LOCALIZATION OF TACHYKININS AND THEIR RECEPTOR mRNAs

IN THE HUMAN HYPOTHALAMUS AND BASAL FOREBRAIN

by

Monica Kapoor Chawla

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF CELL BIOLOGY AND ANATOMY

In Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1996

LOCALIZATION OF TACHYKININS AND THEIR RECEPTOR mRNAs IN THE HUMAN HYPOTHALAMUS AND BASAL FOREBRAIN

by

Monica Kapoor Chawla

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF CELL BIOLOGY AND ANATOMY

In Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1996

UMI Number: 9713402

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Monica Kapoor Chawla

entitled Localization of Tachykinins and their Receptor mRNAs in the Human Hypothalamus and Basal Forebrain

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Paul A. St. John

Paul A. St. John Ph.D. — Date 8/1/96

Jean M. Wilson

Jean M. Wilson Ph.D. — Date 8/1/96

Mark E. Wise

Mark E. Wise Ph.D. — Date 8/1/96

Naomi Rance

Naomi E. Rance M.D., Ph.D. — Date 8/1/96

Nathaniel McMullen

Nathaniel McMullen Ph.D. — Date 8/1/96

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Naomi Rance

Dissertation Director — Date 8/6/96

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation form or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or job judgement the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

Signed: Monica Chawla

ACKNOWLEDGEMENTS

I would like to give my heartfelt thanks to Dr. Naomi Rance, who as my advisor provided not only the expert guidance but also her support and friendship throughout my graduate training, for which I will always be grateful.

I thank all my committee members, Dr. Nathaniel McMullen, Dr. Jean Wilson, Dr. Mark Wise and Dr. Paul St John for their advice regarding my research project and career.

I thank my parents, Inder Prakash Kapoor and Sita Kapoor for instilling in me the value of higher education regardless of gender or race.

My daughters Sarina and Monita have shown tremendous understanding for which I am very grateful. And lastly thanks to my husband Sudesh Chawla without whom none of this would have been possible. His encouragement and support were essential in this endeavor.

TABLE OF CONTENTS

TABLE OF CONTENTS-Continued

TABLE OF CONTENTS-Continued

LIST OF ILLUSTRATIONS

LIST OF TABLES

ABSTRACT

Numerous studies in experimental animals have emphasized the importance of tachykinin peptides in hypothalamic function. There is however, little information on the location of these peptides in the human brain. In the first study, *in situ* hybridization was used to map the distribution of neurons expressing the substance P (SP) or neurokinin B (NKB) gene transcripts. The distribution of neurons containing tachykinin mRNAs was found to be distinct and complementary: SP was the predominant tachykinin in the striatum, posterior hypothalamus and intermediate, ventromedial and mammillary nuclei; there were more NKB mRNA containing neurons than SP neurons in the magnocellular basal forebrain, the bed nucleus, and the preoptic/anterior hypothalamic regions. Comparable numbers of neurons expressing both mRNAs were identified in the infundibular nucleus and amygdala.

Because numerous neurons containing NKB mRNAs were identified in the nucleus basalis of Meynert, it was next determined if NKB mRNA and choline acetyltransferase (ChAT) mRNAs are colocalized in this region. It was found that approximately 30% of the cholinergic neurons in the nucleus basalis also expressed NKB gene transcripts. This is the first identification of peptide colocalization in a significant population of magnocellular cholinergic neurons in the human basal forebrain. The nucleus basalis of Meynert plays an important role in higher brain functions in humans.

There is considerable evidence suggesting that SP and gonadotropin releasing hormone (GnRH) neurons are anatomically and functionally connected in the human brain. In this study, double *in situ* hybridization with ^{35}S-labeled SP receptor (SPR) and digoxigenin-labeled GnRH riboprobes was used to determine if GnRH neurons contain

SPR mRNAs. The radiolabeled GnRH riboprobe hybridized with scattered neurons in the preoptic-septal regions and medial basal hypothalamus. A digoxigenin-labeled GnRH probe labeled cells in the medial basal hypothalamus, the primate control center for reproduction. SPR mRNA was identified in numerous magnocellular basal forebrain neurons, however, GnRH neurons containing SPR mRNAs were not identified. Although the possibility that SPR mRNA may be present in these cells but below the level of detection remains, present data suggests that a link between SP and GnRH neurons does not exist in the human brain.

CHAPTER ONE

INTRODUCTION

In 1931, Von Euler and Gaddum isolated a factor from equine intestine which produced contraction of smooth muscle in the rabbit jejuneum. They named this factor Substance P (SP) (Von Euler and Gaddum, 1931) and unlike aceytylcholine, the spasmogenic activity of SP could not be blocked by atropine (Von Euler and Gaddum, 1931). SP was postulated to be a protein or peptide, but it would take almost 40 years before it was subsequently sequenced and characterized by Leeman et al. who were in the process of isolating a corticotropin releasing factor from bovine hypothalamus (Chang and Leeman, 1970; Chang et al. 1971). Early studies used bioassays of crude extracts from brain and intestine to elucidate the physiological properties of SP, but these studies were complicated by the presence of other tachykinins (Maggio and Mantyh, 1994). The isolation and synthesis of SP has permitted the development of methods for its detailed biochemical, histochemical, physiological, and pharmacological characterization. SP is now known to be an important neurotransmitter in the mammalian central as well as peripheral nervous system (Maggio, 1988).

In 1949, Esparmer isolated eledoisin from the posterior salivary gland of the Mediterranian octopus (Esparmer, 1949). This substance proved to have hypotensive, sialogogic and spasmogenic activities similar to SP. This research paved the way for the discovery of other non-mammalian tachykinins such as physalaemin (Esparmer et al. 1964) and kassinin (Anastasi et al. 1977). While classifying the non-mammalian peptides into tachykinins, bradykinins and bombesins, Esparmer proposed that most of peptides isolated from frog skin had counterparts in mammalian tissues. In 1983-1984, the existence of novel mammalian tachykinins was reported independently by four groups of researchers (Kimura et al. 1983; Nawa et al. 1983; Kangawa et al. 1983; Minamino et al. 1984). Two homologues of SP were described, neurokinin A and neurokinin B. These were decapeptides with the characteristic carboxy-terminal sequence Phe-X-Gly-Leu-Met-NH_2 common to all tachykinins.

Molecular Biology of Mammalian Tachykinins

Recent advances in molecular biology have revealed that the mammalian tachykinins are derived from 2 separate genes, preprotachykinin I (PPT-A) and preprotachykinin II (PPT-B) (Nakanishi, 1987; Krause et al. 1989). The PPT-A gene is approximately 8.4 kb in length and is comprised of seven exons interrupted by 6 intron regions. Exons 2-7 translate into 6 proteins including the signal peptide, SP, two spacer sequences, substance K, and the carboxy-terminal respectively. The PPT-A gene transcribes into 3 different mRNA sequences by alternate mRNA splicing and tissue specific processing, alpha, beta and gamma-PPT-A mRNAs. All three mRNAs can be translated into SP while only beta and gamma-PPT-A forms can be processed into neurokinin A (NKA). Neuropeptide K is derived from beta-PPT-A mRNA while neuropeptide gamma as the name indicates, is derived from gamma-PPT mRNA (Nawa et al. 1984; Nakanishi, 1987; Otsuka and Yoshioka, 1993).

Neurokinin B (NKB) is processed from an entirely different, although structurally related PPT-B gene (Nakanishi, 1987). The PPT-B gene is ~ 8 kb in length and contains 7 exon regions. Although the PPT-B gene is transcribed into 2 mRNA species by the differential usage of promoter sites and alternate RNA spicing, it codes for only one tachykinin peptide (NKB). Both the PPT-A and PPT-B genes seem to have evolved from a common ancesteral gene by duplication as they are structurally similar in the genomic arrangements for gamma-PPT-A mRNA and for the major NKB coding mRNA sequence. Both mRNA sequences are coded by the first, second, fourth, fifth and sixth genomic segments corresponding to the 5'-untranslated region, a signal peptide, a spacer region, the tachykinin sequence, and the carboxy-terminal region. Therefore, not only are the introns similar but the exon sizes are identical between the two genes (Nakanishi, 1987; Krause et al. 1990).

Tissue Distribution of Tachykinins

Because of the diverse functions of tachykinins, there have been numerous studies of the distribution of these peptides in the rat central nervous system using either immunohistochemical (Ljungdahl et al. 1978; Inagaki et al. 1982; Jonakait et al. 1991; Cuello et al. 1985; Marksteiner et al. 1992a; Marksteiner et al. 1992b; Lucas et al. 1992) or *in situ* hybridization (Warden and Young, III, 1988; Harlan et al. 1989; Marksteiner et al. 1992a; Marksteiner et al. 1992b; Lucas et al. 1992) techniques. The early studies were based upon immunocytochemical and radioimmunoassay methods. Because there is considerable homology between the tachykinin peptides due to the shared carboxy-terminal sequence, it was difficult to separate the distribution pattern of SP from other tachykinins using these methods. To circumvent the problem of antibody cross-reactivity, more recent studies in rats have utilized *in situ* hybridization (Warden and Young, III, 1988; Burgunder and Young, III, 1989a; Harlan et al. 1989; Lucas et al. 1992; Zhang and Harlan, 1994). In contrast to the numerous studies in rats, there have been few reports of the distribution of tachykinin-immunoreactive neurons in human brain (Bouras et al. 1986; Mai et al. 1986; Sakamoto et al. 1987). Furthermore, there have been no studies of the location of tachykinin precursor mRNAs in the human.

In the rat central nervous system, radioimmunoassay studies have shown that SP is unevenly distributed, with the highest levels found in the mesencephalon, hypothalamus, preoptic area, spinal cord and brainstem (Pernow, 1983). Within the telencephalon, SP immunoreactivity is present in the olfactory tubercle, amygdala, hippocampus, caudate, putamen, globus pallidus and septum (Pernow, 1983). SP-immunoreactive cells were also identified in the medial hypothalamus, habenula and thalamic nucleus of the rat diencephalon (Ljungdahl et al. 1978; Pernow, 1983). Numerous cells containing SP mRNA were observed in layer II of the olfactory tubercle, in the islands of Calleja, nucleus accumbens, caudate and putamen (Harlan et al. 1989; Warden and Young, III, 1988). Cells containing SP mRNA were also detected in the lateral and medial septum, bed nucleus of stria terminalis, medial and cortical nuclei of amygdala and the hippocampus. In the diencephalon, SP mRNA was identified in the anterior hypothalamus, retrochiasmatic area, ventromedial, arcuate, dorsal pre-mammillary, supramammillary, submammillothalamic and posterior hypothalamic nuclei (Ljungdahl et al. 1978; Warden and Young, III, 1988; Harlan et al. 1989).

In the human, SP immunoreactive fibers were detected in the olfactory bulb, amygdala, hippocampus, caudate, putamen, globus pallidus and septum. Few SP immunoreactive cell bodies, however, were identified (Pernow, 1983; Cooper et al. 1981; Mai et al. 1986). Small numbers of SP immunoreactive cells were identified in the medial and lateral hypothalamus, ventromedial nucleus, supramammillary region, infundibular nucleus, mammillary bodies, and thalamus (Cooper et al. 1981; Mai et al.

1986). A dense plexus of SP immunoreactive fibers surrounding the blood vessels was been reported in the non-human primate median eminence (Hökfelt et al. 1978).

There have been relatively few studies on the distribution of NKB in the central nervous system. In rats, NKB mRNA containing cells were detected in the cerebral cortex, hippocampus, amygdaloid complex, bed nucleus of stria terminalis, ventral pallidum, habenula, medial preoptic area, arcuate nucleus, diagonal band of Broca, nucleus accumbens and lateral mammillary bodies (Warden and Young, III, 1988; Burgunder and Young, III, 1989b; Lucas et al. 1992). Immunochemical detection for NKB cell bodies using an antibody directed against the amino-terminal portion of the precusor protein was consistent with the *in situ* hybridization data (Marksteiner et al. 1992a; Lucas et al. 1992).

Because of the paucity of data regarding the distribution of tachykinins in the human we decided to perform a detailed analysis of the distribution of SP and NKB mRNAs.

Functions of Tachykinins

Physiological studies have revealed that tachykinins have widespread actions in the central and peripheral systems. SP was first shown to have pharmacological effects producing smooth muscle contraction in the intestine and lowering of blood pressure [for reviews see (Pernow, 1983; Otsuka and Yoshioka, 1993)]. Tachykinins have been proposed to be important in the transmission of sensory signals particularly nociceptive (Pernow, 1983). SP is increased in the dorsal horn by noxious stimuli consistent with its participation in nociception (McCarson and Krause, 1994; Brown et al. 1995). SP and NKA evoke a slow depolarization of ganglion cells in the inferior mesenteric ganglion upon stimulation of peripheral visceral c-afferents, and thus may participate in regulation of intestinal motility (Otsuka and Yoshioka, 1993). Tachykinins may also be important in the integration of sensory and motor signals by the extra-pyramidal motor system which includes the striatum and substantia nigra (Pernow, 1983; Otsuka and Yoshioka, 1993). The rodent striato-nigral tract contains a high density of SP immunoreactive fibers which terminate in a dense plexus in the substantia nigra (Ljungdahl et al. 1978). SP and NKA also excite a subpopulation of striatal neurons to evoke the release of dopamine (Petit and Glowinski, 1986; Baruch et al. 1988). In addition, dopamine pathways exert negative feedback effects on tachykinin gene expression via D1 and D2 receptor subtypes in the straitum (Haverstick et al. 1989; Young, III et al. 1986).

Tachykinins may also participate in learning and memory via input to the nucleus basalis of Meynert, an important region implicated in these processes (Murray and Fibiger, 1985; Ridley et al. 1986; Dekker et al. 1991; Dunnett , and Fibiger, 1993; Wainer et al. 1993; Nabeshima, 1993). The nucleus basalis is innervated by a dense network of SP-immunoreactive fibers (Haber and Watson, 1985) and SP-immunoreactive nerve terminals appear closely associated with magnocellular neurons in the human nucleus basalis (Beach et al. 1987). In the rat, ultrastructural studies have confirmed the presence of SP-immunreactive terminals on cholinergic neurons (Bolam et al. 1986; Zaborszky et al. 1991). SP receptor mRNA is also expressed by basal forebrain cholinergic neurons in rats (Gerfen, 1991) and SP receptor immunoreactivity is present in a subset of human magnocellular basal forebrain neurons. Electrophysiological studies have demonstrated an excitatory action of SP on cultured cholinergic basal forebrain neurons (Yamaguchi et al. 1990; Nakajima et al. 1991) and injections of SP in the rat nucleus basalis improves memory (Huston and Oitzl, 1989). In patients with Alzheimer's Disease, SP levels are reduced approximately 20-40% in the cerebral cortex and hippocampus (Murphy, 1992).

SP has also been implicated in the regulation of numerous reproductive functions including gonadotropin secretion, female lordosis and male sexual behavior. These functions in turn are modulated by the levels of gonadal steroids. One important site for the regulation of sexual behavior is the ventromedial nucleus of the hypothalamus. There are many SP immunoreactive neurons in the ventromedial nucleus that contain estrogen receptors (Akesson, 1994). These neurons project to the dorsal midbrain periaquaductal grey and injections of SP into this region facilitates lordosis in the female (Yamano et al. 1986; Dornan et al. 1987; Dornan and Malsbury, 1989; Akesson et al. 1994). In addition, estrogen regulates tachykinin immunoreactivity in the ventromedial nucleus. For example, estrogen replacement in ovariectomized rats increases tachykinin immunoreactivity in the ventromedial nucleus (Akesson, 1994). Male sexual behavior such as mounting frequency, intromission, and ejaculation are mediated by a complex circuitry involving the olfactory bulb, amygdala, hypothalamus. In addition, brainstem nuclei coordinate the motor and autonomic functions essential for copulatory behavior (Meisel and Sachs, 1994). Tachykinins are decreased in these areas following castration, providing evidence that these peptides affect motor and motivational aspects of male sexual behavior (Akesson and Micevych, 1995).

Intraventricular injections of SP, NKA or NKB results in increased blood pressure and heart rate, sympathetic efferent activity, visceral vasocontriction and hindlimb vasodilation (Hall et al. 1989; Nagashima et al. 1989b; Nagashima et al. 1989a; Itoi et al. 1994). The same response can be elicited by microinjecting SP in the ventromedial, paraventicular or anterior hypothalamus, areas that are considered to be centers for integrating neuroendocrine, autonomic and behavioral responses (Swanson and Sawchenko, 1983). SP is also a potent systemic vasodilator (Eklund et al. 1977).

Tachykinin Receptors

Pharmacological and radioligand binding studies have revealed heterogeneity among the tachykinin receptors with varying rank orders of agonist potencies in different tissues and distinct patterns of tissue binding sites (Nakanishi, 1987; Regoli et al. 1987; Regoli et al. 1989). Definitive proof for the subtypes of tachykinin receptors, however, have come from receptor subcloning studies. Three mammalian receptors have been subcloned and sequenced thus far: NK1, NK2 and NK3 receptors specific for SP, NKA and NKB respectively (Nakanishi, 1991; Takahashi et al. 1992; Ohkubo and Nakanishi, 1991).

Amino acid sequences of the 3 rat neurokinin receptors (molecular weight = 46, 44, and 51 Kd) reveals that they are small proteins containing 407, 390 and 452 amino acid residues, respectively. Studies of rat and human tachykinin receptors (Gerard et al. 1990; Buell et al. 1992; Gerard et al. 1993) have shown that there is significant sequence homology with members of the G-protein linked receptor family. They all contain seven hydrophobic transmembrane domains composed of 20-25 uncharged amino-acid residues with the amino-terminus situated extracellularly and the carboxy-terminal in the cytoplasm. In addition, all three receptors possess *N*-glycosylation sites at the amino-termini and numerous serine and threonine residues as possible phosphorylation sites at the carboxy-termini. The highly conserved sequences are the transmembrane domains, especially the seventh. The three tachykinin receptors interact with G-proteins via the basic residues in the third and fourth cytoplasmic loops (Nakanishi, 1987; Shigemoto et al. 1990; Buell et al. 1992). The activation of G-proteins causes an increase in inositol phosphates leading to an accumulation of intracellular calcium.

The genes for the human tachykinin receptors have shown significant homology in their structure. For example, all three receptor subtypes are composed of five exons interrupted with introns at analogous positions. The size of NK1 receptor gene is approximately 60 kb, NK2 is approximately 12 kb and NK3 is approximately 45 kb (Gerard et al. 1993). The NK1 receptor gene has been localized to chromosome 2 (Gerard et al. 1991) and the NK2 gene to the q23-pter region of chromosome 10 (Gerard et al. 1990). Numerous DNA regulatory elements have been identified in the NK1 receptor gene which includes a response element for cAMP, AP-1, NF-kB, AP-2, AP-4, OCT-2 and SP-1 (Gerard et al. 1991; Takahashi et al. 1992). The NK2 receptor gene has a GC box and a modified cAMP response element (Gerard et al. 1990) while the NK3 gene contains AP-1, AP-2, Sp-1 and cAMP response elements (Takahashi et al. 1992).

Localization of tachykinin binding sites in the rodent central nervous system have been demonstrated primarily by receptor autoradiography (Rothman et al. 1984; Shultz et al. 1984; Buck et al. 1986; Danks et al. 1986; Saffroy et al. 1988; Stoessl, 1994). More recently, immunocytochemical methods have been used to determine the location of cells expressing receptor protein (Kaneko et al. 1993; Nakaya et al. 1994), and *in situ* hybridization has been used to identify corresponding cell bodies containing tachykinin receptor mRNAs (Elde et al. 1990; Shigemoto et al. 1990; Tsuchida et al. 1990; Gerfen, 1991; Hershey et al. 1991; Aubry et al. 1993; Maeno et al. 1993; Whitty et al. 1995; Ding et al. 1996). NK1 receptor binding sites have been demonstrated in cortical layers I and

II, the olfactory tubercle, nucleus accumbens, caudate-putamen, globus pallidus, medial and lateral septum, rostral thalamus, medial and lateral preoptic nucleus, arcuate nucleus, amygdala, hippocampus, dorsal raphe and dorsal parabrachial nucleus in the rat (Rothman et al. 1984; Buck et al. 1986; Danks et al. 1986; Saffroy et al. 1988).

A study by Mizuno *et al.* described the distribution of NK1 receptor protein in the rat central nervous system using immunohistochemical methods (Nakaya et al. 1994). Areas with the highest density of labeled cells were found in the cortical amygdaloid nucleus, hilus of dentate gyrus, amygdalo-hippocampal area, medial and lateral habenula, lateral medial-mammillary and supra-mammillary nucleus, posterior hypothalamus, olfactory tubercle, medial and lateral septum, diagonal band of Broca and lateral part of bed nucleus of stria terminalis. A medium density of labeled neurons was found the caudate-putamen, nucleus accumbens, globus pallidus, cerebral cortex (layers II-IV), hippocampus, anterior and lateral hypothalamus, periventricular, caudal arcuate nucleus, ventromedial and dorsomedial nucleus (Nakaya et al. 1994). NK1 receptor-immunoreactivity was identified in large cholinergic neurons and small somatostatin neurons in the striatum (Kaneko et al. 1993).

NK1 receptor gene expression in rats has been studied using Northern blot hybridization and RNase-protection assays (Tsuchida et al. 1990; Hershey et al. 1991; Whitty et al. 1995). These studies showed that NK1 receptor mRNAs are widely distributed in both the central nervous system and peripheral tissues such as striatum, hypothalmus, olfactory bulb, urinary bladder, salivary glands and intestine. Oligonucleotide probes complementary to 2 different regions of rat NK1 receptor gene revealed intense hybridization signals in the basal ganglia, mitral cell layer of olfactory bulb, medial amigdalo-hippocampal nucleus, vertical limb of the diagonal band of Broca, and dorsal tegmentum (Maeno et al. 1993). Moderate to weak signals were seen in the hippocampus, horizontal limb of the diagonal band of Broca, zona incerta, substantia innominata, lateral hypothalamus, dorsal suprachiasmatic nucleus, midbrain, and medula oblongata (Maeno et al. 1993). In rat dorsolateral striatum, medial septum and basal forebrain neurons, NK1 receptor mRNAs were found to be selectively expressed by cholinergic neurons (Gerfen, 1991). NK1 receptor mRNAs colocalize with dopamine D2 receptors in the striatum (Aubry et al. 1993).

NK3 binding sites were identified in cortical layers IV-V, mediolateral septum, supraoptic and paraventricular nuclei, interpeduncular nucleus, ventral tegmental area and substantia nigra (Mantyh et al. 1989). The location of NK3 receptor proteins and mRNAs has been recently described in the rat central nervous using both immunocytochemistry and in situ hybridization histochemistry (Ding et al. 1996). A specific antibody was used which gave coincident results among the immunocytochemical and hybridization methods. Intense labeling was observed in the olfactory bulb, cortical layers IV and V, medial septum, diagonal band of Broca, bed nucleus of stria terminalis, globus pallidus, ventral pallidum, paraventricular and supraoptic nucleus, zona incerta, dorsal, lateral and posterior hypothalamus, amygdaloid and medial habenula nucleus, ventral tegmentum, midbrain periaquaductal grey, substantia nigra, raphe and pontine nucleus and spinal dorsal horn. NK3 receptor mRNAs were found in neurons in the cerebral cortex, hypothalamus and cerebellum while NK2 was restricted to urinary bladder, large intestine, stomach and adrenal glands (Tsuchida et al. 1990).

Very little is known about the localization of neurokinin receptors in the human brain. NK1 binding sites have only been identified in the caudate-putamen, nucleus accumbens, septum, hippocampus, amygdala, locus coeruleus, and spinal cord (Dietl and Palacios, 1991; Nilsson et al. 1991). More recently, NK1 receptor mRNAs were identified in the cholinergic interneurons of human striatum using *in situ* hybridization histochemistry (Aubry et al. 1994). In one study, no NK2 or NK3 receptor binding sites were identified in monkey or human brain (Dietl and Palacios, 1991). Northern blot analysis for NK3 in various human tissues have also failed to show a positive signal (Buell et al. 1992). However, polymerase chain reaction (PCR) analysis on mRNA extracted from human cortex, hippocampus, hypothalamus and striatum showed the presence of NK3 receptor mRNAs in these tissues, indicating that these receptors and their mRNAs may be present but are very low in abundance (Buell et al. 1992).

Regulation of Gonadotropin Secretion in the Female

The female reproductive axis consists of the hypothalamus, the anterior pituitary gland and the ovary. The anterior pituitary gland secretes the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are released into peripheral blood and target the ovary, where they control folliculogenesis, steroid secretion and ovulation (Page, 1994). FSH stimulates the growth and maturation of ovarian follicles whereas LH stimulates the theca and granulosa cells of the follicle to secrete estrogen. A monthly surge of LH causes ovulation of the dominant follicle (Page, 1994). The positive effect of estrogen is manifested by the surge of LH. The gonadal steroids in turn have both positive and negative feedback effects on gonadotropins at the hypothalamic and pituitary level (Knobil and Hotchkiss, 1988).

The menstrual cycle is divided into two phases, a follicular phase characterized by increasing secretion of estradiol by the dominant ovarian follicle, and a luteal phase characterized by secretion of progesterone by the corpus luteum (Filicori et al. 1986). The LH surge and ovulation occurs at the mid-point of the cycle. Progesterone prepares the uterus for implantation of the zygote. If fertilization does not occur, the corpus luteum degenerates after 14 days and the endometrium is shed (menses). In humans and other higher primates, the menstrual cycle is approximately 28 days long (Ferin et al. 1984; Knobil and Hotchkiss, 1988).

The release of LH and FSH from the anterior pituitary gland is controlled by gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus (Carmel et al. 1976; Mais et al. 1986). GnRH is released in a pulsatile fashion, and the GnRH "pulses" correspond to pulsatile release of LH and FSH (Clarke and Cummins, 1982). GnRH is a decapeptide that is synthesized in the hypothalamus in a prohormone form as a 92 amino acid precursor (Seeburg and Adelman, 1984). Post-translational modification results in a 52 amino acid sequence called GAP (gonadotropin associated protein) and a 23 amino acid signal peptide flanking the GnRH sequence (Seeburg and Adelman, 1984; Adelman et al. 1986; Seeburg et al. 1987). The axons of GnRH neurons terminate in the median emminence and release hormones into the primary capillary plexus of the hypothalmic-hypophyseal portal plexus. GnRH neurons are not located in discrete nuclei but are found widely scattered throughout the septal, preoptic and anterior hypothalamic areas (King et al. 1982; King and Anthony, 1984; Silverman et al. 1987; Merchenthaler et al. 1989; Witkin, 1990; Hiatt et al. 1992). In primates, GnRH neurons are located both within the medial basal hypothalamus as well as in rostral regions (Silverman et al. 1977; Silverman et al. 1979; Goldsmith et al. 1983; Rance et al. 1994). In contrast, in rats, GnRH neurons are located almost exclusively in rostral regions including the in the medial preoptic area, septal regions and diagonal band of Broca (King et al. 1982; Witkin et al. 1982; Kalra, 1976; Merchenthaler et al. 1984; Merchenthaler et al. 1989; Silverman et al. 1987).

The pattern of gonadotropin secretion during the menstrual cycle is modified by positive and negative feedback actions of ovarian steroids (McEwen et al. 1982; Plant, 1986) as well as other inputs from the hypothalamus and higher brain centers (Knobil, 1980; Reame et al. 1984; Gross et al. 1985). The sites and mechanisms of action of steroid feedback are important themes in neuroendocrine research and a frequent subject of controversy (Kalra, 1976; Kalra and Kalra, 1986; Karsch, 1987). For example, estradiol may exert a negative feedback effect on the pituitary gonadotrophs by decreasing responsiveness to GnRH or act at a hypothalamic level by inhibiting release of GnRH (Weick et al. 1983; Shivers et al. 1983a; Wilson et al. 1984). Although GnRH neurons are influenced by gonadal steroids (Kalra, 1976; Kalra and Kalra, 1986; King and Seiler, 1988; Witkin, 1989; Kalra and Kalra, 1989; Witkin et al. 1991) they do not express estrogen or progesterone receptors (Shivers et al. 1983b; Fox et al. 1990). Therefore, steroid feedback may involve an interneuron to GnRH neurons which contains a steroid receptor. Alternatively, steroid effects on GnRH neurons may involve non-receptor mediated mechanisms.

The anterior pituitary gland has been postulated to be the primary target of estrogen feedback in primates (Krey et al. 1975). In studies by Knobil and colleagues, GnRH release was blocked by destruction of the arcuate nucleus, and replaced with an exogenous unvarying pulsatile regimen. Positive and negative feedback effects of estradiol could still be demonstrated under these conditions, indicating a pituitary site of action (Krey et al. 1975). However, these studies do not rule out the possibility of additional actions of gonadal steroids at a hypothalamic level in the intact animal. Further evidence for a hypothalamic site of estrogen negative feedback comes from studies showing that microinjections of estradiol into the monkey hypothalamus blocks the pulsatile release of LH (Ferin et al. 1974; Ferin et al. 1979; Ferin, 1983). In addition, physiological levels of estrogen administered to ovarectomized rhesus monkeys causes a significant reduction in frequency of hypothalamic multiunit electrical activity associated with the pulsatile release of LH (Kesner et al. 1987). Therefore, both the pituitary and the hypothalamus may be involved in mediating the effect of estradiol in primates.

Steroid negative feedback in laboratory rats is demonstrated by the increased plasma LH and FSH after ovariectomy (Shaar et al. 1975; Wise and Ratner, 1980), a response that is reversed by estrogen replacement (Judd, 1987). In male rats, both LH and GnRH pulses were increased in frequency following castration indicating a hypothalamic influence (Levine et al. 1991). A hypothalamic site of steroid feedback is also suggested by the observation that GnRH mRNA increases in the hypothalamus of rats after gonadectomy (Selmanoff et al. 1991). However, these data are controversial as other laboratories have found disparate results (Rothfeld et al. 1989).

Modulation of Gonadotropin Secretion by Tachykinins

Numerous lines of evidence indicate that SP may participate in the control of gonadotropin secretion at a hypothalamic level (Tsuruo et al. 1987). SP neurons are located in the medial basal hypothalamus, the control center for reproduction and the site of the "GnRH pulse generator" in the primate (Krey et al. 1975; Knobil, 1980; Wilson et al. 1984). Synaptic contacts between SP and GnRH neurons in the rat hypothalamus have been revealed by a combination of electron microscopy and immunocytochemistry

(Tsuruo et al. 1991). In addition, tachykinin-immunoreactive neurons in the arcuate nucleus of female rats concentrate estrogen (Akesson and Micevych, 1988). Gonadal steroids have a modulatory effect on the biosynthesis, processing and release of tachykinins in the hypothalamus (Vijayan and McCann, 1979; Antonowicz et al. 1982; Tsuruo et al. 1984; Micevych et al. 1988). Furthermore, substance P content in the median eminence varies with the estrous cycle (Antonowicz et al. 1982; Micevych et al. 1988) and levels of hypothalamic substance P in ovariectomized rats correlate with levels of LH in estrogen- primed rats (Jarry et al. 1988).

SP could also exert an effect on gonadotropin secretion at the level of the anterior pituitary gland. Receptors for substance P have been identified in the anterior pituitary using immunocytochemistry (Kerdelhue et al. 1985; Wormald et al. 1989) and in cultured human pituitary cells, substance P was shown to inhibit GnRH stimulated LH release. There is a dense plexus of SP immunoreactive fibers in the median eminence of primates (Ronnekleiv et al. 1984; Everitt et al. 1986) and hypothalamic fibers from the rat hypothalamus to the median eminence contain SP (Makara et al. 1986). Iodinated SP binding to human pituitary cell membranes was shown in a specific and saturable manner (Wormald et al. 1989). SP was able to inhibit luteinzing hormone (LH) secretion from cultured human pituitary cells (Wormald et al. 1989). Other studies have shown a stimulatory action of SP on LH secretion (Ohtsuka et al. 1987; Arisawa et al. 1990).

Physiology of Postmenopausal Females

Menopause occurs in American women between the ages of 48-55 years (Krailo and Pike, 1983). Current census figures (1990) predict that there are 50 million women over the age of 50 years in the United States. Because the average life span of a women is around 80 years, approximately one third of a women's life is spent in the post menopausal period.

Menopause is characterized by ovarian failure due to loss of ovarian follicles resulting in estrogen deficiency. The changes leading to menopause are generally preceded by a period of endocrine, somatic, and psychological changes due to advancing follicular depletion (Gosden, 1987). The degeneration of primordial ovarian follicles starts in fetal life and continues throughout reproductive life. This decline is accelerated in the decade preceding menopause (Richardson et al. 1987). Many of the symptoms of menopause are caused by estradiol deficiency such as as osteoporosis, urogenital atrophy and hot flushes. In addition, profound psychological changes may occur including mood swings, depression, anxiety, irritability and insomnia (Cope, 1976).

In postmenopausal women, the loss of estrogen production by the ovary removes steroid negative feedback in the hypothalamic-pituitary system. The result is a marked increase in pituitary LH and FSH secretion. Gonadotropin hypersecretion also occurs in young women secondary to gonadectomy (Yen and Lein, 1976; Zanisi et al. 1987). Approximately one month after bilateral oophorectomy, the mean serum levels of LH and FSH in young women increase to postmenopausal levels (Monroe et al. 1972).

The gonadotropin hypersecretion in menopause is associated with increased GnRH gene expression in the medial basal hypothalamus suggesting increased activity of these neurons (Rance and Uswandi, 1996). In addition, there is hypertrophy of a subpopulation of infundibular neurons containing estrogen receptor, substance P and neurokinin B mRNAs (Sheehan and Kovacs, 1966; Sheehan, 1967; Rance et al. 1990; Rance and Young, III, 1991). Tachykinin gene expression is also increased in the hypothalamus of postmenopausal women (Rance and Young, III, 1991). Rance et al. have hypothesized that these changes are secondary to removal of the negative feedback effects of gonadal steroids and not due to age per se (Rance and Young, III, 1991; Rance, 1992; Rance and Uswandi, 1996). Because both the tachykinin and GnRH gene expression increase in postmenopausal women, these systems may be functionally connected. In the rat hypothalamus, arcuate tachykinin neurons synapse on GnRH neurons (Tsuruo et al. 1991). It is not known if a similar anatomic connection exists in the human brain.

SPECIFIC AIMS

Specific Aim 1. To map the distribution of neurons containing substance P and neurokinin B gene transcripts in the human hypothalamus and basal forebrain.

Rationale: Previous studies of the distribution of tachykinins in the human brain have used immunohistochemical methods that do not distinguish between substance P and neurokinin B. In this specific aim, we will use oligonucleotide probes targeted to non-homologous regions of the SP and NKB genes to map the differential distribution of these gene transcripts. A clear understanding of the location neurons containing tachykinin gene transcripts will provide insight into the functional roles of these peptides in the human brain.

Specific Aim 2. To determine if NKB and choline acetyltransferase mRNAs are colocalized within magnocellular neurons in the human nucleus basalis of Meynert.

Rationale: The magnocellular basal forebrain system plays an important role in higher brain functions in human and primates. The nucleus basalis of Meynert in particular has been a subject of intense investigation dating from the first descriptions of degeneration of magnocellular cholinergic neurons this area in Alzheimer's

Disease. There have been no reports, however, of neuropeptide colocalization with acetylcholine in this region of the human brain. In our previous specific aim, we observed numerous neurons containing neurokinin B mRNAs in the human magnocellular basal forebrain. In the present study, we will determine if NKB and ChAT mRNAs are colocalized in the human nbM.

Specific Aim 3. To determine if GnRH neurons express SP receptor mRNA in the human hypothalamus.

Rationale: Both tachykinin and GnRH gene expression increases in the hypothalamus of postmenopausal women suggesting that these two systems may be functionally connected. A mechanism for this interaction is suggested by ultrastructural studies in laboratory rats which show SP-immunoreactive synapses onGnRH neurons. It is not known if an analogous anatomic connection exists in the human brain. In this specific aim, we will use double *in situ* hybridization histochemistry to determine if hypothalamic GnRH neurons express SP receptor mRNAs.

CHAPTER TWO

LOCALIZATION OF NEURONS CONTAINING SUBSTANCE P AND NEUROKININ B mRNAs IN THE HUMAN HYPOTHALAMUS AND BASAL FOREBRAIN

ABSTRACT

In situ hybridization histochemistry was used to map the distribution of neurons expressing the substance P (SP) or NKB (NKB) genes in the human hypothalamus and basal forebrain. Hypothalami from 5 adult males were dissected in coronal and sagittal blocks, snap frozen and serially sectioned at a thickness of 20 μm. Every 20th section was hybridized with a ^{35}S-labeled 48-base synthetic cDNA probe. Slides were dipped into nuclear emulsion for visualization of mRNAs at the single cell level. The location of labeled neurons (> 5X background) was mapped using an image-combining computer microscope system. An extensive and complex distribution of labeled neurons was observed. Overall, the distribution of the two tachykinin precursor mRNAs was complementary: NKB was the predominant tachykinin in the rostral hypothalamus whereas SP mRNA predominated in the posterior hypothalamus. Numerous NKB neurons were identified in the magnocellular basal forebrain complex, the bed nucleus of stria terminalis, and the preoptic/anterior hypothalamic area. Scattered NKB neurons were present in the infundibular and paraventricular nuclei, paraolfactory gyrus, posterior hypothalamus, lateral division of the medial mammillary nucleus and extended amygdala. Numerous neurons expressing SP mRNAs were identified in the premammillary, supramamillary and medial mammillary nuclei, posterior hypothalamus and the corpus

striatum. SP neurons were also observed in the preoptic area, the infundibular, intermediate, dorsomedial and ventromedial nuclei, infundibular stalk, amygdala, the bed nucleus of stria terminalis and paraolfactory gyrus. These studies demonstrate distinct and complementary distribution patterns of SP and NKB gene expression in the human hypothalamus and basal forebrain.

INTRODUCTION

Tachykinins are widely distributed in the central as well as peripheral nervous system. The tachykinin family of peptides in mammals includes substance P (SP), neurokinin A (NKA), neurokinin B (NKB), neuropeptide K (NPK) and neuropeptide-gamma (NPγ(Nakanishi, 1987). SP, NKA, NPK and NPγ are derived from the preprotachykinin A (PPT-A) gene by alternate RNA splicing and tissue specific processing (Nawa et al. 1984) whereas NKB is derived from the preprotachykinin B (PPT-B) gene (Kotani et al. 1986; Krause et al. 1990). The tachykinins have a highly conserved carboxy-terminal amino-acid sequence of -Phe-X-Gly-Leu-Met-NH_2, where X is either a branched aliphatic residue (NKA, NPK, NPγ and NKB) or an aromatic one (SP) (Maggio, 1988). Based on the nature of the X residue, tachykinins exhibit different bioactivities (Erspamer, 1971; Lee et al. 1986). Although there is some cross-reactivity, tachykinins bind to 3 distinct classes of receptors in mammalian tissues: NK-1, NK-2 and NK-3; the receptors for SP, NKA and NKB respectively (Gerard et al. 1993). Tachykinin receptors belong to the G-protein coupled sub-family of receptors that contain seven putative transmembrane hydrophobic domains (Takahashi et al. 1992).

Tachykinins participate as neuromodulators or neurotransmitters in a wide variety of physiological processes, for reviews see (Pernow, 1983; Aronin et al. 1986; Maggio, 1988; Otsuka and Yoshioka, 1993). Most attention has been focused on SP, which was isolated from equine intestine in 1931 (Von Euler and Gaddum, 1931) and characterized 40 years later (Chang and Leeman, 1970; Chang et al. 1971). Because NKA, NKB, NPK and NPγ were described more recently (Kimura et al. 1983; Nawa et al. 1983; Kangawa et al. 1983; Minamino et al. 1984; Nawa et al. 1984), there is less information available on the biologic properties of these peptides. The diverse functions of tachykinins have led to numerous studies of their distribution in the rat central nervous system using either immunohistochemical (Ljungdahl et al. 1978; Inagaki et al. 1982; Jonakait et al. 1991; Cuello et al. 1985; Marksteiner et al. 1992a; Marksteiner et al. 1992b; Lucas et al. 1992) or *in situ* hybridization techniques (Warden and Young, III, 1988; Harlan et al. 1989; Marksteiner et al. 1992a; Marksteiner et al. 1992b; Lucas et al. 1992). In contrast, there have been few studies of the distribution of tachykinin-immunoreactive neurons in human brain (Bouras et al. 1986; Mai et al. 1986; Sakamoto et al. 1987) and no studies of the location of tachykinin mRNAs in this species.

In the present study, we used *in situ* hybridization to map the distribution of tachykinin neurons in the human hypothalamus and basal forebrain. We focused on these regions because of the involvement hypothalamic tachykinins in the control of anterior pituitary function (Kerdelhué et al. 1978; Vijayan and McCann, 1979; Eckstein et al. 1980; Dees et al. 1985; Ohtsuka et al. 1987; Arisawa et al. 1990; Picanco-Diniz et al. 1990; Kalra et al. 1991; Debeljuk et al. 1992; Shamgochian and Leeman, 1992), cardiovascular regulation (Hall et al. 1989; Nagashima et al. 1989b; Itoi et al. 1994) and sexual behavior (Dornan and Malsbury, 1989; Kalra et al. 1991; Olster and Blaustein, 1992a; Olster and Blaustein, 1992b; Swann and Newman, 1992; Swann and Macchione, 1992; Akesson, 1993). Previous immunohistochemical studies of the distribution of tachykinins in the human hypothalamus utilized antibodies that do not distinguish between SP and NKB (Mai et al. 1986; Sakamoto et al. 1985; Constantinidis et al. 1983; Bouras et al. 1986; Sakamoto et al. 1987) In this study, we used oligonucleotide probes targeted to non-homologous regions of the SP and NKB genes, to reveal the differential distribution of these gene transcripts. *In situ* hybridization also offers a distinct advantage when studying postmortem human materials because the visualization of neuronal somata does not require pretreatment with colchicine.

MATERIALS AND METHODS

Hypothalami were collected from 5 adult males (16, 19, 23, 52 and 61 years of age) who had died from sudden unexpected causes including acute myocardial infarction, chest trauma or asphyxiation. The postmortem intervals ranged from 4 to 8 hours. Autopsy specimens were collected in accordance with the guidelines set forth in the *Federal Register* and the Human Subjects Committee of the University of Arizona. The subjects had no history of hospitalization or serious illness prior to death. Complete autopsies were performed to rule out any systemic illness other than atherosclerosis.

The brains were bisected and sagittal blocks were dissected by coronal cuts rostral to the optic chiasm and caudal to the mammillary bodies, and a horizontal cut superior to the anterior commissure. A final sagittal cut was made 1 cm lateral to the third ventricle.

The contralateral hypothalamus was divided into anterior and posterior coronal blocks by making a cut intersecting the posterior edge of the anterior commissure and the tuberoinfundibular sulcus (c.f. Rance et al. 1994). All blocks were rapidly frozen in isopentane cooled to -30^{o}C and stored at -80^{o}C until used. Twenty µm thick serial sections were cut in a cryostat and thaw mounted onto gelatin-coated slides. The slides were stored at -80^{o}C until hybridization.

***In Situ* Hybridization Histochemistry**

Every 20th section was hybridized with ^{35}S-labeled 48 base synthetic cDNA probe. The SP probe was complementary to bases 124-171 of rat mRNA (Krause et al. 1987) which is 91% homologous to the human SP gene. The sequence of the NKB probe was: CTGGCTGGACGCTCATCTTGCCCATAAGTCCCACAAAGAAATCATGCA (Dr. T. Bonner, personal communication). The specificity of the probes was tested by Northern analysis at the same conditions of stringency (Rance and Young, III, 1991). The probes were labeled using terminal deoxynucleotidyl transferase (Boehringer-Mannheim, Indianapolis, ID) and ^{35}S dATP (>1000 Ci/mM; New England Nuclear, Boston, MA). The sections were postfixed lightly in 4% formaldehyde for 5 min, dipped in 0.25% acetic anhydride in 0.1M triethanolamine for 10 min and dehydrated in an ascending concentration of ethanol (to 100%), followed by a 5 min rinse in chloroform. The sections were hydridized overnight at 37^{o} C with approximately 10^{6} dpm ^{35}S-labeled probe/50ml hybridization buffer. Posthybridization washes were done with SSC for 1 hr (4 x 15min in 12M NaCl + 1.2M Na citrate) followed by 50% formamide at 40^{o}C and 2 x with SSC (6M NaCl + 0.6M Na citrate) at room temperature. After a brief rinse in water followed by 70% ethanol, the slides were dipped in NTB3 nuclear emulsion (diluted 1:1

with water, Eastman Kodak). The slides were developed after a 4 month exposure using Dektol developer and fixer (Kodak) and counterstained with toluidine blue.

Analysis

All sections were initially scanned with the aid of a Nikon Optiphot microscope, equipped with a 10X Nikon planapo lens and a Darklite condensor (Micro video instruments, Avon, MA). Selected sections were then digitized using an image-combining computer microscope equipped with a motorized stage (Glaser et al. 1983) and Neurolucida software (Microbrightfield Inc., Baltimore, MD). The various hypothalamic nuclei and basal diencephalic boundaries were drawn using a Nikon 4X planapo objective and brightfield illumination. The location of all labeled neurons was manually digitized using a 10X Zeiss planapo objective and a Darklite condensor (Micro Video Instruments, Avon, MA). A neuron was considered labeled if the number of silver grains exceeded 5X background. All labeling was verified with a 25X Zeiss planapo lens under brightfield illumination. To determine profile area of a labeled neuron, the somata of approximately 50 labeled neurons were digitized in each area. The cross-sectional area of a labeled neuron was determined by manually digitizing the outline of the cell body using a 63X oil-immersion objective. Maps of neurons expressing NKB or SP mRNAs were made with Neurorotate (Microbrightfield Inc.) and Corel-draw computer software. The coronal maps were obtained from subject 814, a 52-year old male who died from asphyxiation (4 hour postmortem interval). The sagittal series was from subject 871, a 23-year old male who expired from chest trauma (7 hour postmortem interval). The terminology of Nauta and Haymaker (Nauta , and Haymaker, 1969) and Saper (Saper, 1990) was used to label hypothalamic and basal forebrain structures. More detailed descriptions of the subdivisions can be seen in (Sukhov et al. 1995).

RESULTS

Computer-assisted maps of SP mRNA containing neurons are shown in Figures 2.1 (coronal series) and 2.2 (sagittal series). Coronal and sagittal maps of the distribution of neurons expressing the NKB gene are shown in Figures 2.3 and 2.4, respectively. Numerous tachykinin neurons were identified in nearly all major hypothalamic nuclei. With a few exceptions, the neurons expressing SP and NKB gene transcripts had distinct distributions. Overall, the distribution of the two tachykinin mRNAs was complementary: NKB was the predominant tachykinin in the rostral hypothalamus whereas SP was concentrated in the posterior hypothalamus. The striking complementary distribution of the two tachykinin mRNAs was readily seen when comparing the sagittal maps (Figs. 2.2 and 2.4).

Rostral basal forebrain and extended amygdala

Numerous large NKB mRNA containing neurons were identified in the magnocellular basal forebrain system (Figs. 2.3A-C, 2.4A-D). Rostrally, numerous magnocellular NKB neurons formed a continuous band that extended from the medial septal nucleus (ms), through the diagonal band of Broca (dbB), into the nucleus basalis of Meynert (nbM) (Fig. 2.3A). Magnocellular neurons were also prominent in the posterior portion of the nbM (Fig. 2.3C). Only a few large NKB neurons were identified in the medial septal nucleus (Figs. 2.3A and 2.6C). In the dbB, a mixture of large and medium-sized NKB neurons were labeled. In the nucleus basalis, however, the NKB neurons were predominately magnocellular in size and morphology (Fig. 2.6A and B). Although NKB neurons were evenly distributed throughout most of the nbM (labeling ca. 30%), there was an oval, densely cellular region of magnocellular neurons where very few neurons were labeled (Figs. 2.3A and 2.3B). The mean cross sectional area of NKB neurons in the nbM was 1,299 $\pm$ 48 μm^2. In contrast to the magnocellular NKB neurons in the nbM, the SP neurons were small in size and few in number (Figs. 2.1A-C).

There were numerous small, oval to round NKB neurons in the bed nucleus of the stria terminalis (2.3A-C, 2.6D). In contrast, only a few SP neurons were detected in this region (Fig. 2.1A). Both SP and NKB mRNA was identified in small neurons scattered from the bed nucleus to the amygdala (extended amygdala, Figs. 2.1A-D, 2.3B-D). These neurons were occasionally aligned in small groups along myelinated fiber bundles.

Chiasmatic region

Scattered small SP neurons were observed within the medial and lateral preoptic region (Fig. 1A-B). In addition, SP mRNA was identified within a subpopulation of neurons in the intermediate nucleus (Fig. 2.1C), that were medium sized (mean cell area = 267 $\pm$ 16μm^2), and round to oval in shape (Fig. 2.5C). In rare cases, small NKB neurons were also identified in the region of the intermediate nucleus.

There were numerous, small NKB neurons throughout the medial preoptic region extending into the anterior hypothalamic area (mean area = 165 $\pm$ 8 μm^2). In sagittal sections, the NKB neurons formed a vertical column of cells running lateral and parallel to the paraventricular nucleus (Fig. 2.4B-D). A few scattered NKB neurons were also present in the parvocellular portion of the paraventricular nucleus. No SP or NKB neurons were identified in the supraoptic nuclei, suprachiastic nucleus, lamina terminalis or OVLT.

Tuberal region

Small to medium SP and NKB neurons (mean profile area = 177 ± 7 μm^2) were scattered throughout the infundibular (arcuate) nucleus (Figs. 2.1C, 2.2A, 2.3C, 2.4A-C, 2.5A, 2.6F). Occasional small SP and NKB neurons were also identified within the infundibular stalk (Figs. 2.1A, 2.2C, 2.4A, 2.5B) and retrochiasmatic area. Neurons containing SP but not NKB mRNA were observed in the ventromedial nucleus (VMN) (Figs. 2.2A and B). No tachykinin neurons were present in the lateral tuberal nuclei.

Mammillary Nuclei and Posterior Hypothalamus

Numerous SP mRNA containing neurons were concentrated in the premammillary, supramammillary and medial mammillary nuclei. Many medium to large-sized SP neurons were evenly distributed throughout the medial and lateral subdivisions of medial mammillary nucleus (Figs. 1D, 2.2A-D, 2.5E). The average cell profile area of SP neurons in the medial mammillary nucleus was 449 ± 17μm^2. Although no NKB neurons were identified in the medial subdivision of the medial mammillary nucleus, a small number of medium sized NKB neurons were identified in the lateral subdivision of this nucleus.

On sagittal sections the location of the premammillary nucleus was highlighted by numerous SP neurons (Figs. 2.2C,D). These neurons were small, oval-shaped and oriented parallel to the posterior border of the medial mammillary nucleus (Fig 2.5D). No SP labeled neurons were present in the lateral mammillary nucleus or in the tuberomammillary region. Large SP neurons however, were abundant in the posterior hypothalamus (mean cell profile area = 685 ± 32μm^2) (Figs. 2.1D, 2.2A-D). A few medium to large NKB mRNA containing neurons were also found in the posterior hypothalamus (Figs. 2.3D, 2.4A-D).

Extrahypothalamic regions

A dense population of small SP mRNA containing neurons was present in the caudate and putamen (mean = 130 ± 4μm^2). In contrast, NKB neurons were not identified in the striatum. The paraolfactory gyrus had both SP and NKB neurons which were small in size.

DISCUSSION

The present study revealed an extensive and complex distribution of neurons expressing tachykinin mRNAs in the human hypothalamus and basal forebrain. *In situ* hybridization demonstrated many more tachykinin neurons in the human hypothalamus than previously described on the basis of immunocytochemical methods (Mai et al. 1986; Bouras et al. 1986). Consistent with studies in laboratory rats (Warden and Young, III, 1988; Harlan et al. 1989; Lucas et al. 1992), the distributions of SP and NKB mRNAs are distinct and complementary: SP was the predominant tachykinin in the posterior hypothalamus, corpus striatum, intermediate nucleus, ventromedial nucleus, mammillary nuclei and posterior hypothalamus. In contrast, there were more NKB mRNA containing neurons than SP neurons in the magnocellular basal forebrain system, the bed nucleus of the stria terminalis, and the preoptic/anterior hypothalamic region. Comparable numbers of neurons expressing both mRNAs were identified in the infundibular nucleus and amygdala.

One of the major findings of the present study was the identification of NKB mRNA in the human magnocellular basal forebrain system. The magnocellular neurons of this system are characteristic in appearance due to their large size, densely stained granular Nissl substance and vesicular nucleus containing a distinct and large nucleolus (Hedreen et al. 1984). NKB neurons were identified all three of the major subdivisions of this system: the medial septal nucleus, the dbB, and the nbM (Saper and Chelimsky, 1984; Hedreen et al. 1984). The nbM in particular has been a subject of intense interest dating from the first description of its degeneration in Alzheimer's Disease (Whitehouse et al. 1982). The nbM projects to neocortical areas in a widespread and topographical manner (Mesulam and Van Hoesen, 1976; Mesulam et al. 1983) and approximately 80-90% of the magnocellular neurons within this nucleus are cholinergic (Mesulam and Geula, 1988). Our preliminary studies indicate that approximately 30% of the cholinergic neurons in the human nbM contain NKB mRNA (Chawla et al. 1995). NKB mRNA has also been identified in the magnocellular basal forebrain system of the rat, although in fewer numbers compared to the human (Warden and Young, III, 1988; Burgunder and Young, III, 1989b).

SP neurons were identified in several divisions of the mammillary nuclei including the medial mammillary, premammillary, and supramamillary subdivisions. Of special interest was the novel identification of numerous SP neurons in the human medial mammillary nucleus. Radioimmunoassable SP has been reported in this area in the human (Cooper et al. 1981), but tachykinin immunoreactive-neurons have not been previously identified in the human, monkey, or rat medial mammillary body (Cooper et al. 1981; Ronnekleiv et al. 1984; Warden and Young, III, 1988; Harlan et al. 1989). In addition, the previous *in situ* hybridization studies have not identified tachykinin mRNAs within this nucleus. The medial mammillary body has reached it's highest development in humans, and is part of the classical limbic circuit described by Papez (Papez, 1937). The medial mammillary may be involved in learning and memory processing based upon its functional and anatomical connections with the hipppocampus and anterior thalamic nucleus (Swanson, 1987; Simerly, 1995). This function of the mammillary bodies is further suggested by the symptoms of amnesia in patients with the Wernicke-Korsakoff syndrome with degeneration of the mammillary bodies (Mair et al. 1979; Torvick, 1987; Mayes et al. 1988; Becker et al. 1990).

The numerous SP neurons in the premammillary nucleus facilitated its identification on sagittal sections. These findings are consistent with the identification of SP mRNA in the rat premammillary nucleus (Warden and Young, III, 1988; Harlan et al. 1989). In the rat, this nucleus can be morphologically divided into dorsal and ventral divisions, however in the monkey (Veazey et al. 1982b) as in our material, these divisions are not apparent. Functionally, the dorsal premammillary nucleus of rodents is involved with the integration of goal-oriented behaviors associated with hunger, thirst and reproduction (Canteras and Swanson, 1992) whereas the ventral premammillary nucleus is involved in neuroendocrine and sexually dimorphic circuitry (Canteras et al. 1992).

Another major area containing SP was the posterior hypothalamus. Little is known about the connections of the posterior hypothalamus in man. The afferent and efferent connections of the posterior hypothalamus in cynomolgus monkeys has been described by Veazey et al (Veazey et al. 1982b; Veazey et al. 1982a). There is a significant projection from the medial posterior hypothalamic nucleus to the dorsal, compact portion of locus coeruleus, whereas the lateral part of the posterior hypothalamus projects to the parabrachial nucleus (Veazey et al. 1982a). In addition, both medial and lateral areas of the posterior hypothalamus contribute heavily to the descending medial forebrain bundle projection to the brainstem (Veazey et al. 1982a). The posterior hypothalamus also has widespread, reciprocal connections with the cerebral cortex that are organized in a topographical manner (Saper, 1990). A major function attributed to the posterior hypothalamus is integration of thermal regulation (Hensel, 1973; Boulant, 1980; Hellon, 1981; Halvorson and Thornhill, 1993; Thornhill and Halvorson, 1994; Monda et al. 1994).

Both SP and NKB gene transcripts were located within neurons of the infundibular nucleus. This finding is consistent with previous observations of tachykinin-immunoreactive neurons in this region in the human (Mai et al. 1986) and monkey (Ronnekleiv et al. 1984). The infundibular nucleus of the human is the homologue of the arcuate nucleus of the rat, and contains a heterogeneous population of cells with a wide variety of classical neurotransmitters and peptides (Everitt et al. 1986; Rance and Young, III, 1991). We have previously described hypertrophy and increased gene expression of tachykinin neurons in the infundibular nucleus of postmenopausal females (Rance and Young, III, 1991). We have hypothesized that the hypertrophy and increased gene expression are secondary to ovarian failure and the resultant loss of estrogen negative feedback. Tachykinin-immunoreactive neurons in the rat arcuate nucleus contain receptors for gonadal steroids (Akesson and Micevych, 1988; Ciofi et al. 1994) and the number of arcuate neurons expressing the NKB peptide or its mRNA is modulated by ovarian steroids (Akesson et al. 1991; Rance and Bruce, 1994; Abel et al. 1996). In addition, tachykinin levels in the median eminence, as well as the number and size of arcuate tachykinin-immunoreactive neurons change over the rat estrous cycle

(Micevych et al. 1988; Antonowicz et al. 1982; Tsuruo et al. 1984). A stimulatory role of tachykinins on gonadotropin secretion is suggested by a variety of experimental paradigms (Vijayan and McCann, 1979; Dees et al. 1985; Ohtsuka et al. 1987; Arisawa et al. 1990) but tachykinins may also have an inhibitory effects under certain conditions (Kerdelhué et al. 1978; Picanco-Diniz et al. 1990). Tachykinin immunoreactive neurons in the arcuate nucleus establish synapses on LHRH neurons in the rat hypothalamus (Tsuruo et al. 1991).

SP mRNA was also identified in a subpopulation of neurons in the intermediate nucleus of the preoptic area. This nucleus is located midway between the supraoptic and the paraventricular nucleus and is distinguished by darkly staining cells (Braak and Braak, 1987; Saper, 1990). It has also been refered to as the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Swaab and Fliers, 1985; Hofman and Swaab, 1989), or the interstitial nucleus of the anterior hypothalamus I (INAH-I) (Allen et al. 1989). In humans, the intermediate nucleus is twice as large in males than in females, a difference that may be age dependent (Swaab and Hofman, 1995). Recently, preproenkephalin mRNA positive neurons were identified in the human intermediate nucleus (Sukhov et al. 1995), but enkephalin-immunoreactivity has not been identified in the rat SDN-POA (Simerly et al. 1988).

Another structure which has been shown to be sexually dimorphic in humans is the posteromedial subdivision of the bed nucleus of stria terminalis. The volume of this nucleus is approximately two and a half times larger in males than in females (Allen and Gorski, 1990). A dense population of NKB neurons was noted in the bed nucleus of stria terminalis (bnst) but very few SP neurons were seen in this area.

Sexual dimorphism has also been shown in the volume of rodent ventromedial nucleus (Matsumoto and Arai, 1983). Many neurons with SP mRNA were present in the ventromedial nucleus, whereas NKB neurons were not identified. This is consistent with studies showing SP- immunoreactive neurons in the rat VMN (Takatsuki et al. 1983), where it may be involved in mediating female sexual behaviors (Akesson, 1994). In addition, a sex related difference exists in the regulation of progesterone receptor and proenkephalin gene expression by estrogen in the VMN (Lauber et al. 1991; Romano et al. 1990). The ventromedial nucleus has been implicated in mediating reproductive behaviors in the rodent (Pfaff, 1989).

The high concentration of tachykinin gene transcripts in almost all the major hypothalamic nuclei suggests that tachykinin peptides are important in mediating many autonomic, behavioral and sexual responses. In general, NKB neurons predominated in the rostral part of the medial hypothalamus, whereas the SP neurons were concentrated in the caudal hypothalamus. This rostral-caudal distribution of tachykinins parallels the conceptual division of medial hypothalamic nuclei into rostral and caudal groups based upon their afferent innervation (Swanson, 1987; Canteras and Swanson, 1992; Simerly, 1995). Based on axonal transport studies in rodents, the medial zone nuclei have been divided into a rostral group mediating homeostasis and reproduction, whereas the caudal group is involved in the processing of cortical information (Swanson, 1987; Simerly, 1995).

TABLE 2.1: Distribution Cells Containing SP and NKB mRNAs in the Human Hypothalamus and Adjacent Structures*

Region	Structure	SP	NKB
Basal forebrain	Medial septum	-	+
	Diagonal band of Broca	+	+++
	Nucleus basalis of Meynert	+	+++
Chiasmatic	Supraoptic nucleus, dorsolateral	-	-
	Supraoptic nucleus, ventromedial	-	-
	Medial preoptic area	+	++
	Intermediate nucleus	++	+
	Suprachiasmatic nucleus	-	-
	Paraventricular nucleus	-	+
	Lamina terminalis (LT)	-	-
	Organum vasculosum of LT	-	-
Tuberal	Dorsomedial nucleus	+	-
	Ventromedial nucleus	++	-
	Infundibular nucleus	++	++
	Premammillary nucleus	++	-
	Lateral tuberal nucleus	-	-
	Tuberomammillary nucleus	-	-
Mammillary	Medial mammillary nucleus	+++	-
	Supramammillary nucleus	+++	-
	Lateral mammillary nucleus	-	-
	Posterior hypothalamus	++	+
Adjacent regions	Extended amygdala	+	+
	Bed nucleus of stria terminalis	+	++
	Caudate nucleus	+++	-
	Putamen	+++	-
	Globus pallidus	-	-
	Paraolfactory gyrus	++	++

* Density of cells in an area or nucleus: - none, + < 5% of cells, ++ 5-30 %, +++ > 30%

TABLE 2.2

ABBREVIATIONS

ac	anterior commissure
amyg	amygdala
bnst	bed nucleus of the stria terminalis
c	caudate
dbB	diagonal band of Broca
fx	fornix
gpe	globus pallidus, external segment
gpi	globus pallidus, internal segment
ic	internal capsule
int	intermediate nucleus
inf	infundibular nucleus
is	infundibular stalk
lh	lateral hypothalamus
lt	lateral tuberal nucleus
lv	lateral ventricle
mi	massa intermedia
mb	mammillary body
mt	mammillothalamic tract
nbM	nucleus basalis of Meynert
oc	optic chiasm

ot	optic track
ovlt	organum vasculosum of the lamina terminalis
ph	posterior hypothalamic area
poa	preoptic region
ps	precommissural septum
p	putamen
pvn	paraventricular nucleus
rt	reticular nucleus of thalamus
sep	septal nuclei
son	supraoptic nucleus
st	stria terminalis
ts	tuberoinfundibular sulcus
vmn	ventromedial nucleus
vp	ventral pallidum
III	third ventricle

Figure 2.1: **A through D**: Coronal maps of neurons containing SP mRNA from subject #814, a 52 year old male with a postmortem interval of 4 hours. The maps are plotted at successive anterior to posterior intervals with the section number at the bottom right of each map. See page 48 for abbreviations. Each symbol represents one neuron.

Bar = 0.3 cm

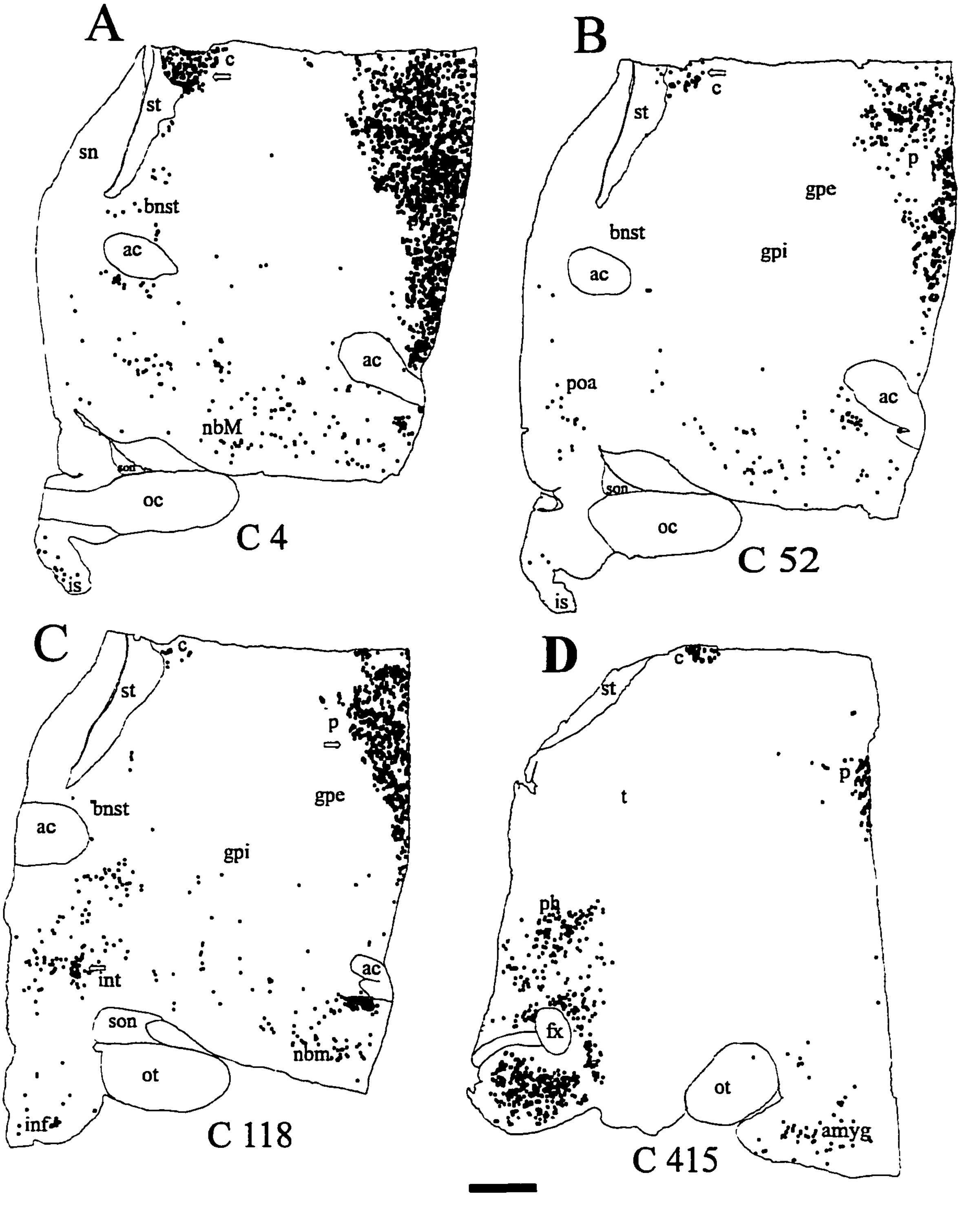

A
c
st
sn
bnst
ac
ac
nbM
son
oc
is
C 4
B
c
st
p
gpe
bnst
gpi
ac
poa
ac
son
oc
is
C 52
C
c
st
p
gpe
bnst
ac
gpi
int
ac
son
nbm
ot
inf
C 118
D
c
st
p
t
ph
fx
ot
amyg
C 415

Figure 2.2: **A through D**: Sagittal maps of neurons containing SP mRNA from subject # 871, a 52 year old male with a postmortem interval of 4 hours. The maps are plotted at successive medial to lateral intervals with the section number at the bottom right of each map. See page 48 for abbreviations. Each symbol represents one neuron. Bar = 0.3 cm

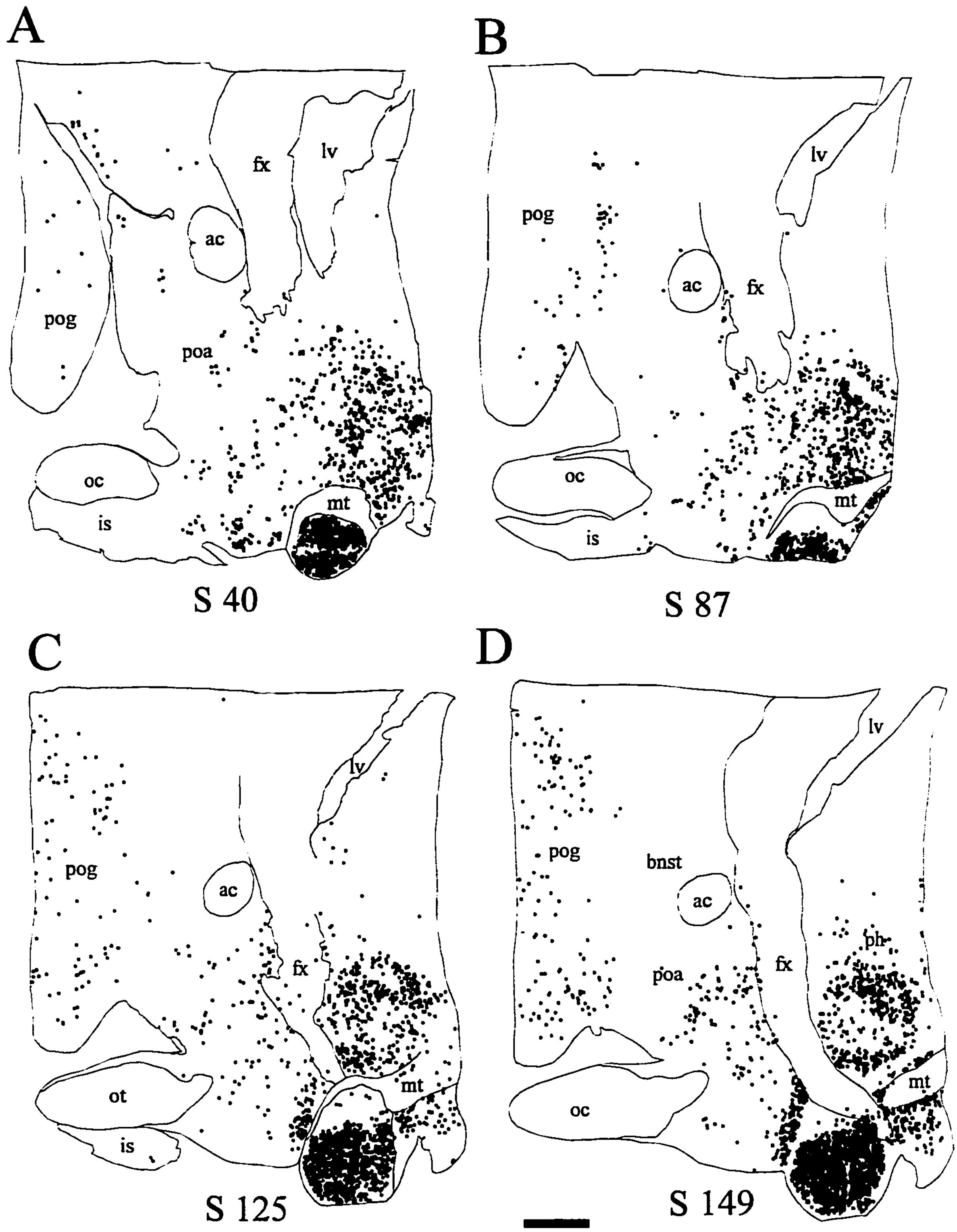

A
pog
poa
ac
fx
lv
oc
is
mt
S 40
B
pog
ac
fx
lv
oc
is
mt
S 87
C
pog
ac
lv
fx
mt
ot
is
S 125
D
pog
bnst
ac
poa
fx
lv
ph
mt
oc
S 149

Figure 2.3: **A through D**: Coronal maps of neurons containing NKB mRNA from subject #814. The maps are plotted at successive anterior to posterior intervals with the section number at the bottom right of each map. See page 48 for abbreviations. Each symbol represents one neuron. Bar = 0.3 cm

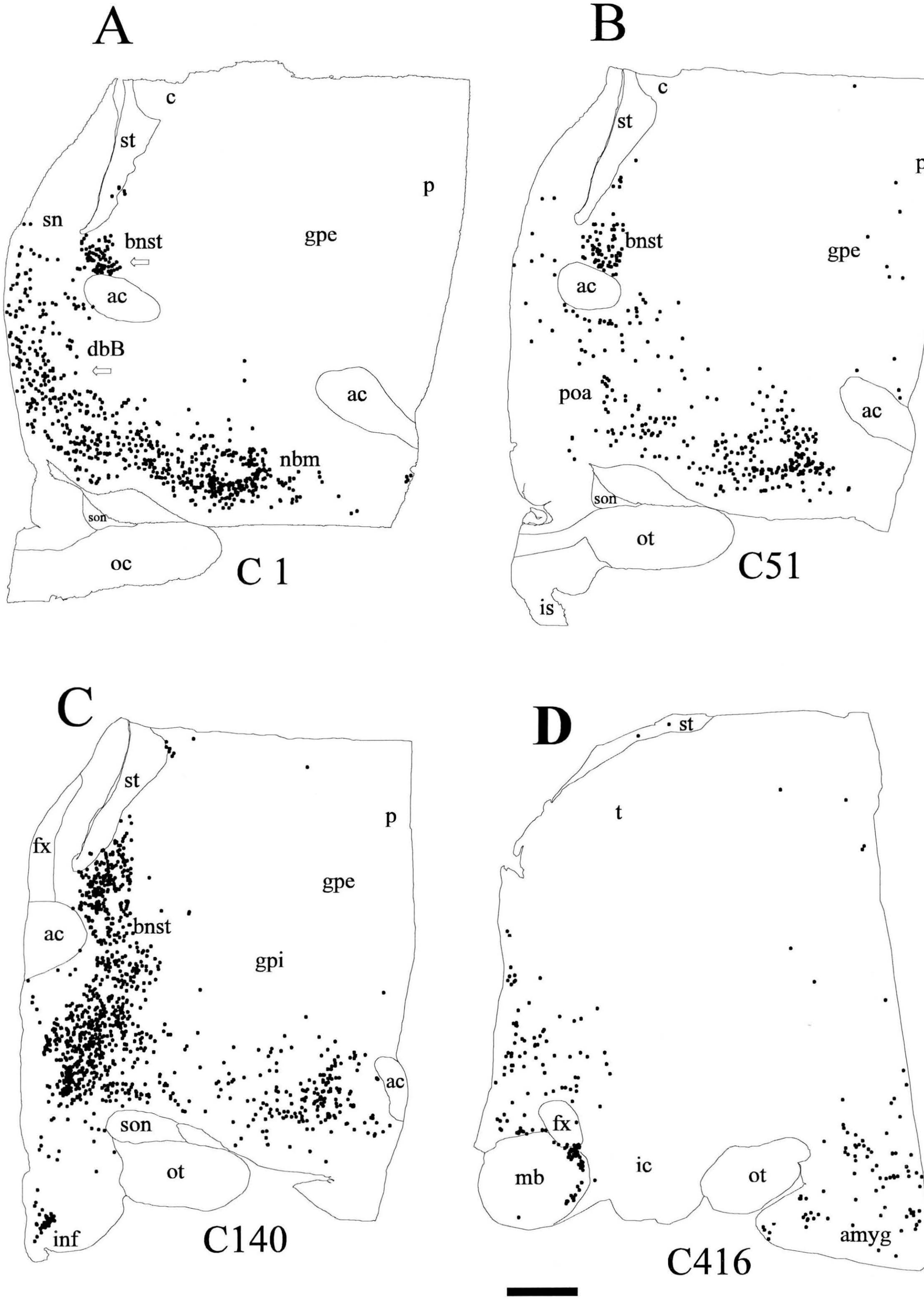

A
B
c
st
p
sn
bnst
gpe
ac
dbB
nbm
son
oc
C 1
poa
ot
is
C51
C
D
fx
gpi
inf
C140
t
mb
ic
amyg
C416

Figure 2.4: **A through D**: Sagittal maps of neurons containing NKB mRNA from subject 871. The maps are plotted at successive anterior to posterior intervals with the section number at the bottom right of each map. See page 48 for abbreviations. Each symbol represents one neuron. Bar = 0.3 cm

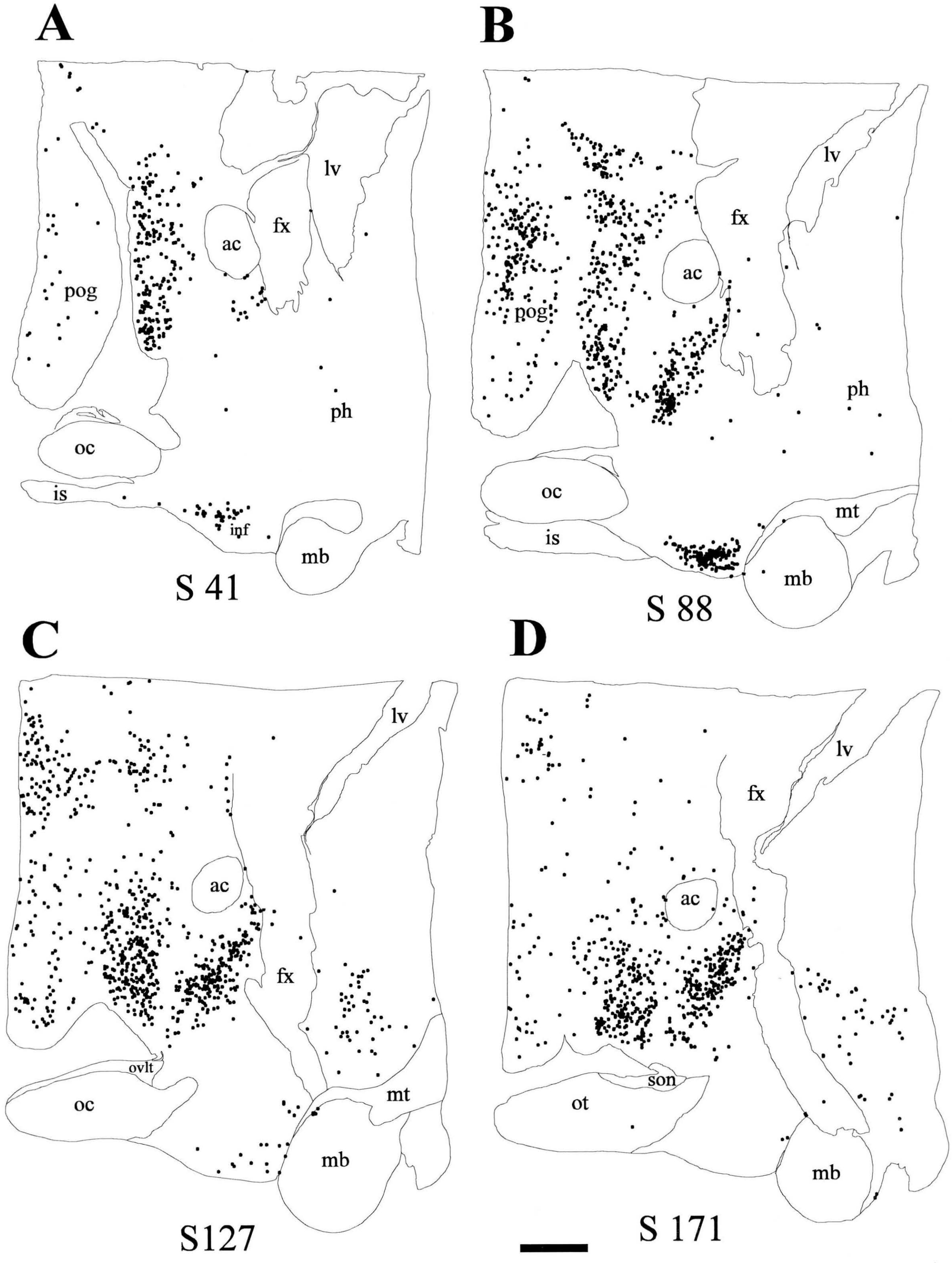

A
lv
fx
ac
pog
ph
oc
is
inf
mb
S 41
B
lv
fx
ac
pog
ph
oc
mt
is
mb
S 88
C
lv
ac
fx
ovlt
oc
mt
mb
S127
D
lv
fx
ac
son
ot
mb
S 171

Figure 2.5: **A through F**: Representative photomicrographs of neurons containing SP mRNA in the infundibular nucleus (A), infundibular stalk (B), intermediate nucleus (C), premammillary nucleus (D), medial mammillary nucleus (E) and posterior hypothalamus (F). Bar = 20 microns.

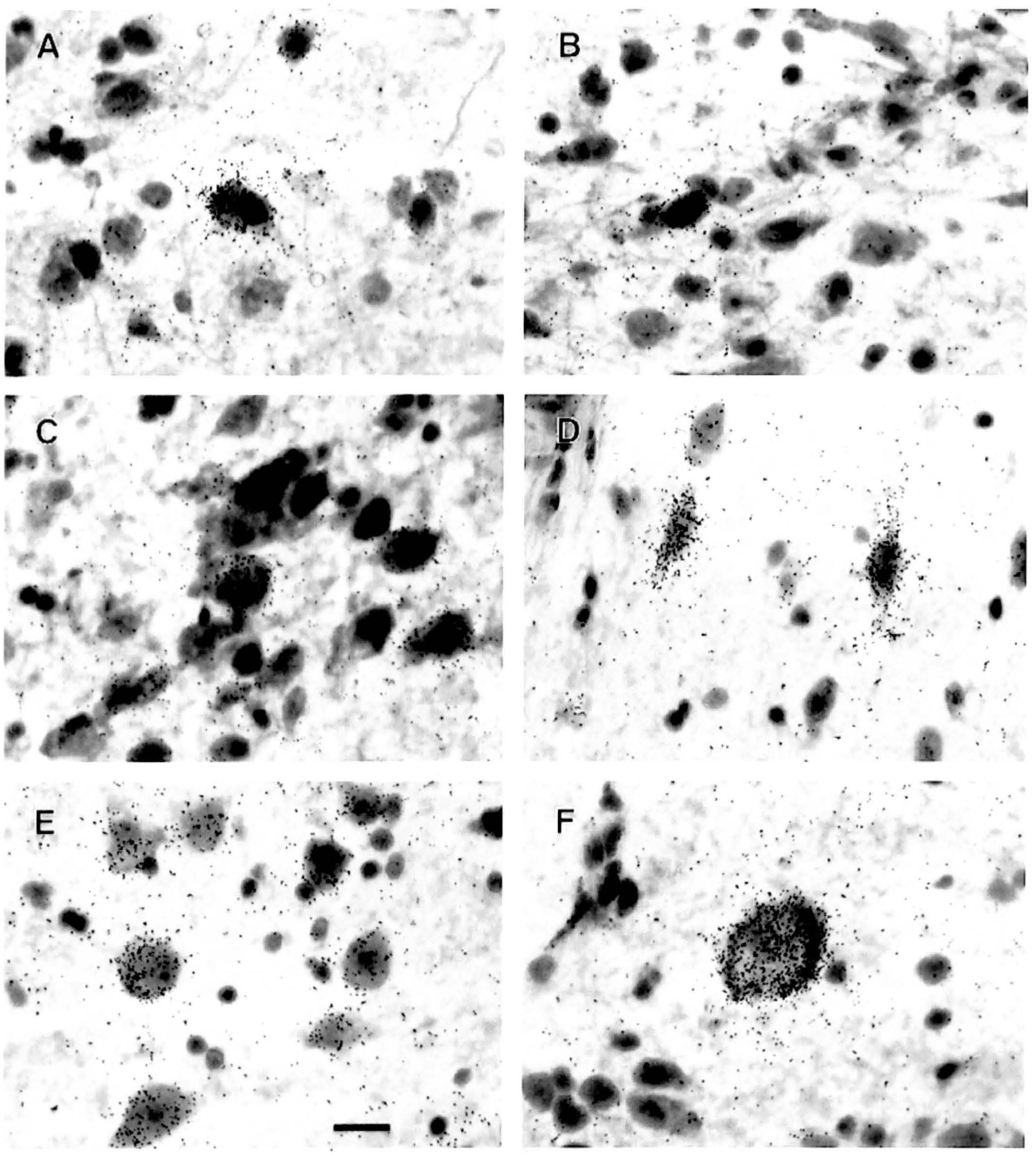
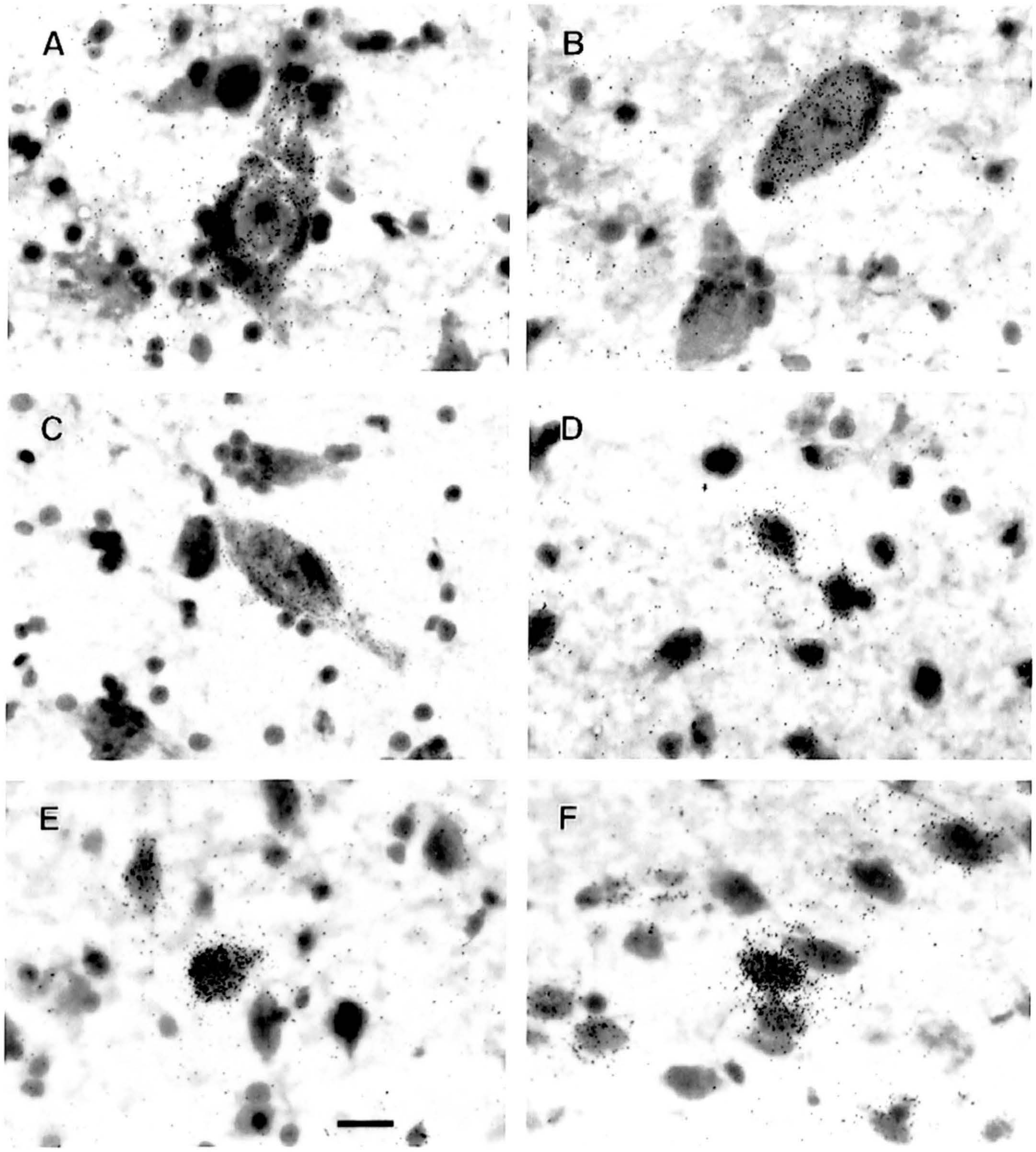

A
B
C
D
E
F

Figure 2.6: **A through F**: Representative photomicrographs of neurons containing NKB mRNA in the nucleus basalis of Meynert (A and B), medial septal nucleus (C), bed nucleus of the stria terminalis (D), preoptic region/anterior hypothalamic area (E) and infundibular nucleus (F). Bar = 20 microns.


A
B
C
D
E
F

CHAPTER THREE

NEUROKININ B AND CHOLINE ACETYLTRANSFERASE mRNAs ARE COLOCALIZED IN NEURONS OF THE HUMAN MAGNOCELLULAR BASAL FOREBRAIN SYSTEM.

ABSTRACT

In previous studies, we identified an extensive population of neurons expressing neurokinin B mRNA in the human magnocellular basal forebrain system. The present study was designed to determine if neurokinin B (NKB) mRNA is colocalized with choline acetyltransferase (ChAT) mRNA in this region. Basal forebrains were collected from 3 adults without histories of neurological disease. Coronal blocks were snap frozen and serially sectioned at 10 µm thickness. *In situ* hybridization was performed on adjacent sections at an interval of ten using ^{35}S-labeled 48-base synthetic cDNA probes. Cells were identified in adjacent sections to determine mRNA colocalization. In the nucleus basalis of Meynert, 93.1% of magnocellular neurons contained ChAT mRNA. Of these neurons, 30.3% also expressed NKB gene transcripts. This study is the first to identify peptide colocalization in a significant population of magnocellular cholinergic neurons in the human nucleus basalis of Meynert.

INTRODUCTION

The human magnocellular basal forebrain system consists of large neurons scattered throughout the medial septum, the horizontal and vertical limbs of the diagonal band of Broca (dbB) and the nucleus basalis of Meynert (nbM) (Saper and Chelimsky, 1984; Hedreen et al. 1984). Immunocytochemical studies have demonstrated that these large neurons contain choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine (Hedreen et al. 1983; Mesulam et al. 1983; Satoh and Fibiger, 1985; Mesulam and Geula, 1988; Walker et al. 1989a). Neurons in the magnocellular basal forebrain system project to neocortical areas in a widespread and topographical manner (Kievit and Kuypers, 1975b; Kievit and Kuypers, 1975a; Jones et al. 1976; Mesulam and Van Hoesen, 1976; Mesulam et al. 1983). The nbM in particular has been a focus of intense investigation, due to the degeneration of this region in patients with Alzheimer's disease (Whitehouse et al. 1981; Whitehouse et al. 1982). Although early studies in experimental animals indicated an important role for basal forebrain cholinergic cells in learning and memory (Murray and Fibiger, 1985; Ridley et al. 1986; Dekker et al. 1991; Dunnett , and Fibiger, 1993; Wainer et al. 1993; Nabeshima, 1993) more recent research has emphasized the importance of this nucleus in the process of attention (Jones et al. 1995).

There have been no reports of neuropeptide colocalization with acetylcholine in the magnocellular basal forebrain of the human. Numerous magnocellular forebrain neurons contain galanin in non-human primates (Melander and Staines, 1986; Walker et al. 1989a; Kordower and Mufson, 1990), but there are only a few neurons expressing this peptide or mRNA in the magnocellular system of the human (Walker et al. 1991). Small numbers of magnocellular neurons that express GAD mRNA (Walker et al. 1989b), luteinizing-hormone releasing hormone mRNA (Rance et al. 1994) or tyrosine hydroxylase-immunoreactivity (Gouras et al. 1992) have been observed in the primate, but it is not known if these substances are colocalized with acetylcholine. We previously observed a large number of neurons containing neurokinin B (NKB) mRNAs in the human magnocellular basal forebrain (Chawla et al. 1994). In the present study we determined if NKB and ChAT mRNAs are colocalized within the magnocellular neurons of the human nbM.

MATERIALS AND METHODS

Basal forebrains were collected from 3 adults (ages 19, 74 and 86 years) who had died suddenly from unexpected causes (acute myocardial infarct, asphyxia and ruptured aortic aneurysm respectively). The post mortem intervals ranged from 8-17 hours. There was no history of systemic or neurological diseases other than atherosclerosis. Autopsy specimens were collected in accordance with guidelines set forth by the *Federal Register* and the University of Arizona.

Each brain specimen was bisected midsagittally and coronal blocks were dissected by cuts rostral to the optic chiasm, caudal to the tuberoinfundibular sulcus and superior to the anterior commissure. These blocks included the anterior region of the nBM and anterior hypothalamus. The tissue blocks were rapidly frozen by immersion in isopentane cooled to -30°C, and then stored at -80°C. Serial sections (10 μm thickness) were cut in a cryostat and thaw mounted onto gelatin coated slides.

In situ hybridization was performed on adjacent sections using oligonucleotides complementary to human ChAT and NKB mRNAs respectively. The ChAT probe was a 45-mer targeted to bases 5683-5727 of human ChAT cDNA (Toussaint et al. 1992). This probe has been previously used to identify cholinergic neurons in human striatum (Aubry et al. 1994). The NKB probe was a 48-mer [see (Rance and Young, III, 1991) for base sequence]. The probes were end labeled with ^{35}S-dATP (New England Nuclear, Boston, MA.) using terminal deoxynucleotidyl transferase (Boerhinger-Mannheim). The slides were brought to room temperature, fixed for 5 minutes in 4% formaldehyde in phosphate buffered saline and then immersed in 0.25% acetic anhydride in triethanolamine for 10 minutes. The slides were dehydrated in a graded series of ethanols and delipidated for 5 minutes in chloroform. After drying, the sections were incubated overnight at 37^{o}C in 50 μl hybridization buffer consisting of 600 mM NaCl, 80 mM Tris-HCl (pH 7.5), 4 mM ethylenediaminetetraacetic acid, 0.1% sodium pyrophosphate, 0.2% sodium dodecyl sulfate, 50% dextran sulfate, 0.2 mg/ml heparin sulfate, 100 mM dithiothreitol and ~ 10^6 dpm of ^{35}S-labeled probe. The hybridization was followed by several washes with 2 X SSC (SSC = 150 mM NaCl and 15 mM Na citrate) at room temperature followed by 2 washes in SSC and 50% formamide at 42^{o}C. The slides were then dried after briefly dipping in 70% alcohol and processed for autoradiography by dipping in NTB-3 emulsion (diluted 1:1 in water, Eastman Kodak). The slides were developed after an exposure time of 4-6 weeks and counterstained with toluidine blue. The specificity for NKB probe was verified by Northern blot analysis on total RNAs isolated from human hypothalamus, hippocampus, and caudate, under identical conditions of stringency(Rance and Young, III, 1991). An sense probe targeted to bases 5683-5727 of human ChAT cDNA (Toussaint et al. 1992) revealed no cell labeling.

Because of the large size of the magnocellular neurons, sections from the same neuron could be identified in the adjacent slides. Sections were examined with a Nikon Labophot microscope equipped with a Nikon drawing tube. Camera lucida drawings were made of all large neurons, blood vessels or other landmarks using a Nikon plan apochromat 10X objective. A 40 X plan apochromat objective was the used to mark labeled cells (defined as >3X background). The tracings of adjacent sections hybridized with either the ChAT or NKB probe were superimposed on one another by alignment of tissue landmarks to facilitate the identification of the same neuron in both sections. Verification of neurons common to adjacent sections was performed with the 40X objective.

RESULTS

Both the ChAT and the NKB probes labeled magnocellular numerous neurons diffusely distributed throughout the anterior nbM. In general, the labeling intensity with the ChAT probe was medium to light, compared with NKB probe which yielded robust labeling (Fig. 3.2). The labeled neurons were magnocellular in morphology, with large central nucleoli and prominent Nissl substance. Rare numbers of small neurons also espressed ChAT or NKB mRNAs. In all three subjects 1181 magnocellular neurons were identified in adjacent sections for subsequent analysis. Approximately 93% of these neurons contained ChAT mRNA (Table 3.1) in agreement with previous immunocytochemical studies (Mesulam and Geula, 1988). A significant number (~ 28 %) contained both ChAT and NKB mRNAs (Table 3.1). No morphological differences were identified between the cells that contained ChAT mRNA vs the cells that expressed both ChAT and NKB mRNAs.

DISCUSSION

The results of this study indicate that a significant percentage of magnocellular cholinergic neurons throughout the anterior nbM contain NKB mRNA. Although the functional implications of this colocalization are unknown, the presence of NKB in cholinergic projection neurons of the nBM raises the possibility of a modulatory role of this peptide on higher cortical functions in the human. Because of the importance of the basal forebrain cholinergic system in cognitive function, and its vulnerability to degeneration in age and disease (Whitehouse et al. 1981; Whitehouse et al. 1982; Collerton, 1986), a substantial effort has been devoted in elucidating the factors which sustain neuronal viability (Bizon et al. 1996) in the basal forebrain. In this regard, it is of interest that NKB has been shown to protect cholinergic neurons from excitatory amino acid-induced neurotoxicity (Wenk et al. 1995). In addition, both NKB and substance P can prevent the toxicity induced by amyloid β-protein to hippocampal neurons in culture (Yankner et al. 1990; Kowall et al. 1991).

Figure 3.1. A: Computer-assisted coronal map of the location of ChAT mRNA containing neurons in the human nucleus basalis of Meynert. B: The box shows the distribution of magnocellular neurons containing ChAT mRNA only (open circles), NKB mRNA only (triangle), ChAT and NKB mRNA (closed circles) and unlabeled (diamond).

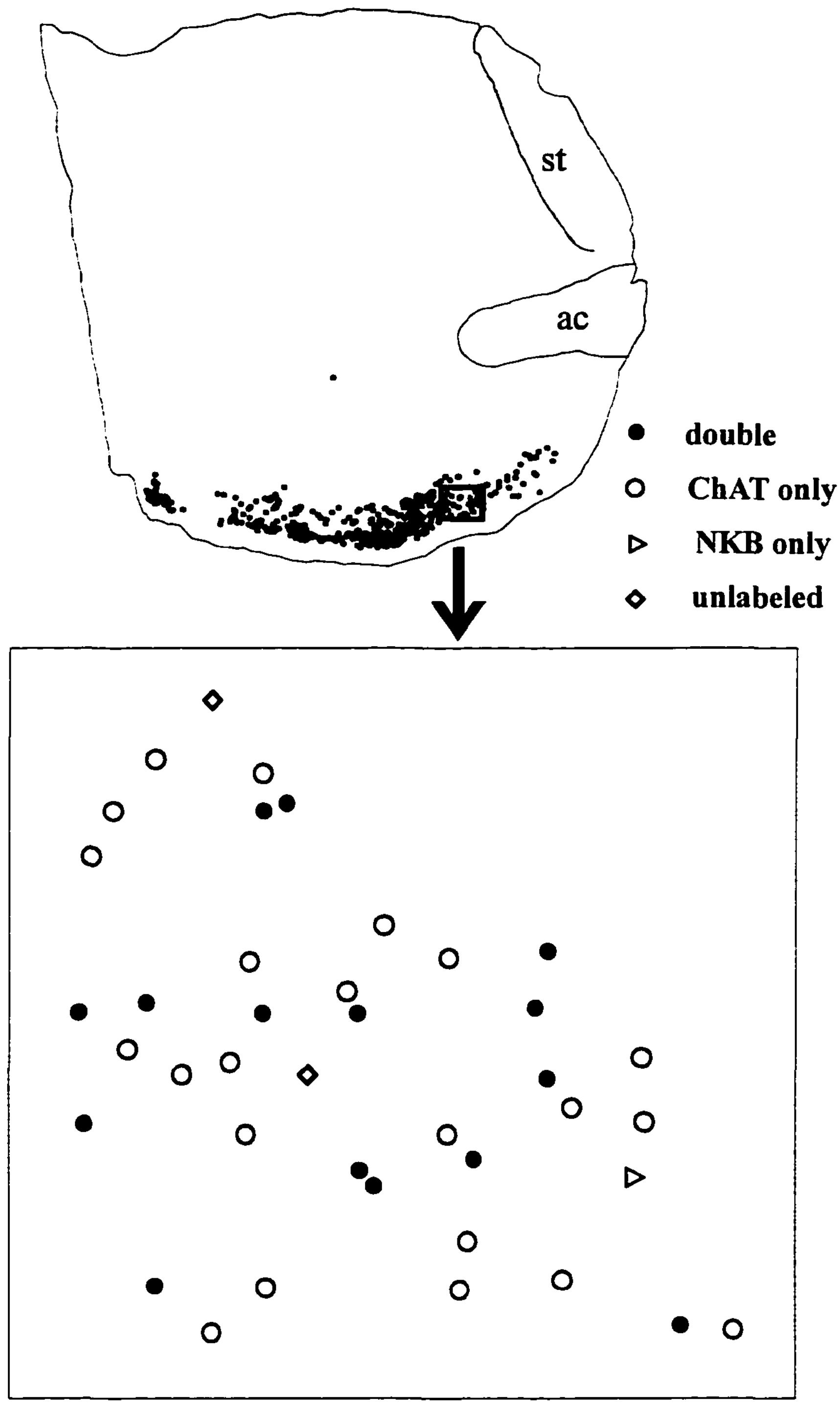

st
ac
double
ChAT only
NKB only
unlabeled

Figure 3.2: Bright-field photomicrographs of a magnocellular neuron (arrows) on adjacent sections containing ChAT (A) and NKB (B) mRNA. Arrowheads show landmarks used to align cell sections. The grains show the location of mRNAs. The neuron is lightly labeled by the ChAT probe in A, compared to dense labeling by the NKB probe.

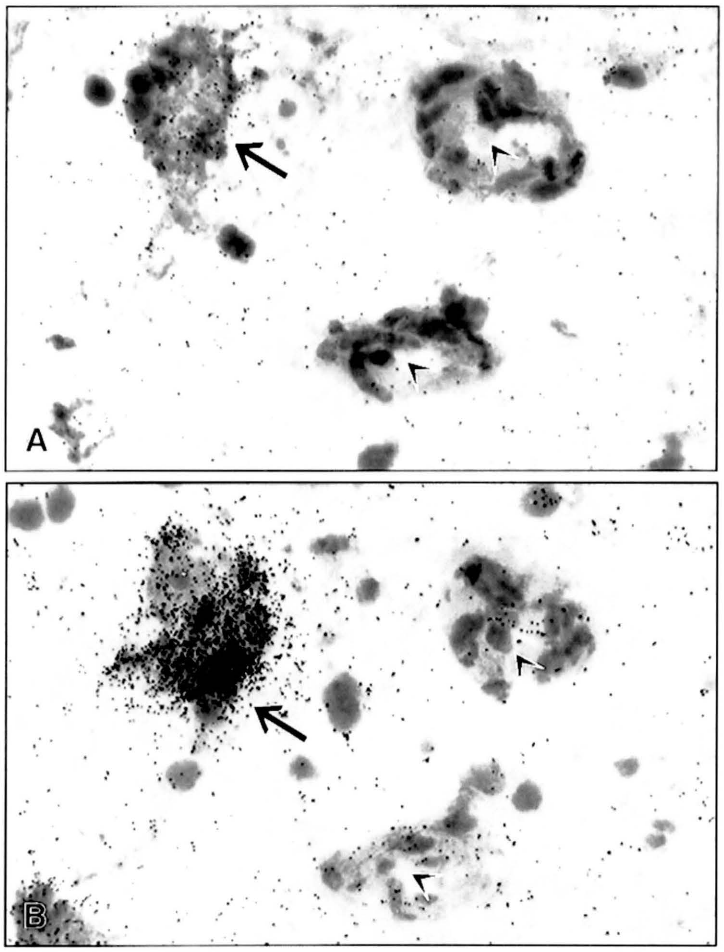

A
B

TABLE 3.1: Labeling of magnocellular neurons identified in adjacent sections located in the nucleus basalis of Meynert.

Subject	# counted	# NKB	# ChAT	# double	# unlabeled	# NKB only	# ChAT only
NR2-92	402	146	369	139	26	7	230
NR18-92	239	55	215	42	11	13	173
NR9-93	540	132	515	125	18	7	390
Total	1181	333	1099	306	55	27	793

TABLE 3.2: Percent of magnocellular neurons containing ChAT or NKB mRNAs in the nucleus basalis of Meynert

Subject	# counted	% NKB	% ChAT	% double	% unlabeled
NR2-92	402	36.3	91.8	34.6	6.5
NR18-92	239	23.0	90.0	17.6	4.6
NR9-93	540	24.4	95.4	23.1	3.3
Total	1181	28.2	93.1	25.9	4.7

CHAPTER 4

GnRH AND SUBSTANCE P RECEPTOR mRNAs ARE NOT COLOCALIZED IN NEURONS IN THE HUMAN MEDIAL BASAL HYPOTHALAMUS

ABSTRACT

Menopause is characterized by ovarian failure and gonadotropin hypersecretion secondary to removal of steroid negative feedback. These changes are associated with increased GnRH and tachykinin gene expression in the hypothalamus of postmenopausal women. The tachykinin neurons contain estrogen receptor mRNA, which suggests that these neurons may function in the hypothalamic circuitry regulating estrogen negative feedback on gonadotropin release. In the present study we determined if GnRH and SP receptor mRNAs are colocalized in neurons in the human hypothalamus. If so, this would provide evidence of an anatomic connection between these two neuronal systems in the human brain. Hypothalami were collected from 5 adults without histories of neurologic or systemic disease. The hypothalami were serially sectioned in a cryostat onto subbed slides. *In situ* hybridization was performed using sensitive cRNA probes. An ^{35}S-labeled GnRH riboprobe labeled scattered neurons in the preoptic-septal regions and medial basal hypothalamus. A digoxigenin-labeled GnRH probe labeled neurons predominately in the medial basal hypothalamus, the primate control center for reproduction. Numerous magnocellular basal forebrain neurons were identified using a ^{35}S-labeled substance P receptor (SPR) cRNA probe. Double *in situ* hybridization was then performed using ^{35}S-labeled SPR and digoxigenin-labeled GnRH cRNA probes. Detection of GnRH mRNA was accomplished using an immunological method while the radiolabeled SPR probe was visualized by autoradiography. These studies did not reveal

the presence of SPR mRNA in GnRH neurons in the human medial basal hypothalamus, indicating that a link between them does not exist.

INTRODUCTION

GnRH is the hypothalamic hormone that controls the secretion of LH and FSH from the anterior pituitary gland (Page, 1994). The gonadotropins control ovarian function and stimulate the secretion of the steroid hormones, estrogen and progesterone. Gonadal steroids, in turn, regulate gonadotropin secretion via positive and negative feedback effects on higher brain centers (McEwen et al. 1982; Plant, 1986; Knobil and Hotchkiss, 1988). Although it is well documented that ovarian steroids influence hypothalamic function, GnRH neurons themselves do not contain estrogen or progesterone receptors (Shivers et al. 1983b; Fox et al. 1990). Therefore, it has been proposed that estrogen feedback effects are mediated by steroid sensitive interneurons that synapse onto GnRH neurons.

Numerous lines of evidence indicate that SP may participate in the regulation of steroid feedback on gonadotropin release. SP neurons are located within the primate medial basal hypothalamus (see chapter 2), the control center for reproduction in the primate (Krey et al. 1975). SP immunoreactive neurons in the rat arcuate nucleus contain estrogen receptors (Akesson and Micevych, 1988), and neurons in the human hypothalamus contain both SP and estrogen receptor mRNAs (Rance and Young, III, 1991). More importantly, hypothalamic tachykinins are modulated by ovarian steroids (Antonowicz et al. 1982; Tsuruo et al. 1984; Jarry et al. 1988; Brown et al. 1990; Akesson et al. 1991) and tachykinin levels in the median eminence, as well as the number and size of arcuate tachykinin-immunoreactive neurons change over the rat estrous cycle (Micevych et al. 1988; Antonowicz et al. 1982; Tsuruo et al. 1984).

A stimulatory role of tachykinins on LH secretion is suggested by a variety of experimental paradigms. For example, intraventricular injection of substance P (SP) stimulates LH release in ovariectomized rats (Vijayan and McCann, 1979; Arisawa et al. 1990) and conversely, injection of SP antiserum inhibits LH release (Dees et al. 1985; Arisawa et al. 1990). In an *in vitro* perfusion system, SP stimulated LH release from a rat hypothalamus and pituitary in a dose dependent manner (Ohtsuka et al. 1987). Moreover, SP induced LHRH release from the isolated medial basal hypothalamus into the perfusion efflux (Ohtsuka et al. 1987). Tachykinins may also have an inhibitory role on LH secretion under certain conditions (Kerdelhué et al. 1978; Picanco-Diniz et al. 1990).

There is also evidence that tachykinins participate in the hypothalamic circuitry regulating steroid feedback in postmenopausal women. Menopause is characterized by ovarian failure and gonadotropin hypersecretion secondary to removal of estrogen negative feedback. These changes are accompanied by hypertrophy of neurons in infundibular nucleus of postmenopausal women (Sheehan and Kovacs, 1966; Sheehan, 1967; Rance et al. 1990; Rance and Young, III, 1991). The hypertrophied infundibular neurons contained estrogen receptor, SP and neurokinin B mRNAs but not GnRH mRNA (Rance and Young, III, 1991). In addition, tachykinin gene expression is markedly increased in the hypertrophied infundibular neurons of postmenopausal women (Rance and Young, III, 1991). More recently, it has been found that GnRH gene expression is also increased in the medial basal hypothalamus of postmenopausal women (Rance and Uswandi, 1994).

The finding of increased gene expression of both tachykinin and GnRH neurons in the hypothalami of postmenopausal women suggests that these two systems may be functionally connected. The anatomic basis for this connection is suggested by the finding that tachykinin-immunoreactive neurons in the arcuate nucleus of the rat establish synapses on LHRH neurons (Tsuruo et al. 1991). If this synapse exists in the human brain, GnRH neurons in the human hypothalamus should express SP receptor (SPR) mRNAs. Therefore, in this study a double *in situ* hybridization procedure was developed to determine if SPR and GnRH mRNAs were colocalized within human hypothalamic neurons.

MATERIALS AND METHODS

Tissue preparation

Hypothalami were collected from 5 adult males. Postmortem intervals were 5-24 hours and complete autopsies revealed no neurological or systemic diseases. Hypothalami were dissected by making coronal cuts rostral to the optic chiasm and caudal to the mammillary bodies. Sagittal cuts were made through the third ventricle and 4 mm lateral to the midline. The blocks were frozen in isopentane and serially sectioned in a cryostat at 20 um thickness. The tissue was thaw mounted onto gelatin-subbed slides and dried briefly on a warming plate (32^{o} C, 1 min). The first 100 sections were stored at -80^{o} C in slide boxes until ready to use. Every 10th section was examined in the procedures described below.

Subcloning of human SPR

Human substance P (1224 bp) cDNA was generously provided by Dr. James E. Krause, (Dept. Anatomy and Neurobiology, Washington University School of Medicine, WA). The plasmid DNA (pBluescript) was cut with BamHI and HindIII restriction enzymes and electrophoresis on a 1% agarose gel was performed to confirm the size of DNA insert. Because tachykinin receptors have homology with each other and with other G-protein coupled receptors, a Gene Bank search was performed using Mac Vector software to identify the region of least homology for subsequent subcloning. The plasmid was cut with PstI and SmaI to obtain a 685 bp fragment, and electrophoresis on a preparative low melting agarose gel was done to separate the fragment. The 685 bp fragment and the pBluescript vector (previously restricted with PstI and Sma I) were dephosphorylated using calf intestinal phosphatase prior to ligation. The ligation reaction was carried out in a 20 μl volume containing 100 mM Tris-HCl (pH7.5), 20mM $MgCl_2$, 20mM DTT, 10 mM ATP, 0.1 - 5 μg DNA, 20-500 U T_4 DNA ligase and allowed to proceed for 12-16 hrs at room temperature. A 1-10 ul of the ligated product was introduced into competent E. coli cells and placed on ice for 30 mins, heat shocked for 30-45 secs, placed on ice for 2 min and added to a small amount of LB medium. Incubation of the transformed E. coli cells was done at 37^{o}C with shaking at 225 rpm for 1 hour. The medium was plated on LB plates containing 100 ug/ml ampicillin and blue/white screening was performed using 40ug/ml X-gal (5-bromo-4-chloro-3-indolyl-ß galactopyranoside) and 0.5 mM IPTG (ß-D-isopropyl-thiogalactopyranoside). Recombinant colonies (white) were picked for amplification of plasmid DNA. After purification of the DNA and restriction with BssHII, electrophoresis on 1 % agarose gel was performed to confirm the fragment size. In addition, the fragment was sequenced using a dideoxy chain termination method to confirm the identity and orientation of the insert DNA.

Subcloning of human GnRH

A 500 bp Human GnRH fragment in a pSP65 plasmid (Promega) was generously provided by Dr. John Adelman, (Vollum Institute, Portland, OR). A 260 bp SacI fragment of the insert was subcloned into pBluescript (Stratagene) to remove a poly-adenylated tail present at the 3' end as described above.

***In situ* hybridization using ^{35}S-labeled GnRH and SPR riboprobes:**

Every tenth slide was processed for *in situ* hybridization. The synthesis of high specific activity radiolabeled cRNA probes was accomplished by *in vitro* transcription. 1.0 ug of GnRH cDNA was restricted with BssHII, extracted with phenol/chloroform and ethanol precipitated. Radioactive cRNA was synthesized in a reaction containing the following reagents: 250 uCi alpha-thio ^{35}S-UTP (NEN Research Products, Boston, MA), 50 uM each rATP, rCTP and rGTP, 1 µg linearized plasmid DNA, 40 mM Tris-HCl (pH7.5), 8 mM $MgCl_2$, 2 mM spermidine, 5 mM NaCl, 10 mM DTT, 1 U/µl RNase inhibitor and 10 U T_3 or T_7 RNA polymerase. The total reaction volume was 10 µl, contained in a 1.5 ml Eppendorf tube. The tube was mixed gently and incubated for 30 min at 37^{o}C. 10 U of RNA polymerase was added to the mix and incubated for another 30 min. To stop the reaction, 20 U RNase and 0.5 U RNase free DNase was added and incubated for 10 min at 37^{o}C. 422 µl TE, 22.5 µl 4 M NaCl, 50 µg tRNA and 1 ml ethanol was added to the Eppendorf , placed on wet ice for 10 min and spun in an Eppendorf centrifuge for 10 min. The resultant RNA pellet was rinsed with 1 ml 70% ethanol (RNase free) and dried. The RNA was reconstituted with 50 µl TE/ 0.2% SDS and 5 µl 5 M DTT.

Tissue sections were taken from -80^{o}C freezer and warmed to RT for 15-20 min. Sections were postfixed in a 4% formaldehyde in PBS solution, then treated with 0.25% acetic anhydride in 0.1M triethanolamine HCl/0.9% NaCl, pH 8, for 10 minutes. The slides were delipidated in ascending alcohols and chloroform. After drying the slides were incubated overnight under paraffin coverslips with 1-2 x 10^6 dpm probe per 60 μl of hybridization buffer which contained 50% deionized formamide, 100 μg/ml salmon sperm DNA, 250 μg/ml yeast total RNA, 20mM Tris-HCl (pH 7.4), 1 mM EDTA, 300 mM NaCl, 10% dextran sulfate, 10 mM DTT, 10% sodium thiosulfate, 10% SDS, 0.02% BSA, 0.02% Ficoll and 0.02% polyvinylpyrrolidone. The coverslips were removed in 4X SSC/1mM DTT for 15 min, replacing with fresh solution 4 times and shaking on the rotator for 5 min at 70 rpm. The slides were then incubated for 30 min at 37^{o}C in 20 μg/ml RNase A dissolved in a buffer containing 500 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 250 μM EDTA. Following the RNase treatment, four 5 min RT rinses in 0.1 x SSC/1 mM DTT and two 30 min rinses at 65^{o}C in 0.1x SSC /1 mM DTT were done. The slides were then placed in 0.1x SSC at RT to cool, followed with 1 min rinses in a graded series of alcohol containing 300 mM ammonium acetate. After dipping in 100% alcohol and dried before processing for autoradiography.

Autoradiographical detection:

Nuclear track emulsion (NBT3, Kodak, Rochester, NY) diluted 1:1 water was heated to 42^{o}C in the dark room. The slides were dipped into the emulsion, taking precautions against the formation of air bubbles. Air drying of the slides was conducted in the dark room for 2-3 hrs and placed in light-tight slide boxes containing dessicant capsules. The slides were stored at 4^{o}C, and test slides developed at intervals to determine optimum exposure times. The slides were developed in Dektol (Kodak) and fixed in Kodak fixer.

***In situ* hybridization using digoxigenin labeled GnRH cRNA probe:**

The probe was made by transcribing the 260 bp GnRH cDNA fragment in the presence of digoxegenin-11 UTP and T7 RNA polymerase. The reaction mixture contained 400 uM digoxegenin-labeled -rUTP (Boehringer Mannheim, Indianapolis, IN), 100 uM unlabeled rUTP, 500 uM each rATP, rGTP, rCTP, 1 µg linearized plasmid DNA, 0.5 unit/µl RNase inhibitor, 40 mM Tris-HCl (pH7.5), 6mM $MgCl_2$, 2 mM spermidine, 5mM NaCl, 10 mM DTT and 500-100 units/ml of bacteriophage DNA dependent RNA polymerase. After incubation at 37^oC for 1 hr, 500-1000 units/ml RNA polymerase were added and incubated another hour. DNase and RNAse inhibitors were added to a final concentration of 0.1 µg/µl and 1 unit/µl respectively and incubated at 37^oC for 10 min. The probe was then purified on a G-50 Sephadex column (BM) and stored at -80^oC.

Immunological detection of digoxigenin :

Hybridization histochemistry using the digoxigenin-labeled GnRH probe was performed as described for the radiolabled probe. For visualization of the digoxigenin label, non-specific binding was first prevented by incubating the tissue in a blocking solution containing 2x SSC, 0.05% Triton X-100 and 2% normal sheep serum (Vector Labs, Burlingame, CA) overnight. The slides were then washed in a buffer containing 100 mM Tris-HCl (pH 7.5) and 100 mM NaCl for 10 min and for 6 hrs in same solution containing anti-digoxegenin-peroxidase (Boehringer Mannheim) diluted 1:200 (0.75 U/ml), 0.2% Triton X-100 and 1.0% normal sheep serum. The sections were incubated for 10-20 min in peroxidase color reagent made by dissolving 10 mg 3,3'-

diaminobenzidine tetrahydrochloride (DAB) in 50 ml 100 mM Tris-HCl (pH 7.6) and 8 μl hydrogen peroxide. The reaction was stopped in 0.1M Tris (pH 7.6), briefly dipped in water and then 70% EtOH. The sections were counterstained with Geimsa.

Double *in situ* hybridization using digoxigenin-labeled GnRH and ^{35}S-labeled tachykinin receptor cRNA probes

In vitro transcription was used to generated a digoxigenin-GnRH riboprobe and and ^{35}S-labeled SPR riboprobe as described above. *In situ* hybridization was performed by incubating the sections with both probes simultaneously, at a concentration of $2x10^6$ DPM (radiolabeled) + 5 μl (digoxigenin-labeled) per 100 μl of the hybridization buffer. The procedures to detect digoxigenin were performed first, followed by dipping in photographical emulsion for autoradiographic detection of the radiolabeled probe.

Controls for specificity

No labeled cells were observed when sections were hybridized with GnRH and SPR sense probes under the same conditions of stringency. In addition, Northern blot analysis was performed on total RNA isolated from human caudate, posterior hypothalamus, hippocampus and substantia nigra using the SPR probe under the same conditions of stringency as the *in situ* hybridization. This procedure revealed the presence of a band of approximately 1.5 kb consistent with the expect size of the SPR (Fig. 4.1).

RESULTS

The radioactive GnRH probe labeled small neurons in the infundibular nucleus, preoptic area, septal region, bed nucleus of stria terminalis, diagonal band of Broca, and large neurons in the nucleus basalis of Meynert (Fig. 4.3). The morphology and distribution of these neurons was similar to previous studies of the human hypothalamus that used oligonucleotide probes (Rance et al. 1994). In addition, after 6 weeks of exposure, large neurons in the diagonal band of Broca were labeled that were not previously observed. The digoxigenin-GnRH cRNA probe labeled neurons primarily in the medial basal hypothalamus (Fig. 4.4). These neurons were small in size, elongated to oval with morphologic features similar to immunoreactive-GnRH neurons previously described in the human hypothalamus (King and Anthony, 1984; Stopa et al. 1991). The DAB reaction product was identified in the cell body and proximal processes of GnRH neurons (Fig. 4.4).

The SPR probe labeled magnocellular neurons in the basal forebrain, including the diagonal band of Broca and nucleus basalis of Meynert (Fig. 4.6). No SPR mRNA containing neurons were found in the hypothalamic regions containing GnRH neurons. This impression was confirmed by the *in situ* hybridization procedure conbining the digoxigenin GnRH riboprobe and the radiolabeled SPR riboprobe. Digoxigenin labeled GnRH neurons were observed in the medial basal hypothalamus, but no silver grains were concentrated over the cell bodies of these neurons (Fig. 4.7).

DISCUSSION

Using sensitive cRNA probes and a double-labeling procedure, we were unable to demonstrate colocalization of GnRH and SPR mRNAs within neurons of the human hypothalamus. We cannot rule out the possibility that SPR mRNA is present in GnRH but below levels of detection using the current methodology. However, SPR mRNA was detected in magnocellular basal forebrain neurons on the same slides, providing evidence of the success of the *in situ* hybridization procedure. It is also possible that the effects are mediated via another subtype of tachykinin receptor such as the NK3 receptor.

Tachykinin receptors bind to G-proteins and stimulate phosphoinositol turnover and intracellular Ca^{2+} release. Recently a putative cAMP response element (CRE) sequence has been identified in the promotor of SPR gene, which implicates autoregulation by the receptor (Foulkes et al. 1991). Receptors for peptides may be up regulated or down regulated depending on the concentration of ligand it is exposed to and the time of exposure. Some receptors are internalized and down regulated by degradation, others are inactivated by phosphorylation (Collins et al. 1992). The activation of PKA due to initial rise in cAMP phosphorylates many intracellular substrates, one of which is the receptor, while another is the cAMP response element binding protein (CREB), whose ability to stimulate target gene transcription is regulated by it's phosphorylation. A CRE sequence has been identified in the promoter region of NK1 receptor gene (Foulkes et al. 1991), but its functional significance remains to be elucidated. CRE/CREB interaction and increased or decreased gene transcription of receptor mRNA may provide the cell with a compensatory mechanism for maintaining receptor number and responsiveness. Thus, our inability to detect tachykinin receptor mRNA in the infundibular nucleus may be a result of receptor downregulation mediated at the transcriptional level.

It is also possible that the increase in SP gene expression in the hypothalamus of postmenopausal women is not related to the increase in GnRH gene expression. There are numerous peptides/and or classical neurotransmitters involved in the regulation of GnRH neurons. For example, ultrastructural studies in laboratory rats have shown that GnRH neurons receive synaptic input from norepinephine, dopamine, serotonin, GABA, neuropeptide Y and beta-endorphin neurons (Chen et al. 1989; Horvath et al. 1993; Kiss and Halasz, 1985; MacLusky et al. 1988; Tsuruo et al. 1991; Tsuruo et al. 1990; Chen et al. 1989). Perhaps steroid negative feedback of GnRH neurons in humans occurs via one of these transmitter systems. Alternatively, SP neurons could modulate GnRH neurons via an interneuron, and thus a direct synaptic connection may not exist.

Because tachykinins are potent vasodilators, perhaps they may be increasing the blood supply to the anterior pituitary and thereby increasing the amount of available GnRH. Finally, it is possible that SP modulates gonadotropin secretion directly at the level of the anterior pituitary gland.

Our finding of NK1 receptor mRNAs in magnocellular neurons in the human basal forebrain is consistent with studies showing immunoreactive SPR-immunoreactivity in this region (Kowall et al. 1993). Immunocytochemical studies have also shown SPR-immunoreactivity in the septal nuclei, diagonal band of Broca and nucleus basalis of Meynert of the rat (Nakaya et al. 1994). The nucleus basalis is innervated by a dense network of SP-immunoreactive fibers (Haber and Watson, 1985) and SP-immunoreactive nerve terminals are closely associated with magnocellular neurons in the human nucleus basalis (Beach et al. 1987). In the rat, ultrastructural studies have confirmed the presence of a synapse of SP-immunoreactive terminals on cholinergic neurons (Bolam et al. 1986; Zaborszky et al. 1991).

Fig. 4.1: Northern blot analysis using the human SPR probe. Lanes 1-4 are total RNA isolated from human cortex, substantia nigra, posterior hypothalamus and striatum respectively. The band at approximately 1.5 kb (indicative of SP receptor mRNA size) is seen in all 4 lanes, although the posterior hypothalamus and the striatum contain a denser band. The second band seen at the bottom of the gel may be accumulation of very small sized RNA.

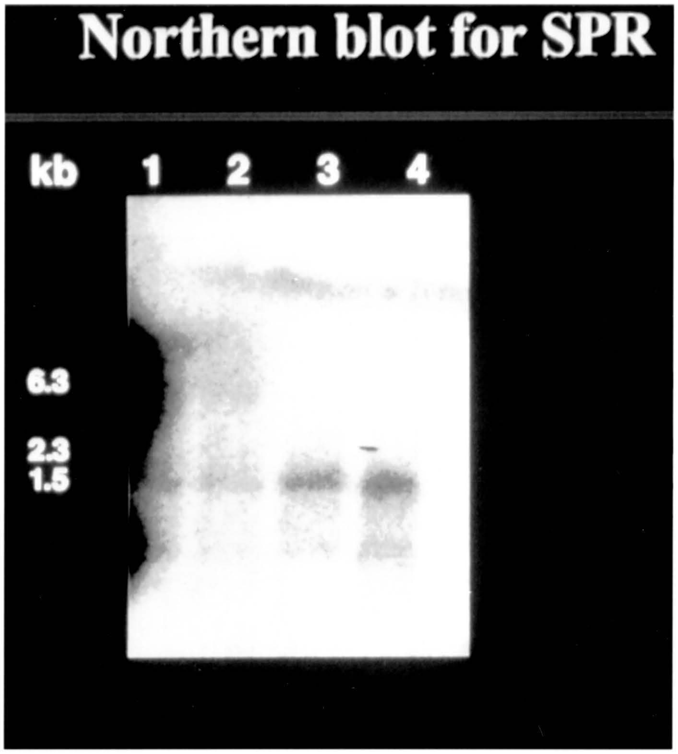

Northern blot for SPR
kb
1
2
3
4
6.3
2.3
1.5

Fig. 4.2: A Southern blot obtained during the subcloning of human GnRH. Panel A contains a photo of the preparative low-melting agarose gel. Lane 1 indicates the DNA size markers, lanes 2-3 indicate the size of the insert DNA after digestion of pSP65 plasmid cut with the restriction enzyme (SacI) into approximately 260 bp and 230 bp fragments. Panel B shows the size of the insert DNA after subcloning into pBluescript plasmid. Lane 1 contains the DNA size markers, lane 2 indicates the size of DNA insert (approximately 260 bp) after restriction with BssHII. This enzyme cleaves the insert DNA and well as the RNA polymerase promoter regions flanking the multiple cloning site.

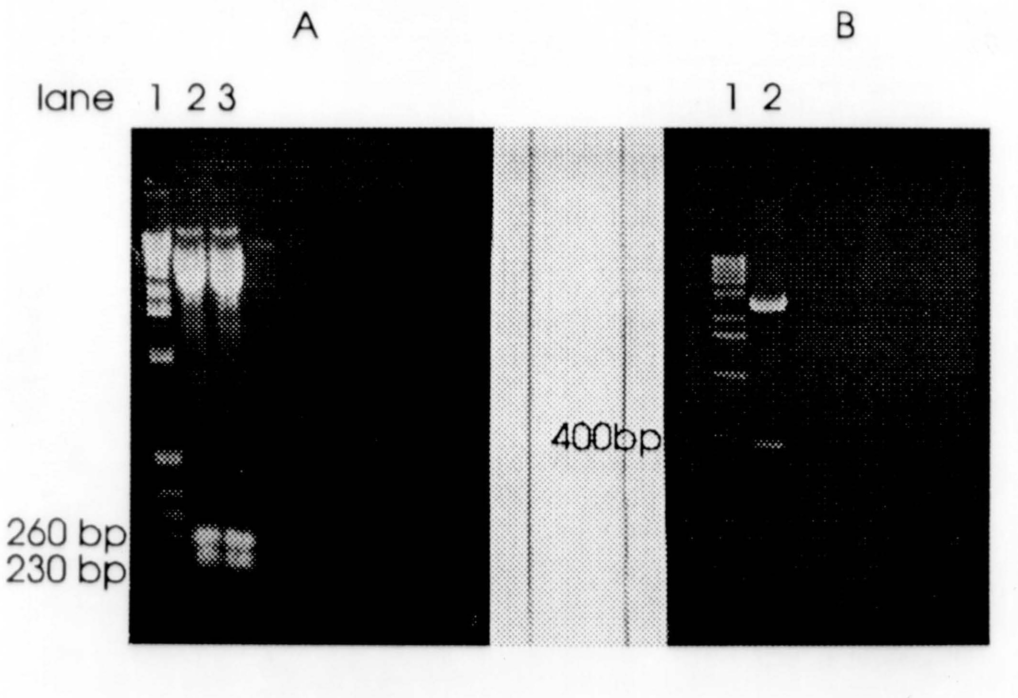

A
B
lane 1 2 3
1 2
400bp
260 bp
230 bp

Fig. 4.3: Photomicrograph of a neuron in the human infundibular nucleus labeled with the ^{35}S-labeled GnRH cRNA probe. Autoradiographic grains mark the location of GnRH mRNA. The cytoplasm of this neuron is not visible due to prior RNase treatment. Nuclei are counterstained with toluidine blue.

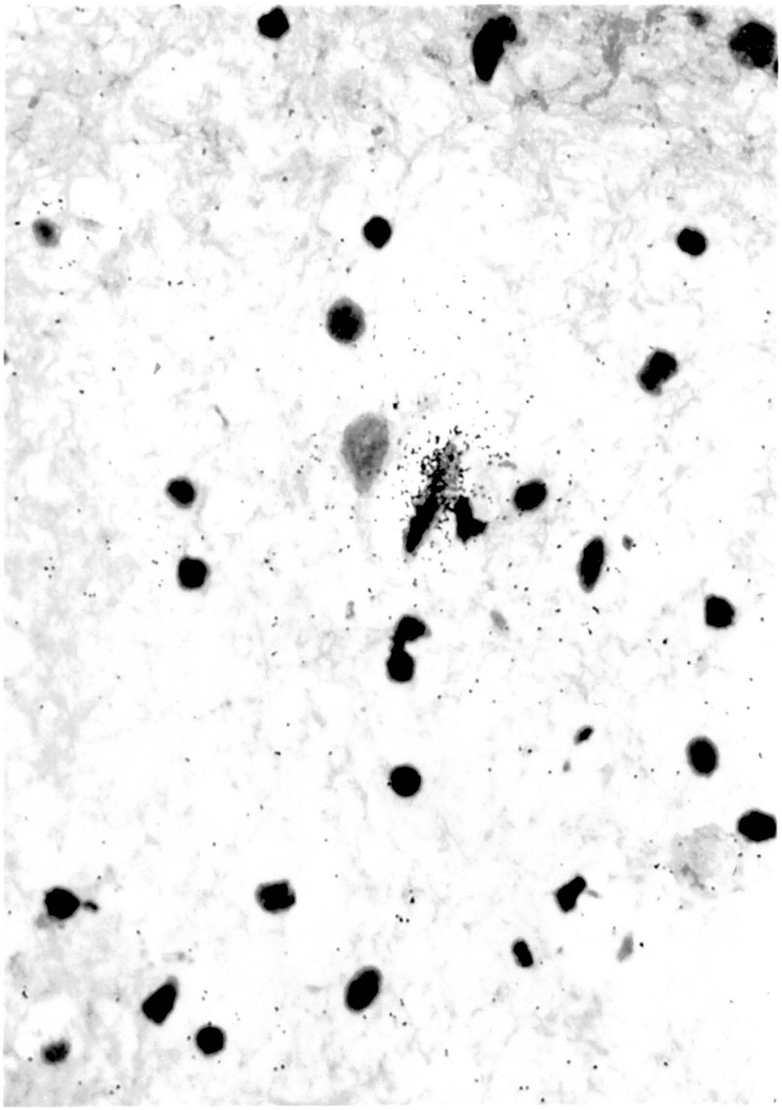


Fig. 4.4: Photomicrograph of a neuron in the human hypothalamus labeled with the digoxigenin-labeled GnRH riboprobe. The brown reaction product in the cytoplasm identifies the location of GnRH mRNA. Cell nuclei are counterstained with methyl-green.

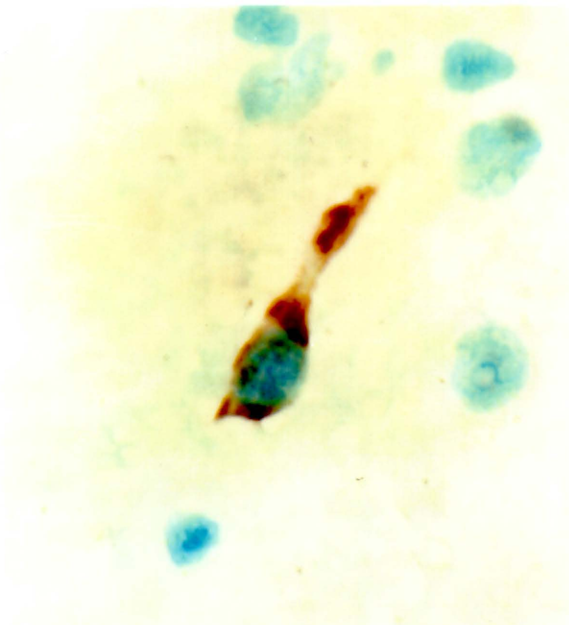


Fig. 4.5: Photomicrograph of neuron labeled by the ^{35}S-labeled SPR riboprobe in the rat striatum. The autoradiographic grains mark the location of SPR mRNA. The nucleus is out of the plane of focus. The cytoplasm of this neuron is not visible due to prior RNase treatment. Nuclei are counterstained with toluidine blue.

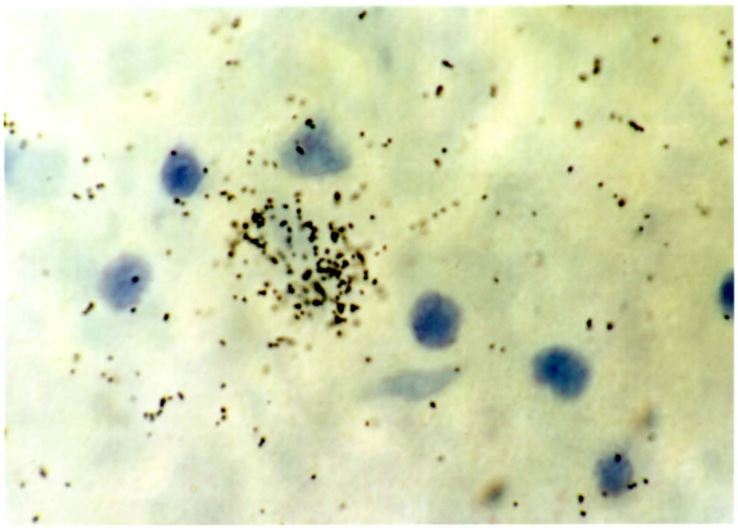


Fig. 4.6: Photomicrograph of a magnocellular neuron in the human basal forebrain labeled with the ^{35}S-labeled SPR probe. The image has been inverted using Adobe Photoshop software to make the autoradiographic grains appear white.

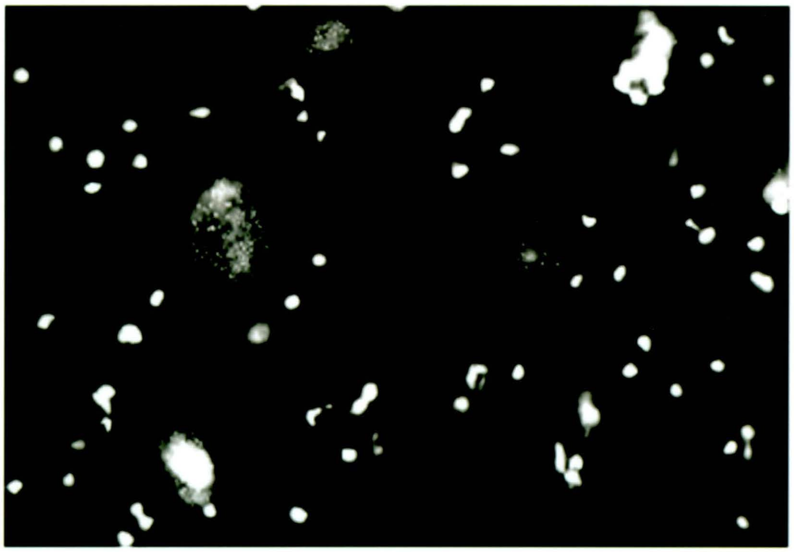
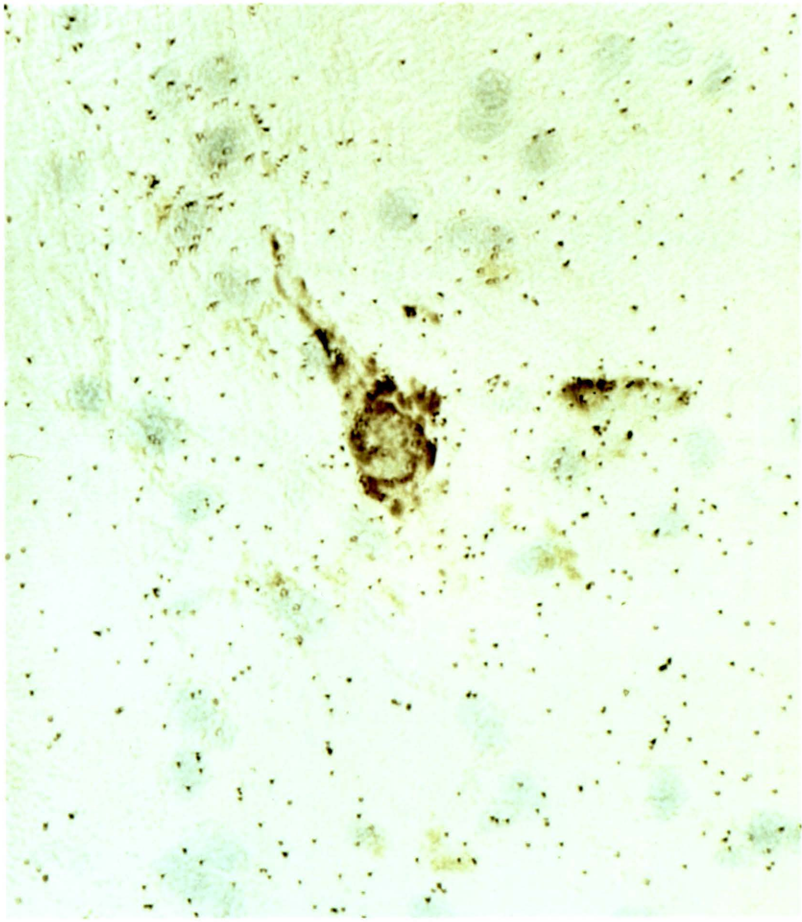


Fig. 4.7: Photomicrograph of a neuron labeled with the digoxigenin-GnRH probe (brown reaction) which was simultaneously hybridized with a ^{35}S-labeled SPR riboprobe. Although background grains are scattered throughout the photomicrograph, no grains are concentrated over the cytoplasm of the GnRH neuron.



CHAPTER FIVE

SUMMARY

The hypothalamus is a complex region of the brain that integrates a wide range of autonomic, endocrine and behavioral responses involved in homeostasis and reproduction. These functions include the regulation of body temperature, cardiovascular responses, ingestive behavior, thirst, osmolality, and sexual and maternal behaviors that are necessary for the survival of the species. The precise localization of the many hypothalamic functions has been traditionally studied in experimental animals because of the inherent difficulties in working with human tissue. To ultimately extrapolate this information to humans, however, research must be performed on humans. In these studies, *in situ* hybridization has been applied to human postmortem tissue to provide some of the first detailed information of the architecture of the tachykinin systems in the human hypothalamus and basal forebrain.

Even though tachykinins were known to be present in high quantities in the human hypothalamus, their exact cellular distribution as well as their subtype specificity was not known. In part, this was due to the limitations of applying immunocytochemistry to human material because the cell somata frequently cannot be visualized without colchicine pretreatment. In addition, there are several different kinds of tachykinins with significant homologies between their genes and amino-acid sequence. All previous human studies utilized antibodies that cross-reacted with the various tachykinin species and differences exist in mammalian species in terms of tachykinin distribution. For example, in the monkey, a dense plexus of SP immunoreactivity is present in the median

eminence, and SP cell bodies are in the infundibular nucleus, while in rodents this area is relatively poor in SP. Therefore, one could not assume that the distribution of tachykinins in experimental animals applied to humans.

We used oligonucleotide probes targeted to non-homologous regions of tachykinin cDNAs and mapped the distribution of these mRNAs with the aid of a computer microscope system. These studies provide the first demonstration of a distinct but complementary distribution of SP and NKB precursor mRNAs in the human hypothalamus and basal forebrain. We found numerous SP neurons in the medial mammillary nucleus, an area which is devoid of SP neurons in rats. Abundant SP neurons were also present in the medial, supra and premammillary nucleus, the intermediate nucleus, posterior hypothalamus and the striatum. In contrast, NKB mRNA was identified in neurons within the magnocellular basal forebrain, bed nucleus of the stria terminalis, and preoptic/anterior hypothalamic region. Both SP and neurokinin B mRNAs were identified in the infundibular nucleus and amygdala.

Overall, NKB mRNA containing neurons were prominent in rostral hypothalamic nuclei whereas, SP was the predominant tachykinin in the caudal nuclei. This distinctive pattern may be a reflection of a general organization of hypothalamic circuits which have divergent rostral and caudal connections with the telencephalic limbic system. The medial preoptic region comprises a major part of the rostral nuclei and is thought to play a critical role in a variety of reproductive, thermoregulatory and ingestive behaviors. The anterior medial zone nuclei receives direct imputs from the olfactory and visceral systems, and indirect imputs via association areas of the cerebral cortex including the limbic regions. Output of information from the anterior medial zone nuclei are conveyed to the telencephalon, diencephalon and brainstem regions that influence neuroendocrine, autonomic and somatomotor responses. It is also important to note that circulating gonadal steroids which bind to cells in this region have a regulatory effect on most of these functions. The caudal nuclei of the medial hypothalamus are mainly associated with cognitive, emotional, learning and memory functions based upon their afferent connections from hippocampus and efferents to the anterior thalamus.

One notable finding of the mapping studies was the identification of a significant population of NKB neurons in the magnocellular basal forebrain system including the nucleus basalis. Because approximately 90% of magnocellular basal forebrain neurons are cholinergic, these data suggested that NKB was colocalized in cholinergic neurons in this region. The nucleus basalis provides the major cholinergic projection system to neocortex, and is thought to play an important role in learning and memory in primates. In addition, this region has been a subject of intense investigation following the demonstration of degeneration of the magnocellular cholinergic neurons in Alzheimer's disease.

Because of the large size of the magnocellular neurons we were able to identify individual neurons on adjacent slides to determine the presence or absence of colocalization. This method was chosen over a double-labeling procedure (chapter 4) so that the sensitivity could be optimized for both probes. We found that approximately 90% of the magnocellular neurons in the nucleus basalis contain ChAT mRNAs, consistent with studies showing that 80-90% of magnocellular neurons are ChAT immunoreactive. Approximately 30% of these neurons also contained NKB mRNAs. These data are the first demonstration of peptide colocalization in magnocellular cholinergic neurons and suggest that NKB may have an important role in the function of the major cholinergic projection system to neocortex. The presence of SP receptor mRNAs in the basal forebrain magnocellular neurons (chapter 4) also suggests a role for SP in the regulation of these neurons.

Earlier experiments had shown that tachykinins as well as GnRH gene expression increases in the medial basal hypothalamus of women in association with ovarian steroid withdrawal of menopause. These data, as well as numerous studies in experimental animals, led to the hypothesis that SP neurons are modulating GnRH neurons in the human medial basal hypothalamus via a synaptic connection. In chapter four, we used a double *in situ* hybridization procedure to investigate whether SPR mRNAs are present in GnRH mRNA containing neurons in the human hypothalamus. A sensitive GnRH riboprobe labeled scattered neurons throughout the rostral and medial basal hypothalamus consistent with previous studies. The digoxigenin-probe was less sensitive: GnRH neurons were only visulalized in the medial basal hypothalamus, the region with highest gene expression. SPR mRNA was identified in magnocellular neurons of the basal forebrain, but we were unable to identify neurons in the medial basal hypothalamus that contained this mRNA. The double-labeling procedure confirmed that medial basal hypothalamic GnRH neurons did not contain tachykinin mRNAs. There are many interpetations of finding that GnRH neurons do not express SPR mRNA in the human hypothalamus that are discussed in detail in chapter 4. We were unable to confirm our hypothesis that SP neurons are affecting GnRH neurons via a synaptic connection.

These studies have provided important new information on the chemoarchitecture of the tachykinin systems in the human hypothalamus. In addition they provide the first demonstration of peptide colocalization in the major cholinergic system of neurons in the human brain. A firm anatomic foundation in the human brain, combined with the numerous anatomic and physiologic studies in laboratory animals, will ultimately shed light on the functions of the tachykinin systems in the human brain.

APPENDIX A

HUMAN SUBJECTS:

This project is exempt from Human Subject Approval under Exemption #5. This research involves the study of pathological specimens in which "information is recorded by the investigator in such a manner that subjects cannot be identified directly or indirectly through identifiers linked to the subjects" (45 Code of Federal Regulations 46.101, Exemption # 5).

REFERENCES

Abel, T.W., M.L. Voytko, and N.E. Rance (1996) Hormone replacement suppresses neurokinin B (NKB) gene expression in the arcuate nucleus of ovariectomized monkeys. Soc.Neurosci.Abstr. (submitted).

Adelman, J.P., A.J. Mason, J.S. Hayflick, and P.H. Seeburg (1986) Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin-release inhibiting factor in human and rat. Proc.Natl.Acad.Sci.USA *83*:179-183.

Akesson, T.R., C. Sternini, and P.E. Micevych (1991) Continuous estrogen decreases neurokinin B expression in the rat arcuate nucleus. Mol.Cell.Neurosci. *2*:299-304.

Akesson, T.R. (1993) Androgen concentration by a sexually dimorphic population of tachykinin-immunoreactive neurons in the rat ventral premammillary nucleus. Brain Res. *608*:319-323.

Akesson, T.R. (1994) Gonadal steroids regulate immunoreactive tachykinin in the ventromedial nucleus of the rat hypothalamus. J.Comp.Neurol. *341*:351-356.

Akesson, T.R., C. Ulibarri, and S. Truitt (1994) Divergent axon collaterals originate in the estrogen receptive ventromedial nucleus of hypothalamus in the rat. J.Neurobiol. *25*:406-414.

Akesson, T.R. and P.E. Micevych (1988) Estrogen concentration by substance P-immunoreactive neurons in the medial basal hypothalamus of the female rat. J.Neurosci.Res. *19*:412-419.

Akesson, T.R. and P.E. Micevych (1995) Sex steroid regulation of tachykinin peptides in neuronal circuitry mediating reproductive functions. In P.E. Micevych and R.P. Hammer (eds): Neurobiological effects of sex steroid hormones. Cambridge University Press: Cambridge, pp. 207-233.

Allen, L.S., M. Hines, J.E. Shryne, and R.A. Gorski (1989) Two sexually dimorphic cell groups in the human brain. J.Neurosci. *9*:497-506.

Allen, L.S. and R.A. Gorski (1990) Sex difference in the bed nucleus of the stria terminalis of the human brain.. J.Comp.Neurol. *302*:697-706.

Anastasi, A., P. Montecuchi, V. Esparmer, and J. Visser (1977) Amino acid composition and sequence of kassinin, a tachykinin dodecapeptide from the skin of African frog *Kassina senegalensis*. Experiential *33*:857-858.

Antonowicz, U., B. Jakubowska-Naziemblo, D. Cannon, and D. Powell (1982) Immunoreactive substance P content in median eminence and pituitary gland during oestrus, dioestrus and after anterior hypothalamic deafferentation. Endokrinol. *79*:25-34.

Arisawa, M., L. De Palatis, R. Ho, G.D. Snyder, W.H. Yu, G. Pan, and S.M. McCann (1990) Stimulatory role of substance P on gonadotropin release in ovariectomized rats. Neuroendocrinology *51*:523-529.

Aronin, N., R. Coslovsky, and S.E. Leeman (1986) Substance P and neurotensin: their roles in the regulation of anterior pituitary function. Annu.Rev.Physiol. *48*:537-549.

Aubry, J.M., M.F. Schulz, S. Pagliusi, P. Schulz, and J.Z. Kiss (1993) Coexpression of dopamine D_2 and substance P (neurokinin-1) receptor messsenger RNAs by a subpopulation of cholinergic neurons in the rat striatum. Neuroscience *53*:417-424.

Aubry, J.M., K. Lundstrom, E. Kawashima, P. Schulz, and V. Bartanusz (1994) NK1 receptor expression by cholinergic interneurons in human striatum. NeuroReport *5*:1597-1600.

Baruch, P., F. Artaud, G. Godehue, L. Barbeito, J. Glowinski, and A. Cheramy (1988) Substance P and neurokinin A regulate by different mechanisms dopamine release from dendrites, and nerve terminals of the nigrostriatal dopaminergic neurons. Neurosci. *25*:889-898.

Beach, T.G., H. Tago, and E.G. McGeer (1987) Light microscopic evidence for a substance P- containing innervation to the human nucleus basalis of Meynert. Brain Res. *408*:251-257.

Becker, J.T., M.R. Furman, M. Panisset, and C. Smith (1990) Characteristics of the memory loss of a patient with Wernicke-Korsakoff's syndrome without alcoholism. Neuropsychologia *28*:171-179.

Bizon, J.L., J.C. Lauterborn, P.J. Isackson, and C.M. Gall (1996) Acidic fibroblast growth factor mRNA is expressed by basal forebrain and striatal cholinergic neurons. J.Comp.Neurol. *366*:379-389.

Bolam, J.P., C.A. Ingham, P.N. Izzo, A.I. Levey, D.B. Rye, A.D. Smith, and B.H. Wainer (1986) Substance P containing terminals in synaptic contact with cholinergic neurons in the neostriatum and basal forebrain: a double immunocytochemical study in the rat. Brain Res. *397*:279-289.

Boulant, J.A. (1980) Hypothalamic control of thermoregulation. In P.J. Morgane and J. Panksepp (eds): Handbook of the hypothalamus. Marcel Dekker, Inc.: New York and Basel, pp. 1-82.

Bouras, C., P.G. Vallet, H. Dobrinov, S.S. Hilaire, and J. Constantinidis (1986) Substance P neuronal cell bodies in the human brain: complete mapping by immunofluorescence. Neuroscience Letters *69*:31-36.

Braak, H. and E. Braak (1987) The hypothalamus of the human adult: chiasmatic region. Anat.Embryol. *175*:315-330.

Brown, E.R., R.E. Harlan, and J.E. Krause (1990) Gonadal steroid regulation of substance P (SP) and SP-encoding messenger ribonucleic acids in the rat anterior pituitary and hypothalamus. Endocrinology *126*:330-340.

Brown, J.L., H. Liu, J.E. Maggio, S.R. Vigna, P.W. Mantyh, and A.I. Basbaum (1995) Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis. J.Comp.Neurol. *356*:327-344.

Buck, S.H., C.J. Helke, E. Burcher, C.W. Schultz, and T.L. O'Donohue (1986) Pharmacologic characterization and autoradiographic distribution of binding sites for iodinated tachykinins in the rat central nervous system. Peptides 7:1109-1120.

Buell, G., M.F. Schulz, S.J. Arkinstall, K. Maury, M. Missotten, N. Adami, F. Talabot, and E. Kawashima (1992) Molecular characterisation, expression and localization of human neurokinin-3 receptor. FEBS *299*:90-95.

Burgunder, J.M. and W.S. Young,III (1989a) Distribution, projection and dopaminergic regulation of the neurokinin B mRNA-containing neurons of the rat caudate-putamen. Neuroscience *32*:323-335.

Burgunder, J.M. and W.S. Young,III (1989b) Neurons with neurokinin B mRNA in the rat magnocellular basal nucleus: distribution, projection and colocalization studies. J.Chem.Neuroanat. *2*:239-251.

Canteras, N.S., R.B. Simerly, and L.W. Swanson (1992) Projections of the ventral premammillary nucleus. J.Comp.Neurol. *324*:195-212.

Canteras, N.S. and L.W. Swanson (1992) The dorsal premammillary nucleus: An unusual component of the mammillary body. Proc.Natl.Acad.Sci.USA *89*:10089-10093.

Carmel, P.W., S. Araki, and M. Ferin (1976) Pituitary stalk portal blood collection in rhesus monkeys: evidence for pulsatile release of gonadotropin-releasing hormone (GnRH).. Endocrinology *99*:243-248.

Chang, M.M., S.E. Leeman, and H.D. Niall (1971) Amino acid sequence of substance P. Nature New Biol. *232*:86-87.

Chang, M.M. and S.E. Leeman (1970) Isolation of a sialogogic peptide from bovine hypothalamic tissue and its characterization as substance P. J.Biol.Chem. *245*:4784-4790.

Chawla, M.K., G.M. Gutierrez, W.S. Young,III, and N.E. Rance (1994) Localization of neurons expressing substance P and neurokinin B mRNAs in the human hypothalamus and basal forebrain. Soc.Neurosci.Abstr. *20*:

Chawla, M.K., G.M. Gutierrez, G.L. Wenk, and N.E. Rance (1995) Choline acetyltransferase and neurokinin B mRNAs are colocalized in neurons of the human magnocellular basal forebrain complex. Soc.Neurosci.Abstr. *21*:

Chen, W.-P., J.W. Witkin, and A.-J. Silverman (1989) Beta-endorphin and gonadotropin-releasing hormone synaptic input to gonadotropin-releasing hormone neurosecretory cells in the male rat. J.Comp.Neurol. *286*:85-95.

Chen, W.P., J.W. Witkin, and J.A. Silverman (1989) Gonadotropin releasing hormone (GnRH) neurons are directly innervated by catecholamine terminals. Synapse *3*:288-290.

Ciofi, P., J.E. Krause, G.S. Prins, and M. Mazzuca (1994) Presence of nuclear androgen receptor-like immunoreactivity in neurokinin B-containing neurons of the hypothalamic arcuate nucleus of the adult male rat. Neurosci.Lett. *182*:193-196.

Clarke, I.J. and J.T. Cummins (1982) The temporal relationship between gonadotropin releasing hormone (GnRH) and luteininzing hormone (LH) secretion in ovariectomized ewes. Endocrinology *111*:1737-1739.

Collerton, D. (1986) Cholinergic function and intellectural decline in Alzheimer's disease. Neuroscience *19*:1-28.

Collins, S., M.G. Caron, and R.J. Lefkowitz (1992) From ligand binding to gene expression: new insights into the regulation of G-protein-coupled receptors. TIBS *17*:37-39.

Constantinidis, J., C. Bouras, and J. Richard (1983) Putative peptide neurotransmitters in human neuropathology: a review of topography and clinical implications. Clinical Neuropathology *2 (2)*:47-54.

Cooper, P.E., M.H. Fernstrom, O.P. Rorstad, S.E. Leeman, and J.B. Martin (1981) The regional distribution of somatostatin, Substance P and neurotensin in human brain. Brain Research *218*:219-231.

Cope, E. (1976) Physical changes associated with menopausal years. In S. Campbell (ed): Management of menopause and post menopausal years. Baltimore: University Park Press, pp. 33

Cuello, A.C., J.V. Priestley, and G. Paxinos (1985) Substance P and enkephalin containing pathways. In G. Paxinos (ed): The Rat Nervous system. Academic Press: Australia, pp. 441-469.

Danks, J.A., R.B. Rothman, M.A. Cascieri, G.G. Chicchi, T. Liang, and M. Herkenham (1986) A comparative autoradiographic study of the distributions of substance P and eledoisin binding sites in rat brain. Brain Research *385*:273-281.

Debeljuk, L., M.A. Villanúa, and A. Bartke (1992) Neurokinin A in the anterior pituitary of female rats: Effects of ovariectomy and estradiol. Peptides *13*:1001-1005.

Dees, W.L., C.W. Skelley, and G.P. Kozlowski (1985) Central effects of an antagonist and an antiserum to substance P on serum gonadotropin and prolactin secretion. Life Sci. *37*:1627-1631.

Dekker, A.J., D.J. Connor, and L.J. Thal (1991) The role of cholinergic projections from the nucleus basalis in memory. Neuroscience and Behavioral Reviews *15(2)*:299-317.

Dietl, M.M. and J.M. Palacios (1991) Phylogeny of tachykinin receptor localization in the vertebrate central nervous system: Apparent absence of neurokinin-2 and neurokinin-3 binding sites in the human brain. Brain Res. *539*:211-222.

Ding, Y.Q., R. Shigemoto, M. Takada, H. Ohishi, S. Nakanishi, and N. Mizuno (1996) Localization of the neuromedin K receptor (NK3) in the central nervous system of the rat. J.Comp.Neurol. *364*:290-310.

Dornan, W.A., C.W. Malsbury, and R.B. Penney (1987) Facilitation of lordosis by injection of substance P into the midbrain central gray. Neuroendocrinology *45*:498-506.

Dornan, W.A. and C.W. Malsbury (1989) Peptidergic control of male rat sexual behavior: The effects of intracerebral injections of substance P and cholecystokinin. Physiol.Behav. *46*:547-556.

Dunnett, S.B. and H.C. Fibiger (1993) Role of forebrain cholinergic systems in learning and memory: relevance to the cognitive deficits of ageing and Alzheimer's dementia. In A.C. Cuello (ed): Progress in brain research. Elsevier Science Publishers B.V.: pp. 413-420.

Eckstein, N., W.B. Wehrenberg, K. Louis, P.W. Carmel, E.A. Zimmermann, A.G. Frantz, and M. Ferin (1980) Effects of substance P on anterior pituitary secretion in the female rhesus monkey. Neuroendocrinology *31*:338-342.

Eklund, B., T. Jogestrand, and B. Pernow (1977) Effect of substance P on resistance and capacitance vessels in the human forearm.. In U.S. von Euler and B. Pernow (eds): Substance P (Nobel Symposium 37). New York: Raven Press, pp. 275-285.

Elde, R., M. Schalling, S. Ceccatelli, S. Nakanishi, and T. Hökfelt (1990) Localization of neuropeptide receptor mRNA in rat brain: initial observations using probes for neurotensin and substance P receptors.. Neurosci.Lett. *120*:134-138.

Erspamer, V. (1971) Biogenic amines and active polypeptides of the amphibian skin. Ann.Rev.Pharmacol. *11*:327-350.

Esparmer, V. (1949) Richercie preliminaria sulla moschatina. Experential *5*:79-81.

Esparmer, V., A. Anastasi, G. Bertaccini, and J.M. Cei (1964) Structure and pharmacological actions of physalaemin, the main active polypeptide of the skin of *physalaemus fuscumaculatus*. Experiential *20*:489-490.

Everitt, B.J., B. Meister, T. Hökfelt, T. Melander, L. Terenius, Ä. Rökaeus, E. Theordorsson-Norheim, G. Dockray, J. Edwardson, C. Cuello, R. Elde, M. Goldstein, H. Hemmings, C. Ouimet, I. Walaas, P. Greengard, W. Vale, E. Weber, J.-Y. WU, and K.-J. Chang (1986) The hypothalamic arcuate nucleus-medium eminence complex:immunohistochemistry of transmitters, peptides and DARPP-32 with special reference to coexistence in dopamine neurons.. Brain.Res.Rev. *11*:97-155.

Ferin, M., P.W. Carmel, E.A. Zimmerman, M. Warren, R. Perez, and R.L. Vande Wiele (1974) Location of intrahypothalamic estrogen-responsive sites influencing LH secretion in the female rhesus monkey.. Endocrinology *95*:1059-1068.

Ferin, M., H. Rosenblatt, P.W. Carmel, J.L. Antunes, and R.L. Vande Wiele (1979) Estrogen-induced gonadotropin surges in female rhesus monkeys after pituitary stalk section. Endocrinology *104*:50-52.

Ferin, M. (1983) Neuroendocrine control of ovarian function in the primate. J.Reprod.Fertil. *69*:369-381.

Ferin, M., D. Van Vugt, and S. Wardlaw (1984) The hypothalamic control of the menstrual cycle and the role of endogenous opioid peptides. Recent Prog.Horm.Res. *40*:441-485.

Filicori, M., N. Santoro, G.R. Merriam, and W.F. Crowley,Jr. (1986) Characterization of the physiological pattern of episodic gonadotropin secretion throughout the human menstrual cycle. J.Clin.Endocrinol.Metab. *62*:1136-1144.

Foulkes, N.S., E. Borelli, and P. Sassone-Coral (1991) CREM gene: Use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. Cell *64*:739-749.

Fox, S.R., R.E. Harlan, B.D. Shivers, and D.W. Pfaff (1990) Chemical characterization of neuroendocrine targets for progesterone in the female rat brain and pituitary. Neuroendocrinology *51*:276-283.

Gerard, N.P., R.L. Eddy,Jr., T.B. Shows, and C. Gerard (1990) The human neurokinin A (substance K) receptor: molecular cloning of the gene, chromosome localization, and isolation of cDNA from tracheal and gastric tissues. J Biol Chem *265*:20455-20462.

Gerard, N.P., L.A. Garraway, R.L. Eddy,Jr., T.B. Shows, H. Iijima, J.-L. Paquet, and C. Gerard (1991) Human substance P receptor (NK-1): Organization of the gene, chromosome localization, and functional expression of cDNA clones. Biochemistry *30*:10640-10646.

Gerard, N.P., L. Bao, H. Xiao-Ping, and C. Gerard (1993) Molecular aspects of the tachykinin receptors. Regulatory Peptides *43*:21-35.

Gerfen, C.R. (1991) Substance P (neurokinin-1) receptor mRNA is selectively expressed in cholinergic neurons in the striatum and basal forebrain. Brain Research *556*:165-170.

Glaser, E.M., M. Tagamets, N.T. McMullen, and H. Van der Loos (1983) The image-combining computer microscope -- an interactive instrument for morphometry of the nervous system. J.Neurosci.Methods *8*:17-32.

Goldsmith, P.C., R. Lamberts, and L.R. Brezina (1983) Gonadotropin-releasing hormone neurons and pathways in the primate hypothalamus and forebrain. In R.L. Norman (ed): Neuroendocrine Aspects of Reproduction. New York: Academic Press, pp. 7-45.

Gosden, R.G. (1987) Follicular states at menopause. Human Reproduction *2*:617-621.

Gouras, G.K., N.E. Rance, W.S. Young,III, and V.E. Koliatsos (1992) Tyrosine-hydroxylase-containing neurons in the primate basal forebrain magnocellular complex. Brain Res. *584*:287-293.

Gross, K.M., A.M. Matsumoto, M.B. Southworth, and W.J. Bremer (1985) Evidence for decreased lutenizing hormone-releasing hormone frequency in men with selective elevations of FSH. J Clin Endocrinol Metab *60*:197

Haber, S.N. and S.J. Watson (1985) The comparative distribution of enkephalin, dynorphin and substance P in the human globus pallidus and basal forebrain. Neuroscience *14*:1011-1024.

Hall, M.E., F.B. Miley, and J.M. Stewart (1989) Cardiovascular effects of substance P peptides in the nucleus of the solitary tract. Brain Res. *497*:280-290.

Halvorson, I. and J. Thornhill (1993) Posterior hypothalamic stimulation of anesthetized normothermic and hypothermic rats evokes shivering thermogenesis. Brain Research *610*:208-215.

Harlan, R.E., M.M. Garcia, and J.E. Krause (1989) Cellular localization of substance P- and neurokinin A- encoding preprotachykinin mRNA in the female rat brain. J.Comp.Neurol. *287*:179-212.

Haverstick, D.M., A. Rubenstein, and M.J. Bannon (1989) Striatal tachykinin gene expression regulated by interaction of D-1 and D-2 dopamine receptors. The Journal of Pharmacology and Experimental Therapeutics *248*:858-862.

Hedreen, J.C., S.J. Bacon, L.C. Cork, C.A. Kitt, G.D. Crawford, P.M. Salvaterra, and D.L. Price (1983) Immunocytochemical identification of cholinergic neurons in the monkey central nervous system using monoclonal antibodies against choline acetyltransferase. Neuroscience Letters *43*:173-177.

Hedreen, J.C., R.G. Struble, P.J. Whitehouse, and D.L. Price (1984) Topography of the magnocellular basal forebrain system in the human brain. J.Neuropathol.Exp.Neurol. *43*:1-21.

Hellon, R.F. (1981) Neurophysiology of temperature regulation: problems and perspectives. Fed.Proc. *40*:2804-2807.

Hensel, H. (1973) Neural processes in thermoregulation. Physiological Reviews *53*:948-1017.

Hershey, A.D., L. Polenzani, R.M. Woodward, R. Miledi, and J.E. Krause (1991) Molecular and genetic characterization, functional expression, and mRNA expression patterns of a rat substance P receptor. Annals of the New York Academy of Sciences *632*:63-78.

Hiatt, E.S., P.G. Brunetta, G.R. Seiler, S.A. Barney, W.D. Selles, K.H. Wooledge, and J.C. King (1992) Subgroups of luteininzing hormone-releasing hormone perikarya defined by computer analyses in the basal forebrain of intact female rats. Endocrinology *130*:1030-1043.

Hofman, M.A. and D.F. Swaab (1989) The sexually dimorphic nucleus of the preoptic area in the human brain: a comparative morphometric study. J.Anat. *164*:55-72.

Horvath, T.L., F. Naftolin, and C. Leranth (1993) Luteinizing hormone-releasing hormone and gamma-aminobutyric acid neurons in the medial preoptic area are synaptic targets of dopamine axons originating in anterior periventricular areas. J.Neuroendocrinology *5*:71-79.

Hökfelt, T., B. Pernow, G. Nilsson, L. Wetterberg, M. Goldstein, and S.L. Jeffcoate (1978) Dense plexus of substance P immunoreactive nerve terminals in eminentia medialis of the primate hypothalamus.. Proc.Natl.Acad.Sci.USA *75*:1013-1015.

Huston, J.P. and M.S. Oitzl (1989) The relationship between reinforcement and memory: parallels in the rewarding and mnemonic effects of the neuropeptide substance P. Neurosci.Biobehav.Rev. *13*:171-180.

Inagaki, S., M. Sakanaka, S. Shiosaka, E. Senba, K. Takatsuki, H. Tagaki, Y. Kawai, H. Minagawa, and M. Tohyama (1982) Ontogeny of substance P-containing neuron system of the rat: Immunohistochemical analysis-I: forebrain and upper brain stem. Neuroscience 7:251-277.

Itoi, K., N. Jost, J. Culman, C. Tschöpe, and T. Unger (1994) Further localization of cardiovascular and behavioral actions of substance P in the rat brain. Brain Res. *668*:100-106.

Jarry, H., A. Perschl, H. Meissner, and W. Wuttke (1988) *In vivo* release rates of substance P in the preoptic/anterior hypothalamic area of ovariectomized and ovariectomized estrogen-primed rats: correlation with luteinizing hormone and prolactin levels. Neurosci.Lett. *88*:189-194.

Jonakait, G.M., L. Ni, P.D. Walker, and R.P. Hart (1991) Development of substance P (SP)-containing cells in the central nervous system: consequences of neurotransmitter co-localization. Prog Neurobiol *36*:1-21.

Jones, D.N.C., J.C. Barnes, D.L. Kirkby, and G.A. Higgins (1995) Age-associated impairments in a test of attention: Evidence for involvement of cholinergic systems. J.Neurosci. *15*:7282-7292.

Jones, E.J., H. Burton, C.B. Saper, and L.W. Swanson (1976) Midbrain, diencephalic and cortical relationships of the basal nucleus of Meynert and associated structures in primates. J.Comp.Neurol. *167*:385-420.

Judd, H.L. (1987) Oestrogen replacement therapy: physiological considerations and new applications. Clin Endocrinol Metab *1*:117-206.

Kalra, P.S. and S.P. Kalra (1986) Steroidal modulation of the regulatory neuropeptides: Luteinizing hormone releasing hormone, neuropeptide Y and endogenous opiod peptides.. J.Steroid Biochem. *25 No.5B*:733-740.

Kalra, S.P. (1976) Tissue levels of luteinizing hormone-releasing hormone in the preoptic area and hypothalamus and serum concentrations of gonadotropin following anterior hypothalamic deafferentation and estrogen treatment of female rats. Endocrinology *99*:101-107.

Kalra, S.P., A. Sahu, M.G. Dube, and P.S. Kalra (1991) Effects of various tachykinins on pituitary LH secretion, feeding, and sexual behavior in the rat. Annals of the New York Academy of Sciences *632*:332-338.

Kalra, S.P. and P.S. Kalra (1989) Do testosterone and estradiol-17 beta enforce inhibition or stimulation of luteinizing hormone-releasing hormone secretion?. Biol.Reprod. *41*:559-570.

Kaneko, T., R. Shigemoto, S. Nakanishi, and N. Mizuno (1993) Substance P receptor-immunoreactive neurons in the rat neostriatum are segregated into somatostatinergic and cholinergic aspiny neurons. Brain Research *631*:297-303.

Kangawa, K., N. Minamino, A. Fukuda, and H. Matsuo (1983) Neuromedin K: a novel mammalian tachykinin identified in porcine spinal cord. Biochem.Biophys.Res.Commun. *114*:533-540.

Karsch, F.J. (1987) Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of luteinizing hormone. Annu.Rev.Physiol. *49*:365-382.

Kerdelhue, B., A. Tartar, V. Lenoir, A. el Abed, P. Hublau, and R.P. Millar (1985) Binding studies of substance P anterior pituitary binding sites: changes in substance P binding sites during the rat estrous cycle. Regul.Pept. *10*:133-143.

Kerdelhué, B., M. Valens, and Y. Langlois (1978) Stimulation de la sécretion de la LH et de la FSH hypothphysaire aprés immunoneutralisation de la Substance P endogen chez la ratte cyclique. C.R.Acad.Sci.Paris D *286*:977-979.

Kesner, J.S., R.C. Wilson, J.-M. Kaufman, J. Hotchkiss, Y. Chen, H. Yamamoto, R.R. Pardo, and E. Knobil (1987) Unexpected responses of the hypothalamic gonadotropin-releasing hormone "pulse generator" to physiological estradiol inputs in the absence of the ovary. Proc.Natl.Acad.Sci.USA *84*:8745-8749.

Kievit, J. and H.G.J.M. Kuypers (1975a) Subcortical afferents to the frontal lobe in the rhesus monkey studied by means if retrograde horseradish peroxidase transport. Brain Research *85*:261-266.

Kievit, J. and H.G.J.M. Kuypers (1975b) Basal forebrain and hypothalamic connections to frontal and parietal cortex in the rhesus monkey. Science *187*:660-662.

Kimura, S., M. Okada, Y. Sugita, I. Kanasawa, and E. Munekata (1983) Novel neuropeptides, neurokinin alpha and neurokinin beta, isolated form porcine spinal cord. Proc.Jpn.Acad. *59*:101-104.

King, J.C., S.A. Tobet, F.L. Snavely, and A.A. Arimura (1982) LHRH immunopositive cells and their projections to the median eminence and organum vasculosum of the lamina terminalis. J.Comp.Neurol. *209*:287-300.

King, J.C. and E.L.P. Anthony (1984) LHRH neurons and their projections in humans and other mammals: species comparisons. Peptides *5*:195-207.

King, J.C. and G.R. Seiler (1988) Ultrastructural evidence suggests variations in biosynthesis and processing within LH-RH neurons as a function of ovariectomy in rats. Brain Res. *452*:127-140.

Kiss, J. and B. Halasz (1985) Demonstration of serotonergic axons terminating on luteinizing hormone-releasing hormone neurons in the preoptic area of the rat using a combination of immunocytochemistry and high resolution autoradiography. Neuroscience *14*:69-78.

Knobil, E. (1980) The neuroendocrine control of the menstrual cycle. Recent Prog.Horm.Res. *36*:53-88.

Knobil, E. and J. Hotchkiss (1988) The menstrual cycle and its neuroendocrine control. In E. Knobil, J.D. Neill, G.S. Greenwald, C.L. Markert, and D.W. Pfaff (eds): The Physiology of Reproduction. New York: Raven Press, Ltd., pp. 1971-1994.

Kordower, J.H. and E.J. Mufson (1990) Galanin-like immunoreactivity within the primate basal forebrain: Differential staining patterns between humans and monkeys. J.Comp.Neurol. *294*:281-292.

Kotani, H., M. Hoshimaru, H. Nawa, and S. Nakanishi (1986) Structure and gene organization of bovine neuromedin K precursor. Proc.Natl.Acad.Sci.USA *83*:7074-7078.

Kowall, N.W., M.F. Beal, J. Busciglio, L.K. Duffy, and B.A. Yankner (1991) An *in vivo* model for the neurodegenerative effects of beta amyloid and protection by substance P. Proc.Natl.Acad.Sci.USA *88*:7247-7251.

Kowall, N.W., B.J. Quigley, J.E. Krause, F. Lu, B.E. Kosofsky, and R.J. Ferrante (1993) Substance P and substance P receptor histochemistry in human neurodegenerative diseases. Regulatory Peptides *46*:174-185.

Krailo, M.D. and M.C. Pike (1983) Estimation of the distribution of age at natural menopause from prevalence data. Am J Epidemiol *117*:356

Krause, J.E., J.M. Chirgwin, M.S. Carter, Z.S. Xu, and A.D. Hershey (1987) Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A. Proc.Natl.Acad.Sci.USA *84*:881-885.

Krause, J.E., M.R. MacDonald, and Y. Takeda (1989) The polyprotein nature of substance P precursors. Bioessays. *10*:62-69.

Krause, J.E., A.D. Hershey, P.E. Dykema, and Y. Takeda (1990) Molecular biological studies on the diversity of chemical signalling in tachykinin peptidergic neurons. Ann.N.Y.Acad.Sci. *579*:254-272.

Krey, L.C., W.R. Butler, and E. Knobil (1975) Surgical disconnection of the medial basal hypothalamus and pituitary function in the rhesus monkey. I. Gonadotropin secretion. Endocrinology *96*:1073-1087.

Lauber, A.H., G.J. Romano, and D.W. Pfaff (1991) Sex difference in estradiol regulation of progestin receptor mRNA in rat mediobasal hypothalamus as demonstrated by in situ hybridization. Neuroendocrinology *53*:608-613.

Lee, C.M., N.J. Campbell, B.J. Williams, and L.L. Iverson (1986) Multiple tachykinin receptors in peripheral tissues and in brain. Eur.J.Pharmacol. *130*:209-217.

Levine, J.E., A.C. Bauer-Dantoin, L.M. Besecke, L.A. Conaghan, S.J. Legan, J.M. Meredith, F.J. Strobl, J.H. Urban, K.M. Vogelsong, and A.M. Wolfe (1991) Neuroendocrine regulation of the luteinizing hormone-releasing hormone pulse generator in the rat. Recent Prog.Horm.Res. *47*:97-153.

Ljungdahl, A., T. Hokfelt, and G. Nilsson (1978) Distribution of substance P-like immunoreactivity in the central nervous system of the rat--I. Cell bodies and nerve terminals. Neuroscience *3*:861-943.

Lucas, L.R., D.L. Hurley, J.E. Krause, and R.E. Harlan (1992) Localization of the tachykinin neurokinin B precursor peptide in rat brain by immunocytochemistry and *in situ* hybridization. Neuroscience *51*:317-345.

MacLusky, N.J., F. Naftolin, and C. Leranth (1988) Immunocytochemical evidence for direct synaptic connections between corticotrophin-releasing factor (CRF) and gonadotropin-releasing hormone (GnRH)-containing neurons in the preoptic area of the rat. Brain Res. *439*:391-395.

Maeno, H., H. Kiyama, and M. Tohyama (1993) Distribution of the substance P receptor (NK-1) in the central nervous system. Mol.Brain Res. *18*:43-58.

Maggio, J.E. (1988) Tachykinins. Ann.Rev.Neurosci. *11*:13-28.

Maggio, J.E. and P.W. Mantyh (1994) The tachykinin receptors. Humana Press: New Jersey.

Mai, J.K., P.H. Stephens, A. Hopf, and A.C. Cuello (1986) Substance P in the human brain. Neuroscience *17*:709-739.

Mair, G.P., E.K. Warrigton, and L. Weiskrantz (1979) Memory disorder in Korsakoff's psychosis. A neuropathological and neuropsychological investigation of two cases. Brain *102*:749-783.

Mais, V., R.R. Krager, N.S. Cetel, J. Rivier, W. Vale, and S.C.C. Yen (1986) The dependency of folliculogenesis and corpus luteum function on pulsatile gonadotropin secretion in cycling women, using a GnRH antagonist as a probe. J Clin Endocrinol Metab *62*:1250

Makara, G.B., I. Kakucska, V. Lenoir, B. Kerdelhue, and M. Palkovits (1986) A substance P-containing hypothalamic neuronal system projects to the median eminence. Brain Res. *374*:399-401.

Mantyh, P.W., T. Gates, C.R. Mantyh, and J.E. Maggio (1989) Autoradiographic localization and characterization of tachykinin receptor binding sites in the rat brain and peripheral tissues. The Journal of Neuroscience *9 (1)*:258-279.

Marksteiner, J., A. Saria, and J.E. Krause (1992a) Comparative distribution of neurokinin B-, substance P-and enkephalin-like immunoreactivities and neurokinin B messenger RNA in the basal forebrain of the rat: Evidence for neurochemical compartmentation. Neuroscience *51*:107-120.

Marksteiner, J., G. Sperk, and J.E. Krause (1992b) Distribution of neurons expressing neurokinin B in the rat brain: immunohistochemistry and in situ hybridization. J.Comp.Neurol. *317*:341-356.

Matsumoto, A. and Y. Arai (1983) Sex difference in volume of the ventromedial nucleus of the hypothalamus in the rat. Endocrinol.Jpn. *30*:277-280.

Mayes, A.R., P.R. Meudell, D. Mann, and A. Pickering (1988) Location of lesions in Korsakoff's syndrome: Neuropsychological and neuropathologial data on two patients. Cortex *24*:367-388.

McCarson, K.E. and J.E. Krause (1994) NK-1 and NK-3 type tachykinin receptor mRNA expression in the rat spinal cord dorsal horn is increased during adjuvant or formalin-induced nociception. J.Neurosci. *14*:712-720.

McEwen, B.S., A. Biegon, P.G. Davis, L.C. Krey, V.N. Luine, M.Y. McGinnis, C.M. Paden, B. Parsons, and T.C. Rainbow (1982) Steroid hormones: humoral signals which alter brain cell properties and functions. Recent Prog.Horm.Res. *38*:41-83.

Meisel, R.L. and B.D. Sachs (1994) The physiology of male sexual behavior. In E. Knobil and J.D. Neill (eds): The physiology of reproduction. Raven Press: New York, pp. 3-105.

Melander, T. and W.A. Staines (1986) A galanin-like peptide coexists in putative cholinergic somata of the septum-basal forebrain complex and in acetylcholinersterase-containing fibers and varicosities within the hippocampus in the owl monkey (Oatus trivirgatus). Neuroscience Letters *68*:17-22.

Merchenthaler, I., T. Gores, G. Setalo, P. Petrusz, and B. Flerko (1984) Gonadotropin releasing hormone (GnRH) neurons and pathways in the rat brain. Cell Tisssue Research *237*:15-29.

Merchenthaler, I., G. Setalo, C. Csontos, P. Petrusz, B. Flerko, and A. Negro-Vilar (1989) A combined retrograde tracing and immunocytochemical identification of luteinizing hormone-releasing hormone and somatostatin-containing neurons projecting to the median emminence of the rat. Endocrinology *125*:2812-2821.

Mesulam, M.-M., E.J. Mufson, A.I. Levey, and B.H. Wainer (1983) Cholinergic innervation of cortex by the basal forebrain: cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (substantia innominata) and hypothalamus in the rhesus monkey. J.Comp.Neurol. *214*:170-197.

Mesulam, M.-M. and C. Geula (1988) Nucleus basalis (Ch4) and cortical cholinergic innervation in the human brain: observations based on the distribution of acetylcholinesterase and choline acetyltransferase.. J.Comp.Neurol. *275*:216-240.

Mesulam, M.-M. and G.W. Van Hoesen (1976) Acetylcholinesterase-rich projections from the basal forebrain of the rhesus monkey to neocortex. Brain Res. *109*:152-157.

Micevych, P.E., D.W. Matt, and V.W. Go (1988) Concentrations of cholecystokinin, substance P, and bombesin in discrete regions of male and female rat brain: sex differences and estrogen effects. Exp.Neurol. *100*:416-425.

Minamino, N., K. Kangawa, A. Fukuda, and H. Matsuo (1984) Neuromedin L: a novel mammalian tachykinin derived from porcine spinal cord. Neuropeptides *4*:157-166.

Monda, M., S. Amaro, A. Sullo, and B. De Luca (1994) Posterior hypothalamic activity and cortical control during the PGE_1 hyperthermia. NeuroReport *6*:135-139.

Monroe, S.E., R.B. Jaffe, and A.R. Midgley,Jr. (1972) Regulation of Human gonadotropins. XIII. Changes in serum gonadotropins in menstruating women in response to oophorectomy. J.Clin.Endocrinol.Metab. *34*:420-422.

Murphy, M. (1992) The molecular pathogenesis of Alzheimer's disease: clinical prospects. The Lancet *340*:1512-1515.

Murray, C.L. and H.C. Fibiger (1985) Learning and memory deficits after lesions of the nucleus basalis magnocellularis: reversal by physostigmine. Neurosci. *14*:1025-1032.

Nabeshima, T. (1993) Behavioral aspects of cholinergic transmission: role of basal forebrain cholinergic system in learning and memory. In A.C. Cuello (ed): Progress in brain research. Elsevier Science Publishers B.V.: pp. 405-411.

Nagashima, A., Y. Takano, K. Tateishi, Y. Matsuoka, T. Hamaoka, and H. Kamiya (1989a) Central pressor actions of neurokinin B: increases in neurokinin B contents in discrete nuclei in spontaneously hypertensive rats. Brain Res. *499*:198-203.

Nagashima, A., Y. Takano, K. Tateishi, Y. Matsuoka, T. Hamaoka, and H. Kamiya (1989b) Cardiovascular roles of tachykinin peptides in the nucleus tractus solitarii of rats. Brain Res. *487*:392-396.

Nakajima, Y., P.R. Stanfield, K. Yamaguchi, and S. Nakajima (1991) Substance P excites cultured cholinergic neurons in the basal forebrain. The Basal Forebrain 157-167.

Nakanishi, S. (1987) Substance P precursor and kininogen: their structures, gene organizations, and regulation. Physiol.Rev. *67*:1117-1142.

Nakanishi, S. (1991) Mammalian tachykinin receptors. Ann.Rev.Neurosci. *14*:123-136.

Nakaya, Y., T. Kaneko, R. Shigemoto, S. Nakanishi, and N. Mizuno (1994) Immunohistochemical localization of substance P receptor in the central nervous system of the adult rat. J.Comp.Neurol. *347*:249-274.

Nauta, W.J.H. and W. Haymaker (1969) Hypothalamic nuclei and fiber connections. In W. Haymaker, E. Anderson, and W.J.H. Nauta (eds): The Hypothalamus. Springfield, Ill.: Charles C. Thomas, pp. 136-209.

Nawa, H., T. Hirose, H. Takashima, S. Inayama, and S. Nakanishi (1983) Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precursor. Nature Lond. *306*:32-36.

Nawa, H., H. Kotani, and S. Nakanishi (1984) Tissue-specific generation of two preprotachykinin mRNAs from one gene by alterative RNA splicing. Nature *312*:729-734.

Nilsson, L., B. Winblad, I. Volkmann, I. Alafuzoff, and L. Bergstrom (1991) Characterization and quantitation of ^{125} I-Bolton Hunter substance P binding sites in human brain. Neurochem.Int. *18*:399-404.

Ohkubo, H. and S. Nakanishi (1991) Molecular characterization of the three tachykinin receptors. Annals of the New York Academy of Sciences *632*:53-62.

Ohtsuka, S., A. Miyake, T. Nishizaki, K. Tasaka, T. Aono, and O. Tanizawa (1987) Substance P stimulates gonadotropin-releasing hormone release from rat hypothalamus in vitro with involvement of oestrogen. Acta Endocrinol.(Copenh.) *115*:247-252.

Olster, D.H. and J.D. Blaustein (1992a) Estradiol pulses induce progestin receptors selectively in substance P-immunoreactive neurons in the ventrolateral hypothalamus of female guinea pigs. J.Neurobiol. *23*:293-301.

Olster, D.H. and J.D. Blaustein (1992b) Progestin receptors in substance P-immunoreactive neurons in the hypothalamus of male guinea pigs after behaviorally effective estradiol pulse treatment. J.Neurobiol. *23*:302-308.

Otsuka, M. and K. Yoshioka (1993) Neurotransmitter functions of mammalian tachykinins. Physiological Reviews *73*:229-308.

Page, R.B. (1994) Hypothalamo-hypophysial complex. In E. Knobil and J.D. Neill (eds): The Physiology of Reproduction. Raven Ltd: New York, pp. 1527-1619.

Papez, J.W. (1937) A proposed mechanism of emotion. Arch.Neurol.Psychiatry *38*:725-743.

Pernow, B. (1983) Substance P. Pharmacol.Rev. *35*:85-141.

Petit, F. and J. Glowinski (1986) Stimulatory effect of substance P on the spontaneous release of newly synthesized [^{3}H] dopamine from rat striatal slices: a tetrodotoxin-sensitive process. Neuropharmacology *25*:1015-1021.

Pfaff, D.W. (1989) Features of a hormone-driven defined neural circuit for a mammalian behavior. Principles illustrated, neuroendocrine syllogisms, and multiplicative steroid effects. Ann.NY Acad.Sci. *563*:131-147.

Picanco-Diniz, D.L.W., M.M. Valenca, C.R. Franci, and J. Antunes-Rodrigues (1990) Role of substance P in the medial preoptic area in the regulation of gonadotropin and prolactin secretion in normal and orchidectomized rats. Neuroendocrinology *51*:675-682.

Plant, T.M. (1986) Gonadal regulation of hypothalamic gonadotropin-releasing hormone release in primates. Endocr.Rev. *7*:75-88.

Rance, N.E., N.T. McMullen, J.E. Smialek, D.L. Price, and W.S. Young,III (1990) Postmenopausal hypertrophy of neurons expressing the estrogen receptor gene in the human hypothalamus. J.Clin.Endocrinol.Metab. *71*:79-85.

Rance, N.E. (1992) Hormonal influences on morphology and neuropeptide gene expression in the infundibular nucleus of postmenopausal women. In D.F. Swaab, M.A. Hofman, M. Mirmiran, R. Ravid, and F.W. van Leeuwen (eds): The Human Hypothalamus in Health and Disease, Progress in Brain Research. Amsterdam: Elsevier, pp. 221-236.

Rance, N.E., W.S. Young,III, and N.T. McMullen (1994) Topography of neurons expressing luteinizing hormone-releasing hormone gene transcripts in the human hypothalamus and basal forebrain. J.Comp.Neurol. *339*:573-586.

Rance, N.E. and T.R. Bruce (1994) Neurokinin B gene expression is increased in the arcuate nucleus of ovariectomized rats. Neuroendocrinology *60*:337-345.

Rance, N.E. and S.V. Uswandi (1994) Luteinizing hormone-releasing hormone (LHRH) gene expression is increased in the hypothalami of postmenopausal women. Soc.Neurosci.Abstr.

Rance, N.E. and S.V. Uswandi (1996) Gonadotropin-releasing hormone gene expression is increased in the medial basal hypothalamus of postmenopausal women. J.Clin.Endocrinol.Metab. *In press*:

Rance, N.E. and W.S. Young,III (1991) Hypertrophy and increased gene expression of neurons containing neurokinin-B and substance-P messenger ribonucleic acids in the hypothalami of postmenopausal women. Endocrinology *128*:2239-2247.

Reame, N., S.E. Saunder, R.P. Kelch, and J.C. Marshall (1984) Pulsatile gonadotropin secretion during the human menstrual cycle: Evidence for altered frequency of gonadotropin releasing hormone secretion. J.Clin.Endocrinol Metab. *59*:328

Regoli, D., G. Drapeau, S. Dion, and P. D'Orl:eans-Juste (1987) Pharmacological receptors for substance P and neurokinins. Life Sci. *40*:109-117.

Regoli, D., S. Dion, N.E. Rhaleb, N. Rouissi, C. Tousignant, D. Jukic, P. D'Orleans-Juste, and G. Drapeau (1989) Selective agonists for receptors of substance P and related neurokinins. Biopolymers. *28*:81-90.

Richardson, S.J., V. Senikas, and J.F. Nelson (1987) Follicular depletion during the menopausal transition: evidence for acclelerated loss and ultimate exhaustion at menopause. J Clin Endocrinol Metab *65*:1231-1237.

Ridley, R.M., T.K. Murray, J.A. Johnson, and H.F. Baker (1986) Learning imapirment following lesion of the basal nucleus of Meynert in the marmoset: modification by cholinergic drugs. Brain Res. *376*:108-116.

Romano, G.J., C.V. Mobbs, A. Lauber, R.D. Howells, and D.W. Pfaff (1990) Differential regulation of proenkephalin gene expression by estrogen in the ventromedial hypothalamus of male and female rats: Implications for the molecular basis of a sexually differentiated behavior. Brain Res. *536*:63-68.

Ronnekleiv, O.K., M.J. Kelly, and R.L. Eskay (1984) Distribution of immunoreactive substance P neurons in the hypothalamus and pituitary of the rhesus monkey. J.Comp.Neurol. *224*:51-59.

Rothfeld, J., J.F. Hejtmancik, P.M. Conn, and D.W. Pfaff (1989) *In situ* hybridization for LHRH mRNA following estrogen treatment. Mol.Brain Res. *6*:121-125.

Rothman, R.B., M. Herkenham, C.B. Pert, T. Liang, and M.A. Cascieri (1984) Visualization of rat brain receptors for the neuropeptide, substance P. Brain Research *309*:47-54.

Saffroy, M., J.C. Beajouan, Y. Torrens, J. Besseyre, L. Bergstrom, and J. Glowenski (1988) Localization of tachykinin binding sites (NK1, NK2, NK3 ligands) in the rat brain. Peptides *9*:227-241.

Sakamoto, N., K. Takatsuji, S. Shiosaka, and M. Tohyama (1985) Evidence for the existence of substance P-like immunoreactive neurons in the human cerebral cortex: an immunohistochemical analysis. Brain Research *325*:322-324.

Sakamoto, N., J.P. Michel, N. Kopp, M. Tohyama, and J. Pearson (1987) Substance P- and enkephalin-immunoreactive neurons in the hippocampus and related areas of the human infant brain. Neuroscience *22*:801-811.

Saper, C.B. (1990) Hypothalamus. In G. Paxinos (ed): The human nervous system. San Diego: Academic Press, pp. 389-413.

Saper, C.B. and T.C. Chelimsky (1984) A cytoarchitectonic and histochemical study of nucleus basalis and associated cell groups in the normal human brain. Neuroscience *13*:1023-1037.

Satoh, K. and H.C. Fibiger (1985) Distribution of central cholinergic neurons in the baboon (Papio papio). I. General morphology. J.Comp.Neurol. *236*:197-214.

Seeburg, P.H., A.J. Mason, T.A. Stewart, and K. Nikolics (1987) The mammalian GnRH gene and its pivotal role in reproduction. Recent Prog.Horm.Res. *43*:69-98.

Seeburg, P.H. and J.P. Adelman (1984) Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. Nature *311*:666-668.

Selmanoff, M., C. Shu, S.L. Petersen, C.A. Barraclough, and R.T. Zoeller (1991) Single cell levels of hypothalamic messenger ribonucleic acid encoding luteinizing hormone-releasing hormone in intact, castrated, and hyperprolactinemic male rats. Endocrinology *128*:459-466.

Shaar, C.J., J.S. Euker, G.D. Riegle, and J. Meites (1975) Effects of castration and gonadal steroids on serum luteinizing hormone secretion and prolactin in old and young rats. J.Endocrinol *66*:45-51.

Shamgochian, M.D. and S.E. Leeman (1992) Substance P stimulates luteinizing hormone secretion from anterior pituitary cells in culture. Endocrinology *131*:871-875.

Sheehan, H.L. (1967) Variations in the subventricular nucleus. J.Path.Bact. *94*:409-416.

Sheehan, H.L. and K. Kovacs (1966) The subventricular nucleus of the human hypothalamus. Brain *89*:589-614.

Shigemoto, R., Y. Yokota, K. Tsuchida, and S. Nakanishi (1990) Cloning and expression of a rat neuromedin K receptor cDNA. J Biol Chem *265*:623-628.

Shivers, B.D., R.E. Harlan, J.I. Morrell, and D.W. Pfaff (1983a) Immunocytochemical localization of luteinizing hormone-releasing hormone in male and female rat brains. Quantitative studies on the effect of gonadal steroids. Neuroendocrinology *36*:1-12.

Shivers, B.D., R.E. Harlan, J.I. Morrell, and D.W. Pfaff (1983b) Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. Nature *304*:345-347.

Shultz, C.W., R. Quirion, B. Chronwall, T.N. Chase, and T.L. O'Donohue (1984) A comparison of the anatomical distribution of substance P and substance P receptors in the rat central nervous system. Peptides *5*:1097-1128.

Silverman, A.-J., L.C. Krey, and E.A. Zimmerman (1979) A comparative study of the luteinizing hormone releasing hormone (LHRH) neuronal networks in mammals. Biol.Reprod. *20*:98-110.

Silverman, A.J., J.L. Antunes, M. Ferin, and E.A. Zimmerman (1977) The distribution of luteinizing hormone-releasing hormone (LHRH) in the hypothalamus of the rhesus monkey. Light microscopic studies using immunoperoxidase technique. Endocrinology *101*:134-142.

Silverman, A.J., J. Jhamandas, and L. Renaud (1987) Localization of luteinizing hormone-releasing hormone (LHRH) neurons that project to the median eminence. J.Neuroscience *7*:2312-2319.

Simerly, R.B., L.D. McCall, and S.J. Watson (1988) Distribution of opiod peptides in the preoptic region: immunohistochemical evidence for a steroid-sensitive enkephalin sexual dimorphism. J.Comp.Neurol. *276*:442-459.

Simerly, R.B. (1995) Anatomical substrates of hypothalamic integration. In G. Paxinos (ed): The rat nervous system. Academic Press: pp. 353-376.

Stoessl, A.J. (1994) Localization of striatal and nigral tachykinin receptors in the rat. Brain Res. *646*:13-18.

Stopa, E.G., E.T. Koh, C.N. Svendsen, W.T. Rogers, J.S. Schwaber, and J.C. King (1991) Computer-assisted mapping of immunoreactive mammalian gonadotropin-releasing hormone in adult human basal forebrain and amygdala. Endocrinology *128*:3199-3207.

Sukhov, R.R., L.C. Walker, N.E. Rance, D.L. Price, and W.S. Young,III (1995) Opiod precursor gene expression in the human hypothalamus. J.Comp.Neurol. *353*:604-622.

Swaab, D.F. and E. Fliers (1985) A sexually dimorphic nucleus in the human brain. Science *228*:1112-1115.

Swaab, D.F. and M.A. Hofman (1995) Sexual differentiation of the human hypothalamus in relation to gender and sexual orientation. Trends Neurosci. *18*:264-270.

Swann, J.M. and N. Macchione (1992) Photoperiodic regulation of substance P immunoreactivity in the mating behavior pathway of the male golden hamster. Brain Res. *590*:29-38.

Swann, J.M. and S.W. Newman (1992) Testosterone regulates substance P within neurons of the medial nucleus of the amygdala, the bed nucleus of the stria terminalis and the medial preoptic area of the male golden hamster. Brain Res. *590*:18-28.

Swanson, L.W. (1987) The hypothalamus. In A. Björklund, T. Hokfelt, and L.W. Swanson (eds): Handbook of chemical neuroanatomy. Elsevier: Amsterdam, pp. 1-124.

Swanson, L.W. and P.E. Sawchenko (1983) Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. Annual Review of Neuroscience *6*:269-324.

Takahashi, K., A. Tanaka, M. Hara, and S. Nakanishi (1992) The primary structure and gene organization of human substance P and neuromedin K receptors. Eur.J.Biochem. *204*:1025-1033.

Takatsuki, K., M. Sakanaka, H. Takagi, M. Tohyama, and Y. Shiotani (1983) Experimental immunohistochemical studies on the distribution and origins of substance P in the medial preoptic area of the rat.. Exp.Brain Res. *53*:183-192.

Thornhill, J.A. and I. Halvorson (1994) Electric stimulation of the posterior and ventromedial hypothalamic nuclei causes specific activation of shivering and nonshivering thermogenesis. Can.J.Physiol.Pharmacol. *72*:89-96.

Torvick, A. (1987) Topographic distribution and severity of brain lesions in Wernicke's encephalopathy. Clinical Neuropathology *6*:25-29.

Toussaint, J.L., V. Geoffroy, M. Schmitt, A. Werner, J.M. Garnier, P. Simoni, and J. Kempf (1992) Human choline acetyltransferase (CHAT): partial gene sequence and potential control regions. Genomics *12*:412-416.

Tsuchida, K., R. Shigemoto, Y. Yokota, and S. Nakanishi (1990) Tissue distribution and quantitation of the mRNAs for three rat tachykinin receptors. Eur.J.Biochem. *193*:751-757.

Tsuruo, Y., S. Hisano, Y. Okamura, N. Tsukamoto, and S. Daikoku (1984) Hypothalamic substance P-containing neurons, sex-dependent topographical differences and ultrastructural transformations associated with stages of the estrous cycle. Brain Res. *305*:331-341.

Tsuruo, Y., S. Hisano, J. Nakanishi, S. Katoh, and S. Daikoku (1987) Immunohistochemical studies on the roles of substance P in the rat hypothalamus: possible implication in the hypothalamic-hypophysial-gonadal axis. Neuroendocrinology *45*:389-401.

Tsuruo, Y., H. Kawano, Y. Kagotani, S. Hisano, S. Daikoku, S. Chihara, T. Zhang, and N. Yanaihara (1990) Morphological evidence for neuronal regulation of luteinizing hormone-releasing hormone containing neurons by neuropeptide Y in the rat septo-preoptic area. Neurosci.Lett. *110*:261-266.

Tsuruo, Y., H. Kawano, S. Hisano, Y. Kagotani, S. Daikoku, T. Zhang, and N. Yanaihara (1991) Substance P-containing neurons innervating the LHRH-containing neurons in the septo-preoptic area of rats. Neuroendocrinology *53*:236-245.

Veazey, R.B., D.G. Amaral, and W.M. Cowan (1982a) The morphology and connections of the posterior hypothalamus in the Cynomolgus monkey (Macaca fascicularis). II. Efferent connections. J.Comp.Neurol. *207*:135-156.

Veazey, R.B., D.G. Amaral, and W.M. Cowan (1982b) The morphology and connections of the posterior hypothalamus in the cynomolgus monkey (Macaca fascicularis). I. Cytoarchitectonic organization. J.Comp.Neurol. *207*:114-134.

Vijayan, E. and S.M. McCann (1979) *In vivo* and *in vitro* effects of substance P and neurotensin on gonadotropin and prolactin release. Endocrinology *105*:64-68.

Von Euler, U.S. and J.H. Gaddum (1931) An unidentified depressor substance in certain tissue extracts. Journal of Physio.London *72*:74-87.

Wainer, B.H., T.L. Steininger, J.D. Roback, M.A. Burke-Watson, E.J. Mufson, and J. Kordower (1993) Ascending cholinergic pathways: functional organization and implications for disease models. In A.C. Cuello (ed): Progress in brain research. Elsevier Science Publishers B.V.: pp. 9-30.

Walker, L.C., V.E. Koliatsos, C.A. Kitt, R.T. Richardson, Ä. Rökaeus, and D.L. Price (1989a) Peptidergic neurons in the basal forebrain magnocellular complex of the rhesus monkey. J.Comp.Neurol. *280*:272-282.

Walker, L.C., D.L. Price, and W.S. Young,III (1989b) GABAergic neurons in the primate basal forebrain magnocellular complex. Brain Research *499*:188-192.

Walker, L.C., N.E. Rance, D.L. Price, and W.S. Young,III (1991) Galanin mRNA in the nucleus basalis of Meynert complex of baboons and humans. J.Comp.Neurol. *303*:113-120.

Warden, M.K. and W.S. Young,III (1988) Distribution of cells containing mRNAs encoding substance P and neurokinin B in the rat central nervous system. J.Comp.Neurol. *272*:90-113.

Weick, R.F., V. Pitelka, and D.L. Thompson (1983) Separate negative feedback effects of estrogen on the pituitary and the central nervous system.. Endocrinology *112*:1862-1864.

Wenk, G.L., W. Danysz, M.K. Chawla, and N.E. Rance (1995) Neuroprotection from NMDA within the nucleus basalis magnocellularis. Soc.Neurosci.Abstr. *21*:

Whitehouse, P.J., D.L. Price, A.W. Clark, J.T. Coyle, and M.R. DeLong (1981) Alzheimer disease: Evidence for selective loss of cholinergic neurons in the nucleus basalis. Ann.Neurol. *10*:122-126.

Whitehouse, P.J., D.L. Price, R.G. Struble, A.W. Clark, J.T. Coyle, and M.R. DeLong (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science *215*:1237-1239.

Whitty, C.J., P.D. Walker, D.J. Goebel, M.S. Poosch, and M.J. Bannon (1995) Quantitation, cellular localization and regulation of neurokinin receptor gene expression within the rat substantia nigra. Neuroscience *64*:419-425.

Wilson, R.C., J.S. Kesner, J.-M. Kaufman, T. Uemura, T. Akema, and E. Knobil (1984) Central electrophysiologic correlates of pulsatile luteinizing hormone secretion in the rhesus monkey. Neuroendocrinology *39*:256-260.

Wise, P.M. and A. Ratner (1980) Effect of ovariectomy on plasma LH, FSH, estradiol and progesterone and medial basal hypothalamic LHRH concentrations in old and young rats. Neuroendocrinology *30*:15-19.

Witkin, J.W., C.M. Paden, and A.-J. Silverman (1982) The luteininzing hormone-releasing hormone (LHRH) systems in the rat brain. Neuroendocrinology *35*:429-438.

Witkin, J.W. (1989) Synaptology of luteinizing hormone-releasing hormone neurons in the preoptic area of the male rat: Effects of gonadectomy. Neuroscience *29*:385-390.

Witkin, J.W. (1990) Access of luteinizing hormone -releasing hormone neurons to the vasculature in the rat. Neuroscience *37*:501-506.

Witkin, J.W., M. Ferin, S.J. Popilskis, and A.-J. Silverman (1991) Effects of gonadal steroids on the ultrastructure of GnRH neurons in the rhesus monkey: Synaptic input and glial apposition. Endocrinology *129*:1083-1092.

Wormald, P.J., R.P. Millar, and B. Kerdelhue (1989) Substance P receptors in human pituitary: A potential inhibitor of luteinizing hormone secretion. J.Clin.Endocrinol.Metab. *69*:612-615.

Yamaguchi, K., Y. Nakajima, S. Nakajima, and P.R. Stanfield (1990) Modulation of inwardly rectifying channels by substance P in cholinergic neurons from rat brain in culture. J.Physiology Lond. *426*:499-520.

Yamano, M., S. Inagaki, S. Kito, and M. Tohyama (1986) A substance P-containing pathway from the hypothalamic ventromedial nucleus to the medial preoptic area of the rat: an immunohistochemical analysis.. Neuroscience *18*:395-402.

Yankner, B.A., L.K. Duffy, and D.A. Kirschner (1990) Neurotrophic and neurotoxic effects of amyloid b protein: Reversal by tachykinin neuropeptides. Science *250*:279-282.

Yen, S.S.C. and A. Lein (1976) The apparent paradox of the negative and positive feedback control system on gonadotropin secretion. Am J Obstet Gynecol *126*:942-954.

Young, W.S., III, T.I. Bonner, and M.R. Brann (1986) Mesencephalic dopamine neurons regulate the expression of neuropeptide mRNAs in the rat forebrain. Proc.Natl.Acad.Sci.USA *83*:9827-9831.

Zaborszky, L., W.E. Cullinan, and A. Braun (1991) Afferents to basal forebrain cholinergic projection neurons : An update. In T.C. Napier (ed): Basal forebrain. Plenum: New york, pp. 43-100.

Zanisi, M., E. Messi, and L. Martini (1987) Ultrashort feedback control of luteinizing hormone-releasing hormone secretion in vivo. Endocrinology *121*:2199-2204.

Zhang, L. and R.E. Harlan (1994) Ontogeny of the distribution of tachykinins in rat cerebral cortex: Immunocytochemistry and in situ hybridization histochemistry. Dev.Brain Res. 77:23-36.