women include the postmenopausal life span each species undergoes, the seasonal menstrual cycles in the

monkey and some perimenopausal events leading to the cessation of menses. One disadvantage of using the nonhuman primate as a model for menopause is that by the time the monkey is postmenopausal, age is a major factor determining physical strength. As a result, monkeys are very weak and do not tolerate most experimental procedures [for review see (Bellino and Wise 2003)]. Therefore, the young ovariectomized cynomolgus monkey is currently being used as a model to study the effects of hormonal manipulation on the physiology of the reproductive axis. For the aim of this study, we considered the young monkey as a model of menopause based on changes in hypothalamic neuropeptide gene expression after ovarian failure (Abel *et al* 1999,Dong *et al* 1996,Krajewski *et al* 2003). This model has the advantages of removing the aging factor and allowing the analysis of the direct effects of ovarian steroids on the neuroendocrine system. The disadvantage is that this model is not useful in understanding the perimenopause transition time. However, ovariectomy of the nonhuman primate has been proven useful in providing information about specific questions of women's health. For this doctoral dissertation, we propose to use the nonhuman primate model of menopause to determine if the hypothalamic changes already observed in postmenopausal women are reproduced in the monkey as a consequence of ovarian failure. This model gives us the opportunity to separate the events that occur after steroid withdrawal from those due to aging.

Rodent

The need for answers about human reproductive aging has lead to intense research using rodents as an animal model. Many questions have been addressed using the rodent model, although there is considerable debate about the validity of the conclusions because of the significant differences in the reproductive system between humans and rodents. The most critical differences are the absence of menstrual cycles in rodents and the lack of a true menopause. Rodents, however, undergo irregular estrous cycles at middle age. Despite these facts, there are many similarities in both species that make possible to address particular questions.

In humans, one of the earliest events of the perimenopause is the increase in FSH during the periovulatory phase of the menstrual cycle, possibly due to a decrease of inhibin B. Similarly, middle-aged rats exhibit elevated FSH during the afternoon of estrous (DePaolo 1987). During the early perimenopausal stage, estradiol is unchanged or increases slightly, whereas progesterone and LH remain at normal concentrations. In the rat, these hormones behave similarly during the transition to irregular cyclicity (Lu *et al* 1985). The ability of estradiol to induce LH surges is attenuated in both perimenopausal women and middle-aged rats (van Look *et al* 1977,Wise and Camp 1984). In addition, there is similarity in the pulsatile pattern of LH (Fox and Smith 1985). In regularly cycling middle-aged women, the duration of LH pulses increases and the frequency decreases (Matt *et al* 1998). This phenomenon also occurs in middle-aged regularly cycling rats (Scarbrough and Wise 1990). For the concerns of this study, there

are patterns in neuropeptide gene expression and hormonal secretion in the postmenopausal women that are mimicked in the gonadectomized rat. Gonadectomy in male and female rats increases NKB gene expression in the arcuate nucleus of the hypothalamus, mimicking the occurrence in postmenopausal women (Danzer *et al* 1999,Rance and Bruce 1994). The rat model is also suitable for manipulation of NKB gene expression and receptor function with the use of pharmacological tools. Using the rat model, we propose to determine the participation of NKB and its receptor in gonadotropin secretion.

The present studies aim to broaden our understanding of alterations in hypothalamic gene expression and their translation into physiological events. The postmenopausal period is marked by profound changes and symptomology that affect the quality of life. With the appropriate animal models, we aim to provide important new information to deepen our understanding of the menopausal process; information that may be used to prevent or ameliorate menopause-associated health problems.

Figure 1.1. Schematic representation of the hormones and feedback control mechanisms in the reproductive axis. LH, luteinizing hormone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; +, stimulatory signal; -, inhibitory signal.

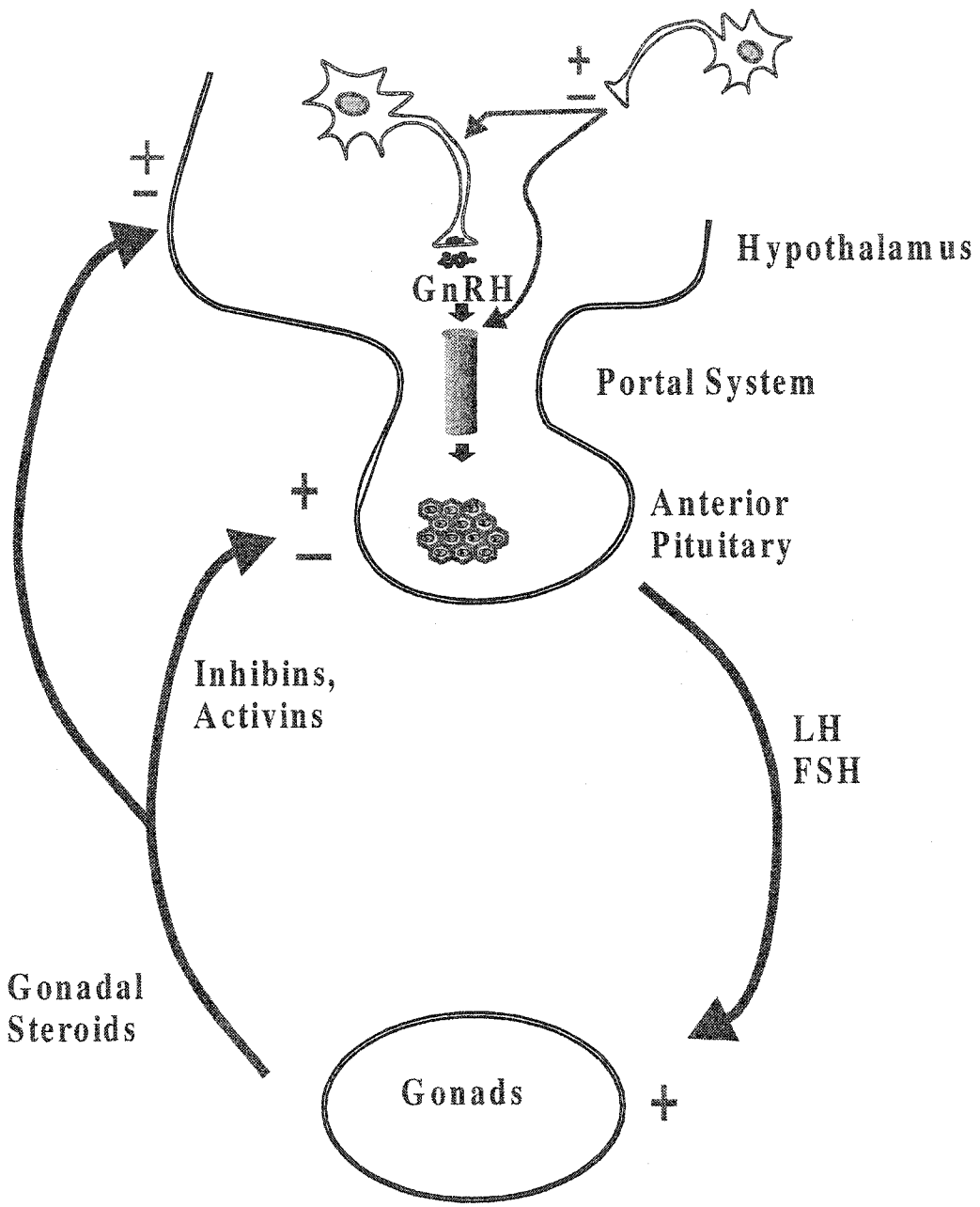
+
−
+
−
Hypothalamus
GnRH
Portal System
+
−
Anterior
Pituitary
Inhibins,
Activins
LH
FSH
Gonadal
Steroids
Gonads
+

Table I. Major nuclei of the hypothalamus.

Hypothalamic subdivision	Nucleus
Preoptic	Vascular organ of the lamina terminalis
	Median preoptic nucleus
	Preoptic periventricular nucleus
	Anteroventral periventricular nucleus
	Lateral preoptic area
Anterior	Suprachiasmatic nucleus
	Anterior periventricular nucleus
	Anterior hypothalamic nucleus
	Paraventricular nucleus
	Subparaventricular zone
	Supraoptic nucleus
	Retrochiasmatic area
	Lateral hypothalamic area
Tuberal	Intermediate periventricular nucleus
	Arcuate nucleus
	Ventromedial nucleus
	Dorsomedial nucleus
	Lateral hypothalamic area
	Ventral premammillary nucleus
Mammillary	Posterior periventricular nucleus
	Posterior hypothalamic nucleus
	Dorsal premammillary nucleus
	Mammillary nuclei
	Tuberomammillary nuclei
	Supramammillary nuclei
	Lateral hypothalamic area

CHAPTER TWO

EFFECTS OF OVARIECTOMY ON THE NEUROENDOCRINE AXES REGULATING REPRODUCTION AND ENERGY BALANCE IN YOUNG CYNOMOLGUS MACAQUES

Abstract

Degeneration of the ovary in middle-aged women results in castrate levels of ovarian steroids and increased gonadotropin secretion from the anterior pituitary gland. Aging in women is also accompanied by significant changes in energy homeostasis. We have observed alterations in hypothalamic morphology and gene expression in older women, including hypertrophy and increased gene expression of neurokinin B (NKB) neurons, elevated levels of gonadotropin releasing-hormone (GnRH) mRNA and decreased numbers of neurons expressing proopiomelanocortin (POMC) mRNA. To determine if loss of ovarian steroids could produce comparable changes in gene expression in young primates, we measured the effects of ovariectomy on NKB, GnRH and POMC gene expression in young cynomolgus monkeys. We also measured serum leptin and body weight to examine the consequences of ovariectomy on energy balance. NKB neurons in the infundibular nucleus of ovariectomized monkeys were larger, more numerous and displayed increased levels of NKB mRNA compared to those of intact controls. Moreover, ovariectomy increased the number of neurons expressing GnRH gene transcripts and elevated serum LH. In contrast, several parameters related to energy

balance, including POMC gene expression, serum leptin and body weights, were unchanged by ovariectomy. Thus, the rise in NKB and GnRH gene expression in older women was simulated by ovariectomy in monkeys but the changes in POMC gene expression and energy balance were not. This study provides strong support for the hypothesis that ovarian failure contributes to the increased NKB and GnRH gene expression observed in postmenopausal women.

Introduction

The menopausal transition marks a fundamental shift in ovarian function from active reproduction to follicle depletion and cessation of menstrual cycles (Greendale and Sowers 1997). The ovarian failure of menopause leads to castrate levels of estrogen and progesterone and a secondary rise in gonadotropin secretion (Chakravarti *et al* 1976). The profound loss of gonadal steroids has repercussions throughout the body (Greendale *et al* 1999). Our understanding of the effects of menopause, however, is confounded by the extended duration of the postmenopausal phase. This long phase of life is accompanied by aging, which leads to alterations in biological rhythms, endocrine control mechanisms and the regulation of energy homeostasis (Lamberts *et al* 1997).

We have observed dramatic changes in neuronal morphology and neuropeptide gene expression within the hypothalamus of older women. In the infundibular (arcuate) nucleus, neurons containing NKB and substance P mRNA hypertrophy and express

increased levels of tachykinin gene transcripts (Rance and Young 1991). The hypertrophied neurons contain estrogen receptor-alpha mRNA, and thus are target tissues for estrogen action (Rance *et al* 1990,Rance and Young, 1991). In addition, GnRH gene expression is modestly increased in a separate subpopulation of neurons within the basal hypothalamus of older women (Rance and Uswandi 1996).

In contrast to the elevated hypothalamic NKB and GnRH gene expression in older women, POMC mRNA-expressing neurons are reduced in number in the infundibular nucleus (Abel and Rance 1999). POMC neurons in the infundibular/arcuate nucleus regulate both reproduction and energy balance in experimental animals (Kalra *et al* 2002). POMC is cleaved to β-endorphin, a peptide that strongly inhibits gonadotropin secretion (Ferin *et al* 1984). Another cleavage product of POMC, α-MSH, reduces food intake through its interactions at multiple hypothalamic sites (Ahima *et al* 2000,Kalra *et al* 2002). Thus, a decline in the activity of hypothalamic POMC neurons could contribute to the increased LH secretion as well as the increase in body weight that has been described in older women (Avis and Crawford 2001).

We have hypothesized that the alterations in hypothalamic gene expression in older women are secondary to the ovarian failure of menopause. Stereological analysis of neurons in the infundibular nucleus revealed no cell loss, suggesting that infundibular cell death was not the underlying cause (Abel and Rance 2000). Other confounding factors due to aging, however, cannot be ruled out. Due to limitations in the collection of

human autopsy specimens, it has not been possible to assess the effects of ovarian failure on hypothalamic gene expression in groups of age-matched women. Therefore, in the present study, we use a primate model to determine the effects of ovariectomy on hypothalamic gene expression. The ovarian endocrine profile (gonadotropin hypersecretion and ovarian steroid withdrawal) is similar among young ovariectomized monkeys, oophorectomized women and postmenopausal women (Chakravarti *et al* 1976,Chakravarti *et al* 1977,Knobil and Hotchkiss 1988,Yen and Tsai 1971). For this reason, the intact and ovariectomized monkey has served as a useful model to study the repercussions of hormone withdrawal and replacement on the cardiovascular and skeletal systems (Jerome *et al* 1997,Wagner *et al* 1992). In the present study, we examine the effects of ovariectomy on hypothalamic NKB, GnRH and POMC gene expression in young cynomolgus monkeys. Serum leptin or body weights were also measured, because of the involvement of POMC neurons in the regulation of energy balance and the significant weight gain associated with aging in women. If the changes in hypothalamic gene expression in older women were secondary to ovarian failure, then ovariectomy might produce comparable changes in young cynomolgus monkeys.

Materials and Methods

Animal protocol. All live animal work was performed at the Comparative Medicine Clinical Research Center of the Wake Forest University School of Medicine. Animal treatments were carried out in compliance with state and federal laws, standards of the Department of Health and Human Services, and the guidelines of the Institutional Animal

Care and Use Committees at both the Wake Forest University and the University of Arizona. Eighteen female cynomolgus monkeys (*Macaca fascicularis*), 8 to 10 years of age, were obtained from Primate Products Inc. (Miami, FL). The animals were fed ad libitum. The Diet Laboratory of the Wake Forest School of Medicine prepared a monkey chow that contained all appropriate calories and nutrients, but was low in naturally occurring plant estrogens (phytoestrogens). The monkeys were housed individually to prevent the stress and ovarian dysfunction that results from the formation of hierarchical social groups in restricted environments (Clarkson *et al* 1995,Kaplan *et al* 1991).

After three months of quarantine, the monkeys were randomly assigned to one of two groups: intact, cycling controls (INTACT, n = 9) or ovariectomized (OVX, n = 9). Ovariectomies were performed under ketamine (15mg/kg i.m.) and butophanol (0.025 mg/kg, i.m.) anesthesia. The menstrual cycles of INTACT animals were monitored by visual inspection and vaginal swabbing. Body weights were measured every two weeks until 10 weeks after group assignment, and then again at 20 and 54 weeks. To allow matching of OVX with INTACT animals in the mid-follicular phase of the menstrual cycle at the time of sacrifice, the animals were sacrificed from 10 to 14 months after group assignment. At sacrifice, the animals were deeply anesthetized with sodium pentobarbital (35 mg/kg, i.m.). Blood samples were obtained, centrifuged, and the serum was collected and frozen for subsequent measurements of LH and leptin. The animals were perfused with cold, 0.01 M phosphate buffer saline (pH 7.4) and the brains were rapidly removed and sliced coronally into 1 cm slabs with the aid of a monkey brain

matrix. Hypothalamic blocks were snap-frozen, packed in dry ice and shipped to the University of Arizona where they were stored at –80°C until sectioning. The blocks were serially sectioned in a cryostat at a thickness of 12 μm, thaw mounted onto gelatin-coated slides and stored at -80°C until hybridization. Every tenth slide was stained with toluidine blue to assist with subsequent matching of the sections to a hypothalamic atlas. One animal from the INTACT group was excluded from the study because of sectioning artifact.

In situ hybridization. For hybridization with the NKB probes, 4 representative sections of the infundibular nucleus from each animal were matched to section 840 of a monkey hypothalamic atlas (Bleier 1984). For hybridization with the GnRH probe, six adjacent sections were selected corresponding to plate 790 of the monkey hypothalamic atlas. This region was chosen to sample the greatest number of type I GnRH neurons (Krajewski *et al* 2003), the neuronal subtype that undergoes changes in gene expression in postmenopausal women (Rance and Uswandi 1996). For hybridization with the POMC probe, we matched sections to two levels of the infundibular nucleus (plates 840 and 884 of the monkey hypothalamic atlas), because the changes in POMC gene expression in postmenopausal women were most pronounced caudally (Abel and Rance 1999).

The *in situ* hybridization procedure used has been described elsewhere (Rance *et al* 1990,Rance *et al* 1994). Oligodeoxynucleotide probes were synthesized and purified

by PAGE gels (Sigma Genosys, The Woodlands, TX). The probes were targeted to bases 331 to 378 of the human preprotachykinin B gene (Page *et al* 2000), bases 1128 to 1175 of the human GnRH gene (Seeburg and Adelman 1984) or bases 7106 to 7153 of the human POMC gene (Takahashi *et al* 1983). Genebank searches showed no significant homology to other mammalian CNS genes for any probe.

For each probe, all slides were processed within the same hybridization procedure. The probes were end-labeled using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN) and [^{35}S] deoxy-ATP (>1000Ci/mmol/L; Perkin Elmer, Boston MA). After prehybridization steps, overnight hybridization and stringent washes, the slides were dipped into NTB-3 nuclear emulsion (diluted 1:1 with water, Eastman Kodak Co., Rochester, NY) for visualization of mRNAs at the single cell level. Test slides were developed at different times to determine the optimum exposure length for each probe. For the GnRH probe, the development was optimized for quantification of the grains associated with type I GnRH neuron, because this subtype of GnRH neuron displayed increased gene expression in postmenopausal women (Rance and Uswandi 1996). Slides were developed after eight days (POMC and GnRH) and fourteen days (NKB) of exposure. Sections were counterstained with toluidine blue. Control slides hybridized with either sense or scrambled probes resulted in the absence of neuronal labeling.

Computer microscope analysis. To analyze NKB and POMC neurons, the perimeter of the infundibular nucleus (unilaterally) was manually digitized using an image-combining computer microscope equipped with a Nikon Optiphot Microscope and Neurolucida Software (Microbrightfield, Colchester VT). The infundibular nucleus was systematically scanned using a 40X Nikon planapochromatic objective during which every labeled neuron (> 5X background) was marked and counted. For image-analysis, twenty NKB or POMC neurons were selected by a systematic random-sampling design using Stereo Investigator software (Microbrightfield, Baltimore, MD). Images of these neurons were digitized for subsequent analysis of autoradiographic grains.

To map the location and count labeled type I GnRH neurons, the six hypothalamic sections (level described above) were unilaterally scanned ventral to the paraventricular nucleus and medial to the lateral edge of the optic chiasm using darkfield illumination and a 10X Nikon planapochromatic objective. All labeled (> 5X background) GnRH neurons within this region were verified using bright-field illumination and a 20X Nikon objective. Images of 20 randomly-selected GnRH neurons were digitized for quantitative analysis of grain numbers. If less than 20 GnRH neurons were present on the six scanned sections, all GnRH neurons were digitized.

Images of neurons were acquired with a Dage-MTI CCD-100 camera (Michigan City, IN) using a 60X Nikon oil-immersion objective. The images were imported into Simple PCI software (Compix Inc., Cranberry Township, PA) for quantitative analysis of autoradiographic grain numbers and manual tracing of cell perimeters. For each slide, background grains were measured, and this value was subtracted from the number of grains for each neuron. Cell perimeters were used to calculate cell profile areas.

Hormone assays. Biologically active LH was quantified at the Endocrine Services Lab at the Oregon National Primate Research Center (Portland, Oregon) using a dispersed mouse Leydig cell bioassay (Ellinwood and Resko 1980). The LH standard was partially purified cynomolgus monkey pituitary preparation (RP-1) made by Dr. Bill Peckham and distributed by the NICHD through the National Pituitary Hormone Distribution Program. The minimum detectable level of LH in this assay was 1-2 ng/ ml with a 6% intra-assay coefficient of variation. Serum leptin was measured using a primate leptin RIA kit (Linco Research Inc. St. Charles, MO) in the laboratory of Dr. Henryk Urbanski at the Oregon National Primate Research Center. An antibody for primate leptin raised in rabbit was used with recombinant human leptin as the standard. The minimum detectable concentration of this assay was 0.7 ng/ml with a 6% intra-assay coefficient of variation. For each hormone, the serum samples were measured in duplicate within the same assay.

Statistical Analysis. The numbers of labeled cells, autoradiographic grains/neuron, and cell profile areas were calculated for each animal and these values were used to compute

the mean and SEM of each experimental group. Group means were compared statistically using t-tests (α = 0.05). Serum LH and leptin were compared in the same manner. Body weight data was analyzed by two-factor ANOVA (treatment x time) with repeated measures followed by Tukey's multiple comparison procedures (α = 0.05).

Results

Hypothalamic neuropeptide gene expression in INTACT vs. OVX cynomolgus monkeys: NKB mRNA-expressing neurons were located predominantly in the infundibular nucleus (at the level examined) in a distribution similar to that described in the human, rat and monkey hypothalamus (Abel *et al* 1999,Chawla *et al* 1997,Rance and Bruce 1994,Rance and Young, 1991). Ovariectomy induced a marked increase in NKB gene expression (Figure 2.1). The number of neurons expressing NKB mRNA in the infundibular nucleus doubled, from 33.8 $\pm$ 3.0 neurons/section in the INTACT group (n = 8) to 67.9 $\pm$ 6.5 neurons/section in the OVX group (n = 9, $p < 0.001$, Figure 2.2). There was also a nearly 4-fold increase in the number of autoradiographic grains/cell (INTACT: 120.5 $\pm$ 10.8 grains/neuron, n = 8 vs OVX: 442.8 $\pm$ 27.0 grains/neuron, n = 9, $p < 0.001$, Figure 2.2). The increase in NKB gene expression after ovariectomy was accompanied by cellular hypertrophy (Figure 2.3). The mean profile area of NKB neurons increased 61%, from 115.5 $\pm$ 4.2 μm^2 in the INTACT animals to 185.7 $\pm$ 7.0 μm^2 in the OVX group, ($p < 0.001$). The morphological features of the enlarged NKB neurons included larger nuclei and nucleoli, and increased Nissl substance (rough endoplasmic reticulum).

Two morphological subtypes of GnRH neurons were seen; small, heavily labeled, oval neurons scattered within the basal hypothalamus, and magnocellular neurons in the nucleus basalis of Meynert. These neurons displayed the morphological features of the type I and type III GnRH neuronal subtypes (respectively) described in studies of the human and monkey CNS (Krajewski *et al* 2003,Rance *et al* 1994,Rance and Uswandi 1996). With the short exposure time used in the present study (8 days), type II GnRH neurons were not visualized in either the INTACT or OVX groups (Krajewski *et al* 2003,Rance *et al* 1994). Ovariectomy increased the detection of type I GnRH neurons in the monkey basal hypothalamus from 2.0 $\pm$ 0.3 neurons/hypothalamic section (unilaterally) to 3.8 $\pm$ 0.6 neurons/hypothalamic section (n = 8 for both groups, P = < 0.05, Figure 2.4). There was no effect of ovariectomy, however, on the number of autoradiographic grains associated with each type I GnRH neuron (INTACT: 467 $\pm$ 59.7 grains/neuron vs OVX: 486.6 $\pm$ 69.52 grains/neuron) or the profile area of these neurons (INTACT: 129.7 $\pm$ 12.8 μm^2 vs OVX: 147.0 $\pm$ 21.9 μm^2).

There was no effect of ovariectomy on the gene expression of POMC neurons in the young cynomolgus monkey. Figure 2.1 (C and D) shows the distribution of neurons expressing POMC mRNA in the infundibular nucleus at a level corresponding to plate 840 of the monkey hypothalamic atlas (Bleier 1984). This distribution was similar to previous reports of POMC neurons in both human and monkey hypothalamus (Abel *et al* 1999,Abel and Rance 1999,Sukhov *et al* 1995). The POMC neurons in the infundibular

nucleus of the OVX animals appeared to be dispersed compared to INTACT animals. Quantitative analysis, however, revealed no significant change in the number of POMC neurons in a unilateral section of the infundibular nucleus (INTACT: 152.6 ± 18.8 neurons/section, n = 8; OVX: 197.1 ± 17.2 neurons/section, n = 9). Furthermore, there were no changes between groups in the number of autoradiographic grains (INTACT: 169.7 ± 22.9 grains/neuron; OVX: 210.2 ± 23.5 grains/neuron) or profile area (INTACT: 166.9 ± 9.4 μm^2; OVX: 189.8 ± 7.1 μm^2) of neurons expressing POMC mRNA. In the posterior level of the infundibular nucleus, the cell number, the number of grains and the cell profile area of POMC neurons were also unaffected by ovariectomy (data not shown).

Serum LH, leptin and body weight: At the time of sacrifice, the mean serum LH (± SEM) of seven of the INTACT monkeys was 17.5 + 4.8 ng/ml, consistent with the follicular phase of the menstrual cycle (Kerdelhué *et al* 2000). A single INTACT monkey had a serum LH value of 305.4 ng/ml, a level suggesting that this animal was sacrificed during the LH surge (Kerdelhué *et al* 2000). Overall, there was a significant increase in serum LH in the OVX group (INTACT: 53.5 ± 36.2 ng/ml, n = 8; OVX: 432.6 ± 43.0 ng/ml, n=9, mean ± SEM, P = <0.001). In contrast, serum leptin was not significantly altered by long-term ovariectomy (INTACT: 2.3 ng/ml ± 0.4; OVX: 3.3 ng/ml ± 0.6). There was no significant difference in body weight between the INTACT and OVX groups for up to 54 weeks after ovariectomy (Table II).

Discussion

Menopause signals the end of reproductive cycles and the beginning of a long period of life in which circulating ovarian hormones are reduced to castrate levels. To gain insight into the repercussions of ovarian failure in the primate, we evaluated the effects of ovariectomy on hypothalamic gene expression in young cynomolgus monkeys. Intact and ovariectomized monkeys were used to model the ovarian status of pre-and postmenopausal women while removing the factor of age.

Ovariectomy resulted in hypertrophy and increased gene expression of NKB neurons in the infundibular nucleus of young cynomolgus monkeys, similar to the changes observed in postmenopausal women (Rance and Young, 1991). These findings add strong support to the hypothesis that the increase in size and gene expression in hypothalamic NKB neurons in postmenopausal women is secondary to ovarian failure. We have previously shown that estrogen treatment of ovariectomized monkeys dramatically reduces hypothalamic NKB gene expression below the levels of detection of our *in situ* hybridization procedure. In this previous experiment, because no neurons were identified in the estrogen-treated group, we were unable to determine if estrogen replacement altered NKB cell size (Abel *et al* 1999). Thus, the present study provides the first demonstration of ovariectomy-induced hypertrophy of NKB neurons in the primate hypothalamus. The hypertrophied NKB neurons exhibited nuclear enlargement as well as increased Nissl substance, similar to the appearance of NKB neurons in the

postmenopausal human. These morphological features are characteristic of neurons that have been stimulated to produce increased amounts of peptides (Theodosis 2002).

The responsiveness of infundibular NKB neurons to ovariectomy and estrogen replacement in multiple species suggests that these cells participate in the steroid-dependent regulation of gonadotropin secretion (Abel *et al* 1999,Akesson *et al* 1991,Danzer *et al* 1999,Pillon *et al* 2003,Rance and Bruce 1994). This hypothesis is supported by preliminary studies that have shown NKB-immunoreactive terminals in preoptic area of the ewe that lie in close apposition to GnRH somata, suggesting a synaptic relationship between these neurons (Goubillon *et al* 2000b). In addition, a very high proportion of NKB neurons in the infundibular nucleus co-express the estrogen receptor-α protein or mRNA indicating they are direct targets for estrogen action (Goubillon *et al* 2000b,Rance and Young, 1991). Evidence for the responsiveness of NKB neurons to steroids was provided by Page et al., and Goubillon et al; the NKB gene contains five sequences indicative of estrogen response elements (ERE) as well as half sites suggesting imperfect palindromic EREs (Page *et al* 2001). NKB-immunoreactive neurons in the female ewe are larger and more numerous than in the male, and this sexual dimorphism was abolished by prenatal androgen treatment (Goubillon *et al* 2000b).

Long-term ovariectomy of cynomolgus monkeys induced more than an 8-fold elevation in bioactive serum LH. These data illustrate the reciprocal relationship between ovarian hormones and gonadotropin secretion (now known as steroid negative feedback)

that was first elucidated in 1932 (Moore and Price 1932). Coincident with the increase in serum LH was a modest elevation in the number of neurons expressing GnRH gene transcripts. The rise in GnRH gene expression occurred in the same subpopulation of neurons (type I) that have been shown to exhibit increased GnRH gene expression in postmenopausal women (Rance and Uswandi 1996). These findings agree with the demonstration of increased GnRH mRNA in the basal hypothalamus of orchidectomized rhesus monkeys (El Majdoubi *et al* 2000) and, conversely, suppression of hypothalamic GnRH mRNA by estrogen in the ovariectomized female monkey (El Majdoubi *et al* 1998,Krajewski *et al* 2003). Using indirect pharmacological methods, Gill et al. showed that estrogen negative feedback on GnRH secretion is still preserved in older women (Gill *et al* 2002). In sum, these findings suggest that ovarian failure is an important factor contributing to the rise in hypothalamic GnRH gene expression and gonadotropin hypersecretion in postmenopausal women (Rance and Uswandi 1996).

Although the number of neurons expressing POMC mRNA was reduced in older women (Abel and Rance 1999), long-term ovariectomy of young cynomolgus monkeys had no significant effect on POMC gene expression at two different levels of the infundibular nucleus. These data are consistent with the failure of hormone replacement to modify POMC gene expression in ovariectomized cynomolgus monkeys (Abel *et al* 1999). Furthermore, gonadectomy of male cynomolgus monkeys did not result in a significant change in POMC gene expression at comparable levels of the infundibular nucleus (Adams *et al* 1991). POMC gene expression, however, was decreased in the

medial basal hypothalamus of orchidectomized rhesus monkeys (El Majdoubi *et al* 2000). This discrepancy could be due to a number of factors, including different methodologies (RNAse protection assay vs. *in situ* hybridization), gender or anatomical localization. In addition, the effect of orchidectomy on POMC gene expression in the rat has been shown to be markedly time-dependent (Wardlaw and Blum 1990). In the study of El Majdoubi et al., POMC gene expression was assayed 41 days after gonadectomy, compared to 10-14 months described here. We intentionally performed our assays after a long period of ovariectomy in order to model the extended duration of hormone depletion in postmenopausal women and to avoid postoperatory stress that could affect hypothalamic gene expression.

Along with the stable levels of POMC gene expression, ovariectomy did not change body weight or serum leptin in the cynomolgus monkey. Our observations are in agreement with several studies in which long-term ovariectomy of cynomolgus monkeys (Jerome *et al* 1997,Stavisky *et al* 1999) or estrogen replacement therapy (Cefalu *et al* 1994,Wagner *et al* 1991) had no effect on body weight. These data suggest that long-term withdrawal of ovarian steroids does not have a major influence on body weight regulation in young cynomolgus monkeys. In contrast, ovariectomy induces a remarkable weight gain in the rat that is reversed by estrogen replacement (Chu *et al* 1999,Kakolewski *et al* 1968,Kimura *et al* 2002,Watanobe and Suda 1999). Thus, the effect of gonadectomy on the regulation of body weight varies widely among species.

Although an age-associated increase in body weight has been documented in women (Davies *et al* 2001,Lamberts *et al* 1997,Ley *et al* 1992), the contribution of ovarian failure to this weight gain has been a source of controversy [for review see (Avis and Crawford 2001)]. A number of studies have failed to show an association between body mass index and menopausal status or hormone replacement (Haffner *et al* 1997,Havel *et al* 1996,Lindquist 1982). These findings have been confirmed in recent longitudinal studies of large groups of women (Blümel *et al* 2001,Davies *et al* 2001). Similarly, many studies have shown serum leptin levels in women to be independent of the transition to menopause or estrogen replacement therapy (Blümel *et al* 2001,Castracane *et al* 1998,Hadji *et al* 2000,Haffner *et al* 1997,Havel *et al* 1996,Kohrt *et al* 1996). These studies suggest that, similar to the cynomolgus monkey, ovarian failure does not significantly alter body weight regulation in humans.

Research on the physiology of human menopause has been hampered by a limited availability of suitable animal models. Although widely used as a model for menopause, many rodent species diverge from the human by displaying persistent ovarian function after the loss of estrous cycles in middle age (Finch and Gosden 1986,LaPolt and Lu 2003). Nonhuman primates such as baboons, chimpanzees and rhesus monkeys experience a menopause characterized by ovarian failure, but this event occurs relatively late in their lifespan (Bellino and Wise 2003). Consequently, by the time these primate species go through menopause, they are relatively ill because of age-associated pathological changes. The question of whether the young ovariectomized

monkey is a suitable model for postmenopausal women has not been adequately addressed (Bellino and Wise 2003).

In the present study, we found that ovariectomy of young monkeys only partially duplicates the patterns of hypothalamic gene expression observed in older, postmenopausal humans (mean age of 70 years). There was a comparable elevation in NKB and GnRH gene expression in the young cynomolgus monkey and postmenopausal women (Rance and Uswandi 1996,Rance and Young 1991), but the drop in POMC gene expression that was observed in older women (Abel and Rance 1999) was not simulated by ovariectomy of young cynomolgus monkeys. On the basis of the present data, we cannot distinguish whether this disparity in POMC gene expression is due to a species difference or the presence of aging in the postmenopausal women. We favor an aging effect, because a consistent age-associated decrease in POMC gene expression or immunoreactive neurons has been observed in laboratory rodents (Lloyd *et al* 1991,Miller *et al* 1995). It is also possible that aging modifies the response to steroid withdrawal in either monkeys or humans. Thus, the etiology of the drop in hypothalamic POMC gene expression in postmenopausal women remains unresolved.

The postmenopausal phase of life in humans lasts for decades and is accompanied by many gradual changes in endocrine function, including those of the reproductive axis (Gill *et al* 2002,Lamberts *et al* 1997,Rossmanith *et al* 1991). Thus, the young ovariectomized monkey may provide a model that is more closely matched to women

within a short period of time after the menopausal transition, or premenopausal women undergoing surgical oophorectomy, rather than aged, postmenopausal women. On the other hand, study of ovariectomized animals appears to have great value for understanding the repercussions of ovarian steroid withdrawal, such as the hypertrophy of NKB neurons. The profound hypoestrogenic state will have an impact on the physiology of women throughout the extended duration of the postmenopausal phase.

Figure 2.1. Darkfield photomicrographs of adjacent hypothalamic sections from INTACT (A,C) or OVX (B, D) cynomolgus monkeys hybridized with either NKB (A, B) or POMC (C, D) probes. The outlines of the base of the brain, pituitary stalk (PS) and third ventricle (3V) have been superimposed on these photomicrographs. Note the marked increase in the number of labeled NKB neurons in the OVX animals. Scale bar = 0.5 mm on all photomicrographs.

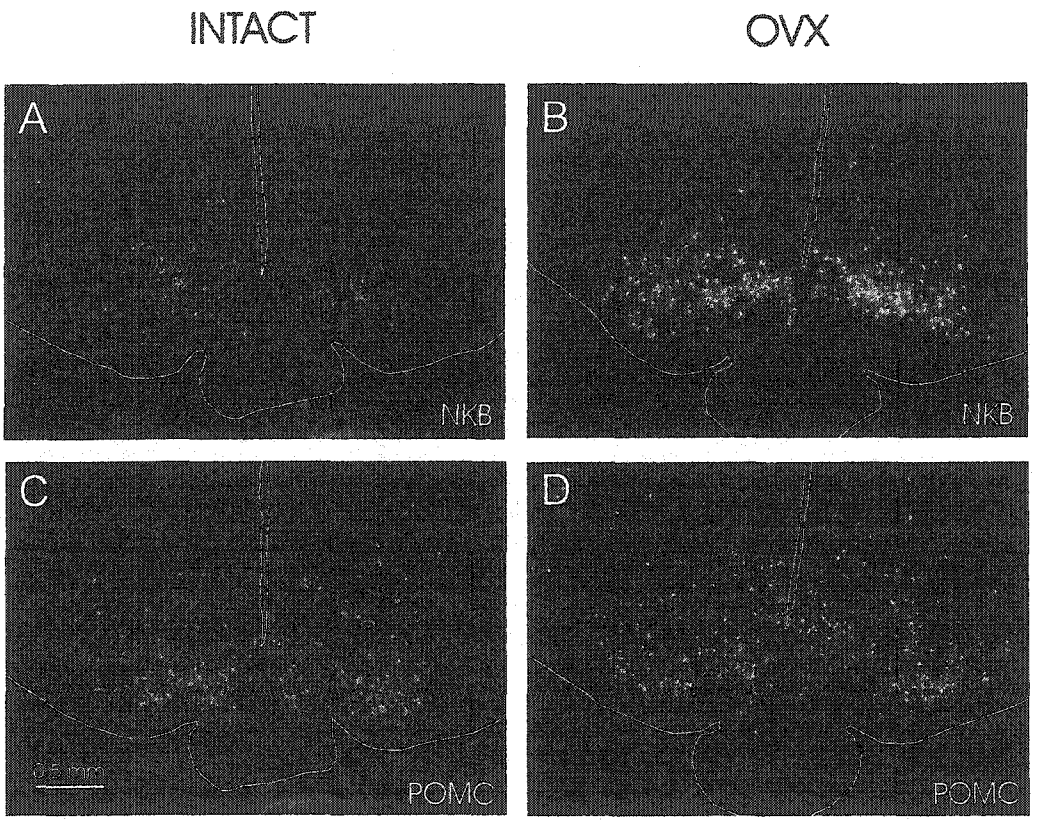
INTACT
OVX
A
NKB
B
NKB
C
POMC
D
POMC

Figure 2.2. Effects of ovariectomy on NKB neurons in the infundibular nucleus of young cynomolgus monkeys. All values are expressed as the mean $\pm$ SEM. The graphs show the number of neurons expressing NKB mRNA in a unilateral section of infundibular nucleus (A), the number of autoradiographic grains for each labeled NKB neuron (B) and the profile area (μm^2) of NKB neurons.

* significantly different from INTACT, P = < 0.001.

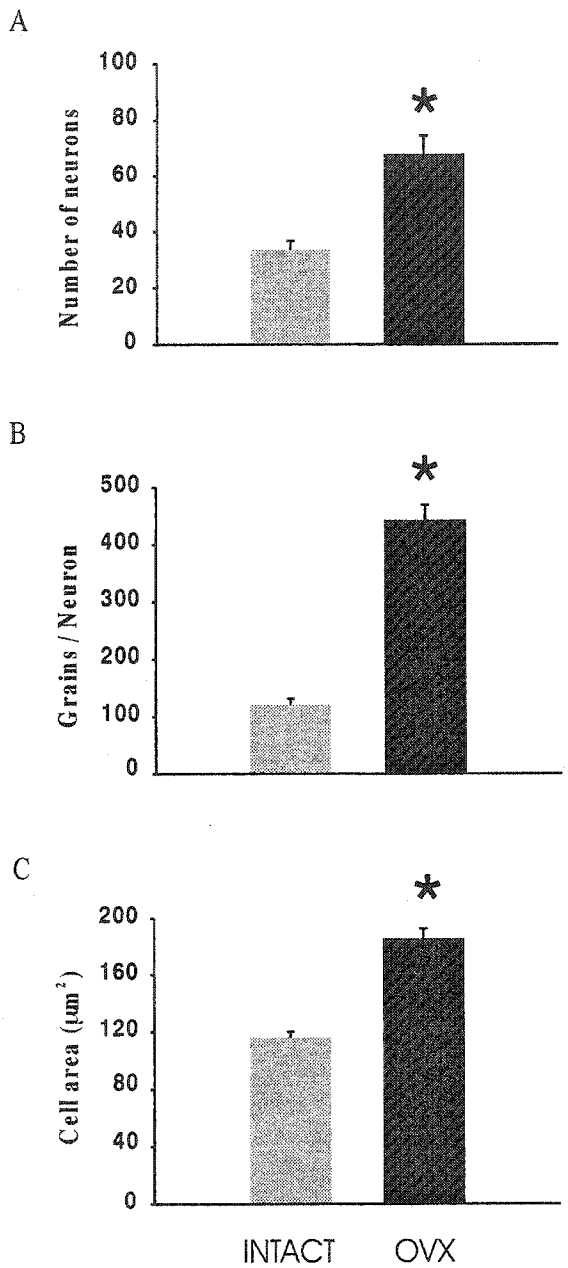
A
Number of neurons
100
80
60
40
20
0
*
B
Grains / Neuron
500
400
300
200
100
0
*
C
Cell area (μm²)
200
160
120
80
40
0
*
INTACT
OVX

Figure 2.3. Photomicrographs of neurons in the infundibular nucleus of INTACT (A) or OVX (B) cynomolgus monkeys labeled with the NKB probe. The autoradiographic grains mark the location of NKB mRNA and the sections have been counterstained with toluidine blue. The neuronal hypertrophy in ovariectomized monkeys was accompanied by larger nuclei and increased numbers of autoradiographic grains. Scale bar = 25 μm on both photomicrographs.

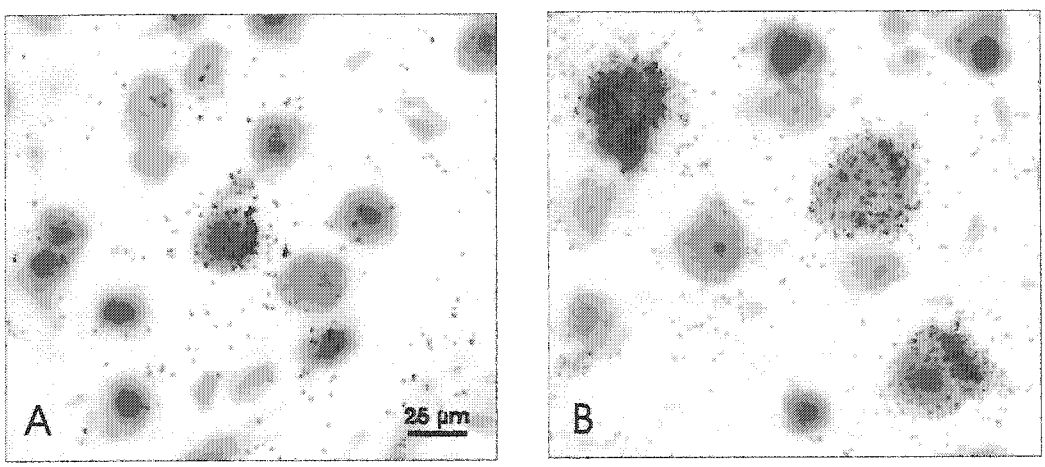
A
25 µm
B

Figure 2.4. Effects of ovariectomy on GnRH neurons in the hypothalamus of young cynomolgus monkeys. All values are expressed as the mean ± SEM. The graphs show the number of neurons expressing GnRH mRNA in a unilateral section of hypothalamus (A), the number of autoradiographic grains for each labeled GnRH neuron (B) and the profile area (μm^2) of GnRH neurons.

* Significantly different from INTACT, P = < 0.05.

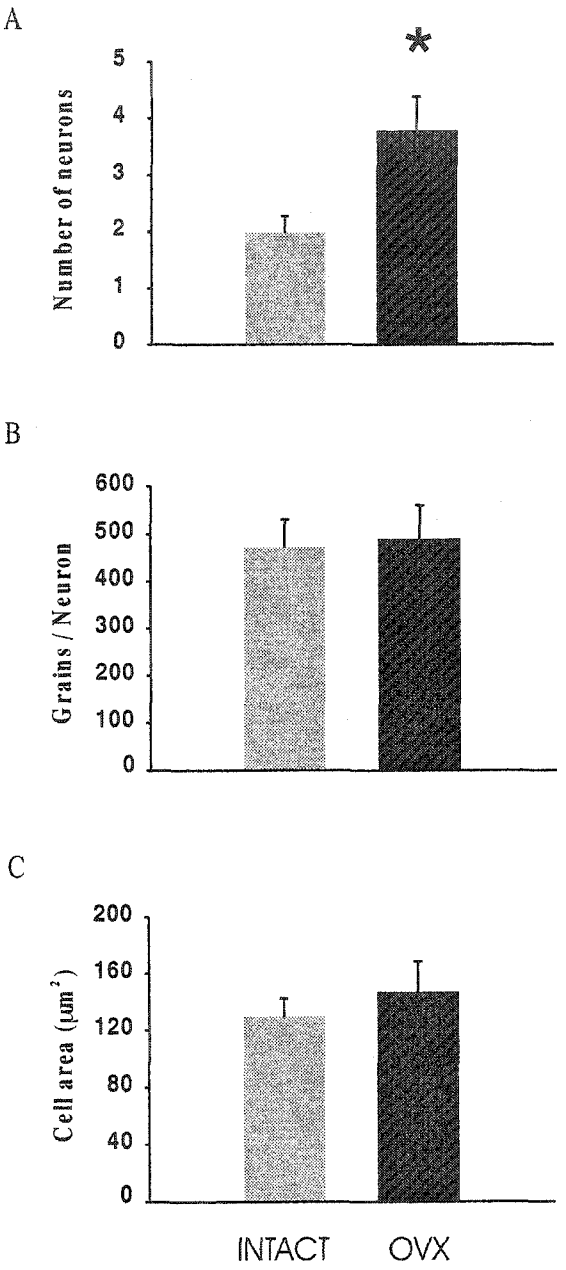
A
*
Number of neurons
5
4
3
2
1
0
B
Grains / Neuron
600
500
400
300
200
100
0
C
Cell area (μm²)
200
160
120
80
40
0
INTACT
OVX

Table II. Body weights (Kg) of young female cynomolgus monkeys (INTACT) compared to a matched group of ovariectomized animals (OVX).

	12 weeks prior to OVX	1 week prior to OVX	1 week after OVX	8 weeks after OVX	20 weeks after OVX	54 weeks after OVX
INTACT	3.09 ± 0 .15	3.23 ± 0.14	3.23 ± 0.15	3.10 ± 0.11	3.17 ± 0.15	3.03 ± 0.12
OVX	2.88 ± 0.12	3.04 ± 0.12	2.90 ± 0.10	3.00 ± 0.11	2.9 ± 0.12	2.84 ± 0.17

All values are means ± SEM. There was no significant difference between INTACT and OVX groups at any time point or within INTACT or OVX groups across time points.

* n = 8 INTACT and n = 9 OVX for all time periods except at 54 weeks where n = 6 INTACT and n = 7 OVX.

CHAPTER THREE

CENTRAL INJECTION OF SENKTIDE OR NEUROPEPTIDE Y (NPY) INHIBITS LH SECRETION BUT INDUCES DIFFERENT PATTERNS OF FOS EXPRESSION IN THE RAT HYPOTHALAMUS

Abstract

Arcuate neurokinin B (NKB) neurons in several species express estrogen receptor-α and are strongly modulated by gonadal steroids in several species. Although these findings suggest that NKB neurons participate in the reproductive axis, there is no information on the regulation of LH secretion by NKB or its receptor, Neurokinin 3 (NK_3). In the present study, we determined whether of central injection of senktide, a selective NK_3 receptor agonist, altered serum LH in ovariectomized estrogen-treated rats. We also compared the effect of senktide injection to that of Neuropeptide Y (NPY), a well-characterized modulator of LH secretion. Senktide, NPY or vehicle was injected into the lateral ventricle of awake, freely moving rats and serial blood samples were collected for LH radioimmunoassay. The rats were sacrificed 90 minutes after injection and the brains removed and processed for Fos immunocytochemistry. A significant inhibition of serum LH was observed for up to 90 minutes after injection of either senktide or NPY. In the senktide-injected rats, the inhibition of LH was accompanied by increased Fos expression in the medial preoptic area and the arcuate, paraventricular and

supraoptic nuclei. In contrast, injection with NPY significantly increased Fos only in the paraventricular and supraoptic nuclei. Thus, although senktide and NPY both inhibited LH secretion, they induced different patterns of hypothalamic Fos induction. This study provides the first demonstration of modulation of LH secretion through NK_3 receptor activation.

Introduction

The regulation of LH secretion by gonadal steroids involves complex hypothalamic circuits that converge on neurons secreting GnRH (Evans 1999,Horvath *et al* 1997). One of the key regulatory centers for reproduction is located within the hypothalamic arcuate (infundibular) nucleus. Neurons within the arcuate nucleus are express neurokinin B, a tachykinin peptide that shares a common C-terminal end with substance P and substance A, but whose mRNA is transcribed from a different preprotachykinin gene. Neurokinin B neurons in the arcuate nucleus have been shown to express receptors for gonadal steroids in a variety of species, including human, rat and sheep (Ciofi *et al* 1994,Goubillon *et al* 2000b,Rance and Young, 1991). The high degree of estrogen receptor and NKB colocalization in the arcuate nucleus provides evidence that these neurons are target tissues for estrogen action (Goubillon *et al* 2000b).

In the infundibular (arcuate) nucleus of postmenopausal women, NKB neurons undergo hypertrophy and display increased levels of tachykinin gene transcripts (Rance and Young, 1991). Ovariectomy induced similar changes in young cynomolgus

monkeys, suggesting that the hypertrophy and increased NKB gene expression in older women is a consequence of the ovarian failure of menopause (Sandoval-Guzmán *et al* 2003). Indeed, there are numerous studies showing changes in arcuate neurokinin B neurons in response to ovariectomy and steroid replacement (Abel *et al* 1999,Akesson *et al* 1991,Danzer *et al* 1999,Pillon *et al* 2003,Rance and Bruce 1994,Sandoval-Guzmán *et al* 2003). Furthermore, the levels of NKB mRNA vary with the rat estrous cycle (Rance and Bruce 1994). In the sheep arcuate nucleus, NKB expressing cells are sexually dimorphic. Furthermore, NKB-immunoreactive terminals lie in close proximity to GnRH cell bodies in the rostral hypothalamus and GnRH terminals in the median eminence (Goubillon *et al* 2000b). Although these studies suggest that arcuate NKB neurons play an important role in the sex-steroid regulation of reproduction, the relationship between NKB receptor activation and LH secretion has not been explored.

The mammalian tachykinins consist of substance P (SP), NKA, two elongated forms of NKA (neuropeptide K and neuropeptide-gamma) and NKB (Severini *et al* 2002). SP, NKA and NKB are the preferential ligands of three distinct classes of G-protein coupled receptors, NK_1, NK_2 and NK_3 respectively. Previous studies have demonstrated either inhibitory, stimulatory or no effects of central tachykinin peptide injections on LH release in a variety of experimental paradigms (Arisawa *et al* 1990,Battmann *et al* 1991,Kalra *et al* 1992a,Sahu and Kalra 1992). However, interpretation of these studies is complicated by the potential interactions of the naturally

occurring tachykinins with all three receptor types, and their susceptibility to proteases (Helke *et al* 1990,Maggi and Schwartz 1997,Regoli *et al* 1994,Severini *et al* 2002).

In the present study, we determined if central injections of senktide, an NK_3 receptor agonist, would modulate LH secretion in ovariectomized estrogen-treated rats. Senktide has proven to be a useful tool to elucidate the distribution, regulation and function of NK_3 receptors in the central nervous system and peripheral tissues because of its selectivity and potency (Laufer *et al* 1986,Mileusnic *et al* 1999a). The effects of senktide were compared with NPY, a well-characterized modulator of gonadotropin secretion (Crowley *et al* 1987,Sahu *et al* 1987). To gain insight into possible neural sites underlying the effects of senktide or NPY, immunocytochemical methods were used to identify the induction of Fos. Fos is the protein product of the immediate early gene *c-fos*, that is expressed after synaptic stimulation and is used as a marker for cell activation (Curran *et al* 1984,Curran and Morgan 1985,Dragunow and Faull 1989,Hoffman and Lyo 2002).

Materials and Methods

Animals: Twenty-four 250-300 g female Sprague-Dawley rats (Harlan Sprague-Dawley, Houston Texas) were used in these studies. Animals were housed with controlled temperature and humidity under 12 h light and dark cycles with free access to commercial food and water. Animal protocols were approved by the University of Arizona Institutional Animal Care and Use Committee and conformed to NIH guidelines.

Twelve days before the experiment, the rats were anesthetized with 0.60 ml/kg (i.m. injection) of a cocktail of ketamine (33.3 mg/ml), xylazine (10.7 mg/ml) and ace promazine (1.3 mg/ml) and ovariectomized using a dorsal surgical approach (Fig. 1). They were then transferred to a stereotaxic apparatus and a guide cannula (Plastics One, Roanoke, Va., USA) was implanted into the lateral ventricle (coordinates from Bregma: -0.6 mm AP, +1.1 mm ML and -4.8 mm DV from skull) and secured with dental cement. A dummy cannula was placed until the day of the experiment. On the morning of day 10, the rats were subcutaneously implanted with 30 mm silastic capsules (Dow Corning, 1.57 mm ID, 3.18 mm OD) containing 180 μg estradiol/ml sesame oil (Legan and Karsch 1975,Wise *et al* 1981). These capsules were incubated in saline at 37°C overnight before placement. This treatment was designed to standardize estrogen levels at low constant physiologic levels (Dubal *et al* 1998,Wise *et al* 1981). On day 11, an indwelling jugular vein catheter (Braintree Scientific, Braintree, MA) was inserted under general anesthesia. The distal end of the catheter was exteriorized dorsally and flushed with heparinized saline (20 U/ml).

On the morning of day 12, the animals were divided into three groups; Senktide (600 pmol, Sigma, St. Louis, MO); NPY (500 pmol, Peninsula Laboratories Belmont, CA) or vehicle (saline) injection. The dosages were based on previous studies of i.c.v. injections of senktide or NPY (Ding *et al* 2000,Kalra *et al* 1992a,Kalra and Crowley 1984). All injections were performed between 9:00 and 9:30 a.m. The dummy cannula

was replaced with an injection cannula under light isoflurane anesthesia (< 3 minutes duration). The injection cannula was connected by a tube to a Hamilton syringe in a microinjector pump, which delivered 4 μl at a constant rate of 1 μl/min. The animals were awake and freely moving throughout the experiment. Behavioral responses consisting of either wet-dog shakes (senktide) or food consumption (NPY) were observed to verify the effectiveness of drug delivery. Blood samples of 250 μl were withdrawn through the indwelling jugular catheter immediately before i.c.v. injection and 15, 30, 60 and 90 minutes after. An equivalent volume of saline was replaced after each blood collection. The blood samples were refrigerated for an hour and then centrifuged for 15 minutes at 3000 rpm. Serum was collected and stored at -20ºC until LH radioimmunoassay.

Ninety minutes after the injections, the rats were deeply anesthetized and perfused via the ascending aorta with 0.01 M phosphate buffered saline (PBS, pH 7.4) followed by ice-cold 4% paraformaldehyde in phosphate buffer. Brains were removed, postfixed for 24 h in 4% paraformaldehyde and then cryoprotected for 48 h in 30% sucrose in phosphate buffer. Brains were frozen and coronally sectioned (40 μm thickness) with a sledge microtome. Anterior sections were mounted and stained with cresyl violet for verification of cannula placement in the lateral ventricle. The remaining sections were stored in cryoprotectant solution (Watson, Jr. *et al* 1986) until immunocytochemical procedures.

Immunocytochemistry: Every tenth section throughout the hypothalamus (seven rats/group) was processed for immunocytochemistry using a Fos antibody (AB-5, Santa Cruz Biotechnology. Santa Cruz, CA) that has been previously characterized (Caston-Balderrama *et al* 1998). Sections from all animals were immunostained concurrently. The free floating-sections were rinsed 3 times for 10 minutes in 0.01 M PBS and then incubated for 30 minutes with 0.3 % H_2O_2 in phosphate buffered saline (PBS) for endogenous peroxidase inhibition. Sections were blocked with 3% normal goat serum and 0.3% triton X-100 in 0.01 M PBS for two hours then incubated with a rabbit c-Fos antibody (1:20,000) for 48 h at 4°C. The sections were then rinsed and incubated with biotinylated goat anti-rabbit IgG (1:250; Vector Laboratories) for 2 hours. Sections were then reacted with the Elite ABC Kit (Vector Laboratories) for 90 min. The bound peroxidase was visualized by reaction with 0.02% diaminobenzidine (DAB) and 0.003% H_2O_2 for 4 min. Sections were mounted on gelatin-coated slides and coversliped.

Tissue analysis: Analytical procedures were performed without knowledge of the experimental group. Hypothalamic nuclei were identified with the aid of a rat brain atlas (Paxinos and Watson 1986): medial preoptic area (MPOA, Plate 18 and 19), anteroventral periventricular preoptic area (AVPv, Plate 18), suprachiasmatic nucleus (SCN, Plate 23), supraoptic nucleus (SON, Plate 23 or 24), paraventricular nucleus (PVN, plate 25), and the arcuate nucleus (ARC, plates 29-31). The boundaries of the nuclei were outlined using an image-combining computer microscope using Neurolucida Software (Microbrightfield, Colchester VT) and with a 10X Nikon objective. The

MPOA was circumscribed by a rectangle extending dorsal-ventrally from the anterior commissure to the optic chiasm and 1 mm lateral to the third ventricle and the AVPv was outlined as described by Le et al., (Le *et al* 2001). These hypothalamic regions were systematically scanned with a 20X planapo Nikon objective. Every Fos-immunoreactive neuron within the boundaries was marked and counted.

Radioimmunoassay: Serum LH concentrations were measured by radioimmunoassay using NIDDK kits kindly provided by Dr. A. F. Parlow. This RIA has an intra-assay variation of 6%. Serum LH concentration was expressed in terms of LH RP-3. All samples were measured in duplicate within the same assay.

Statistical analysis: The values of serum LH concentrations were used to calculate the mean and standard error of the mean (SEM) of each time point of each experimental group. Group means were statistically compared using two-way analysis of variance (ANOVA) with repeated measures. For post-hoc analysis, Tukey's methods were used with $\alpha = 0.05$. The numbers of Fos-immunoreactive cells/region were compared using one-way ANOVA and Tukey's method for post-hoc analysis, except for the SON that was analyzed with the Student-Newman-Keuls method for post-hoc analysis ($\alpha = 0.05$).

Results

Serum LH concentrations at successive time points following the injection of senktide, NPY or saline into the lateral ventricle of ovariectomized-estrogen treated rats is shown in Figure 2. The intraventricular injection of the NK_3 receptor agonist, senktide, to rats resulted in a maximum of 39% decrease of serum LH at 60 minutes. The inhibition of serum LH by senktide was significant at 30, 60 and 90 minutes after injection. The NPY injection also resulted in decreased serum LH and this effect was significant at 15 min and continued for 30, 45, 60, and 90 minutes post-injection (Figure 2). Behavioral responses (wet-dog shakes) were observed in all animals receiving senktide. The animals exhibited a variety of normal behaviors between the wet dog-shakes, including grooming, eating, drinking and burrowing. Approximately 10 minutes after NPY injections, nearly all the animals (7/8 rats) started eating rat chow and they continued to eat for the full 90 minutes of the experiment.

Low numbers of Fos-immunoreactive neurons were observed throughout the brain in the saline injected rats. In the rats injected with senktide, significantly increased numbers of Fos immunoreactive neurons were detected in the MPOA, SON, PVN and ARC nuclei (Table III). In the PVN of senktide-injected rats, Fos was detected in both the parvocellular and magnocellular divisions. Fos expression was also significantly increased in the PVN and SON of NPY injected rats relative to controls. Fos detection in AVPv was not significantly different between saline, senktide and NPY injected rats.

There was no difference in the number of Fos immunoreactive neurons between NPY-injected rats and controls in the other nuclei analyzed.

Discussion

NKB neurons in the arcuate/infundibular nucleus have been postulated to play an important role in the sex-steroid regulation of reproduction, given their location within reproductive control centers, their high degree of steroid receptor colocalization (Ciofi *et al* 1994,Goubillon *et al* 2000b,Rance and Young, 1991) and responsiveness to estrogen treatment in ovariectomized rodents, sheep and monkeys (Abel *et al* 1999,Danzer *et al* 1999,Pillon *et al* 2003,Rance and Bruce 1994). In addition, the changes in NKB gene expression in the postmenopausal human hypothalamus (Rance and Bruce 1994,Rance and Young, 1991) suggests that these neurons may play a role in human reproductive physiology as well. In the present study, we found that central injection of senktide, a selective and potent NK_3 receptor agonist, inhibited LH secretion in estrogen-treated, ovariectomized rats. In addition, senktide induced Fos expression in several hypothalamic nuclei that are considered regulatory centers for gonadotropin secretion. The most selective of the tachykinin receptors is NK_3, with highly preferential binding and activation by NKB (Maggi and Schwartz 1997). Therefore, these data support the concept that NKB neurons are involved in the regulation of mammalian reproduction

Fos expression was induced in the MPOA, SON, PVN and ARC after senktide injection, a finding consistent with previous studies (Smith and Flynn 2000). These

regions express NK_3 receptor immunoreactivity, NK_3 receptor mRNA or [^{3}H] senktide receptor binding (Langlois *et al* 2001,Mileusnic *et al* 1999a,Mileusnic *et al* 1999b) suggesting that the action of senktide was due to a direct effect on the NK_3 receptor. Although senktide and NPY decreased serum LH in a similar manner, they produced divergent patterns of Fos expression in hypothalamic nuclei, as well as different behavioral effects. These results are consistent with the different anatomical localization and functions of NK_3 and NPY receptors in the central nervous system.

Numerous studies over the last three decades have implicated the MPOA and the arcuate nucleus as key regulatory centers of the reproductive axis (Barraclough 1973,Simerly 2002). Neurons in both the MPOA and ARC express GABA, an inhibitory neurotransmitter that modulates GnRH secretion (Horvath *et al* 1992,Lopez and Negro-Vilar 1990). The ARC also expresses peptides that have inhibitory effects on the reproductive axis, such as the opioid peptides, β-endorphin, dynorphin and enkephalin (Ferin *et al* 1984). Both the MPOA and ARC project to the site of GnRH neurons in the rostral MPOA/septal region. Thus, we hypothesize that the suppressive effect of senktide on LH secretion is due to activation of inhibitory neurons projecting to GnRH neurons. A direct effect of senktide on GnRH neurons or gonadotrophs in the anterior pituitary gland, however, is not excluded by these data.

The finding of senktide-induced Fos expression in the SON and PVN is consistent with previous studies implicating NK_3 receptors in posterior pituitary function (Ding *et*

al 1999,Polidori *et al* 1989,Smith and Flynn 2000). Magnocellular SON and PVN neurons express high levels of NK_3 receptor-immunoreactivity and NK_3 receptor binding. Furthermore, senktide stimulates vasopressin and oxytocin release into peripheral plasma (Ding *et al* 1999,Takano *et al* 1986). Estrogen also influences the activity of vasopressin and oxytocin neurons but the nature of this interaction is not well understood (Voisin *et al* 1997). Vasopressin inhibits the secretion of LH secretion, although this effect has been contradictory in some studies (DePaolo *et al* 1986,Heisler *et al* 1994,Palm *et al* 1999,Palm *et al* 2001). The regulation of LH release by vasopressin may also depend on different factors, as the presence of oxytocin synergizes with the inhibitory effect of vasopressin on LH release (Hipkin 1970). Thus, there is a possibility that senktide might reduce LH through the release of vasopressin from PVN or SON.

Future studies will be needed to address the precise relationship between arcuate NKB neurons and reproductive axis. For example, it is not known if arcuate NKB neurons project to the hypothalamic nuclei that are activated by senktide administration. Furthermore, the effects of NKB release from endogenous axon terminals is probably much different than the global activation of NK_3 receptor sites after intraventricular injections of senktide. Finally, the effects of tachykinin receptor stimulation are likely to be modified by gender or hormonal status. Indeed, in a previous study, the central administration of antisense oligomers targeted to the NKB gene caused a reduction in serum LH in orchidectomized rats, indicative of a stimulatory effect of NKB on LH secretion in this animal model (Sandoval-Guzmán *et al* 2000). These data are

characteristic of the reproductive axis, in which steroid hormones modulate neuronal function in a complex and sometimes paradoxical fashion.

Similar to the effects of senktide, NPY administration inhibited LH secretion in ovariectomized estrogen-treated rats. However, NPY induced Fos expression only in the PVN and SON, consistent with previous studies (Li *et al* 1994). The inhibition of LH by NPY is in contrast to reports in which central injection of NPY stimulated LH in ovariectomized, steroid-treated rats (Kalra *et al* 1992b). This discrepancy is most likely explained by the different paradigms of steroid replacement, as NPY reverts to an inhibitory effect in the rat when steroids are omitted (Kalra *et al* 1992). In the previous study, rats were primed by s.c. bolus injection of estradiol benzoate and progesterone, compared to implantation of silastic capsules containing low concentrations of estradiol in the present study. Inhibitory effects of central NPY injections have also been documented in ovariectomized estrogen-treated sheep (McShane *et al* 1992) and goats (Ichimaru *et al* 2001). The inhibitory actions of NPY may play an important role in the suppression of GnRH secretion during lactation (Li *et al* 1999) and as a mechanism to reduce reproductive competency when body energy stores are depleted (Ichimaru *et al* 2001). The inhibitory effect of NPY on LH secretion was accompanied by increased Fos expression in the PVN. These data are in agreement with previous studies showing NPY receptors in the nucleus and the induction of Fos predominantly in the PVN after NPY injection. However, the mechanism of action of NPY may also be explained by a direct effect of NPY on GnRH neurons. NPY neurons in the arcuate nucleus project to the

preoptic area and their axons lie in close proximity with GnRH neurons (Li *et al* 1999). Furthermore, GnRH fibers in the median eminence co-express NPY1 receptors (Li *et al* 1999).

In summary, the present study showed that central injection of senktide, an NK_3 receptor agonist, inhibits LH secretion in estrogen-treated ovariectomized rats. Senktide also induced Fos expression in various hypothalamic nuclei including the ARC and MPOA, two important regulatory centers of the reproductive axis. Although NPY inhibited LH secretion in a similar fashion, it produced different patterns of Fos activation as well as divergent behavioral effects. The demonstration of modulation of LH secretion by NK_3 receptor activation provides a critical piece of information in support of the hypothesis that NKB neurons are involved in the sex steroid-responsive regulation of gonadotropin secretion. Understanding the precise role of NKB neurons in this process may ultimately shed light on the complex steroid feedback mechanisms that control the reproductive axis.

Figure 3.1. Schematic representation of the experimental protocol. E_2, estradiol 17-β; I.c.v., intracerebroventricular; NPY, neuropeptide Y; OVX, ovariectomized; ∇, time of blood withdrawal.

Experimental Protocol

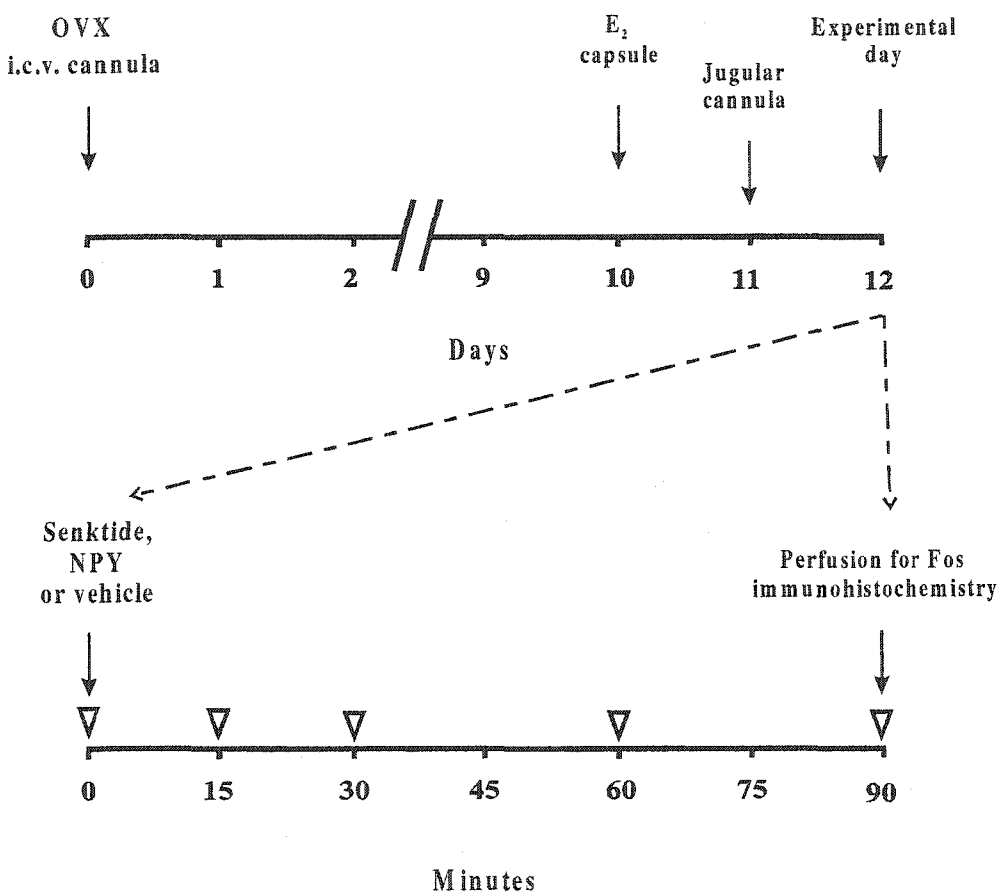

Figure 3.2. Serum LH concentration at different time points after injection of senktide or NPY into the lateral ventricle of ovariectomized estrogen-treated rats. All values are expressed as the mean $\pm$ SEM.

* Significantly different from saline injected rats, $P = < 0.05$.

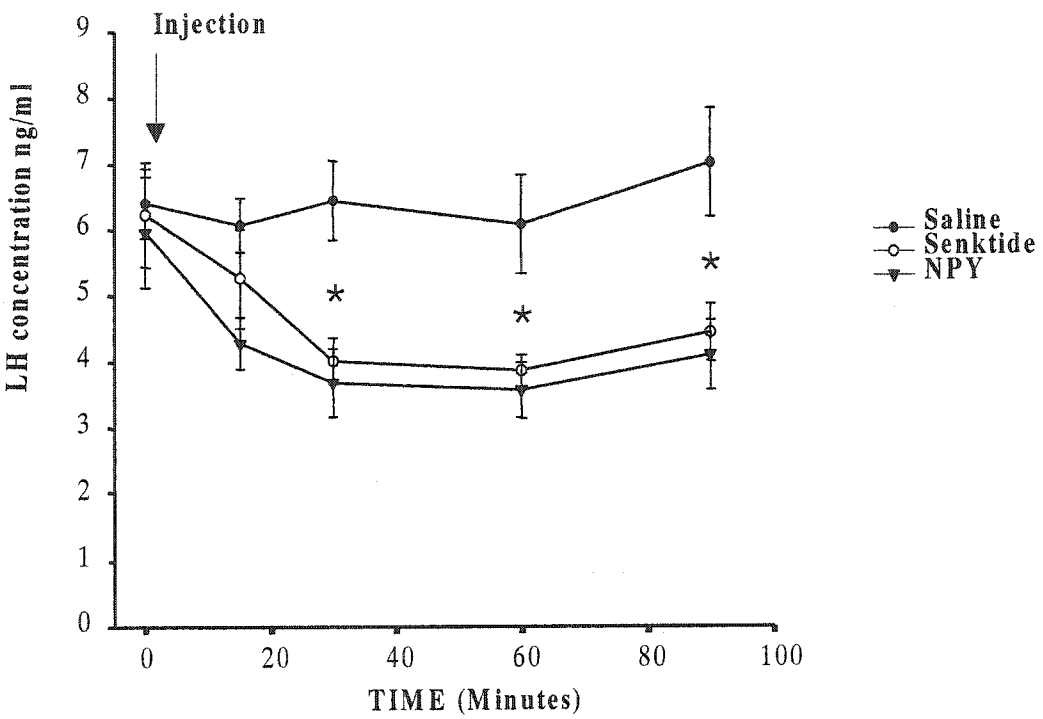
Injection
LH concentration ng/ml
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
100
TIME (Minutes)
*
*
*
Saline
Senktide
NPY

Table III. Fos-like immunoreactivity in different hypothalamic nuclei after lateral ventricular injection of saline, senktide or NPY.

Treatment	MPOA	AVPv	SCN	SON	PVN	ARC
Saline	79.3±16.9	1.5±0.6	6.5±1.5	4.2±1.4	34.4±5.0	6.5±1.5
Senktide	133.8±16.1[ab]	2.0±0.3	NA*	80.2± 12.1[ab]	106.5±19.4[a]	18.1±3.9[a]
NPY	80.4±9.5	0.8±0.3	12.6±3.8	33.0±4.5[a]	81.1±10.4[a]	14.1±1.1

Abbreviations: anteroventral periventricular preoptic area, AVPv; medial preoptic area, MPOA; the suprachiasmatic nucleus, SCN; the supraoptic nucleus, SON, paraventricular nucleus; the arcuate nucleus, ARC; neuropeptide Y, NPY.

*NA, not analyzed

[a] P = <0.05, statistical comparison to the vehicle treated group.

[b] P = <0.05, statistical comparison to the NPY treated group.

CHAPTER FOUR

INTRAVENTRICULAR ADMINISTRATION OF A NEUROKININ B ANTISENSE OLIGONUCLEOTIDE REDUCES SERUM LH IN THE GONADECTOMIZED RAT

Abstract

Human menopause is associated with ovarian failure, gonadotropin hypersecretion and increased hypothalamic NKB gene expression. The increased NKB gene expression and elevated LH secretion is simulated by gonadectomy in both rodents and cynomolgus monkeys. These data suggest that increased activity of NKB neurons contributes to the gonadotropin hypersecretion observed after removal of steroid negative feedback. There is virtually no information, however, on whether pharmacological manipulation of NKB or the NK_3 receptor affects the secretion of LH in gonadectomized animals. In the present study, 20-mer phosphorothioate-end-modified oligodeoxynucleotides (ODN) targeted to NKB or NK_3 receptor mRNAs were infused for two days into the lateral ventricle of gonadectomized rats. Control rats were infused with either 20-mer ODNs with four mismatches, or vehicle alone. Serum LH was determined by RIA. We also determined the effects of subcutaneous injections of an NK_3 receptor antagonist, SB-222200, into gonadectomized male and female rats. NKB antisense-treated rats exhibited a 25% reduction in serum LH was observed in the (4.08 ± 0.46 ng/ml, mean ± SEM) compared to mismatched controls (5.49 ± 0.44 ng/ml) or vehicle

controls. However, serum LH of male and female rats treated with NK_3 receptor antisense ODNs was not significantly different from serum LH of control rats. Similarly, injections of the NK_3 receptor antagonist had no effect on serum LH concentrations in male and female rats. These data provide evidence that NKB neurons contribute to the increased gonadotropin secretion in gonadectomized rats. The effect, however, does not appear to be mediated by NK_3 receptors.

Introduction

The ovarian failure of menopause results in a loss of estrogen negative feedback and hypersecretion of LH and FSH from the anterior pituitary gland. These hormonal changes are accompanied by significant structural and functional changes in the hypothalamic arcuate/infundibular. One of the predominant changes is the hypertrophy of neurons containing neurokinin B (NKB), substance P (SP) and estrogen receptor alpha (Rance and Young, 1991). The hypertrophy of hypothalamic neurons is associated with a marked increased in NKB gene expression in the human infundibular nucleus (Rance and Young, 1991). These changes appear to be due to ovarian failure, because gonadectomy induces similar changes in the arcuate/infundibular of young cynomolgus monkeys (Sandoval-Guzmán *et al* 2003) and rodents (Danzer *et al* 1999,Rance and Bruce 1994). In support of this hypothesis, arcuate NKB gene expression is suppressed after steroid replacement in both the nonhuman primate (Abel *et al* 1999) and the rat (Danzer *et al* 1999). The responsiveness of NKB gene expression to the steroid environment is also evident during the estrous cycle (Rance and Bruce 1994). Therefore, we have

proposed that arcuate NKB neurons participate in the hypothalamic circuitry regulating LH secretion. In our previous chapter, we demonstrated that intracerebral injection of senktide, an NK_3 receptor agonist, reduced serum LH concentration in the ovariectomized estrogen-treated rat. These data provided strong support to the hypothesis that NKB participates in regulation of LH, but does not address the role of NKB in the gonadotropin hypersecretion induced by removal of steroid negative feedback.

We hypothesized that increased NKB activity contributes to the increase in serum LH in gonadectomized animals. To test this hypothesis, we determined the effects of antisense oligodeoxynucleotides (ODNs) targeted to the NKB gene on LH secretion in orchidectomized rats. We also used antisense ODN methodology to target the NK_3 receptor as well as NK_3 receptor antagonists to determine if this receptor is involved in the effects of NKB.

Materials and Methods

Sprague-Dawley rats weighing 250-300 g were used for these experiments. Animals were housed at controlled temperature and humidity under 12-hr light and dark cycles with free access to commercial food and water. Animal protocols were approved by the University of Arizona Institutional Animal Care and Use Committee and conformed to NIH guidelines. For gonadectomy and stereotaxic surgery the rats were

anesthetized with of a cocktail (0.60 mg/kg i.m.) consisting of ketamine (33.3 mg/ml), xylazine (10.7 mg/ml) and ace promazine (1.3 mg/ml).

Oligodeoxynucleotides (ODNs): Twenty-mer ODNs were synthesized by Sigma Genosys (Woodlands, Texas) and purified by high-performance liquid chromatography. Oligos were phosphorothioate-end modified to improve resistance against nucleases. The NKB antisense ODN (aNKB) was complementary to bases 151-170 of the NKB gene (Bonner *et al* 1987). Three NK_3 receptor antisense ODNs (aNK_3-1, aNK_3-2 and aNK_3-3) were complementary to bases 245- 264, 911- 930 and 494-513 of the NK_3 receptor gene, respectively (Shigemoto *et al* 1990). A genebank search was performed to ensure no significant homology of the ODNs with other known rat CNS genes. The ODNs had either very weak or no secondary structure and no formation of primer-dimers. The mismatch ODNs consisted of 4 flipped base pairs. All ODNs were dissolved in artificial CSF (Harvard apparatus) at a final concentration of 5 μM.

Experiment 1: Nineteen rats were orchidectomized, and five days later they were placed in a stereotaxic brain apparatus (Kopf Instruments) for the implantation of a brain cannula (Plastics One) connected to an ALZET minipump (model 1007D, infusion rate of 5 μl/hr). Animals were divided in three groups, one receiving the antisense NKB (aNKB, n = 7), the second receiving a mismatch NKB ODN (mNKB, n = 7) and a third group receiving artificial CSF (n = 5). The cannula was implanted into the right lateral ventricle (coordinates: 0.5 mm caudal to Bregma, 1.1 mm lateral to the midline, and 3.8 mm

vertical from the dural surface). Daily body weight measures were taken. Two days after continuous ODN infusion, the animals were decapitated under metophane anesthesia. The brains were rapidly removed and snap frozen in isopentane and trunk blood was collected. For all experiments, blood was refrigerated for an hour and then centrifuged at 3000 rpm for 15 minutes. Serum was stored at –20°C until LH RIA. To measure tissue NKB concentrations, 1 mm slabs were dissected through the arcuate nucleus with the aid of a rat brain matrix (ASI Instruments, Warren, MI). The frozen slabs were placed on an aluminum stage cooled with dry ice and the arcuate median eminence was dissected by with a tissue punch (0.69 mm i.d., Fine Science Tools). Tissue samples were expelled into an Eppendorf tube with 240 μl of 0.1 N HCl and sonicated. Tubes were centrifuged for 20 min at 10,000 rpm and the supernatant was removed, aliquoted and stored at -80°C. The aliquots of supernatant were freeze-dried, centrifuged and resuspended in 100 μl of assay buffer for RIA (NKB RIA kit, Peninsula Labs). The pellet was dissolved in 500 μl of 3% NaOH and stored at 4°C for protein assay (DC Protein II assay kit, BioRad, Richmond, CA).

Experiment 2: This experiment utilized an identical infusion protocol to that described above. Animals were orchidectomized and five days later, infused for two days with the antisense ODN targeted to the NK_3 receptor (aNK_3-1). Control groups consisted of mismatched ODN (mNK_3-1) and vehicle (n = 10 animals/group).

Experiment 3: This experiment determined the effects of ODN infusion targeting two different regions of the NK_3 receptor on serum LH in female rats. The infusions of ODNs started 11 days after ovariectomy (procedures described above). There were two antisense groups (aNK_3-2 and aNK_3-3), two mismatched groups (mNK_3-2 and mNK_3-3) and a vehicle control group (n = 6 animals/group).

Experiment 4: Pilot study with eight 250 g male rats (Sprague-Dawley) that were orchidectomized and seven days later, at 7:30 am, a jugular catheter (Braintree Scientific) was implanted into the vein for blood collection. At 11:00, a baseline blood sample was taken. One group of animals (n = 4) were given a first injection (subcutaneous) of the NK_3 receptor antagonist, SB-222200 (Tocris Cookson Inc.). This antagonist is approximately 60-fold more selective for the NK_2 receptor than NK_3 receptor, and is 100,000-fold more selective for NK_3 than NK_1 (Sarau *et al* 2000). SB-222200 was diluted in PEG-400 to a final volume of 500 µl and a dose of 10 mg/kg. A second group of animals (n = 4) were given injections of vehicle as a control. Blood samples (300 µl) were taken at 15, 30, 45, 90, and 120 minutes after the first injection. At 60 minutes after the first injection, the animals received a second injection of SB-222200 10 mg/kg. Based on previous studies (Sarau *et al* 2000), we used two s.c. injections of 10 mg/kg that were 1 hour apart. After withdrawal of every blood sample, the same volume of physiological saline was replaced, in addition, a heparin-lock solution (20 Units/ml) was used to fill the cannula and avoid coagulation inside the tubing. Collected blood was

refrigerated for an hour and then centrifuged at 3000 rpm for 15 minutes, plasma was stored at –20ºC until RIA.

Experiment 5: Twenty female Sprague-Dawley rats of 250-300 g were ovariectomized. On day thirteen after ovariectomy, a catheter (Braintree Scientific) was implanted into the jugular vein for blood collection with a heparin-lock solution (20 U/ml). The following morning, animals received a subcutaneus injection of SB-222200 (n = 10) diluted in PEG-400 at a final volume of 500 μl and at a dose of 20 mg/kg. Blood samples (300 μl) were taken right before the antagonist injection and then at 30, 60 and 90 minutes after injection. Control animals received only vehicle injections (n = 10). After withdrawal of every blood sample, an identical volume of physiological saline was replaced. Collected blood was refrigerated for an hour and then centrifuged at 3000 rpm for 15 minutes, plasma was stored at –20ºC until RIA.

LH Radioimmunoassay: Serum LH was measured in duplicate or triplicate samples by radioimmunoassay using NIDDK kits (intra-assay variation of 6%) kindly provided by Dr. A. F. Parlow. Serum LH concentration was expressed in terms of LH RP-3.

Statistics: Statistical comparisons of serum LH values in experiments 1-3, were performed using one way analysis of variance ($\alpha = 0.05$). Newman-Keuls Methods were used for post-hoc analysis with $\alpha = 0.05$. In experiments 4 and 5, serum LH was

compared between groups using two-way analysis of variance with post hoc analysis set at $\alpha = 0.05$.

Results

Experiment 1: Central infusion of NKB antisense ODNs in orchidectomized rats significantly reduced serum LH to 4.08 ng/ml ± 0.46 (mean ± SEM) compared to mismatched NKB (5.49 ng/ml ± 0.44) or vehicle (6.4 ng/ml ± 0.77. Figure 4.1). NKB concentrations in the arcuate/median eminence were lower in the rats receiving antisense NKB ODN (148.3 pmol/g ± 21.490) compared to mismatched NKB ODN (187.8 pmol/g ± 45.9) although this difference did not achieve statistical significance. After two days of continuous infusion, there was no difference in body weight between experimental and control groups.

Experiment 2: Two days of infusion of an antisense ODN targeted to the NK_3 receptor (aNK_3-1) did not alter serum LH compared with mismatched ODN (mNK_3–1) and vehicle (aNK_3-1, 6.8 ng/ml ± 0.58; mNK_3-1, 5.3 ng/ml ± 0.73; vehicle, 5.9 ng/ml ± 1.08, mean ± SEM).

Experiment 3: After two days of infusion of two different antisense ODNs targeted to different regions of the NK_3 gene, serum LH concentrations were not significantly different from controls. Serum LH concentrations were 6.75 ± 1.08 ng/ml in rats receiving aNK_3-2 compared to 6.93 ± 1.32 ng/ml (mNK_3-2 group) and 4.59 ± 0.54 ng/ml

(vehicle control). Similarly, two days of aNK_3-3 resulted in serum LH concentrations (6.49 ± 0.55 ng/ml) that were not significantly different than mNK_3-3 (5.25 ± 0.52 ng/ml) and vehicle control.

Experiment 4: We compared serum LH concentrations of rats injected with SB-222200 with concentrations of rats injected just with the vehicle (Table IV). Two injections of the antagonist of NK_3 receptor, SB-222200 into orchidectomized rats, did not alter significantly serum LH.

Experiment 5: The injection of the antagonist of NK_3 receptor, SB-222200 into ovariectomized rats, did not alter significantly serum LH. We compared serum LH concentrations of rats injected with SB-222200 with concentrations of rats injected with vehicle alone. We did not find a statistically significant change in serum LH between the treatment groups at any time after injection (Table V).

Discussion

Luteinizing hormone secretion in both males and females is strongly influenced by negative feedback effects of gonadal steroids. Steroid negative feedback has been classically demonstrated by removal of the gonads, which results in a marked increase in LH secretion from the anterior pituitary gland, and steroid replacement, which suppresses LH secretion back to intact levels. In parallel with the rise and fall of LH secretion after gonadectomy and steroid replacement, there is a rise and fall in NKB gene expression

(Danzer *et al* 1999,Sandoval-Guzmán *et al* 2003). Furthermore, NKB gene expression is increased in the human hypothalamus in association with the gonadal failure of menopause (Rance and Young 1991). These findings, and the high degree of estrogen receptor colocalization (Goubillon *et al* 2000b,Rance and Young 1991), suggest that NKB neuron activity contributes to the rise in LH secretion after gonadectomy.

In the present study, we showed that central infusion of antisense ODN to downregulate NKB, results in a decreased serum LH in orchidectomized rats. These data support our hypothesis that NKB may exert a stimulatory effect on gonadotropin secretion in the absence of gonadal steroids. The reduction of LH by the antisense infusion of NKB is not simply due to a nonspecific effect of ODN infusion, because the mismatched ODN infusion had no effect on serum LH.

The finding that antisense NKB ODN infusion inhibits LH secretion provides indirect evidence that NKB may stimulate LH secretion in the absence of gonadal steroids. These data are in contrast to our previous study, in which the injection of senktide, a specific NK_3 receptor agonist, inhibited serum LH in ovariectomized estrogen-treated rats. The contrasting effects of NKB on LH secretion is typical of the effects of other hypothalamic regulatory peptides on LH secretion. For example, neuropeptide K (NPK) inhibits LH release in orchidectomized rats (Sahu and Kalra 1992), but exerts a stimulatory action on LH release in intact males (Kalra *et al* 1992). Another example is substance P (SP), which suppresses LH when administered to

ovariectomized rats but increases LH release in ovariectomized estrogen-primed rats (Arisawa *et al* 1990). Similarly, whether NPY has a positive or negative effect on LH secretion varies, depending on the steroid environment (Kalra *et al* 1992b). Therefore, the apparent contradictory effects of NKB on LH secretion is in keeping with the effects of numerous other regulatory peptides on the reproductive axis.

We were unable to demonstrate that antisense ODN infusion reduced NKB peptide concentration in arcuate/median eminence tissue. Although the levels of NKB were lower in the antisense group, this finding was not statistically significant. There may be several explanations for this finding. Specific data on NKB turnover is lacking, making difficult to predict the time course of NKB pool depletion that would occur after translation arrest. Thus, it is possible that antisense ODNs might not affect old reservoirs of the peptide and a smaller amount of the newly synthesized NKB may be responsible for gonadotropin secretion. In addition, the arcuate nucleus receives tachykininergic innervation from other brain areas, including the brainstem (Magoul *et al* 1993), that are farther away from the site of infusion or the ventricles. Thus, the detection of a change in peptide content may be masked by the presence of distant NKB fibers projecting to the arcuate nucleus. Finally, the NKB antibody supplied in the commercial RIA kit may have cross-reacted with other tachykinins, and may have overwhelmed the ability to detect a specific effect on NKB.

In our second set of studies, we used antisense methodology to target NK_3, the receptor for NKB. In contrast to the effects of targeting the NKB peptide, we found no significant change in serum LH after infusion of an antisense NK_3 receptor ODN. One explanation for this negative result is that the endogenous mRNA is folded, and the antisense NK_3 ODN targeted an inaccessible mRNA sequence. Therefore, we designed two additional ODNs to target different parts of the NK_3 gene. These ODNs also had no effect when infused into gonadectomized female rats. Thus, three different antisense ODNs targeting the NK_3 receptor had no effect on serum LH. The possibility that the knockdown was not sufficient or that disrupting the translation of NK_3 does not alter the already expressed receptor cannot be ruled out. Alternatively, these data may indicate that the stimulatory effect of NKB on gonadotropin secretion is mediated through a receptor other than NK_3.

To explore the possibility that NK_3 might not be the receptor involved in the simulation of LH secretion by NKB in gonadectomized animals, we conducted experiments using a more traditional pharmacological approach. We infused a highly specific NK_3 receptor antagonist, SB-222200. This nonpeptidergic antagonist penetrates the blood brain barrier and thus could be administered via subcutaneous injections (Michl *et al* 2001,Sarau *et al* 2000). Our pilot experiments showed no significant effect of SB-222200 on serum LH in gonadectomized male rats. Similarly, in gonadectomized females, SB-222200 did not alter LH secretion for up to 90 minutes after injection.

These data support the hypothesis that the stimulatory effects of NKB are through a receptor other than NK_3.

In Chapter Three, we presented evidence that the NK_3 receptor was involved in inhibition of LH release in ovariectomized estrogen-treated rats. In contrast, in the current chapter, we present evidence that in gonadectomized rats, the effects of NKB on serum LH are mediated by receptors other than NK_3. These data suggests that the effect of NKB in gonadotropin secretion is mediated through different receptors, depending on the steroid environment. That is, when the effect is inhibitory (in gonadectomized estrogen-treated rat), NK_3 mediates the response, and when the effect is stimulatory (in gonadectomized rat), other tachykinin receptors may mediate the response. This is plausible because the three major tachykinin receptors are not highly selective (Helke *et al* 1990,Maggi 1995a). NKB can bind and activate NK_1 receptor with a relatively high affinity (Maggi and Schwartz 1997) and NK_1 is located in the central nervous system and (Tsuchida *et al* 1990) and the anterior pituitary gland (Winkler *et al* 1995). NK_2 receptors are primarily located outside the CNS but are also located in the anterior pituitary gland (Pisera *et al* 2003,Tsuchida *et al* 1990). Thus, NKB could modulate gonadotropin function through the NK_1 or NK_2 receptors. Alternatively, NKB could be modulating LH secretion through a tachykinin receptor that has not yet been described. For example, there has been an additional NK_3 receptor isolated from human brain called the NK_3 receptor homologue (Krause *et al* 1997). This receptor has not yet been characterized in other species.

In summary, we provide indirect evidence that NKB stimulates serum LH in the gonadectomized rat, but this stimulation may not involve the NK_3 receptor. These findings are important for understanding the possible contribution of NKB neurons to regulation of the reproductive axis. Ultimately, these data will provide insight into the physiological relevance of the increase in NKB gene expression in postmenopausal women.

Figure 4.1 Serum LH concentrations of gonadectomized rats infused with an antisense oligonucleotide targeted to NKB, a mismatch oligonucleotide or the vehicle. The bars show the group mean $\pm$ SEM.

* Significantly different from mismatch and vehicle, P = < 0.001.

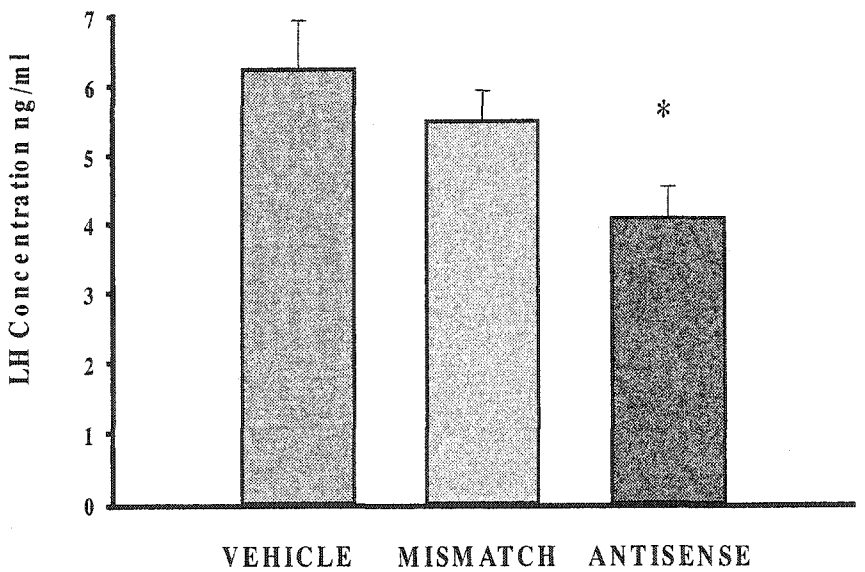
LH Concentration ng/ml
7
6
5
4
3
2
1
0
*
VEHICLE
MISMATCH
ANTISENSE

Table IV. Mean serum LH concentration of gonadectomized rats injected with the NK_3 antagonist, SB-222200. Values are expressed in ng/ml ± SEM.

Minutes after first injection	Vehicle	SB-222200
0 (before First injection)	4.59 ± 0.54	6.52 ± 0.65
15	3.87 ± 0.56	6.07 ± 0.59
30	3.73 ± 0.23	5.89 ± 0.75
45	3.93 ± 0.32	5.80 ± 0.38
60 (Second injection)	NA	NA
90	4.21 ± 1.12	5.38 ± 0.34
120	4.47 ± 0.82	6.02 ± 0.89

Table V. Mean serum LH concentration of ovariectomized rats injected with the NK_3 antagonist, SB-222200. Values are expressed in ng/ml ± SEM.

Minutes after first injection	Vehicle	SB-222200
0	7.91 ± 0.48	7.7 ± 0.36
30	8.64 ± 0.34	8.02 ± 0.56
60	7.66 ± 0.5	6.98 ± 0.48
90	7.09 ± 0.58	6.79 ± 0.72

Data analyzed using two way analysis of variance with $\alpha = 0.05$.

SUMMARY

Ovarian steroid hormones influence a variety of processes essential for the functioning of the central nervous system. They induce long-term changes in neuronal structure (organizational effects) and rapid changes in gene expression, protein synthesis and electrical properties (activational effects). Steroid secretion from the gonads also regulates hypothalamic GnRH activity for the maintenance of reproductive cyclicity.

In humans, reproductive aging is characterized by a gradual decrease in the ovarian follicular pool, leading to loss of menstrual cycles, a decline in fertility and depletion of ovarian steroids. The postmenopausal loss of ovarian steroids is accompanied by remarkable changes in the human brain. There is an increase in GnRH gene expression in a subpopulation of neurons within the medial basal hypothalamus (Rance and Uswandi 1996). Estrogen replacement decreases GnRH gene expression in ovariectomized monkeys, suggesting that the changes in GnRH neurons observed in postmenopausal women are related to ovarian failure (Krajewski *et al* 2003).

In addition to changes in GnRH neurons in postmenopausal women, hypertrophy occurs in the hypothalamic infundibular (arcuate) nucleus. The hypertrophied neurons display morphologic features of activated neurons such as enlarged nuclei and nucleoli, and increased Nissl substance (rough endoplasmic reticulum). These neurons express neurokinin B (NKB), substance P (SP) and estrogen receptor (ER) mRNA and the

changes in neuronal size are accompanied by increased in NKB gene expression. The projections and the function of the hypertrophied NKB neurons in the infundibular nucleus are not known. However, in primates, ewes and rats, these neurons are highly responsive to steroids suggesting a regulatory role for NKB in the reproductive axis (Abel *et al* 1999,Danzer *et al* 1999,Pillon *et al* 2003).

Proopiomelanocortin (POMC) mRNA, an opioid precursor, decreases in the infundibular nucleus of postmenopausal women (Abel and Rance 1999). Among the POMC products, β-endorphin is an inhibitory signal for GnRH gene expression (Wardlaw and Ferin 1990). Thus, the decrease of POMC in postmenopausal women could contribute to the elevation of GnRH and gonadotropin hypersecretion.

Two important questions arise from this background. Are the changes in neurokinin B gene expression in the postmenopausal human hypothalamus secondary to ovarian failure? Do neurokinin B neurons participate in the hypothalamic circuitry regulating the reproductive axis? We addressed the first question in chapter two, and the second question in chapters three and four.

Monkeys are commonly used as models for humans because they have similar endocrine profiles and symptomology. In chapter two, we described experiments using ovariectomized young cynomolgus monkeys to mimic human ovarian failure. *In situ* hybridization and computer microscopy was used to measure changes in GnRH, NKB

and POMC gene expression and cell morphology after ovariectomy. The medial basal hypothalamus (MBH) was examined because this is the integrative center of reproduction in the primate. We also monitored leptin in serum and body weight because of the participation of POMC neurons in energy balance.

We found that NKB neurons in the infundibular nucleus of ovariectomized monkeys were larger, more numerous and displayed increased levels of NKB mRNA compared to those of intact controls. Moreover, ovariectomy increased the number of neurons expressing GnRH gene transcripts and elevated serum LH. In contrast, the parameters related to energy balance, including POMC gene expression, serum leptin and body weights, were unchanged by ovariectomy. Thus, the rise in NKB and GnRH gene expression in older women was simulated by ovariectomy in monkeys but the changes in POMC gene expression and energy balance were not as they do in aged humans (Abel and Rance 1999,Messinis *et al* 1999). This study provides strong support for the hypothesis that ovarian failure contributes to the increased NKB and GnRH gene expression observed in postmenopausal women. Gene expression of POMC neurons was unchanged after long-term ovariectomy in contrast to the decreased POMC gene expression in postmenopausal women. This result suggests that the decreased POMC gene expression in older women is due to other factors such as aging. However, the possibility of a species difference or modulation of the response to steroids by aging could not be excluded.

This study provides important insights in the understanding of the CNS after ovarian failure. Hormone replacement therapy is administered to prevent cardiovascular disease, weight gain, osteoporosis and degenerative brain diseases (Speroff 1993). However, controversy exists in terms of the efficacy of the hormone therapy for the broad physiological events postmenopausal women experience.

In Chapter Three, we tested the hypothesis that NKB participates in the reproductive axis regulating gonadotropin secretion. We determined if intracerebral injection of a selective NK_3 receptor agonist (senktide) altered serum LH in the ovariectomized estrogen-treated rat. We also compared the effect of senktide injection to that of Neuropeptide Y (NPY), a well-characterized modulator of LH secretion (Kalra and Crowley 1984). Senktide, NPY or vehicle was injected into the lateral ventricle of awake, freely moving rats and serial blood samples were collected for LH radioimmunoassay. The rats were sacrificed 90 minutes after injection and the brains removed and processed for Fos immunocytochemistry. Intracerebral injection of the agonist senktide decreased serum LH to concentrations comparable to those that NPY injection produced. This inhibition was accompanied by significant changes in Fos expression in various hypothalamic nuclei. We found that although both senktide and NPY decreased serum LH to similar concentrations, they produced different patterns of cell activation. When the rats were injected with senktide we observed Fos activation in MPO, SON, PVN and AN. These areas contain the NK_3 receptor as shown by previous

studies (Mileusnic *et al* 1999b). When the rats were injected with NPY we observed Fos in the PVN and SON. The nuclei activated after senktide injection participate in pituitary and hypothalamic endocrine regulation. This study provides evidence that stimulation of the NK_3 receptor inhibits LH secretion possibly through activation of hypothalamic neurons.

Although we used a synthetic agonist of the NK_3 receptor, we propose that activation of the receptor *in vivo* is mainly due to NKB since the NK_3 receptor is the most selective of the tachykinin receptors (Maggi and Schwartz 1997). The distribution patterns of Fos expression after senktide injection is the same even when using different concentrations of senktide (Ding *et al* 2000), which suggests that the activation we observed in our experiment is not the result of a high dose. To better explain the mechanism of gonadotropin regulation by senktide, future studies will be necessary to define the neurotransmitter content of the activated neurons. A direct effect of senktide on the pituitary gland or on GnRH neurons also cannot be ruled out.

In Chapter Four, we further investigated the role of NKB and its receptor in gonadotropin regulation. We designed an antisense oligonucleotide (ODN) targeted to the coding region of the NKB gene. The antisense was infused for two days to determine if down-regulation of NKB would affect LH secretion the gonadectomized rat. Luteinizing hormone was decreased by 25% in the group of rats that received the antisense ODN targeted to NKB. This result suggests that NKB may stimulate LH

secretion in the gonadectomized rat, and agrees with our hypothesis that NKB contributes to gonadotropin hypersecretion after steroid withdrawal.

In contrast to the apparent stimulatory effect of NKB on LH secretion in gonadectomized rats, in Chapter Three, we demonstrated an inhibitory effect of NK_3 receptor activation on LH secretion in the ovariectomized, estradiol-treated rat. These opposing effects may be due to the different steroid environment between the two experiments. Several peptides that affect gonadotropin secretion, including substance P, neurokinin A, neuropeptide K, neuropeptide Y and galanin, also have different effects depending on the ovarian steroid milieu (Sahu *et al* 1992, Kalra *et al* 1992, Arisawa *et al* 1990, Sahu *et al* 1987). We hypothesize that these divergent effects are mediated through different receptors or a different site of action depending on the steroid environment. Other factors, such as gender or circadian rhythms, may also play a role in possible inhibitory versus stimulatory effects of neuropeptides on LH secretion

In the second part of Chapter Four, we explored the role of the NK_3 receptor on LH secretion in gonadectomized rats. We designed two different antisense oligonucleotides targeted to the NK_3 receptor gene and infused them in an animal model of increased NKB, the gonadectomized rat. After two days of continuous antisense infusion we measured serum LH concentration. We found that in both, male and female gonadectomized rats, downregulation of the NK_3 receptor did not altered LH concentration. Furthermore, we injected a selective NK_3 receptor antagonist, into the

lateral ventricle of gonadectomized rats. The antagonist SB-222200, is a potent and selective nonpeptide tachykinin receptor antagonist that crosses the blood brain barrier in the rat (Sarau *et al* 2000). However, we found that injection of the antagonist had no effect on LH release. Thus far, our results suggest that in the gonadectomized rat, NKB has a stimulatory effect on gonadotropin secretion and this stimulation seems to be through a different receptor than NK_3. More experiments are needed to substantiate these findings.

The tachykinin receptors and their affinity for endogenous and synthetic ligands has been extensively studied (Helke *et al* 1990,Maggi 1995b,Maggi and Schwartz 1997). Interestingly, the absence of one neurokinin receptor can be compensated by the presence of the other two receptors. To determine the participation of each tachykinin receptor it may be necessary to test the effects of specific antagonists for each of the three neurokinin receptors. In addition, administration of a combination of antagonists might be necessary to clarify the contribution of crossreactivity of tachykinins and its receptors in gonadotropin regulation. The effects of tachykinin receptor antagonists may be also modified by gonadal steroids. Regulation of tachykinin receptors by estradiol has been studied in peripheral tissues. In the rat uterus, estradiol stimulates the NK_1 receptor, decreases the NK_3 receptor but has no effect on NK_2 (Pinto *et al* 1999,Villablanca and Hanley 1997). Thus, ovarian steroids differentially regulate neurokinin receptors in the periphery, however, it remains to be determined if ovarian steroids modulate tachykinin

receptors in CNS. Changes in the tachykinin receptors after the ovarian failure of menopause might provide insights into consequences of steroid withdrawal in the CNS.

The finding that NKB regulates LH sheds light on previous studies of changes with NKB gene expression and provides direction for future studies. The physiological relevance of these findings lies in the high probability that arcuate NKB neurons are an important site for steroid feedback. However, it remains to be determined if NKB regulates LH secretion through direct actions on GnRH neurons, via pituitary gonadotrophs or indirectly through hypothalamic neural circuits.

The dramatic increase in NKB mRNA after ovarian failure may provide insight into the clinical symptoms of postmenopausal and oophorectomized women. NKB may contribute to the increased GnRH and gonadotropin secretion. The consequences of hypersecretion of gonadotropins are not well understood. There is evidence, however, that in addition to the classical targets of LH action (ovary and testis), other tissues contain functional LH receptor. Luteinizing hormone receptors are found in different brain structures like pyramidal cell layers in the hippocampus, hypothalamus, cerebellum, area postrema, ependyma and choroid plexus of the lateral ventricle. They have also been found in spinal cord where they may have a neurotrophic function. In peripheral tissues LH receptors are also found, including the uterus, placenta, fallopian tubes and lymphocytes, in epithelial cells of normal mammary gland but also in malignant breast tumors and in several cancer lines.

What are other possible consequences of increased NKB mRNA in postmenopausal women? NKB increases blood pressure via the release of vasopressin from the magnocellular neurons of the paraventricular and supraoptic nuclei (Ding *et al* 1999,Polidori *et al* 1989). Another consequence of the release of vasopressin is the inhibition of water and salt intake (Flynn and Smith 1998,Massi *et al* 1988). However, it is not known if these effects are mediated by arcuate NKB neurons. There is a possibility that hypertrophied NKB neurons in the arcuate nucleus regulate parvocellular PVN function as well. These hypotheses are also based in pharmacological and molecular studies that have implicated central NKB in cardiac function, anxiety and water intake (Ding *et al* 1999,Flynn and Smith 1998,Teixeira *et al* 1996). Neurons from dorsal, ventral and posterior parvicellular subnuclei of the PVN project to centers in the brainstem and spinal cord that modulate the activity of the autonomic nervous system (Swanson and Kuypers 1980). Neurons in the medial parvicellular subdivision contain somatostatin, corticotropin-releasing factor and other releasing hormones that send axons to median eminence. Thus, the parvocellular PVN area of hypothalamus is of critical importance for the maintenance of homeostasis. Arcuate neurons project to PVN and the parvocellular PVN is activated after senktide injection (Chapter Three) and expresses NK_3 receptors (Baker and Herkenham 1995). However, to define the role of arcuate NKB neurons it will be necessary to conduct track-tracing experiments to elucidate the projections of this specific group of neurons.

In this dissertation, new insights are provided concerning the hypothalamic changes after ovarian failure and the regulation of gonadotropin release. Most importantly, we provide information indicating a role for NKB in regulation of the reproductive axis. Although there are a considerable number of peptides/neurotransmitters that regulate gonadotropin secretion, it seems likely that NKB is one of the most important modulators of reproduction. Evidence to substantiate this assumption includes (1) Neurokinin B is located in one of the reproductive centers (the arcuate nucleus) of the hypothalamus; (2) this subpopulation of neurons has the highest degree of estrogen receptor coexpression (3) the NKB neurons are exquisitely responsive to ovarian steroids. Furthermore, the changes in NKB gene expression in postmenopausal women indicate that these data would apply for the understanding of human physiology. The intense study of NKB, its projections and function may provide important information on brain modulation by steroids and the central control of reproduction.

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