The Stimulation of Luteinizing Hormone Secretion from Anterior Pituitary Cells in Culture by Substance P: A Dissertation

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THE STIMULATION OF LUTEINIZING HORMONE SECRETION FROM ANTERIOR PITUITARY CELLS IN CULTURE BY SUBSTANCE P: A POSSIBLE PHYSIOLOGICAL ROLE IN REPRODUCTIVE FUNCTION

A DISSERTATION PRESENTED BY

Maureen Shamgochian

Submitted to the Faculty of the
University of Massachusetts Medical School
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Sciences
THE STIMULATION OF LUTEINIZING HORMONE SECRETION FROM ANTERIOR PITUITARY CELLS IN CULTURE BY SUBSTANCE P: A POSSIBLE PHYSIOLOGICAL ROLE IN REPRODUCTIVE FUNCTION

A Thesis

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ABSTRACT

The observations that substance P (SP) is localized in the anterior pituitary gland (AP) and is regulated by the hormonal status of the animal, as well as the demonstration of SP binding sites in the AP, have led to the idea that SP may participate in the regulation of AP function. Numerous and sometimes contradictory reports of SP effects on AP hormone secretion, particularly on luteinizing hormone (LH), left the question of whether SP acts directly at the level of the AP to regulate LH secretion still unanswered. To investigate a possible physiological function of SP in the AP, the effects of exogenous SP on LH secretion from AP cells from adult and prepubertal male and female rats in short term culture were studied. It was found that SP (100nM-1µM) significantly stimulates LH release in cultured AP cells and that this effect varies as a function of age and sex. SP has no significant effect on LH release from AP cells of male and female prepubertal rats. After day 30 a sharp increase in the response to SP occurs in both sexes. This level of responsiveness continues through adulthood in AP cells from the female rat. In contrast, AP cells from male rats failed to respond during adulthood (over 50 days of age) but were
highly responsive during the peripubertal period (30-35 days). The possibility that the responsiveness to SP is influenced by the endocrine status of the animal was investigated by exposing AP cells from responding animals to androgens in vivo and in vitro. It was found that AP cells from female rats treated with androgen were less responsive to 100nM SP but did respond at higher doses of SP. SP effects on AP function were further analyzed in experiments using radioligand binding assays to assess possible changes in SP receptor number or affinity as related to age and sex. In AP membranes from female rats, maximum binding is 8-fold higher (Bmax=4.2 pmol/mg membrane protein) than in AP membranes from male rats (Bmax=560fmol/mg membrane protein). These studies suggest a role for SP as a secondary regulator of LH secretion with possible physiological significance for reproductive function.
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ABBREVIATIONS

AP-Anterior Pituitary
APSP-Substance P content in the Anterior Pituitary Gland
BHSP-Bolton Hunter group conjugated to Substance P
$B_{\text{MAX}}$-Estimated Maximum Binding Capacity (R°)
GnRH-Gonadotropin Releasing Hormone
H-Hour
$IC_{50}$-Concentration at 50% Inhibition
$K_D$-Dissociation Constant for Receptor-Ligand Interaction
LH-Luteinizing Hormone
PPT-Preprotachykinin
PRL-Prolactin
RIA-Radioimmunoassay
RRA-Radioreceptorassay
SP-Substance P
SPLI-Substance P-like-immunoreacttivity
$T_4$-Thyroxine
TRH-Thyrotropin-Releasing Hormone
TSH-Thyroid Stimulating Hormone
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I INTRODUCTION

Discovery, Isolation and Characterization of Substance P

Substance P (SP) is a peptide that was first detected in extracts of equine brain and intestine in 1931 by von Euler and Gaddum (von Euler and Gaddum 1931). While attempting to demonstrate release of acetylcholine from intestine, they found that a standardized preparation of intestinal extract, designated "P" for preparation, caused a transient hypotension when injected into anesthetized rabbits and stimulated contraction in isolated jejunum in vitro. The contraction of intestinal tissue was not inhibited by atropine and after elimination of other compounds known to cause intestinal contraction it was concluded that these effects were due to an unknown substance. This substance in the preparation that caused the activity was named substance P. It was determined that the substance had a low molecular weight and the biological activity was destroyed by proteolytic digestion (von Euler 1936). A study of extracts from different organs showed that SP was present in significant quantities in the brain. Later studies by Pernow
(1953), extensively mapping the substance in various tissues, revealed a high concentration of the substance in the hypothalamus and in the dorsal horn of the spinal cord.

It was not until 1967, in the course of an attempt to purify a corticotropin releasing factor from bovine hypothalamic extracts, that Leeman and Hammerschlag reported the discovery of a peptide which caused salivation when injected intravenously (Leeman and Hammerschlag 1967). This sialogogic peptide was subsequently characterized as SP. The peptide was purified by Leeman and Chang (1970), and the amino acid sequence was determined to be H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2 (Chang et al 1971).

Additional studies done by Studer et al (1973) and Carraway and Leeman (1979) confirmed the sequence and the identity between the sialogogic peptide and SP.

In the intervening years between the discovery of SP and its purification and sequencing, Espamer et al (1949), discovered active components of octopus salivary glands with hypotensive, sialogogic and spasmogenic activity that could not be attributed to other known substances. These active principles, eledoisin and physaleamin and kassinin, were purified, characterized and sequenced and found to share similar biological activity and chemical structure including the C-terminal sequence -Phe-X-Gly-Leu-Met-NH2 (Anastasi and Espamer 1970; Anastasi 1977). Because the active peptides showed a characteristic fast onset of action on tissues of
the gut, the name tachykinin, which literally means "fast movement", was given to the peptides of the group.

Until recently SP was the only known mammalian tachykinin, however in recent years two other mammalian tachykinins have been isolated. After the development of an RIA for kassinin, it was found that extracts of spinal cord contained abundant kassinin-like immunoreactivity, which was attributed to a novel peptide. This peptide was named substance K, and was sequenced by Kimura et al (1983). At about this time, another group, Kangawa et al (1983) in search of novel neuropeptides sequenced a peptide they called neuromedin K, which has also been called neurokinin B, and a peptide they called neuromedin L which proved to be substance K. Table 5 illustrates the amino acid sequences of some of the tachykinins referred to above.

The three mammalian tachykinins, SP, SK and neuromedin K are derived from two different genes which are structurally related. Nawa and coworkers (1983) cloned a cDNA encoding SP and discovered that the cDNA cloned from bovine striatum encoded not only SP but also encoded substance K in the same precursor. This was called the preprotachykinin-A (PPT-A) gene. Krause et al (1987) isolated a rat PPT cDNA which structurally resembles bovine PPT-A cDNA. The PPT-B gene encodes the precursor for neuromedin K. Analysis of these cDNAs and the proteins they encode made possible the study of the regulation of PPT gene expression.
SP Receptors

As with other neurotransmitter and peptide hormones there is evidence that SP exerts its diverse effects by interacting with specific receptors located on the plasma membrane of the target cell. The potency differences among the tachykinins and their fragments in bioassays on mammalian tissues led to the concept that multiple subtypes of SP receptors existed in those tissues. Further pharmacological studies suggested the presence of at least three distinct types of binding sites, each of which exhibited a preferential affinity for one of the three known mammalian tachykinins. The current nomenclature has classified mammalian tachykinin receptors as NK1, NK2 and NK3 receptor subtypes, based on whether the preferential endogenous ligand is SP, SK or neurokinin B (neuromedin K) (Henry 1987; Regoli et al 1988). These tachykinin binding sites are saturable, of low capacity and high affinity (Burcher 1988; Regoli 1987; Quirion 1985; Cascieri 1983). However it must be kept in mind that binding sites are not always receptors, and in comparing data from radioligand binding and pharmacological effects it has become clear that in vitro, one exogenously applied tachykinin can act as an agonist at another's receptor. If two or three tachykinin receptor subtypes are present in an
organ, two or three different processes could contribute to the final biological effect.

Receptor characterization of most of the classical transmitters has relied on the use of specific high affinity antagonists to distinguish between receptor subtypes. For the tachykinins, unfortunately, as with many peptides, no suitable antagonists have been discovered. The SP antagonists which have been reported are neither specific nor high affinity and frequently have agonist properties. Therefore, the study of tachykinin receptor subtypes has been carried out using the natural, but nonselective agonists. Recently, selective agonists for the tachykinin receptors have provided pharmacological tools for the characterization of receptor subtypes (Regoli 1988). Specific chemical modifications and amino acid replacements within the SP molecule or its fragments increased the selectivity for the NK1, NK2 and NK3 receptors. These selective agonists have made it possible to distinguish between tachykinin receptor subtypes involved in physiological function. For example, the NK1 selective compound, [Pro⁹, Met(O₂)¹¹]SP is a very potent promoter of salivary secretion while the selective agonists for the NK2 and NK3 receptors, [Nle¹⁰]NKA(4-10) and [MePhe⁷]NKB respectively, are completely inactive. This would imply that SP action on NK1 receptors mediates salivary secretion.

Two of the tachykinin receptors, NK1 and NK2 are thought to be coupled to a G-protein that activates a phosphatidyl-
inositol, calcium second message pathway (Buck 1986). The amino acid sequences of these two receptors have recently been described on the basis of the sequence analysis of the molecular clones (Yokota 1989). This information was confirmed when the expressed receptor selectively bound SP. The amino acid sequence shows similarities to other G-protein-coupled receptors and possesses seven membrane spanning domains. The amino- and carboxyl-terminal regions differ between the two receptors which may contribute to their specificities.

Distribution and Physiology of SP in the Hypothalamic-Pituitary Axis

SP was first isolated and characterized from extracts of bovine hypothalamus and is found in high concentrations in that tissue. Since neuroendocrine cells in the hypothalamus which synthesize and secrete regulatory peptides are so important in the regulation of anterior pituitary function, many workers postulated that hypothalamic SP might influence the secretion of anterior pituitary hormones. In addition SP is present in the anterior pituitary itself which raises the possibility that SP could be released from cells within the anterior pituitary to act in a paracrine or autocrine way to influence anterior pituitary function.
SP in the Hypothalamus.

Hypothalamic regulation of anterior pituitary function can occur at many sites, including neural afferent inputs to the hypothalamus, the neuroendocrine cells, hypothalamic interneurons and the neural capillary interface of the median eminence.

SP producing neurons in the hypothalamus are typical in location and morphology to other neurosecretory neurons which influence anterior pituitary function. Hypothalamic-peptide-producing neurons exhibit a common organizational pattern in that most of their cell bodies are located in the paraventricular nucleus, the periventricular area or the arcuate nucleus, with smaller populations of neurons in the medial preoptic area (Weiner 1988). Cell bodies containing SP are found in the arcuate nucleus and medial preoptic area in the rat (Ljungdahl 1978). Immunohistochemical studies identified SP containing fibers in the stria terminalis which projects to neuroendocrine cells in the hypothalamus (Palkovits 1979). Neuronal fibers which stain for SP are found in the same regions that contain SP-positive staining cells. These fibers are especially prominent in the medial preoptic area.
The external lamina of the median eminence is the site of termination of neuroendocrine axons onto capillaries of the hypothalamic-hypophyseal portal system. SP-positive fibers are found in the external lamina of the median eminence in the monkey and in the rat (Hokfelt 1978; Tsuruo 1983). These fibers appear to terminate on the pericapillary space of the portal vessels. More recently, Palkovits et al (1989) demonstrated that SP containing nerve fibers in the median eminence of the rat originate in the ventromedial arcuate nucleus. Such findings support the possibility that SP could be secreted from neuroendocrine cells into the portal circulation and eventually to the anterior pituitary, although attempts to measure SP in the portal blood have been unsuccessful.

There is much evidence which suggests that SP may participate in the control of gonadotropin secretion by acting at the hypothalamic level. Nerve terminals in the medial preoptic area of the mouse which contain SP appear to synapse on GnRH cell bodies (Hoffman 1985). SP also increases GnRH release from medial basal hypothalamus in vitro, resulting in LH release from the pituitary. Estrogen but not progesterone is involved in the release of GnRH by SP (Ohtsuka 1987). Additionally, lateral ventricular injection of an antiserum to SP or an analogue of SP with antagonistic properties has been shown to significantly suppress LH levels in the circulation (Dees 1985). However, studies of this type
involve many potential problems including the specificity of the action of SP and the possibility that the observed effects represent a summation of actions at multiple sites.

Hypothalamic SP-containing neurons in rats display a sex difference in appearance, and in females the ultrastructural morphology of the perikarya varies during the estrous cycle (Tsuruo 1987). At diestrus, the terminals contain large cored vesicles, whereas at estrus they contain small clear vesicles. These studies further demonstrated that SP-containing cell bodies in the arcuate nucleus increase in number during the early stages of pregnancy but decrease in number at the end of pregnancy and during lactation. Decrease in synthesis or increase in release from the nerve terminals in the median eminence may be the cause of this decrease in immunoreactivity in the perikarya and cell bodies. Frankfurt et al (1986), found alterations in SP concentrations in estrogen concentrating areas of rat brain, particularly in the medial preoptic area and the medial ventral hypothalamus, throughout the estrous cycle. The study found that SP concentrations were significantly higher at diestrus and estrus than at proestrus when LH levels are highest.

It is tempting to theorize from these studies that SP may be involved in the hypothalamic-hypophyseal-gonadal axis, however, it is not clear from the results of these studies whether SP is involved in regulation of AP hormone secretion or whether SP may be involved in other events that take place.
during the estrous cycle. More direct tests of SP effects on the neurosecretory cells of the hypothalamus are needed to clarify the putative role of SP in neuroendocrine regulation.

**SP in the Anterior Pituitary**

The possibility that SP, originating in the anterior pituitary, acts there in a paracrine or autocrine way to influence anterior pituitary secretion is supported by several kinds of evidence. SP is present in the anterior pituitary glands of rats, guinea pigs, monkeys and humans as determined by immunocytochemistry and/or radioimmunoassay of whole AP extracts. The immunoreactive SP found in rat anterior pituitary has been found to behave identically to synthetic SP on high pressure liquid chromatography. In regard to the question of what cell types contain SP, Morel and coworkers (1982) found that SP immunoreactivity was present in the lactotroph and gonadotroph in both prepubertal and adult rats. De Palatis et al (1982), using a different immunocytochemical technique, found that SPLI was located in a subpopulation of thyrotrophs in the guinea pig. It has been demonstrated that SP can be released from hemipituitaries incubated in vitro with high potassium concentrations (Aronin et al 1984). Whether SP is released in vivo under physiological conditions, a prerequisite for a paracrine role
for SP, remains to be determined. Evidence to support the idea that SP is synthesized in the anterior pituitary was provided by Jonassen et al (1987) who demonstrated the presence of PPT-A mRNA in rat anterior pituitary.

The SP content in the anterior pituitary is not constant but is highly subject to variation by hormonal treatment. Studies by Aronin et al (1984) revealed that the concentration of SP in the rat anterior pituitary is affected by thyroid status. SP is increased in the anterior pituitaries of hypothyroid rats and is decreased after treatment with excess T4. Whether the increase in the anterior pituitary SP content after thyroidectomy is related to an increase in the number of thyrotrophs that contain SP or to an increase in SP per cell is not known. However, since T4 causes a marked decrease in SP content within two days it is unlikely that this is due to a decrease in the number of thyrotrophs suggesting that thyroid hormones may have an effect on SP content in the anterior pituitary unrelated to thyrotrope number. Alternatively, SP contained in lactotrophs or gonadotrophs could also respond to T4.

Expression of PPT-A gene in rat anterior pituitary is increased after thyroidectomy and decreased by administration of thyroid hormone (Jonassen 1987). Jonassen et al demonstrated the presence in the anterior pituitary of another mammalian tachykinin, SK. SP and SK concentrations are regulated in parallel by thyroid hormone status. These
results strongly suggest that SP and SK are synthesized in
the rat anterior pituitary.

There are also age, sex and gonadal steroid related
changes in SP concentrations in the anterior pituitary. There
is a significant difference in the APSP concentrations of
adult male and female rats. Males have 2-3 fold more APSP
than female rats (De Palatis 1982, Abstract; Coslovsky 1984).
This sex related difference in SP levels is dependent upon
the age of the rat in that no significant difference in APSP
levels between prepubertal males and females is found,
whereas at two months of age, male APs had significantly
higher concentrations of SP. Neonatal exposure to
testosterone (Yoshikawa 1983) appears to be important in
determining the male levels of APSP, as gonadectomy of older
rats did not significantly lower APSP concentrations.

Further insight into the importance of gonadal steroids
in mediating the changes in APSP came with reports by
Coslovsky (1984) and DePalatis (1985) which showed that
ovariectomy of adult female rats causes a significant
increase in SP content, whereas administration of estrogen to
gonadectomized rats of both sexes causes a reduction.
Administration of the 5-α-reduced metabolite of testosterone,
5-α-dihydrotestosterone (DHT), markedly increases SP
concentrations in both gonadectomized sexes. However
testosterone is ineffective in elevating SP concentrations in
the AP suggesting the possibility that aromatization of testosterone to estradiol may attenuate the response to the portion of testosterone that is reduced to DHT.

It is not known whether gonadal steroids act directly at the level of the AP to mediate changes in SP concentrations in vivo. The AP has receptors for androgens, including DHT, and estrogen as well as 5-α-reductase activity, but does not have the aromatase enzyme. It is possible, therefore that gonadal steroids could act directly at the AP level.

Sensitivity to gonadal steroids requires a maturational process and becomes evident during the juvenile stage of development (Coslovsky 1985). In contrast, hypo- or hyperthyroidism alter SP levels in the anterior pituitary of infantile rats to the same extent as in adult rats. The maturation process required for gonadal steroid regulation of SP concentrations could be a result of the reported changes in sensitivity to testosterone and estrogen during development (Negro-Vilar 1973). This report shows that the sensitivity of the hypothalamic-pituitary unit to the negative feedback effect of testosterone declines during development, whereas sensitivity to T4 may not undergo such changes. DHT administration to hypothyroid infantile rats leads to a significant increase in SP content in the anterior pituitary, suggesting that thyroid hormones may somehow regulate the timing of the sensitivity to androgens in the
AP. An alternative explanation of this additive effect of DHT is that androgenic hormones are known to decrease $T_4$ binding capacity of thyroid binding globulins resulting in abnormalities of the plasma transport of $T_4$, and perhaps an exacerbation of the existing hypothyroid condition which is not specifically related to the action of DHT on the anterior pituitary.

There is also a clear sex difference in anterior pituitary PPT-A mRNA levels, and gonadal steroids appear to play a regulatory role (Jonassen et al 1988; Brown et al 1990). In female rats androgen increases whereas estradiol decreases anterior pituitary PPT-A mRNA. Normal male rats have higher anterior pituitary PPT-A mRNA abundance yet appear to be less sensitive to gonadectomy than females, suggesting that either estrogen is the important gonadal steroid regulating SP concentrations, or, as Yoshikawa and Hong suggested, neonatal exposure to testosterone is the determining factor in adult male APSP concentrations.

**Effects of SP on Anterior Pituitary Hormone Release**

Numerous reports suggest that SP may affect anterior pituitary hormone release. The effects of SP in many reports vary depending on the site, route or method of administration
of the peptide. Tables 1-4 summarize the reported effects. Intravenous and intracerebroventricular injection of SP in rats stimulates prolactin release (Vijayan 1979; Kato 1976). In vitro incubation of hemipituitaries with SP also produces an increase in prolactin release.

SP also alters growth hormone release although its effects are inconsistent. Intravenous injection (Vijayan 1979; Rivier 1977) of synthetic SP increased plasma concentrations of growth hormone. Similarly, injection of SP into the third ventricle of conscious, normal or ovariectomized rats resulted in an increase in plasma growth hormone (Vijayan 1980). In contrast, reduced levels of growth hormone have been reported with SP injection into the lateral ventricle in urethane anesthetized male rats (Chihara 1978).

The studies reported above do not address the question of whether the effects of SP were directly on the anterior pituitary or on the hypothalamus, nor do they suggest whether SP effects require an intermediate mediator. Chihara et al (1978) proposed that SP injected intraventricularly acts via somatostatin because pretreatment with somatostatin antiserum abolished the SP effect. Further, in a separate report (Sheppard 1978) found that SP applied to incubated hypothalami caused an increase in somatostatin release.

Incubations of pituitary segments with SP inhibits ACTH secretion (Jones 1978). Intravenous injection of SP stimulated β-endorphin and β-lipotropin release in rats as
measured in serum, however the source of release was not clear as β-endorphin is present in other tissues besides the pituitary. In vitro studies showed that SP induced β-endorphin release from dispersed anterior pituitary cells in a dose related manner (Matsumura 1982) suggesting that SP is acting directly at the level of the anterior pituitary.

The effects of SP on luteinizing hormone (LH) are of particular interest since SP levels in the AP are regulated by gonadal steroid hormones, yet these studies have not conclusively shown the site of action and appear to be dependent on the route of administration. For example, intravenous injection of SP resulted in reduced plasma gonadotropin levels in ovariectomized female rats, while intracerebroventricular injection of SP resulted in an increase in LH levels (Vijayan 1979). In the same report pituitaries incubated with SP in vitro resulted in no change in LH release. These data suggest that the effects of SP observed in vivo were mediated via gonadotropin releasing hormone.

Other in vitro studies have shown conflicting results, for example, a stimulatory effect of SP on LH release has been reported by Fisher (1974) using in vitro pituitary incubations from 20 day old female rats. In contrast, SP significantly inhibited the ability of gonadotropin releasing hormone (GnRH) to stimulate release of LH from primary anterior pituitary cell cultures prepared from rats in
diestrus I and proestrus, but not at diestrus II or estrus (Kerdelhue 1978).

Intracerebroventricular administration of SP antiserum increases LH secretion before and after the surge with no effect on the magnitude of the LH surge itself, implying that SP may be inhibiting LH secretion at selective times during the cycle. The location of the effect can not be determined from this experiment, however.

In another report (Dees 1985) lateral ventricular injection of an antiserum to SP resulted in significantly suppressed LH levels in castrated male rats, suggesting that SP may have a stimulatory action. An antiserum to SP administered intravenously reduced LH secretion within 24 hours in male rats treated with estradiol whereas this effect was not seen in intact male rats of comparable size. This finding suggests that SP may stimulate LH secretion, although it is not possible to determine at what site the antiserum is blocking SP action. This study also suggests that the LH stimulating effect of SP depends on the hormonal status of the animal. Alternatively, estradiol could be acting to alter the clearance rate of the antiserum thereby prolonging the effect.

What emerges then, from these studies is that SP shows effects that may be different depending on the site of action and also on the hormonal state of the animal. To date it has not been possible to define with precision the role SP plays
in the control of anterior pituitary hormone secretion. It is difficult to interpret studies in which SP is administered peripherally because peptides of similar molecular weight have been shown to cross the blood brain barrier only to a limited extent leaving questions about the site of action unanswered.

Intracerebroventricular injections of microgram quantities of SP may result in nonphysiological effects on hypothalamic or other brain regions which may receive neuronal SP input or regions which do not normally have access to SP.

Studies using SP antisera are problematical because of confusion as to where these large proteins are diffusing to and acting. The antisera may not be specific for SP or may be contaminated with large enough amounts of the peptide to cause an effect. Further, antisera to SP may not necessarily block SP action but could act in an opposite way by stimulating the aggregation of receptors. Anti-insulin antibodies enhance the biological activity of submaximal doses of insulin by stimulating the clustering of insulin receptors (Kahn 1978).

While intriguing these studies raise many questions. Are the effects of SP strictly pharmacologic? Where does SP act, directly on the anterior pituitary or indirectly at the hypothalamic level or both? Why does hormonal status influence SP concentration in the anterior pituitary gland
but not in the hypothalamus and does hormonal status influence SP actions?

Other Peptides Implicated in Anterior Pituitary Regulation

Secretion of AP hormones is controlled by a complicated interplay of neural factors and negative and positive feedback signals from the target endocrine glands. More recently, it has been shown that in addition to the classical hypothalamic releasing factors or primary stimulators of AP hormone secretion, other peptides including neuropeptide Y (Kalra et al 1983; Crowley and Kalra 1987), neurotensin (Vijayan et al 1988), angiotensin II (Steele et al 1985), opiate agonists (Blank et al 1986; Cicero et al 1986), and vasoactive intestinal peptide (Abe et al 1989) affect the release of AP hormones. Many of these peptides are present in the hypothalamus and there is evidence that some may affect the release of hypothalamic releasing factors or may be released into median eminence blood vessels (Kalra 1983; Crowley 1987). In addition these peptides may be synthesized and stored within the pituitary and released to act in a paracrine or autocrine way (Abe et al 1985; Nagy et al 1988).

Several peptides in addition to gonadotropin releasing hormone (GnRH) affect gonadotropin release and may act as
secondary modulators (Kow et al 1988; Crowley et al 1987; Chabot et al 1988; Blank et al 1986). Neuropeptide Y (NPY), a 36 amino acid peptide isolated from bovine brain, is one such peptide prominent in the literature. Depending on the gonadal hormone milieu, intracerebroventricular injection of NPY has been reported to inhibit (Kerkerian et al 1985) and stimulate (Kalra et al 1984) LH release. It is thought that the actions of NPY may have physiological significance because of findings that NPY concentrations in certain hypothalamic regions are regulated by ovarian steroids and because the peptide stimulates the release of GnRH from medial basal hypothalamus (Crowley et al 1985; Crowley et al 1987).

Evidence suggests that NPY may be a unique modulator of LH secretion in that it promotes GnRH release and may be secreted into the portal blood to enhance GnRH stimulated LH release. NPY-like immunoreactivity has been demonstrated in corticotrophs, lactotrophs, somatotrophs and gonadotrophs (Chabot et al 1988). These researchers have shown a direct effect of NPY (1μM) on the pituitary to stimulate LH. Perhaps it will be found that other peptides play important roles at both CNS and AP levels in the complex neuroendocrine communication which must be required for fine tuning of anterior pituitary hormone secretion.

Another peptide which plays an important role in pituitary regulation is vasoactive intestinal peptide (VIP).
VIP has been detected in lactotrophs (Morel et al 1982) and causes release of PRL in vivo and in vitro (Frawley and Neill 1981; Samson et al 1980). VIP content in the anterior pituitary is increased in hypothyroidism, an effect which was reversed by T₄ replacement (Lam et al 1989). In this aspect the regulation of VIP content resembles that of SP in the anterior pituitary.

**Rationale and Objectives**

The discovery of SP in hypothalamus and anterior pituitary, and the finding that SP concentrations in anterior pituitary are regulated by the hormonal status of the animal generated the idea that there may be a specific functional significance of this peptide in the anterior pituitary. Several types of experiments suggest that SP can affect AP hormone secretion; however, striking differences in the effects of SP have been reported depending on the experimental protocol and the method of administration. This has resulted in a confusing literature with the question of whether SP acts at the level of the anterior pituitary to regulate AP hormone secretion still unanswered.

The sex differences in APSP concentrations and the regulation of those concentrations by gonadal steroids are supportive of the notion that SP may play a role in regulating gonadotropin secretion. The experiments which
address the question of the effect of SP on LH secretion are particularly confusing. Although significant regulatory control of LH secretion takes place in the central nervous system, mechanisms within the anterior pituitary allowing for local control cannot be ruled out. For these reasons I decided to focus on the effect of SP on LH secretion. In this thesis I propose the hypothesis that SP may play a functional role in the regulation of LH secretion by an action within the anterior pituitary.

**Approach**

Evidence that SP mediates a particular biological effect is usually based on the following lines of evidence: 1) presence, immunohistochemistry shows immunoreactive SP at the appropriate location, 2) release, SP can be released by the appropriate tissue, 3) efficacy, exogenous SP elicits the effect, 4) demonstration of receptors, binding and/or autoradiographic studies show SP receptors at the appropriate target site (Maggio 1988).

The presence of SP and its biosynthesis in the anterior pituitary gland has been established (Morel 1982; DePalatis 1982; Aronin 1984; Jonassen 1987). The release of SP from hemipituitaries in a K+-stimulated, Ca++-dependent manner has been established (Aronin 1984), however, a physiological stimulus for release or the possibility of release of SP in
vivo has not been established. The ability of SP to modulate LH release by acting directly on the anterior pituitary was evaluated using short term primary cultures of anterior pituitary cells with the goal of satisfying the third of the above criteria. In order to satisfy the fourth criteria and to correlate the sex and age differences in the SP biological effect to differences in SP receptor affinity or number, SP binding was studied in anterior pituitary membrane preparations. To examine the possibility that SP may be released from anterior pituitary cells to act directly on its own or surrounding cells experiments were done using SP antibody to alter endogenous SP effects.

**Choice of Assay**

The use of cell cultures of pituitaries for studying pituitary hormone secretion provides a number of advantages over in vivo experimentation or in vitro systems using hemipituitaries. Primary cultures of pituitary cells allow the complete isolation of the pituitary from neural and humoral influences and the addition of test substances under a controlled environment. The reproducibility of LH release is much higher in cell cultures than in hemipituitaries because cell cultures are prepared from several animals and individual differences tend to be damped out. Problems with uniform dispersion of test substances, oxygen and CO₂ are
difficult to overcome in incubations of hemipituitaries.

There are also disadvantages to use of primary culture systems for physiological studies. First, it is not a physiological model to be compared with an in vivo model. The isolation of the tissue from neural and humoral influences could result in artifactual results which do not reflect the in vivo response. Also the preparation of cells is heterogeneous introducing questions about what cell type may be affected by a test substance. There have been no reports of a continuous culture line that releases gonadotropins in response to GnRH which would provide a more homogeneous system for the study of receptor and signal transduction mechanisms. Separation of pituitary gonadotrophs on the basis of their larger size has been achieved with moderate success, however this process requires extremely large numbers of animals to get a reasonable yield for experimentation.

In contrast, preparation of primary cultures is a relatively simple procedure. Anterior pituitaries are removed and treated with enzymes (collagenase and hyaluronidase) in order to break down intracellular connections, subjected to mechanical disruption and placed in suitable culture dishes in growth medium. Independent tests of cell viability, such as stimulation of hormone release, maintenance of normal cell function and morphology and trypan blue exclusion can then be performed. Cells which have been in culture for between 18 hours and 4 days possess excellent responsiveness to GnRH in

Studies have shown that 1-3 day pituitary cell cultures maintain many of the characteristics of the animal from which they were prepared and pituitary cells from different animal models behave differently in both a physical and physiological sense. Denef et al (1978) found differences in the gravity sedimentation rate of gonadotrophs from 14 day old males, 14 day old females, and adult males and also found that each of these models responded to GnRH differently. Tang and Tang (1979) have reported that GnRH stimulation in cultured pituitary cells from adult females induced larger LH responses than in cell cultures from adult males. The authors interpret this result to indicate differences in gonadotroph population.

It has also found that pituitary cells from adult female rats released more LH than did pituitary cells from adult male rats (Nakano, 1976). Snyder et al (1976) found that basal PRL secretion differed in cells from normal and castrated adult female rats indicating that the effects of gonadal steroids on PRL secretion remain after cells are placed in culture. It is well known from in vivo studies that changes in the responsiveness of the pituitary to GnRH occur throughout the female reproductive cycle (Arimura, 1977). In 18 hour pituitary cell cultures taken from adult female rats at different stages of the estrous cycle LH responses to GnRH were significantly greater in cells taken from diestrus-II
and proestrus animals compared with those from estrus or diestrus-I (Baldwin 1981). O'Connor et al (1980), in a study using 3 day cultures of pituitary cells derived from intact and ovariectomized females, found that ovariectomy increased the GnRH induced LH release by more than 2-fold. However, Reel et al (1978) reported that female rat anterior pituitary cells, taken from rats at different stages of the estrous cycle, lose the characteristic responsiveness to GnRH when placed in culture for 1-4 days.

Because cultured cells provide a simple and direct way of assessing regulation of hormone secretion in anterior pituitary and maintain physiological differences between male and female, I decided to use the short term primary culture model system for defining the role of SP in the anterior pituitary. Using the information from the above reports and a report by Hopkins (1977), which states that a 48 H recovery period is needed after dispersion in order to regain response capability, the secretion experiments in this project were designed to use 48-72 H cultures of AP cells.

In the attempt to further characterize the responses of anterior pituitary cells to SP, radioligand binding studies of SP binding sites in the anterior pituitary were done to examine possible alterations in SP binding parameters (changes in number or affinity of the receptors) in the anterior pituitary. This binding assay protocol (described in Materials and Methods) is almost identical to the method used
in characterizing the GnRH receptor and many other anterior pituitary receptors (Clayton 1981).

Specific and saturable high affinity binding sites for SP have been demonstrated in the anterior pituitary using different ligands: $^3$H-SP or the analog $^{125}$I-D-Tyr$^0$-NorLeu$^{11}$-SP (Kerdelhue et al, 1985). In this study the $K_d$ is between 1.5 and 20 nM, similar to that reported for brain cortex and parotid cells. Further there is a similar index of specificity for binding of SP fragments and analogs. These authors report that the number of adenohypophyseal SP receptors was altered during the estrus cycle of the rat, being highest on the afternoon of proestrus during the LH surge. More recently, SP binding sites were described in whole anterior pituitary cells using $^{125}$I-BHSP as the ligand (Larsen 1989). They report a $K_d$ of 0.48nM with a Bmax of 1187 sites/cell and demonstrate that SP is internalized and degraded after binding to a cell surface receptor. These results imply that SP effects at the level of the anterior pituitary are mediated via specific receptors on the cell surface.
Summary of Goals

The goal of the present studies was to clarify the effects of SP on the secretion of LH, to characterize the binding of radiolabeled SP to AP membranes and to explore the possibility of regulation of the response and binding parameters by the hormonal status of the animal.
<table>
<thead>
<tr>
<th>EXPERIMENTAL SITE OF INJECTION</th>
<th>SP DOSE</th>
<th>EFFECTS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral Ventricle</td>
<td>100 nM</td>
<td>Serum GH suppression of PRL</td>
<td>Eckstein, 1980</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serum PRL elevation of LH</td>
<td>Vijayan, 1979</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serum LH and PRL elevation of PRL</td>
<td>Vijayan, 1979</td>
</tr>
<tr>
<td></td>
<td>10 µg</td>
<td>Serum GH suppression on suppression of LH</td>
<td>Chithara, 1978</td>
</tr>
<tr>
<td></td>
<td>10 µm solution</td>
<td>Serum GH suppression on suppression of PRL</td>
<td>Chithara, 1978</td>
</tr>
</tbody>
</table>

Table 1: Reported effects of Substance P on anterior pituitary secretion
Table 2: REPORTED EFFECTS OF SUBSTANCE P ON ANTERIOR PITUITARY SECRETION

<table>
<thead>
<tr>
<th>EXPERIMENTAL MODEL</th>
<th>SITE OF INJECTION</th>
<th>SP DOSE</th>
<th>EFFECT</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Urinary anesthetized</td>
<td>Jugular Vein</td>
<td>5 - 50 ~g</td>
<td>Elevation of serum PRL (L-Dopa blocked response)</td>
<td>Kato, 1976</td>
</tr>
<tr>
<td>2. Urethane anesthetized</td>
<td>Jugular Vein</td>
<td>25 - 149</td>
<td>Elevation of serum Ch</td>
<td>Rivier, 1977</td>
</tr>
</tbody>
</table>

*Elevation of serum GH* and *Elevation of serum PRL* refer to changes in hormone levels following intravenous injections of Substance P.
### Table 3: Reported Effects of Substance P on Anterior Pituitary Secretion

<table>
<thead>
<tr>
<th>Reference</th>
<th>Effect</th>
<th>SP Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kerdelhue, 1978</td>
<td>LH release at proestrus stimulated</td>
<td>1 - 100m in incubation medium</td>
</tr>
<tr>
<td>Fisher, 1974</td>
<td>Increased FSH release</td>
<td>100m in incubation medium</td>
</tr>
<tr>
<td>Matsumura, 1982</td>
<td>Inhibited ACTH release</td>
<td>10m in incubation medium</td>
</tr>
<tr>
<td>Nicholson, 1984</td>
<td>ACTH release</td>
<td>10-10^2 M in incubation medium</td>
</tr>
<tr>
<td>Vijayan, 1980</td>
<td>OR TSH release</td>
<td>150 - 200 ng / ml</td>
</tr>
<tr>
<td>Vijayan, 1979</td>
<td>Increased PRL release</td>
<td>1 µL - 10 µL in incubation medium</td>
</tr>
<tr>
<td>Vijayan, 1979</td>
<td>No change in GH, LH</td>
<td>1 µL - 10 µL in incubation medium</td>
</tr>
<tr>
<td>Kerdelhue, 1978</td>
<td>Increased FSH release</td>
<td>100m in incubation medium</td>
</tr>
<tr>
<td>Nicholson, 1984</td>
<td>ACTH release</td>
<td>10m in incubation medium</td>
</tr>
<tr>
<td>Kerdelhue, 1978</td>
<td>LH release at proestrus stimulated</td>
<td>1 - 100m in incubation medium</td>
</tr>
<tr>
<td>Reference</td>
<td>Site of Injection</td>
<td>Effect</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Kerdelhue, 1978</td>
<td>Lateral Ventricles</td>
<td>Decrease in serum LH before and after LH surge</td>
</tr>
<tr>
<td>Dees, 1985</td>
<td>Lateral Ventricles</td>
<td>Increase in serum LH</td>
</tr>
<tr>
<td>Debeljuc, 1987</td>
<td>Intravenous</td>
<td>Decline in serum LH</td>
</tr>
</tbody>
</table>

Table 4: Reported Effects of Immunoneutralization of Substance P on Anterior Pituitary Secretion
### Table 5: Selected Tachykinin Sequences

<table>
<thead>
<tr>
<th>Mammalian</th>
<th>Substance P</th>
<th>Substance Kα</th>
<th>Substance Kβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-Pro-Val-Asp-Val-Glu-Leu-Met-NH₂</td>
<td>H-Asp-Lys-Thr-Asp-Ser-Val-Gly-Leu-Met-NH₂</td>
<td>H-Arg-Pro-Lys-Pro-Gln-Gln-Pro-Val-Phe-Leu-Met-NH₂</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nonmammalian</th>
<th>Physalaemin</th>
<th>Kasigin</th>
<th>Eledoisin</th>
</tr>
</thead>
</table>

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*Table note:* X is an aromatic or branched aliphatic amino acid.

*Alternate names:* neuromedin B, neurokinin B, neuropeptide S, neuropeptide L.

A C- terminal carboxyamide, B C- terminal pyroglutamic acid moiety.

*Note:* Terminal carboxyamide, N-terminal pyroglutamic acid moiety.
II MATERIALS AND METHODS

Materials

Substance P and analogs of SP were purchased from Bachem Inc. Selective SP receptor agonists were a gift from Dr Domenico Regoli. GnRH was purchased from Sigma Chemicals or Boehringer Mannheim Biochemicals. Collagenase was purchased from Cooper Biomedical. Hyaluronidase and DNase were purchased from Sigma Chemicals. Dulbecco’s Modified Eagles Medium with glucose and L-glutamine without phenol red, horse serum, fetal calf serum, gentamycin sulfate and non-essential amino acids were purchased from Sigma Chemicals. LH radioimmunoassay materials were donated by the National Institute of Diabetes, Digestive & Kidney Diseases (NIDDK) through the National Hormone and Pituitary Program at the University of Maryland School of Medicine. SP Radioimmunoassay materials were provided by Dr. Susan Leeman. Goat anti-rabbit IgG for use as a second antibody in the LH RIA was purchased from Antibodies Inc.

Chemicals used in homogenization, binding and physiological buffers such as Tris, Hepes, dithiothreitol,
BSA, EDTA, sodium, magnesium and calcium chloride, sodium and potassium phosphates etc. were all purchased from Sigma Chemicals.

**Animals**

Sprague Dawley rats, purchased from Charles River Breeding Labs or Harlan Sprague Dawley, of different age and sex depending on the experiment, were used in all binding and pituitary culture experiments. In some experiments rats were made hypothyroid by replacing the drinking water with a solution of 0.05% methimazole and glucose for a period of 21 days. Hypothyroidism was verified by TSH RIA initially and then by SP RIA of anterior pituitary homogenates. The latter method proved to be a reliable test of hypothyroidism in rats. Adult female rats were used at random stages of the estrous cycle. In some experiments, intact female rats were surgically implanted with silastic tubing of 20mm lengths which were either packed with androgen (5-α-dihydrotestosterone, Sigma) or empty for controls. These implants were left in place for a period of 6 weeks and were intact at the time of sacrifice of the rats. Androgenization of females was verified by SP RIA of anterior pituitary homogenates.
Preparation of Anterior Pituitary Membranes for Radioligand Binding Assay

Rats were killed by asphyxiation with CO₂ and then decapitated. The anterior pituitaries were rapidly removed and placed in ice cold 25 mM Hepes buffer containing 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 0.36 mM CaCl₂ and 10 mM glucose. The tissue was placed in a glass homogenizer in 30 mls of a homogenization buffer (pH 7.4) containing 10 mM Tris-HCl, 1 mM DTT, 5 mM EDTA and 0.1% BSA and homogenized on ice with 20 passes. The resulting tissue extract was centrifuged at 1000 g for 10 minutes at 4°C. The supernatant was then centrifuged at 10,000 g for 20 minutes at 4°C. The resulting pellet was washed 2 times by the same procedure. The pellet was resuspended in 1 ml of homogenization buffer with 250 mM sucrose, frozen in liquid nitrogen and stored at -80°C until the time of assay. An aliquot of the membrane preparation was saved for Lowry protein determination.

Preparation of Dispersed Anterior Pituitary Cells

Rats were killed by asphyxiation with CO₂ and decapitated. Anterior pituitaries were removed and placed in Hepes buffer (described above) warmed to 37°C. This and all
other buffers and media used in preparation of cells for
culture were sterilized by filtration through a 0.2
micrometer membrane filter. Pituitaries were rinsed with
buffer and incubated in the above mentioned Hepes buffer
containing collagenase (3 mg/ml) and hyaluronidase (1 mg/ml)
to digest connective tissue holding cells together, DNase
(.02 mg/ml) to digest any DNA that had been released from
broken or damaged cells, and BSA (3%). The tissue was digested
for 60 to 90 minutes in a shaker bath at 37C. Pituitaries
were then mechanically dispersed in this solution by gentle
trituration with sterile, plastic disposable pipets. The
cells were collected by centrifugation at 70 g for 10
minutes, washed 3 times in Hepes buffer and once in DMEM
culture media. Cells were then either counted using a
hemocytometer and cell viability determined by trypan blue
exclusion (consistently greater than 90%) or directly
aliquoted (.25-.3 pituitary equivalents/well) into sterile 24
well tissue culture plates (Falcon) and incubated in 1ml DMEM
with 10% horse serum, 2.5% fetal calf serum (both types of
serum were treated with dextran coated charcoal to remove
steroids and thyroid hormones), gentamycin (10mg/ml) and non
essential amino acids (2mM) for 48 hours in a metabolic
incubator at 37 C with 5% CO₂ and 95% air. One anterior
pituitary from an adult male rat consistently yielded 1.5-
2.5x10⁻⁶ cells Preliminary studies using this experimental AP
cell model revealed that cells were viable, attached to wells
in a monolayer with a low basal release of LH and were highly responsive to GnRH. An incubation period of 4 H was used in order to achieve the maximal LH response to GnRH and to SP. Hopkins (1977) and Baldwin (1984) have shown that GnRH induced LH release is biphasic with an initial release within 2 min. followed by a sustained release during the next 90-120 min. Hopkins also reports that cells become refractory to GnRH after 4 H.

**Radioligand Binding Assay**

To examine the binding of SP to AP membranes, we prepared an iodinated ligand, $^{125}$IBHSP. This ligand is more easily synthesized and has a higher specific radioactivity than $^3$H-SP. In a comparison study, the specific binding (disintegrations/min) of $^3$H-SP (S.A.=27.6 Ci/mmol, 2.4x10^{-9}M) was only 0.6% that of $^{125}$IBHSP (S.A.=1500 Ci/mmol, 3.4x10^{-10}M) (Cascieri and Liang, 1983). More importantly, $^{125}$IBHSP retains normal biological activity. One of the drawbacks is that the Bolton-Hunter group, which is a 3-[(3-iodo-4-hydroxyphenyl)propionyl] group attached to what was a free amino group on the native peptide, results in changes in charge, size and hydrophobicity creating the possibility that the radioligand may not have the same binding specificity as the native peptide. However, this consideration is more important in experiments on receptors for aliphatic
tachykinins such as SK and neurokinin B, where the Bolton-Hunter group may yield a peptide with significantly greater affinity for the receptor than the native peptide (Lee 1986).

$^{125}\text{I}}$-BHSP (S.A. = 2000 Ci/m mole) was iodinated by conjugation of SP to an iodinated Bolton Hunter reagent ($^{125}\text{I}$-N-hydroxysuccinimide ester of p-hydroxypropionic acid, NEN) $^{125}\text{I}$ labeled Bolton Hunter reagent (2 mCi) was flushed with nitrogen to evaporate the solvent. SP (5 micrograms in 40 microliters borate buffer, pH 8.5) was added to the tube and incubated at 0°C for 1 hour and at 4°C for 18 hours. The active ester acylates terminal or lysine amino groups with the iodinated p-hydroxyphenylpropionic residue. The iodinated peptide was purified by HPLC separation using a reverse phase column (Bondapak C$_{18}$, Waters). Approximately 150,000 cpm of this radioligand was incubated with the membrane preparation in the presence of increasing concentrations of various peptides in polypropylene tubes in a final volume of 0.500 ml.

For binding studies, an aliquot of tissue suspension equivalent to 0.250-0.350 mg protein was incubated in a total volume of 0.500 ml of homogenization buffer adjusted to contain 1 mM 1-10 phenanthroline in the presence of $^{125}\text{I}$ BHSP (0.1-0.2nM) with or without increasing concentrations of unlabeled SP for 90 minutes at 4°C (Kerdelhue, 1985). The incubation was terminated after 90 minutes by the addition of 3 ml of ice cold 10 mM Tris-HCl (pH 7.4) to each assay tube. The reaction mixture was immediately filtered
using a Brandel vacuum cell harvester, through glass fiber filters (GF-C, Whatman) pre-soaked in 0.01% poly-ethylimmine to reduce nonspecific binding to the filter. Tubes and filters were then washed 4 times with 3 ml ice cold wash buffer. The entire filtration sequence took 12-15 sec for each tube. The radioactivity trapped on the filter discs was measured by gamma counting. Samples were assayed in triplicate. Under these experimental conditions the amount of radioactivity retained on the filters in the absence of membranes was less than 0.01% of the total radioactivity added, and this was not displaced by unlabeled SP.

**SP Stimulation of LH Secretion**

To determine whether SP had a direct effect on gonadotropin secretion we exposed anterior pituitary cells which had been in culture for 48 hours to various doses of SP. Cultured cells were first washed 3 times with serum free DMEM after which fresh medium (DMEM without serum) containing the test substances was applied. After a 4 hour incubation in a metabolic incubator at 37C in an atmosphere of 5% CO₂ and 95% air, medium was collected, centrifuged at 10000 rpm in a Beckman micro centrifuge and stored frozen at -20C for determination of gonadotropin content by LH RIA. Cells were quickly rinsed in PBS and stored frozen at -80C for
determination of protein content by Lowry protein assay or LH RIA.

It became necessary during the course of the experiments to determine the manner in which the results should be expressed and how results should be normalized. Table 6 indicates the release of LH from normal adult female rat AP cultures in response to GnRH and SP on the basis of nanograms per ml of media (which is the same as ng per culture per 4 h) as well as on the basis of hormone released per mg of protein. It may be seen that the changes in LH release in response to GnRH and SP remain the same whether results are compared on the basis of ng per ml or ng per mg protein, therefore the validity of the results expressed as ng per ml is substantiated. Data from individual experiments were normalized by presenting the results as a percentage of control wells (in parallel cultures). This is a widely used method of normalizing data from pituiary cell culture secretion experiments. In individual experiments the standard errors of treatment replicates was consistently less than 10%, however interassay variability was greater than within assay variation. To minimize the effect of biological variation and variability between experiments, large numbers of animals were used and experiments were repeated several times.
LH Radioimmunoassay

LH concentration in media was measured by using an RIA kit supplied by the NIADDK containing rat luteinizing hormone reference preparation RP-3 as a standard and rat luteinizing hormone antiserum from rabbit. $^{125}$ILH was purchased from NEN Research Products. All reagents were added to 12x75 polystyrene RIA tubes in the sequence 1) buffer (PBS + 0.1%BSA), 2) cold standard or unknown, 3) antiserum diluted to a final tube dilution of 1:180000, 4) radioiodinated LH (NEN). The reagents were then incubated at room temperature for 24 hours followed by the addition of second antibody (goat anti-rabbit IgG), which facilitated separation of bound LH from free LH, and another 24 hour incubation at room temperature. Intra and inter assay variation was less than 10%. Three to six wells were used for each treatment in all experiments, and all samples from the same experiment were assayed in duplicate in the same assay. Results of LH assays were expressed as ng/ml/well/4 h. Data from individual experiments were normalized by presenting the results as net increases in LH secretion or as a percentage of basal LH release in parallel cultures. Protein determinations of homogenized AP cells from random wells was done in each experiment, using the method of Lowry (1951), to assure equal
distribution of cells.

**Incubation of AP cells with SP antibody**

An anti-SP antiserum (SPAb) was a gift from Dr. Susan Leeman. In crossreactivity studies the antibody had no crossreactivity with neurotensin or TRH, and less than 0.05% with SK. Preliminary studies to assess the ability of this antibody to inhibit SP-induced LH release using non immune rabbit serum as a control indicated nonspecific inhibition of LH release by the non immune serum (NRS) as well as the anti-SP serum. It was not clear whether this was due to a crossreaction in the LH RIA or an action on the cultured cells to inhibit LH release. Thus a partial purification of the antibody was undertaken wherein an ammonium sulfate precipitation was done on both sera. The resulting precipitate was dissolved in water and dialyzed to remove ammonium sulfate. The purified SPAb and NRS at concentrations used in the cell incubations (1:100 dil of sera) did not crossreact in the LH RIA.

**Statistics**

The Student's unpaired t test was used to test for significant differences between two means. When more than two means were being statistically compared, one-way or two-way
analysis of variance was performed, followed by Duncan's new multiple range test and/or Scheffe's F-test of multiple comparisons. Values in the text are expressed as the mean +/- SEM.
Table 6. LH released from pituitary cell cultures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LH Released</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml media</td>
<td>ng/mg prot</td>
</tr>
<tr>
<td>Control</td>
<td>146.3 ± 52</td>
<td>1.6 ± .6</td>
</tr>
<tr>
<td>GnRH (10nM)</td>
<td>5591 ± 771</td>
<td>62.1 ± 8.5</td>
</tr>
<tr>
<td>SP (100nM)</td>
<td>2713 ± 385</td>
<td>30.1 ± 4.3</td>
</tr>
<tr>
<td>GnRH (10nM)+SP (100nM)</td>
<td>5936 ± 884</td>
<td>64.8 ± 9.8</td>
</tr>
</tbody>
</table>

Cells were obtained from the anterior pituitary glands of 30-35 day old normal male rats. After dispersion and 48 hours of culture cells were rinsed and incubated for 4 hours in 1ml media with or without treatment. Media was collected and assayed for LH by RIA. Results are expressed as both nanograms of hormone per ml of media and nanograms of hormone per mg of protein ± SEM of six replicates.
III RESULTS

Experiment #1- SP Stimulation of LH Secretion from Anterior Pituitary Cells in Culture

In order to determine whether SP can affect LH secretion from AP hormone secretion by acting directly at the level of the AP gland, we examined the effects of exogenous SP on LH secretion from anterior pituitary cells in culture.

Since the AP cell culture system used in these studies had not been previously established in our laboratory, the purpose of the first set of experiments was to confirm that the AP cells in culture were viable and responsive to GnRH in a dose dependent manner and to determine the maximally effective dose for subsequent experiments. In preliminary experiments (data not shown) GnRH at $10^{-9}$ to $10^{-7}$ M significantly enhanced LH release into the media in a dose dependent manner with a maximal response obtained at $10^{-8}$ M GnRH after 4 hours of incubation. This is in agreement with reports in the literature of GnRH dose and incubation time for maximum response.

Previous studies have demonstrated that prepubertal rats of both sexes have the same concentration of APSP. The
concentration of APSP in male rats increases from the onset of puberty until adulthood when males have 2-3 fold more APSP than females. Since rats of different age and sex have different SP concentrations in the AP, it was reasoned that AP cells in culture prepared from rats of different age and sex may respond differently to SP. AP glands from adult and juvenile (30 days) male and female rats were dispersed into single cells and kept in culture for 48 hours prior to incubation in 100nM SP, 10nM GnRH and both of these substances together. These were doses of GnRH and SP that Kerdelhue (1978) reported which suggested that SP may inhibit GnRH stimulated LH release in 7 day cultures of AP cells from proestrus rats.

Figure 1 shows the pooled results of 4-6 experiments for each age group and sex. In each experiment we compared the GnRH stimulated LH release from treated wells to that seen in untreated control wells and results were expressed as % of control wells. In contrast to the findings reported by Kerdelhue, we found that SP failed to inhibit GnRH stimulated LH release from AP cells derived from either male or randomly cycling female rats. Rather there was a small albeit statistically insignificant increase in LH secretion in the presence of SP that could be seen in cells from adult female and to a lesser extent from juvenile male anterior pituitaries. Given these findings we examined the effects of SP on LH release from adult female AP cells in more detail.
FIGURE 1. LH secretion in response to GnRH and SP in AP cells in culture. 48 hour cultures of AP cells from adult and juvenile male and female rats were incubated for 4 hours in the presence of SP and/or GnRH at the indicated concentrations. Error bars represent standard errors of the mean LH values from 4-6 experiments.
Experiment #2 - Sex Difference in the Stimulation of LH Release by SF in Cultured AP Cells from Adult Rats

Anterior pituitaries of adult female rats (175-200 g) were dispersed to single cells and placed in culture for 48 hours prior to incubation in 100nM SP. Separate wells contained 10nM GnRH to assess cell viability and responsiveness. A total of 8 separate experiments were carried out. As a comparison, Figure 2 also shows results of 7 separate experiments in which cultured AP cells from adult male rats were stimulated with 100nM SP. In the preliminary experiments shown in Figure 1 standard errors were so large that significant differences could not be seen in the female AP cells. In this experiment attempts were made to standardize the assay and fewer cells were aliquoted into each well (approx. 1/4 AP/well). Individual Student's t tests were performed comparing stimulated to control values.

As shown in Figure 2, SP at a concentration of 100nM significantly stimulated LH release (p<.01) from 48 hour cultures of anterior pituitary cells from female rats but had no effect on LH release from male anterior pituitary cells. In these studies GnRH at a concentration of 10nM was effective in stimulating LH release from female (1526+/-377%) and male (1134+/-363%) cells and was used at this concentration in all subsequent experiments to stimulate LH secretion as a measure of cell viability and responsiveness. Basal or control values, (defined as ng of LH secreted into
the media in parallel wells that did not have SP or GnRH added) were 380+/−41 ng/ml media for the female AP cells and 477+/−178 ng/ml media for male AP cells.

The absence of LH release in response to 100nM SP in the AP cells from male rats could suggest a lack of, or fewer SP receptors on AP cells, presumably gonadotrophs, in the male rat. Gonadotrophs represent approximately 14% of anterior pituitary cells in both males and females, however there is a sex difference in the percentage of multihormonal (LH/FSH) cells in the rat (Tougard 1988). Multihormonal cells account for 37-40% of gonadotrophs in the female as compared with 70% in the male. Perhaps it is the monohormonal cell which responds to SP. Follicle stimulating hormone (FSH) was not assayed in this experiment, but could be in future experiments to determine whether there is a sex difference in FSH release. Cicero (1986) reported a sex difference in LH content in APs of adult rats. Females have 5 times less LH content than comparably aged males. One might expect then that in the female rat there is less LH to be released. LH content of cultured AP cells used in this experiment were the same in males and females (approx. 26+/−8 μg/well).
SEX DIFFERENCE IN THE STIMULATION OF LH RELEASE BY SP IN CULTURED AP CELLS FROM ADULT RATS

FIGURE 2. Sex difference in the stimulation of LH release by SP in cultured AP cells from adult rats. 48 hour cultures of AP cells from adult male and female rats were incubated for 4 hours in the presence or absence of a final dilution of 100 nM SP in culture media. Error bars represent the standard error of the mean of 7 (male) and 8 (female) separate experiments. Basal values are 380+/−41 (female AP cells) and 477+/−178 (male AP cells). (*p<.01 versus control values).
Experiment#3-Dose Response Relationship of SP Stimulated LH Release

The dose response relationships for the SP stimulation of LH release was measured in anterior pituitary cell cultures from adult male and female rats. As shown in Figure 3A in AP cells from adult females SP stimulated LH release in a dose dependent manner. In contrast, cells obtained from adult male rats were less responsive to SP at doses higher than 100 nM SP and remained significantly lower up to 1.0 µM SP. Cultured AP cells from adult males do begin to respond at a dose of 1 µM SP, whether this is a physiological or more a pharmacological response is not known. Later results in this thesis show that 1 µM SP can displace $^{125}$I GnRH from anterior pituitary membranes, suggesting that SP at high doses may cross react at the GnRH receptor. Statistical analysis was done using factorial ANOVA followed by Scheffe's F-test.

Preliminary experiments indicated that peripubertal male rats also responded to SP, so it was decided to examine the dose response relationship in 30-35 day old male rats. Figure 3B shows that AP cells from peripubertal male rats were highly responsive to SP. Statistical analysis was done using ANOVA followed by Duncan's new multiple range test.
FIGURE 3. SP dose response in cultured AP cells from adult male and female rats. Parallel wells were incubated with the indicated concentrations of SP for 4 hours. In wells incubated with 10nM GnRH LH values were 1438+/−394% of control in cells from female and 1330+/−546% of control in the cells from male. At doses over 100nM SP there is a significant stimulation of LH release into the media (p<.001 versus control values) in AP cells from female rats. Error bars represent the standard error of the mean of LH values from 4 (male) and 6 (female) separate experiments. At 1μM SP, LH release from AP cells of male rats is significantly (p<.05) different from control values. At values of 1μM SP, however, stimulation of LH release from cells of female rats is significantly greater (p<.001) than LH release from cells of male rats. Basal values are 350+/−27 (female AP cells) and 287+/−89 (male AP cells). (*p<.01,**p<.001).
FIGURE 3 B. SP DOSE RESPONSE RELATIONSHIP IN THE STIMULATION OF LH RELEASE IN CULTURED AP CELLS FROM JUVENILE MALE RATS.
Experiment #4 - Effect of Age on the Sex Difference in the Stimulation of LH Release by SP from Cultured AP Cells

The previous experiments established that AP cell cultures from female rats released more LH than AP cells prepared from adult male rats. Yet AP cells prepared from younger male rats were highly responsive to SP (Fig. 3A + 3B). These results introduced the idea that there may be differences in the response depending on the age of animals of a given sex. In order to determine the effect of age on SP stimulated LH release, cultured anterior pituitary cells from male and female rats of different ages were incubated for 4 hours with 100nM SP. Figure 4 shows that in both males and females between the ages of 28 and 35 days there is a sharp rise in SP stimulated LH release. This response remains high in cells from female rats throughout the ages studied, whereas in cells from male rats the response diminishes to prepubertal levels by 60 days of age. Although the AP cells of young male rats are highly responsive to SP, there is a small window of time of responsiveness, and even at the time of maximum responsiveness (35 days) there is a wide variability between results from separate experiments as reflected by the large standard error. For these reasons, unless otherwise specified, adult female rats (150-200g) were used to prepare AP cell cultures to further characterize the response to SP.

It is interesting to note that the increases in
responsiveness to SP noted in the AP cultures from 30-40 day male and female rats correlate with the changes in LH regulation that occur at the onset of puberty in the rat. According to the classification of Ojeda et al (1980) postnatal development in the rat can be divided into four phases: the neonatal period, which extends from birth to the end of the first week of life; the infantile period, which extends from day 8 to day 21; a juvenile or prepubertal period, which ends around day 30; and a peripubertal period, starting at 30-32 days and terminating at the end of puberty with first ovulation (35-45 days) in the female and with sperm release from the germinal epithelium (40-45 days) in the male rat. In the male the maximum LH response to GnRH occurs between 35 and 45 days of age (Ojeda 1988). Whether this similarity between SP and GnRH in regard to age of maximum response is simply correlational or functionally related is a question of interest.
DEVELOPMENT OF THE SEX DIFFERENCE IN THE STIMULATION OF LH RELEASE BY SP IN CULTURED AP CELLS

FIGURE 4. Development of the LH release response to SP. 48 hour cultures of AP cells from male and female rats of the indicated ages were incubated with 100nM SP for 4 hours and LH released into the media was measured. Error bars represent the standard error of the mean of 3-6 separate experiments at each age. After 50 days of age there is a significant (p<.01) difference between LH release in AP cells from male and female rats.
Experiment #5 - Characterization of the Specificity of SP to Stimulate Release of LH from AP Cells in Culture

In order to determine the specificity of SP and the potency of active analogs of SP to cause LH release from cultured AP cells, adult female rats were exposed to 1 µM SP and 1 µM doses of analogs of SP. The analogs that were tested included NK1, NK2, and NK3, selective agonists for neurokinin receptors. The synthetic analog NK1 ([Sar9, Met(O2)11]SP) has been shown to be selective for SP binding sites in both receptor and bioassays (Regoli 1988). The other chosen analogs were substance K (SK) and neurokinin B (NKB) the other two mammalian tachykinins, which are known to crossreact at SP receptors, to provide information on the specificity of SP as opposed to the other tachykinins. We also assessed the effects of SP free acid (SPFA), a non-amidated form of SP with a free carboxyl terminus, and SP(1-9) an amino terminal fragment of SP, to provide information on the section of the peptide that is important in eliciting LH release. Finally we examined the efficacy of an unrelated peptide, neurotensin.

Figure 5 shows that SP is most potent after GnRH, in stimulating LH release but that SK, SPFA and SP(1-9) also significantly stimulate LH release. Neurotensin neurokinin B and the selective agonists for the SK receptor (NK2) and the NKB receptor (NK3) were not effective in stimulating LH release in these cells.

The NK1 selective agonist has been tested in bioassays
in dog carotid artery for bioactivity and is reported to be potent and selective with a high affinity for the NK1 receptor. The fact that it is a much weaker agonist than SP in this system would argue that SP may be acting on a new type of tachykinin receptor in the anterior pituitary. Alternatively the NK1 selective agonist may not be as effective in this system because of the chemical modifications at amino acids 9 and 11. The activity of SK to cause LH release and the inability of the NK2 selective agonist (selective for SK receptor) to cause LH release suggests that SK may be crossreacting at the SP binding site. SPFA and SP(1-9) are both analogues of SP which have intact N-terminal regions. The ability of these compounds to stimulate LH release implies that it is the N-terminal portion of the SP molecule which is important in causing the response. The fact that NKB and NT do not have any activity shows that SP does have some specificity in causing LH release.
FIGURE 5. Stimulation of LH release from AP cells by SP and other peptides. AP cell cultures from adult female rats were incubated in test substances at final concentrations of 1μM. Separate wells were incubated with 10nM GnRH as a comparison. SP stimulates LH release significantly (**p<.001) as do NK1, SK, SPFA and SP (1-9) (*p<.05).
Experiment#6-Stimulation of LH Release by SP in Cultured AP Cells from Hypothyroid Female Rats.

Since hypothyroidism leads to increased SP concentrations in the anterior pituitary gland of female rats the question was asked whether raising the level of SP in the anterior pituitary of the female rat by hormonal manipulation would act to attenuate the LH release in response to SP administration. Adult female rats were made hypothyroid by the addition of methimizole to the drinking water for 21 days. In this experiment SP content was measured by RIA in anterior pituitaries from normal and hypothyroid females as a measure of the hypothyroid response pertaining to SP in the anterior pituitary. The values for SP levels in hypothyroid females approach normal male values. The mean SP value for normal female anterior pituitary glands was $89.5\pm29$ fmol SP/gland ($n=4$) and for hypothyroid female glands $488\pm96$ fmol SP/gland ($n=4$), whereas values for the intact adult male are approximately 600 fmol SP/gland. As shown in figure 6 the response to SP at doses over 100 nM was attenuated significantly in cultured AP cells from the hypothyroid female rats. Statistical analysis was done by factorial ANOVA followed by Duncan's new multiple range test.

However, the LH response to GnRH was also blunted in AP cells from hypothyroid female rats. Although this decrease in GnRH stimulated LH was not significant. There are many other changes that take place in the anterior pituitary in
hypothyroid animals complicating the interpretation of these studies. LH content measured in the cells collected after these experiments shows a decrease in LH content in the hypothyroid cells. AP cells from untreated female rats contained 22.4+/-1.1 µg/well whereas AP cells prepared from hypothyroid rats contained 10.8+/-3.5 µg/well. It has been shown that in adult rats thyroidectomy facilitates LH release and leads to a reduction in LH content in the AP, an effect which is reversed by treatment with T3 or T4 (LaRochelle 1974). Therefore it is not possible to conclude from this experiment whether there is a specific change in responsiveness to SP or whether the reduction in the response to SP is secondary to other changes produced by hypothyroidism in the mechanism regulating LH release.
FIGURE 6. Stimulation of LH release by SP in cultured AP cells from hypothyroid female rats. AP cell cultures from normal and hypothyroid rats were incubated in increasing concentrations of SP. At SP doses above 100nM there is a significant difference in LH release into the media in response to SP between normal and hypothyroid cells (*p<.05). LH appears to be blunted in the hypothyroid cells in separate wells incubated in 10nM GnRH, this is not a significant effect.
Experiment#7-Gonadal Steroid Treatment In Vivo and In Vitro

In order to shed further light on whether the sex difference in the response to SP is mediated by gonadal steroids, AP cells were prepared from intact female rats which had been treated with DHT. In vivo studies indicate that both estrogens and androgens can alter SP concentrations in the rat AP. In particular androgen increases whereas estrogen decreases APSP in female rats (Coslovsky 1984). In vivo stimulation of APSP content by testosterone was less than that by DHT, suggesting that aromatization of testosterone to estrogens could complicate the interpretation of data. While the AP does not contain the aromatizing enzyme, in vivo administration of androgen, which may be aromatized in other tissues, could act on the AP. Therefore, for in vivo experiments, the non aromatizable metabolite of testosterone, DHT was used in order to be sure that the effects measured were due to androgen and not estrogen.

Androgen was administered by surgically implanting silastic tubing of 20mm lengths which had been filled with dry crystalline DHT under the skin of the back. Implants were left in the animal for 6 weeks. This method of administration of steroids has been shown to result in a sustained release (approximately 40 μg/24 hours), which is more biologically effective than the conventional once a day subcutaneous injection (Kincl 1970).
As illustrated in figure 7 AP cells prepared from DHT implanted female rats were less responsive to incubation with 100nM SP in 4 separate observations, although at higher doses (500nm and 1μM SP) there was no difference in LH released between rats with empty implants and DHT implants (data not shown). A Student's t test was done to compare LH release in treated (DHT implanted) and untreated (control implanted). This result would suggest that androgens affect the sensitivity of the responsiveness of the AP to SP but doesn't completely cause a conversion of the female response pattern to the male pattern.

Gonadal steroids have also been shown to modify the AP by acting directly on the gland (Kingsley 1973). For this reason it was thought that incubation of cultured AP cells with gonadal steroids would be a direct and simple way to explore whether gonadal steroids administered directly to the AP can alter the stimulation of LH release in response to SP. The question of whether the response to SP could be diminished in peripubertal males by exposure to androgens was approached in this way. For these experiments cultured AP cells from juvenile male rats of different ages were exposed to 100 nM DHT or testosterone diluted in 100% EtOH (final dilution of EtOH in each well was 0.001% and in preliminary experiments had no discernable effect on cells) for 48 hours prior to incubation with SP. It has been shown (Drouin 1976) in AP cells from adult female rats, that 48 hours of exposure
to DHT or testosterone inhibits the LH response to GnRH.

Table 7 shows results from the experiment in which AP cells from peripubertal male rats were exposed to DHT, testosterone or EtOH vehicle alone. These values from 3 separate experiments are expressed as ng LH/ml media because of the increase in basal LH secretion after exposure of cells to DHT or testosterone. Basal release is increased compared to control (EtOH vehicle) in AP cells treated with DHT or testosterone. The reason for this is unknown. In adult rats androgens have the opposite effect, LH secretion in response to GnRH is reduced in response to androgen exposure, with no effect on basal release. However, there is evidence that the sensitivity of the hypothalamic pituitary unit to the negative feedback effect of androgens declines during sexual development and that this change, which is especially noticable at puberty, may be responsible for maintenance of LH release in the face of rising testosterone levels and may play a role in the induction of puberty (Negro-Vilar 1973). This decreased sensitivity to the negative feedback action of androgens may be in evidence in the androgen exposed AP cells from peripubertal male rats. It could be termed a positive feedback action of androgens in that basal levels are increased. The reduced effect of SP and GnRH to stimulate LH release above basal, is perplexing and may be evidence of a maximal stimulation of LH release.
FIGURE 7. SP Stimulation of LH Release from AP Cells of DHT Implanted Rats. AP cell cultures prepared from androgenized rats were exposed to SP for 4 hours. LH released into the media was measured. For controls AP cells were prepared from rats implanted with empty capsules. Basal values for LH were 457+/−92 (control AP cells) and 375+/−36 (DHT AP cells). (*p<.05)
TABLE-7 LH SECRETION IN AP CELLS PREPARED FROM 30-35 MALE RATS- EFFECT OF ANDROGEN TREATMENT OF CELLS

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>LH IN MEDIA (NG/ML MEDIA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ETOH</td>
</tr>
<tr>
<td>CONTROL</td>
<td>1061+-178</td>
</tr>
<tr>
<td>SP (100NM)</td>
<td>3443+-1137</td>
</tr>
<tr>
<td>SP (1μM)</td>
<td>4564+-1385</td>
</tr>
<tr>
<td>GNRH (10NM)</td>
<td>5570+-2042</td>
</tr>
</tbody>
</table>

Values are given in ng LH/ml media and represent mean+- SEM of 3 observations.

ETOH=EtOH vehicle, DHT=dihydrotestosterone, T=testosterone.
Experiment#8-Substance P Antibody Inhibition of LH Release in Cultured AP Cells

SP is synthesized and its concentrations are regulated hormonally within the anterior pituitary. SP is releasable from hemipituitaries in a Ca++ dependent-K+ stimulated manner (Aronin 1984). These observations support the view that SP may be released in response to an unknown physiological stimulus to act on its own or on neighboring cells in a paracrine or autocrine way. In order to explore this possibility cultured AP cells from female rats were incubated for 4 hours in the presence of SP Ab to inhibit any action of endogenous SP on LH release into the media. SPAb and normal rabbit serum (NRS) were tested for binding by incubating in the presence of 125I-Tyr-SP and/or unlabeled SP. SPAb specifically bound 20% of the counts added, these counts were displaced by unlabeled SP. NRS did not bind any counts. Both purified sera were checked in the LH RIA for interference or crossreactivity. At the dilutions used in the bioassay neither compound interfered in the LH RIA. In preliminary studies a dose response curve for antibody-SP interaction showed that a SPAb dilution of 1:100 maximally bound SP but did not interfere in the LH RIA. The SP Ab or NRS (at a final dilution of 1:100) was then added to cultured AP cells from adult female rats for a period of 4 hours. Media was then collected and assayed by LH RIA.

The results of four separate experiments, illustrated in
Figure 8, show that in the presence of the SP Ab, LH released into the medium was significantly elevated. This is an unexpected result. If SP is stimulating LH release into the medium, it would be expected that added SP Ab would bind any released endogenous SP thereby preventing any action of SP to stimulate release of LH into the medium. The question of whether the SP antisera may have been contaminated with enough SP to cause a response was approached by acid-acetone extracting an amount of the SP antisera, equal to what was used in the secretion assay, for measurement of SP in the SP RIA. The results of the SP RIA showed that the SP antisera was contaminated with SP but in an amount which would not be capable of causing LH release. The amount of SP Ab used in the secretion assay contained 56.8 fmol SP. In 1 ml of incubation media this would be a concentration of 0.056 nM, a concentration too low to cause a stimulation of LH release. Previous results reported here indicate a concentration of at least 50 nM is needed to stimulate LH release.

There are some problems associated with using antibodies as neutralizing tools. In some situations antibodies have been reported to cause a clustering of receptors which is an important first step in internalization of hormone receptor complexes. For example, in an effort to develop pure antibodies against the GnRH receptor, Conn (1986) incubated GnRH antagonist dimers (2 molecules of GnRH pure antagonist crosslinked with ethylene glycol bis succimidyl succinate)
with immunoglobulin G antibodies which crossreact with the 
antagonist, with the result that a dimer is bound at each Fab 
terminus of the antibody molecule. GnRH antagonist and the 
dimer are pure antagonists and do not evoke LH release. 
However, when the antibody-dimer complex is incubated with 
anterior pituitary cell cultures it acts as an agonist and 
results in LH release. It is thought by the author (Conn 
1986) that the antibody-antagonist complex is binding to cell 
surface receptors for GnRH via an interaction of the 
antagonist bound at Fab terminals with the receptor. The 
antibody acts as a crosslinker to effect the aggregation of 
receptors which stimulates the biological response. 
Similarly, when cells are exposed to submaximal doses of 
insulin, addition of anti-insulin antibodies enhances the 
biological activity of insulin (Kahn 1978). 

In the experiment reported here, SPAb may be increasing 
the biological effect of the submaximal amount of SP already 
bound to the antibody (56.8 fmol) by causing an aggregation 
of receptors. Alternatively, SPAb may bind SP released from 
AP cells or SP already bound to SP receptors and subsequently 
act to cluster SP binding sites to result in a biological 
effect, LH release. An alternative hypothesis could be that a 
closely related analog of SP actually inhibits LH release. 
The increase caused by SP may be a result of competition with 
the inhibitor or antagonist.
It is planned in the future to do an experiment in which increasing doses of SP, with or without SPAb, will be added to cultured AP cells from female rats. If SP Ab is enhancing the biological response of the added SP, the dose response curve to which SP Ab has been added will be shifted to the left.
FIGURE 8. Effects of immunoneutralization on basal LH release from AP cells in culture. Short term AP cell cultures were incubated in the presence or absence of SPAb (1:100 final dil) or NRS (1:100 final dil) for 3 hours, media was collected and LH measured. Error bars represent the standard error of the mean of 4 separate experiments. SPAB significantly stimulates LH secretion (*p<.05 versus NRS or control wells).
Experiment#9-SP binding studies of AP membranes

Results of the preceding experiments show that SP effects the release of LH from cultured AP cells depending on the age and sex of the rat used to prepare the cells. To explore the possibility that these effects are mediated by a receptor for SP, radioligand binding studies were done on AP membrane preparations. Two separate reports (Kerdelhue 1985; Larsen 1989) have demonstrated high affinity, saturable and specific binding sites for SP in AP membranes from male and female rats. Adenohypophyseal SP binding in females and males of different ages was examined to determine whether differences in SP binding could account for differential LH release caused by SP. Experiments were carried out using the protocols and conditions outlined by Kerdelhue as described in the Materials and Methods. Shown in figure 9 are representative curves from each animal model as fit by GraphPad (ISI). The curves represent the non-linear least squares regression fit of the data points. The data shown are representative of 6-10 separate binding experiments for each animal model. Binding results were analyzed by composite analyses of competition binding experiments using the LIGAND (Munson, 1983) program. Raw data was pooled before analysis. The composite analysis of the competition data from female AP membranes in 6 separate experiments yielded a $K_D=135$ nM and an estimated capacity of 4.225pmol/mg protein. Binding data from
10 experiments with male AP membranes yeild a $K_0=43\text{nM}$ and a capacity of 565 fmol/mg protein, and analysis of 6 experiments using juvenile male AP membranes yeilded a $K_0=52$ and a capacity of 624 fmol/mg protein.

Results of secretion experiments shown in the previous pages demonstrate that in the female rat the concentration required to stimulate LH secretion is lower than that required in the male rat. Such data, particularly when combined with the binding results of this experiment, are compatible with the presence of spare SP receptors in AP glands of female rats.
TABLE 8

COMPARISON OF THE $K_D$ AND $R^o$ FOR SP IN AP MEMBRANES FROM MALE, FEMALE, JUVENILE AND HYPOTHYROID RATS.

<table>
<thead>
<tr>
<th>AP Membranes</th>
<th>$K_D$ (nM)</th>
<th>$R^o$ (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Male</td>
<td>46</td>
<td>560</td>
</tr>
<tr>
<td>Adult Female</td>
<td>135</td>
<td>4200</td>
</tr>
<tr>
<td>Juvenile Male</td>
<td>52</td>
<td>620</td>
</tr>
<tr>
<td>Hypothyroid Male</td>
<td>100</td>
<td>1300</td>
</tr>
<tr>
<td>Hypothyroid Female</td>
<td>48</td>
<td>1900</td>
</tr>
</tbody>
</table>

The dissociation constants ($K_D$) and the number of binding sites ($R^o$) were determined by a composite computer analysis (LIGAND) of competitive displacement experiments in which data points from several experiments were pooled. The number of experiments for each determination were: adult male-10, adult female-6, juvenile male (30 days of age)-6, hypothyroid male-6, hypothyroid female-1.
Experiment#10-Inhibition of $^{125}$IBHSP binding by SP analogs

To determine the specificity of the SP binding site in AP membranes, competition of unlabeled SP and selected analogs was studied. The concentration of peptides required to inhibit $^{125}$IBHSP by 50% (IC$_{50}$) was determined and these are listed in the accompanying table of rank order of ligand potencies (% of IC$_{50}$ for SP) for the analogs used in the study. Selected titration curves are shown in figure 10. The peptides studied inhibited $^{125}$IBHSP binding in a competitive manner, SP being the most potent, followed by SPFA (SP free acid), a C-terminal modification of the SP molecule.

The inhibition of $^{125}$I BHSP binding to AP membranes by SPFA raises a question as to whether the receptor is of the NK1 subtype which has been described in brain, olfactory and salivary gland. The modified C-terminal portion of the peptide significantly reduces its ability to displace the radioligand from the receptor. This would suggest that the C-terminal portion of the peptide is important in its binding to receptor. The ability of SPFA to displace the radioligand from binding sites on AP membranes as well as its ability to cause LH release from AP cells in culture would indicate that the C-terminal portion of the peptide is not as important for binding of the peptide as it is in brain, olfactory and salivary membranes. In addition, studies of the NK1 receptor
reveal a different specificity profile in that physalaemin displaces ligand for the NK1 site in brain or salivary membranes with almost equal potency as SP. In this experiment physalaemin did not displace with any appreciable potency. This would also suggest that the binding site being described may be different from the NK1 receptor.
TABLE 9.

RECEPTOR BINDING AFFINITIES OF SP AND ANALOGUES

<table>
<thead>
<tr>
<th>Substance</th>
<th>IC$_{50}$ (M)</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance P</td>
<td>3.0x10$^{-8}$</td>
<td>1</td>
</tr>
<tr>
<td>SPFA</td>
<td>6.0x10$^{-7}$</td>
<td>0.05</td>
</tr>
<tr>
<td>GnRH</td>
<td>8.0x10$^{-6}$</td>
<td>0.0037</td>
</tr>
<tr>
<td>Substance K</td>
<td>&gt;10$^{-5}$</td>
<td>&lt;.0005</td>
</tr>
<tr>
<td>Physalaemin</td>
<td>&gt;10$^{-5}$</td>
<td>&lt;.0005</td>
</tr>
<tr>
<td>Eledoisin</td>
<td>&gt;10$^{-5}$</td>
<td>&lt;.0005</td>
</tr>
<tr>
<td>SP (1-4)</td>
<td>&gt;10$^{-5}$</td>
<td>&lt;.0005</td>
</tr>
<tr>
<td>SP (7-11)</td>
<td>&gt;10$^{-5}$</td>
<td>&lt;.0005</td>
</tr>
<tr>
<td>Kassinin</td>
<td>&gt;10$^{-5}$</td>
<td>&lt;.0005</td>
</tr>
</tbody>
</table>

FIGURE 10. Inhibition of $^{125}$IBHSP by SP and selected analogs. Different unlabeled analogs of SP were used at increasing concentrations to displace $^{125}$IBHSP from AP membranes from male rats. Curves represent typical experiments. Each experiment was repeated at least 3 times.
Experiment#11-Inhibition of $^{125}$IBHSP binding by divalent cations

In order to further examine the suspected differences in SP binding characteristics between NK1 receptor and the putative SP receptor in AP membranes, the effects of divalent cations on $^{125}$IBHSP binding were examined. Binding conditions for NK1 receptor sites in CNS and salivary gland differ greatly from conditions used for the binding experiments in AP membrane preparations in this study. The binding conditions used to study SP receptor characteristics by Kerdelhue (1985), and which were followed in this study included no divalent cations and contained 5mM EDTA in the buffer. However, the NK1 receptor in brain cortex membranes and salivary gland membranes binds SP minimally in the absence of Mn$^{++}$ or Mg$^{++}$, which stimulate specific binding of SP by increasing the number of high affinity receptors.

Another reason behind this study was that in order to determine whether the interaction of SP with its AP binding site is modulated by guanyl nucleotides as are NK1 receptors and other receptors which are coupled to G-protein, it would be necessary to include Mg$^{++}$ in the assay buffer to increase the receptor coupling to G-protein.

In this experiment specific SP binding was measured in the presence of increasing concentrations of the divalent cations Mg$^{++}$, Mn$^{++}$ and Ca$^{++}$. 

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The results shown in Figure 11 illustrate that Mn++, Mg++, and Ca++ all inhibit $^{125}$IBHSP binding to AP membranes at quite low concentrations, with Mn++ being most potent, and Ca++ the least potent. In the previously described NK1 receptor in brain and salivary membranes, SP binding is increased by divalent cations with Mn++ being the most potent and Ca++ the least. Binding was inhibited at room temperature (not shown) and at 4°C equally. The divalent cations used inhibited total and specific binding.
FIGURE 11. Inhibition of $^{125}$IBHSP binding by divalent cations. Effect of increasing concentrations (0-1mM) of the indicated divalent cations on specifically bound $^{125}$IBHSP presented as % of control (no divalent cation) binding. Shown is a representative experiment, one of 5 repetitions, error bars represent the standard error of the mean of triplicates.
Experiments# 12 and 13-Inhibition of $^{125}$IBHSP binding by guanyl nucleotides

The NK1 receptor recently cloned contains seven membrane spanning domains and shows a sequence similarity to the members of G-protein coupled receptors (Yokota 1989; Hershey and Krause 1989). Evidence for coupling of receptors to G-proteins has been obtained from radioreceptor assays in which guanyl nucleotides inhibit the binding of agonists to their receptors. The observation that SP binding in brain and salivary gland is inhibited by guanyl nucleotides lends support for a coupling of those receptors to a G-protein. No such effects of guanyl nucleotides on SP binding in AP membranes have been reported. In order to examine this question the effect on SP binding of selected nucleotides was studied. These experiments were done in the presence of .01mM Mg$^{++}$ which is the highest concentration that does not inhibit SP binding and 100µM guanyl nucleotides. The results shown in Figure 12 show that the binding of the radiolabeled agonist is not inhibited by the nonhydrolyzable analogs of GTP, but was slightly inhibited by GTP. To determine whether GTP changes the affinity or the number of sites, the competitive displacement of the agonist in the presence or absence of 100µM GTP was studied. This experiment is shown in Figure 13. A computer analysis of this data gives $K_D$ values of 30 and 67 nM in the absence or presence of GTP respectively, and
estimated $B_{\text{max}}$ values of 530 and 670 fmol/mg protein in the absence or presence of GTP respectively. The lack of greater effect of GTP on $K_D$ or $B_{\text{max}}$ discouraged further experimentation in this area, but does not definitely rule out a possible G-protein coupled mechanism in SP receptor binding.
Inhibition of $^{125}$IBHSP Binding by Guanine Nucleotides

**FIGURE 12.** Effect of guanyl nucleotides on $^{125}$IBHSP binding to AP membranes. The data is presented as the % of specifically bound counts in the absence of nucleotide. The nucleotides were tested at 100μM and the $^{125}$BHSP was tested at .2nM and 1nM.
FIGURE 13. Competitive displacement of $^{125}$IBHSP by SP in the absence and presence of GTP. Increasing concentrations of SP were used to displace labeled agonist in the presence and absence of 100μM GTP and 0.01mM Mg$^{++}$. Binding was done at 4°C. The curves represent the computer fitting of data points from one experiment.
Experiment#14-Inhibition of $^{125}$IBHSP binding in AP membranes by selective agonists for neurokinin receptors

It has recently been shown (Regoli et al 1988) that the natural neurokinin peptides are not selective for the different neurokinin receptor subtypes. If more than one type of neurokinin receptor is present in the anterior pituitary it would be difficult to distinguish whether the observed effects of SP in the AP are due to SP acting at an NK1 receptor site or SP acting at a SK or NKB receptor site. To determine whether the observed SP binding in AP membranes is due to SP binding to an NK1, NK2 or NK3 neurokinin receptor subtype, the recently developed selective agonists, [Sar$^9$, Met($^2$)$_{11}$]SP (selective for the NK1 site), [Nle$^{10}$]NKA(4-10) (selective for the NK2 site) and [MePhe$^7$]NKB (selective for the NK3 site) were used in competitive displacement experiments. The representative experiment in figure 14 shows that the selective agonists for the NK1 site and the NK3 site displace $^{125}$IBHSP on AP membranes. These agonists have been reported to discriminate quite well between the three receptor subtypes and are being used widely to classify neurokinin receptors. The results of this experiment suggest that there are at least two neurokinin receptor subtypes in the anterior pituitary, the NK1 subtype and the NK3 subtype. This result is surprising because SK, the endogenous ligand at the NK2 site, stimulated LH release from cultured AP cells.
from female rats, yet the NK2 selective agonist did not displace the radioligand. This would imply that SK may be crossreacting at the SP binding site to cause the effect on LH secretion. It is not known whether NKB has any effects on AP hormone secretion. In this study it did not affect LH secretion.
FIGURE 14. Competitive displacement of $^{125}$IBHSP binding by neurokinin receptor selective agonists. Increasing concentrations of synthetic agonists selective for the NK1, NK2 and NK3 neurokinin receptors were used to displace $^{125}$IBHSP binding to AP membrane preparations along with increasing concentrations of SP as a comparison. The curves represent the computer fit of the data points of a representative experiment which was repeated 4 times.
Experiment #15-Competitive displacement of $^{125}$I-BHSP by unlabeled SP in AP membranes from normal and hypothyroid rats.

SP concentrations in the AP are increased in hypothyroidism, however, LH release in response to SP in cultured AP cells from hypothyroid female rats is significantly reduced. In order to determine whether the reduced LH response in AP cells from hypothyroid rats is due to a change in binding parameters ($K_D$ or $B_{max}$), a competitive displacement of labeled SP binding to hypothyroid AP membranes was carried out with normal AP membranes as a control. An anterior pituitary gland from each group was extracted in 2N acetic acid and assayed for SP using SP RIA. Hypothyroid glands contained 579 fmol SP/gland and normal glands contained 270 fmol/gland. A composite analysis of the data from 4 separate experiments using the LIGAND program resulted in a $K_D = 35$ nM and a $B_{max} = 553$ fmol/mg of protein for the SP binding in control or normal AP membranes and a $K_D = 100$ nM and $B_{max} = 1.325$ pmol/mg protein. An aliquot of the AP membranes were extracted in 2N acetic acid and assayed for SP to determine whether membranes from hypothyroid animals were contaminated with endogenous SP which would interfere with SP binding experiments. It was found that hypothyroid membranes contained more SP than membranes from normal rats.
Hypothyroid membranes contained 163 fmol SP/mg of protein and control membranes contained 53 fmol SP/mg protein. This difference did not interfere significantly with binding.
FIGURE 15. Displacement of $^{125}$IBHSP by unlabeled SP in normal and hypothyroid animals. Increasing concentrations of unlabeled SP were used to displace .2nM $^{125}$IBHSP binding from AP membranes of normal and hypothyroid rats. Curves represent a computer fit of the data points from a representative experiment which was repeated 4 times.
Experiment#16-Displacement of $^{125}\text{IGNRH}$ binding to AP membranes by unlabeled SP

The question of whether SP acts to stimulate the secretion of LH in cultured AP cells by acting at a GnRH receptor was addressed in this experiment by examining the displacement of $^{125}\text{IGNRH}$ by increasing concentrations of unlabeled SP in AP membranes from female rats. The $^{125}\text{IGNRH}$ was purchased from NEN and is the radioiodinated native GnRH molecule not one of the newer synthetic analogs which have a higher affinity for the binding site and do not degrade during the course of the incubation. The use of labeled analogs of GnRH abolished the problem of low affinity binding in GnRH radioreceptor assays (Clayton and Catt, 1981). For the purposes of this study, to determine whether SP could displace GnRH at its receptor site with any potency, the native molecule was an appropriate ligand. The assay was carried out using the procedure of Clayton and Catt (1981) which was very similar to the procedure used for SP binding in AP in the previous experiments. The results show that SP has very little potency to displace GnRH at its binding site with an $IC_{50}=10\mu M$. This would probably not be a physiological concentration of SP in the anterior pituitary. This experiment provides evidence that SP is probably not acting at a GnRH receptor to stimulate LH secretion.
FIGURE 16. Displacement of $^{125}$I-GnRH binding by SP in AP membranes. Increasing concentrations of SP were used to displace labeled GnRH from AP membranes. The curve represents the best computer fit of the data points of a representative experiment which was repeated 3 times. Each data point is the average of triplicates.
**TABLE 10.**

COMPARISON OF SP BINDING CHARACTERISTICS IN ANTERIOR PITUITARY AND SALIVARY GLAND MEMBRANES

<table>
<thead>
<tr>
<th>Binding Parameter</th>
<th>AP Membranes (Male Rat)</th>
<th>Salivary Membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_D$</td>
<td>50 nM</td>
<td>1 nM</td>
</tr>
<tr>
<td>$B_{MAX}$</td>
<td>565 fmol/mg</td>
<td>550 fmol/mg</td>
</tr>
<tr>
<td>Divalent Cation</td>
<td>Inhibits Binding (&gt;0.05 mM)</td>
<td>Increases Binding (2.5-10 mM)</td>
</tr>
<tr>
<td>(Mg$^{++}$,Ca$^{++}$,Mn$^{++}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanyl Nucleotides</td>
<td>No Effect</td>
<td>Reduce Affinity</td>
</tr>
<tr>
<td>Second Messenger</td>
<td>Unknown</td>
<td>Phosphatidyl Inositol</td>
</tr>
<tr>
<td>Specificity</td>
<td>SP&gt;SPFA&gt;SK&gt;SP&gt;Physalaem &gt;</td>
<td>SK&gt;SPFA</td>
</tr>
</tbody>
</table>

This table was compiled to examine the differences between the SP binding site in the AP and NK1 receptor in salivary gland membranes. The comparison points out the many dissimilarities between the two binding sites and could argue for a novel sub type of SP receptor.
IV DISCUSSION

The Effect of SP on LH Release from AP Cells in Culture

This thesis clearly demonstrates that SP can stimulate LH release in short term cultures of anterior pituitary cells and that the SP stimulation of LH release varies as a function of age and sex. In cultured AP cells from prepubertal female rats there is no significant effect of SP on LH release. However, after day 30, coincident with the onset of puberty, an increase in the response to SP occurs which continues throughout adulthood. AP cells from male rats also become highly responsive during the peripubertal stage (ages 30-40 days) but fail to respond after the animal reaches adulthood (ages 50 days and above).

These results differ from those reported by Kerdelhue (1979). He reported no stimulation of LH secretion by SP at doses up to 1μM using AP cells from adult female rats. However, his cells were kept in culture for 7 days prior to stimulation with SP. It was subsequently shown (Baldwin and Downs 1981) that cultured cells only maintain their characteristic estrus cycle responsiveness for 18 to 24
hours. After 7 days in culture AP cells may have lost certain secretory characteristics and responsiveness that they may have had in vivo or after shorter periods in culture. Kerdelhue also found that in anterior pituitary cultures from female rats at different stages of the estrus cycle, SP (100nM) inhibited GnRH (10nM) stimulated LH release from AP cells of proestrus rats. The inhibition by SP of GnRH stimulated LH release in AP cells from proestrus rats was not seen in AP cells from random cycling females which were used in the present study. This inhibitory effect of SP could have been obscured by the pooling of rats from different stages of the estrus cycle.

The results of the present study do agree with those of Fisher and colleagues (1974) who reported that SP (10μM) stimulated LH release in hemipituitaries of female rats. Also, there is a supporting in vivo experiment (Debeljuk 1987) in which SP antiserum injected intravenously results in reduced LH secretion in estrogen treated male castrates but not in untreated intact males. Although it shed no light in terms of the site of action, this experiment supports the findings reported in this thesis and also suggest that the LH stimulating effect depends on the hormonal status of the animal.

Among the tachykinins studied, SP is the most effective in inducing LH secretion by AP cells in culture. NK1, the selective agonist for the SP receptor (Regoli et al, 1988)
stimulated LH release only slightly. It was expected that, if SP were stimulating LH release via a typical NK1 specific receptor NK1 agonist should stimulate release with almost equal potency as SP. This raises questions about the potency of the NK1 selective agonist and also suggests the type of binding site being characterized in the present study may be different from NK sites previously described. The NK2 and NK3 selective agonists do not stimulate LH release, although the NK3 agonist does displace binding of $^{125}$IBHSP in AP membranes. There may be a fourth kind of tachykinin binding site or the response may not involve binding to a true receptor. SSPFA, which has little potency to displace labeled ligand from brain cortex and salivary gland SP specific receptors, has bioactivity and also displaces labeled ligand with almost equal potency to SP, suggesting that the native intact C-terminal is not important in binding or bioactivity. This implies that the SP binding site in anterior pituitary could be different from previously described SP receptors. SK stimulates LH release, however, it does not displace labeled ligand in SP binding studies. It is possible that SK (an aliphatic tachykinin) has difficulty in displacing $^{125}$IBHSP (a more hydrophobic molecule) which may have a higher affinity for the receptor. If this is the case SK may be acting as an agonist at a SP receptor, as tachykinins are known to be not perfectly specific.
Effects of the Hormonal Milieu on the SP Response

Gonadal Steroids

Attempts were made in the present study to manipulate the hormonal milieu of anterior pituitary cells in culture in order to determine the effects of gonadal steroids on SP stimulated LH release in both male and female rats of different ages. Other researchers have had much success in studying the regulation of LH secretion with direct incubations of cultured pituitary cells in estradiol or DHT (Ramey, 1987; Tang, 1978; Hubert, 1988). The advantage of this method is that direct effects of gonadal steroids at the level of the anterior pituitary can be studied. The disadvantage is the difficulty of simulating the appropriate levels of gonadal steroids in order to analyze a true physiological response. In the present study attempts were made to affect the lower sensitivity of AP cells from adult male rats by a direct action of androgens at the level of the AP by incubating AP cells from peripubertal male rats in the presence of androgens. This experiment shed no light on whether the response to SP can be regulated by gonadal steroids because basal levels of LH secretion were so high that even the response to GnRH was obscured. This result is interesting in that it could be a hint of a positive feedback action of androgens at the level of the AP during the onset
of puberty in male rats. This has never been reported, although there are reports of reduction in the sensitivity to the negative feedback action of androgens at the time of puberty.

In experiments where DHT silicon capsules were implanted into female rats at 24 days of age the result was a decreased sensitivity to 100nM SP. This would suggest that circulating androgens can affect the sensitivity of the responsiveness of the AP to SP. However, at higher doses of SP there was no difference in the response to SP between AP cells from control and DHT treated animals. This would suggest that a complete conversion to the male pattern had not been affected. In future experiments longer times of DHT exposure will be used for this experiment and SP binding studies will be carried out on AP membranes from the experimental groups to determine whether any differences in sensitivity could be correlated with differences in binding parameters.

**Thyroid Status**

The thyroid status of the animal has been shown to regulate the amount of SP present in the anterior pituitary (Aronin et al, 1984). Chemically induced hypothyroidism can raise the SP levels in female rat anterior pituitaries to that of the level of SP in adult males. It was reasoned that
if the higher SP concentrations in the adult male rat anterior pituitary gland are in some way linked to the lack of LH release in response to SP in that animal, AP cells from hypothyroid females may respond similarly to AP cells from adult males. Cultured AP cells from female rats made hypothyroid experimentally by addition of methimazole to their drinking water for 21 days were treated with SP in order to determine whether hypothyroidism has any effect on SP stimulated LH release. The dose response to SP in AP cells from hypothyroid female rats resembled that of the normal adult male, in that the LH response to SP was attenuated. Yet in the dose response to SP in cells from intact male and females there was no difference in the LH released in response to GnRH. Therefore only a very weak correlation could be made with the attenuated LH response in cells from normal male rats which contain, as in the hypothyroid female, higher concentrations of SP in the anterior pituitary gland, but whether these two phenomena are causally related or simply nonspecific in nature is not known.

The response to GnRH is also decreased in AP cells from hypothyroid female rats. This raises the possibility that the reduction in LH response to SP is nonspecific in nature and may be due to other effects of hypothyroidism. A lower LH content in cultured AP cells from the hypothyroid animal explains the reduction in LH secretion in response to GnRH and SP.
Hypothyroidism in female rats has been shown to reduce LH and FSH release and to disrupt ovulation (Hagino, 1971). In male rats thyroidectomy results in reduced LH and FSH release and reduced serum testosterone (Bruni et al, 1975). In a contrasting report, Kalland et al (1978), found no difference in basal serum LH, FSH and testosterone levels between euthyroid and hypothyroid males. However, in both male and female rats, hypothyroidism results in higher serum LH and FSH levels after gonadectomy (LaRochelle and Freeman, 1973; Bruni et al, 1975), or at 15 minutes after acute GnRH administration (Kalland et al, 1978). Taken together these data imply a relationship in the secretory control mechanisms between gonadotrophs and thyrotrophs. The mechanism by which thyroid hormone acts to modulate gonadotropin release is not known. Available evidence is consistent with the speculation that SP may be a common anatomical (SP has been localized in both thyrotrophs and gonadotrophs) and perhaps functional element in both thyrotrophs and gonadotrophs (Morel, 1982; DePalatis, 1984), the levels of which are regulated differently by thyroid and gonadal status (Coslovsky et al, 1985). The results presented in this thesis demonstrate that hypothyroidism in female rats shifts the bioactivity profile for SP to one resembling that seen in the normal adult male rat. This is consistent with the finding that in thyroidectomized females there is a reduction in serum LH and impaired ovulation and fertility.
Measurment of Basal LH Release in the Presence of a Neutralizing Antibody to SP

The lack of an antagonist to SP or a known physiological stimulus for release of endogenous SP has made it difficult to show experimentally that APSP could be acting in a paracrine or autocrine way to affect LH regulation. The question of whether SP can be released from AP cells to act in a paracrine or autocrine way to cause LH release was approached in this study by incubating cultured AP cells from adult females in the presence of a purified SP antiserum with the expectation that this would bind released endogenous SP thereby reducing basal LH secretion. However, SPAb added to AP cell cultures for 4 hour incubations had the unexpected effect of stimulating LH release rather than reducing it. Much preliminary work was done to insure that this effect was not artifactual. Equivalent wells were incubated with non immune rabbit serum as a control. This treatment did not stimulate LH release. In order to eliminate proteins other than immunoglobulins in the SP antiserum and the normal rabbit serum, an ammonium sulfate precipitation of antibody was done on both serums. The purified SPAb and NRS were checked for crossreactivity or interference in the LH assay. It was found that at the concentrations used in the cell incubations there was no interference in the LH assay with

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either compound. Therefore, it appears that SPAb itself affects LH secretion. The SPAb was contaminated with a small amount of SP (60 fmol) but certainly not enough to cause a stimulation of LH secretion which requires 50 to 100 nM SP. Alternatively, SPAb could be binding endogenously released SP, each arm of the immunoglobulin binding one SP molecule stimulating a capping or bridging of receptors and potentiating what might in the absence of SPAb be a small effect. Precedence for this kind of phenomenon comes from studies on GnRH receptor microaggregation for which a molecule consisting of a GnRH pure agonist bound to an antibody which crossreacts with the antagonist was used to probe receptors (Conn, et al, 1986). Surprisingly the molecule behaved as an agonist. When the antibody was cleaved and reduced, the monofunctional antibody-antagonist complex returned to antagonistic behavior. This experiment showed that an antagonist could be converted into an agonist by linking the two antagonist molecules giving them the ability to stimulate microaggregation of receptors. If SPAb is binding the C-terminal ends of endogenously released APSP, the extended N-terminal ends of the peptide would still be available to bind to receptor and the Ab bridge could stimulate receptor microaggregation. The specificity data reported in this thesis, which showed that C-terminal modifications did not reduce the biological effect or the binding of SP to AP membranes, support the concept that the
receptor could be stimulated by the N-terminal portion of the molecule.

**SP Binding Sites in the Anterior Pituitary Gland**

Like other neurotransmitter and peptide hormones, the effects of SP are initiated by binding to target cell receptors. It is possible that one of the sites of action of gonadal steroids is at the membrane receptor level to modulate the extent of SP binding and LH release in the female. A correlation between receptor regulation and target organ receptiveness has been demonstrated for several hormones, including gonadotropins (Savoy-Moore et al, 1980), somatostatin (Schonbrunn and Tashjian, 1978), and TRH (Perrone and Hinkle, 1978). It was hypothesized that the enhanced LH response to SP in female anterior pituitary cells was due to an increase in the affinity or number of SP receptors in the female. SP binding sites in female rat anterior pituitary membranes were initially reported by Kerdelhuet al (1985). Results presented in this thesis are consistent with some of the findings of Kerdelhuet al although estimates of receptor affinity and density were somewhat different. They report a $K_d$ = 1.5-20 nM and a $B_{max}$ = 160 fmol/mg. In the present work, a composite analysis of SP binding to anterior pituitary membranes from male rats using
the LIGAND computer program generated a $K_d=42\text{nM}$ and a $B_{\text{max}}=570$ fmol/mg, whereas analysis of SP binding to anterior pituitary membranes of female rats yielded a $K_d=130$ and a $B_{\text{max}}=4.2$ pmol/mg. These binding characteristics differ from those reported by Kerdelhue. These differences could be explained by the different radioligands used in the two studies. Kerdelhue did not use $^{125}\text{I}B\text{SP}$, but instead used $^3\text{HSP}$ or a $^{125}\text{I}\text{Tyr-SP}$. The different radioligands could be binding to different SP binding sites in the AP or binding with more or less affinity to the same binding site. However, allowing for the differences in $K_d$ and $B_{\text{max}}$ the results of the present study confirm the hypothesis that there is an increase in the number of binding sites in the female rat anterior pituitary which could be responsible for the enhanced effect of SP in these animals. A direct correlation is confounded by the slightly decreased affinity of SP binding in the female rat. However, the large increase in receptor number could explain the difference in response, whereas the slight decrease in affinity could be due to an increase in the number of both high and low affinity sites, with the large number of low affinity sites obscuring the ability of the assay to detect the higher affinity sites.

The finding that AP cells from females have greater sensitivity to SP and a far greater number of receptors is in keeping with the concept of spare receptors (Nickerson 1956).
Spare receptors modulate the hormone sensitivity of the cell. The kinetics of hormone receptor binding follow the laws of mass action, to illustrate: \( H + R \rightarrow HR \rightarrow \) Biological Response. The critical concentration is that for HR and this is determined by concentration of both the hormone \([H]\) and receptor \([R]\). By increasing the \([R]\) the reaction can be driven to the right. The higher \([R]\) is, the less \([H]\) is required to reach the same concentration of HR. As a result the cell is more sensitive to \( H \). A teleological explanation of this phenomenon was given by Goldstein (1974). He suggests that in circumstances where a rapid onset of response and a rapid termination of response is required, a spare receptor capacity provides a mechanism for obtaining a response at a very low concentration of agonist which has a relatively low affinity for the receptor. The low affinity of the agonist assures a rapid rate of dissociation. If high affinity of the agonist for the receptor was used to achieve sensitivity then the rate of reversal of the effect would be slow.

There was no evidence of a second binding site in any of the animal models studied (the inclusion of a second binding site did not improve the computerized fitting of the data) although the displacement curve is shallow giving the illusion of multiple binding sites. One of the problems with the assay is that native SP is susceptible to proteolysis, even at 4°C. Thus it is difficult to accurately set the concentration of SP at each point. Furthermore degradation
probably occurs at different rates at different concentrations of SP resulting in the shallow appearance of the curve. Peptidase inhibitors were not used in the assay as they decreased SP binding.

Direct correlation of binding characteristics with biological effects is difficult in the case of the SP binding experiments in AP membranes from hypothyroid male rats as well. As mentioned above, there are conflicting reports on whether hypothyroidism in male rats causes an increase, a decrease or no change in serum LH levels. The present SP radioreceptor assay study of AP membranes from hypothyroid male rats showed a doubling of receptor number and a slight decrease in affinity of the receptor. Binding studies done on AP membranes from hypothyroid female rats resulted in a $K_d=48\,\text{nM}$ and $B_{\text{max}}=1.9 \, \text{pmol/mg prot}$. These values are different from control female values ($K_d=135$ and $B_{\text{max}}=4.2 \, \text{pmol/mg}$) and are changing in the direction of the binding parameters in male AP membranes. This correlates with the reduced LH response to SP in AP cells from hypothyroid female rats. This would suggest that SP may be partially responsible for the reduced LH secretion in hypothyroid female rats, however this does not explain the reduction of LH release in response to GnRH.

Radioreceptor assay does not distinguish between functional and nonfunctional receptors and may not be
accurate enough to distinguish between two or more distinct subtypes of tachykinin receptor. The sensitivity of the radioreceptor assay approach may not be optimal for the quantitation of changes in SP receptors in different physiological states because of the lack of a highly purified, synthetic, non-degradable analog of SP which could be used as a probe for high affinity SP binding sites.

Very similar problems were encountered in the attempt to establish a radio receptor assay for GnRH. Early investigations were done with iodinated GnRH as the radioligand (Clayon and Catt, 1981). It was found that labeled GnRH interacted predominantly with a very low affinity (K_d=10^{-6} M) binding site with a very high capacity for GnRH. Only a small percentage of tracer bound to sites of reasonably high affinity (K_d=10^{-8} M). It was found that there was significant tracer degradation even when assay incubations were done at 4°C (Clayton et al, 1979). Since then agonist and antagonist analogs of GnRH have been synthesized for biological and binding studies. These analogs had a slower rate of degradation by pituitary peptidases, prolonged action on LH release and interacted selectively with specific high affinity binding sites. Hormone analogs have been validated as probes for analysis of several physiological receptors and have been of primary importance in the field of hormone receptor research.
Effect of Divalent Cations on SP Binding in AP

In spite of the lack of sensitivity of the radio receptor assay for studying the regulation of high affinity binding sites, the present binding studies have provided some important and novel observations. Attempts were made to examine the possibility that the action of SP may be mediated through coupling with a guanine nucleotide binding protein. It was found in the course of these studies that SP binding is inhibited over 80% by 1 mM magnesium and 95-98% by 5 mM magnesium, concentrations that are required for optimal high affinity binding of SP in brain tissue (Cascieri and Liang, 1983). While it is possible that magnesium enhances the activity of peptidases in the pituitary, causing the ligand and perhaps the receptor to degrade, it is not likely since magnesium inhibits binding equally at 37°C and 4°C and peptidases are inactive at 4°C. Another possibility could be that Mg²⁺ promotes coagulation of vesicles effectively masking binding sites. To determine whether this was true anterior pituitary membranes were pretreated with saponin. Saponin pretreatment had no effect on control binding or on the inhibitory effects of magnesium (data not shown). The present studies also demonstrate that SP binding is inhibited by calcium to a lesser extent than magnesium and by manganese.
which is even more potent than magnesium. Perhaps the SP receptor is a calcium channel or is coupled to a calcium channel resulting in a competition for binding at the site or an uncoupling of SP-bound receptor in the presence of calcium, magnesium or manganese. Alternatively magnesium and calcium may stimulate release of stored SP in synaptic vesicles in the anterior pituitary membrane preparation resulting in a competition for binding with exogenous ligands.

The inhibition of binding by divalent cations, particularly Ca\(^{++}\) and Mg\(^{++}\) was a common characteristic of GnRH receptor binding (Marian and Conn, 1980). This study demonstrates that Ca\(^{++}\) inhibits binding of a superactive analog of GnRH in spite of the fact that GnRH does not stimulate LH release in the absence of extracellular calcium. This suggests that the action of Ca\(^{++}\) occurs after hormone receptor binding. This is consistent with the fact that increasing intracellular calcium by means of ionophores or depolarization alone is sufficient to cause LH release. TRH binding to bovine anterior pituitary membranes is inhibited by Ca\(^{++}\) at all concentrations tested and by Mg\(^{++}\) and Mn\(^{++}\) at concentrations greater than 2.5mM as reported by Labrie et al (1972). This characteristic of releasing factor receptor binding has not been adequately explained and the mechanism of divalent cation inhibition of binding at the level of the receptor is still unknown, and is particularly perplexing.
because of the absolute requirement for Ca++ during signal transduction and for the final biological response.

**Differences Between SP Binding Sites in the AP and the NK1 Receptor**

Some of the above mentioned characteristics of the SP binding site in the anterior pituitary distinguish it from the classical NK-1 site which has been previously described in brain, salivary and submaxillary gland membranes (Bahouth and Musacchio, 1985; Cascieri and Liang, 1983; Lee, et al., 1983). Some of these differences include: 1) K_d and B_{max}, the classical NK-1 site is a higher affinity (1nM) lower capacity site; 2) G-protein coupling, it has been confirmed that guanine nucleotides have an inhibitory effect on SP binding to the classical NK-1 site, whereas guanine nucleotides do not appear to inhibit SP binding to the binding site in AP membranes; 3) divalent cations, Mn^{++}, Mg^{++} and Ca^{++} increase SP binding in brain, salivary and submaxillary membranes with maximum effects at 5mM, whereas in AP membranes SP binding is significantly inhibited in the presence of divalent cations even at concentrations as low as .1 mM; 4) specificity, for the NK-1 receptor the rank order of potencies is SP>SK>neurokinin B, and the nonmammalian peptides physalaemin (almost equal potency to SP)>eledoisin>kassinin, however in
the AP membrane physalaemin has almost no potency to displace SP, nor does SK, the C-terminal modification of SP in SPFA does displace SP with an IC$_{50}$ of 10$^{-6}$ as does GnRH with an IC$_{50}$ of 10$^{-5}$. These differences in receptor characteristics raise the question of whether this binding site is an unknown tachykinin receptor subtype or another receptor with some affinity for SP. There is an increasing body of evidence that strongly supports the existence of an N-terminal directed SP receptor which mediates distinct biological activities of N-terminal metabolites of SP (Stewart 1982; Skilling 1990).

In light of the similarities between the SP binding site in the AP and the GnRH receptor and the differences between the APSP binding site and the salivary gland SP receptor, the question of whether SP may be acting at a GnRH receptor was addressed. Indeed, some conformational and structural aspects of the undecapeptide, SP, may provide slight acceptability at the receptor site for the decapeptide, GnRH. It has been postulated that the driving force for the formation of hormone-receptor complex in GnRH interaction with its receptor is probably an ionic interaction between the amino acid Arg in position 8, which is positively charged, and the negatively charged carboxyl groups in the binding site. The hormone-receptor complex may be stabilized by aromatic π-π interactions between the histidine, tryptophan and tyrosine moieties of the hormone and the tyrosine and tryptophan moieties of the binding site (Hazum and Conn, 1988). The Arg
moiety of SP in the protonated form could be very important for activity and potency because it contributes an ionic nature to the molecule, the Lys moiety could make a similar contribution. The Phe-Phe moieties could contribute structural specificity to molecule and allow possible π-π interactions with the GnRH receptor site. Indeed, SP has some potency to displace 125IGnRH from AP membranes (IC_{50}=10\mu M) suggesting that at higher concentrations SP could act at a GnRH receptor.

The physiological relevance of the effect of SP on LH release from anterior pituitary cells has been addressed from the aspect of whether local SP concentrations in the anterior pituitary could possibly get to 100nM, the concentration used to stimulate LH release from cells in culture and the IC_{50} in competition binding curves. In the case of a paracrine or autocrine effect SP would be released to act directly in the area of release. It has been shown that SP can be released in a K^+ stimulated, Ca^{++} dependent manner from hemipituitaries, and that the amount released was almost equal to the total amount of SP contained in the gland before stimulation (Aronin 1984). In other words, upon appropriate stimulation a pituitary cell could release its entire contents of SP into the extracellular space. The concentration of SP in the anterior pituitary is \approx 200 fmol per gland in the female rat and \approx 400 fmol per gland in the male rat. There are
approximately 4.0x10^6 cells per pituitary (Ben-Jonathan et al, 1983) and these cells are approximately 10-15 μm in diameter. Cell volume can be estimated using the formula for the volume of a sphere (.5pL) and assuming the 1:1 relationship between cell volume and the trapped volume between cells, the extracellular volume would be estimated to be .5pL. The volume of the extracellular space in an entire anterior pituitary gland would be 4.0x10^6 cells/pituitary x .5x10^-12 L = 2x10^-6 L or 2μL. Assuming 200x10^-15 moles of SP was released into the extracellular volume of 2μL (200x10^-15/2x10^-6) a concentration of 100nM would be reached assuming uniform dispersion. Thus, it is possible that SP can reach levels sufficient to activate LH release in vivo.

**A Possible Physiological Releasing Factor for SP**

A physiological factor which stimulates SP release from AP is not known. However, a recent abstract (Calvo 1989) reports a significant stimulation of SP release when compared to basal release from perifused anterior pituitaries from normal male rats in response to 10 minute infusions of TRH (10nM-1μM). GnRH was also tested and no effect was observed on SP release. I have also observed SP release in response to TRH in AP cells in culture from both intact males and hypothyroid males. However, out of 10 experiments, 5 resulted
in SP release in response to TRH (100nM) and 5 were negative (data not shown). I also observed no SP release in response to GnRH (10nM and 100nM). In future I would like to pursue these experiments in animal models which have higher SP levels in the AP to increase the chance of SP being released. I will also try AP cells from rats which are more sensitive to SP, such as female or peripubertal male rats, with the idea that since AP cells from these animals can respond to SP in vitro maybe these models have release mechanisms for SP already in operation in vivo.

Another method of optimizing the cell's ability to release SP in response to TRH, would be to attempt to increase the number of TRH receptors on AP cells. Prolactin release by TRH is enhanced by estrogen, presumably by an action of the steroid on the number of TRH binding sites on lactotrophs (Gershengorn 1979). Also, TRH receptor number fluctuates during the rat estrous cycle being minimal at diestrus and maximal on the evening of proestrus (De Lean 1977). Perhaps the use of AP cells from rats at proestrus or the treatment of AP cells with estrogen would increase the chances of observing SP release from AP cells in response to TRH.

If TRH does act as a physiological releasing factor for SP, estrogen, by increasing TRH receptors, may be a factor involved in this action. This idea would be consistent with the lower SP concentrations in the anterior pituitary in the
female rat. An increased rate of SP release could be one of the mechanisms keeping SP concentrations lower than in the male. This concept would also be compatible with the increased sensitivity to SP observed in AP cells from female rats. In other words estrogen may be the common factor, which increases TRH receptor number, SP release and SP binding sites and thereby allowing the action of SP to increase LH release in the female rat. Future studies will involve the treatment of estrogen primed AP cells with TRH, perhaps using a perifusion apparatus rather than a static culture system to facilitate observations at shorter time points. TRH receptors may also prove to be the link between thyroid status and LH secretion.

Speculation on the Factors which could be Responsible for the Sex Difference in SP Stimulated LH Release

The regulation of gonadotropin secretion is mediated by a combination of sexually dimorphic and developmental neuroendocrine mechanisms. However, it is difficult to determine whether apparent sex and age differences in the sensitivity to SP are caused by developmental and sexual dimorphisms (such as differences in LH content or number of responding gonadotropes) or whether SP plays an important role in the maintenance of the sex and age differences in LH.
regulation.

At the AP level there have been numerous reports of sex differences in the content of AP hormones. However, since hormone content at any time is the result of a dynamic process of synthesis and secretion these reports shed little light on the process of LH regulation at the level of the AP. Cicero et al (1986) reports that adult male APs contain 5 times more LH than female APs. Whether this higher content is a result of lower secretion of LH by males is not addressed in the that study. Other regulatory peptides show content differences depending on the hormonal status of the animal.

Morphological differences in gonadotropes and differences in cell number have been reported in relation to sex, estrous cycle and age (Tougard and Tixier-Vidal 1988). Although gonadotropes represent approximately 14% of the anterior pituitary cell population of normal adult male and female rats, there is a sexual dimorphism in the size of gonadotropes. In male rats gonadotropes range in size from 30-160μm² in area while in the female rat gondotropes are from 130-170μm². There is also a sex difference in the percentage of multihormonal LH/FSH cells in the rat. In the female multihormonal cells account for 37-40% of gonadotropes as compared with 70% in the male. Perhaps it is the monohormonal cells which are more abundant in the female which are sensitive to SP. Although there is no change in the absolute number of gonadotropes throughout the estrous cycle,
a decrease in the percentage of monohormonal cells at proestrus suggests that these cells are involved in the LH surge.

Layered onto this morphological complexity is the complexity of differential sensitivity of gonadotropes to circulating regulatory factors such as gonadal steroids, GnRH, other hypothalamic peptides and AP peptides which may be released by neighboring cells to modulate the release of LH. The speculation that the multihormonal and monohormonal gonadotropes are regulated differently (perhaps mediated by differential sensitivity to regulatory peptides) would be compatible with the results of the present study. There are changes in gonadotrope cell number and size during development. Gonadotropes increase during the first week of life to a percentage which is higher than the adult, with a higher cell number in females than in males. It is not known whether these are multihormonal or monohormonal. A higher percentage of gonadotrophs which are responsive to SP at the time of puberty in the male rat would be consistent with the findings reported in this thesis.
Regulation of LH Secretion independent of Mediation by GnRH.

The focus of the ensuing paragraphs will be on the mechanisms by which the gonadal steroid environment can regulate LH secretion from the anterior pituitary, independent of variations in GnRH secretion, in an attempt to evaluate the possibility that a paracrine or autocrine action of SP may provide an intrapituitary mechanism for LH regulation. The evidence from GnRH receptor work indicates that the regulation of GnRH receptor is not a major mechanism for hormonal modulation of gonadotroph sensitivity. This is thought to be conferred by changes in post-receptor events and perhaps other mechanisms which have not been defined as yet (Clayton, et al, 1985).

Stimulation of LH Release by SP in Female Rats

There are several reports indicating that the pituitary is the site of estrogen and progesterone action regulating the release of LH. This would require gonadal steroid regulatable mechanisms within the anterior pituitary. Peptides which are synthesized and whose concentrations are regulated by gonadal steroids in the anterior pituitary would be ideal candidates to mediate such mechanisms. Sustained
estrogen action has been shown to increase the releasable stores of LH in the pituitary (Kalra and Kalra 1974; Pickering and Fink 1979), and the gradual enhancement in LH secretion in response to GnRH stimulation is directly related to the concentration and duration of pituitary exposure to estrogen. On the other hand, a progesterone milieu during the afternoon of estrus through noon of diestrus II, results in the gradual decline in pituitary responsiveness to exogenous GnRH. In a study investigating the change in responsiveness of dispersed pituitary cells in perfused columns, Speight and Fink, (1981) demonstrated that the LH response to GnRH from cells prepared from rats at proestrus were much greater than that of rats killed at other times of the cycle. This difference was seen when cells were stimulated with 60 mM K+ suggesting that at least part of the increase in pituitary responsiveness at proestrus is not dependent on changes in specific GnRH receptors. Perhaps an increase in the releasable pool of LH and/or increased sensitivity to regulatory peptides could account for the extra LH released over and above LH release mediated through GnRH receptors. Tang et al (1982) have shown that estrogen effects on pituitary LH responses to GnRH in vitro can be both inhibitory and stimulatory depending on the concentration and time of the estrogen exposure. They demonstrate that the estrogen priming effect on pituitary LH response to GnRH is initially associated with an increase in cellular LH content,
then with increases in GnRH binding and in an index of cell proliferation which may include LH producing cells.

More evidence of the pituitary's participation in the feedback effects of estrogen include in vitro studies which show that estrogen enhances both basal and GnRH induced LH release (Tang et al 1982; Greeley et al 1975; Drouin and Labrie 1981; Drouin et al 1976). There have also been reports of ovarian steroid binding sites located intracellularly (Stumpf 1971) and on rat pituitary membranes (Bression 1986).

There is some evidence which is compatible with the idea that the "priming" effect of GnRH on gonadotropes may be mediated by peptides which are regulated within the AP. Exposure of the rat AP gland in vivo to low (doses too low to produce a detectable increase in plasma LH) continuous concentrations of GnRH or to small pulses of GnRH can result in a surge of LH which is similar in magnitude to the spontaneous ovulatory surge of LH (Fink et al 1976). This phenomenon is known as the priming effect of GnRH and demonstrates how small pulses of GnRH could produce an ovulatory surge of LH. Studies on the mechanism of the priming effect have been done using short term incubations of hemipituitary glands (Pickering and Fink 1976). These studies showed that there are major differences between the releasing action and the priming action of GnRH. The priming action of GnRH develops at an age at least 12 days after the onset of the releasing action of GnRH. The priming action is enhanced
by estrogen and does not depend on extracellular Ca++ but is
dependent on protein synthesis and the integrity of the
microfilaments. Several experiments showed that the new
protein synthesized as part of GnRH priming is not LH
(Pickering and Fink 1979), but rather a protein with a
molecular weight (MW) of about 69,000 (Curtis, Lyons and Fink
1984) which may be related to the hypothalamic estrogen
stimulated 70,000 MW protein thought to be a heat shock
protein. Heat shock proteins are highly conserved throughout
evolution and are thought to be the forerunners to specific
cell surface receptors. Solubilized AP membranes subjected to
polyacrylamide gel electrophoresis after exposure to
radiolabeled SP results in a radiolabeled band determined to
be 70,000 MW which is not seen in the presence of unlabeled
SP (preliminary findings, data not shown). This protein was
later found to be a heat shock protein (Barry Oblas, personal
communication) this would suggest that SP can bind
specifically to heat shock proteins. There is also synthesis
of an 87,000 MW protein stimulated by GnRH incubation of
dispersed pituitary cells. It is tempting to speculate that
these newly synthesized proteins may be heat shock type
proteins which may act as receptors for regulatory peptides.

How do the results presented in this thesis relate to
the control of LH release in normal intact female rats? In AP
cells from normal random cycling female rats SP causes a
significant stimulation of LH release. The finding that SP is
Effective in stimulating LH release in pituitary cell cultures from adult females but not adult males implicates this peptide as a possible intrapituitary regulatory factor for variant LH secretion. The preponderance of evidence suggests both negative and positive feedback actions of estrogen can be exerted at the level of the anterior pituitary gland and suggests that there are mechanisms at that level to regulate the amount of LH released other than decreased or increased sensitivity to GnRH, which depends in large part on GnRH up regulation or down regulation of its own receptor. Although GnRH stimulates LH release in both male and female pituitaries, differences in LH secretion patterns exist. It has been demonstrated using a pituitary cell culture system that there is a sex difference in LH release in response to GnRH stimulation (Tang, 1978). The results demonstrate the ability in female rat pituitary cultures to release a significantly greater percentage of total LH in response to GnRH, and suggest that this may provide the essential mechanism required for the preovulatory LH surge, whereas the lower response in the male may be sufficient for tonic LH secretion.

Another intrapituitary mechanism independent of GnRH which increases LH release and where SP may play a role is the employment of different mechanisms for release of newly synthesized and stored pools of the hormone. Ramey and colleagues (1987) have reported that estradiol sensitizes the
gonadotroph to GnRH for stimulation of LH biosynthetic processes and that regulation of the release of newly synthesized LH is different from that for previously stored LH. They report that the differences in GnRH stimulated LH synthesis caused by the estrogen environment could not fully account for differences in GnRH stimulated LH release. Therefore, it would seem that estrogen has separate effects on the mechanisms regulating GnRH stimulated LH release and LH biosynthesis. Conclusions by Ramey and Liu and Jackson (1979) suggest that there are two pools of LH, and that under non GnRH-stimulated conditions newly synthesized LH accounts for a relatively greater percentage of the total LH released compared to that released in response to GnRH, suggesting that there may be more than one mechanism of release. Hiatt and Schwartz (1989), in a recent study of basal LH secretion from pituitary fragments taken at different stages of the estrus cycle, demonstrate that in the presence of an antagonist to GnRH, basal LH secretion is higher in proestrus pituitaries than in metestrus pituitaries, suggesting that there may be a higher basal release independent of GnRH stimulation at proestrus. My results are consistent with the possibility that SP may be an active substance, released in response to an unknown physiological stimulus, acting in a paracrine or autocrine fashion to stimulate an augmented release of LH in females. This putative mechanism would be particularly important at the time of the LH surge. One
future experiment will involve comparing the sensitivity of 24 hour cultures of AP cells from female rats at different stages of the estrous cycle to determine whether there are cycle differences in the ability of SP to stimulate LH release.

**Stimulation of LH Release by SP in Male Rats**

AP cell cultures from the adult male rat are significantly less sensitive to SP, although SP concentrations in the adult male AP are 2 to 3-fold higher than in the female rat AP. The higher concentration of SP, if the peptide is released basally, could act to desensitize the AP to SP. Binding studies described here, which show a decrease in the number of SP receptors in the AP membranes of male rats, would be consistent with this hypothesis. Future experiments will focus on this question by attempting to desensitize responding AP cells from female rats by a constant exposure of cells to low doses of SP.

In the present study AP cell cultures from adult male rats were less sensitive to exogenous SP than were AP cells from adult females. The present results and other studies demonstrate SP binding in both male and female rats (Larsen, 1989; Kerdelhue, 1985). Results of the present SP
binding study show that SP receptors have an 8-fold lower capacity in the male than in the female, this could be the result of an 8-fold difference in the number of cells which have binding sites for SP, nevertheless the end result is more receptors in the female and a more sensitive response in the female as measured by LH release from AP cells. These findings are compatible with the presence of spare receptors in the AP glands of female rats allowing for an increased sensitivity to lower doses of SP.

Alternatively, SP binding in the male may be uncoupled to a physiological second messenger stimulating LH release or perhaps is coupled to a different signal system. It remains a distinct possibility that in vivo there could be some mechanism to inhibit binding or that there could be two distinct types of SP receptor in the pituitary the ratio of which could be regulated by the gonadal steroid environment and which are obscured by in vitro methods of analysis. Improved techniques in manipulating the steroid environment and increased sensitivity in SP binding studies in the anterior pituitary will aid in the attempts to answer these questions.
Stimulation of LH Release by SP during Puberty

The development of responsiveness to SP in AP cell cultures from both males and females closely parallels the onset of puberty. The results of the present study demonstrate that SP stimulation of LH secretion varies as a function of age and sex. Before puberty AP cells from both males and females are unresponsive to SP. However, at 30-35 days of age there is a steep increase in SP stimulated LH release in both sexes. This response continues in the female until sexual maturity and at all ages studied. In contrast to these results AP cells from males failed to respond after 40 days of age and remained relatively unresponsive throughout maturity at all ages studied.

The onset of puberty involves a complex array of neuroendocrine regulatory mechanisms. The first hormonal signal begins after 28 days of age. In the female rat a diurnal pattern of LH release starts around day 30 in which both basal LH levels and LH pulse amplitude are greater in the afternoon than in the morning. These augmented afternoon pulses result in significantly more estrogen and progesterone release from ovaries in vitro (Ojeda and Urbanski, 1988). In some rats afternoon minisurges of LH occur at this time. The LH minisurges are estrogen dependent whereas the increase in LH pulse amplitude is estrogen independent and thought to be centrally driven (Urbanski and Ojeda, 1987). The increase in
basal LH release during the peripubertal period has been studied in the rhesus monkey (Teresawa et al, 1984). It was found that the amplitude of the difference in LH levels between evening and morning in ovariectomized animals was greatest when basal LH reached its highest level, which occurred immediately before the first ovulation. They note that first ovulation occurs when all of the LH parameters, including basal LH release, circadian LH fluctuations, pulsatile LH, and biological activity of LH are maximally active. SP could be contributing to this maximal LH output effort by acting in a paracrine or autocrine way to stimulate basal LH release at the time of puberty leading to first ovulation.

Much less is known regarding the mechanisms controlling puberty in the male rat. There have been conflicting reports on LH secretion in the peripubertal male, some reporting enhanced and others reporting decreased LH secretion during this period. The inconsistency probably arises because of the pulsatile manner of LH release at this time. In the present study the response to SP in peripubertal males was highly variable and may reflect the rapid changes occurring in the secretory patterns of LH during peripubertal maturation. Brown-Grant (1976) claims that the prepubertal rat is a bad model for the study of LH secretion and cites studies of several researchers who report wide variability in both baseline and stimulated LH concentrations in plasma which
could only be reduced by using large numbers of rats or pooling serum from several rats. Only with these measurements have meaningful trends been established for the developmental changes in LH secretion in pubertal and prepubertal male rats.

It remains to be established whether changes in the pattern of LH secretion play a role in modulating prepubertal steroidogenesis in the male. It is clear from the present study that AP cells from peripubertal males release LH in response to SP and that this occurs only during the period of puberty in the male rat. It could be postulated that this mechanism allowing for augmented LH release is needed at this time to initiate and fine tune steroidogenesis of the testes. GnRH stimulation has been shown to result in the release of stored hormone preferentially, whereas secretion of newly synthesized hormone is thought to be regulated differently (Ramey et al, 1987). The paracrine action of SP could provide a mechanism for release of a larger percentage of total hormone than that which would be released upon GnRH stimulation alone.
Summary of Conclusions

The results of the present study show:

1. SP can have an effect on LH release by acting directly on AP cells in culture.
2. The effect of SP on LH release is dependent on the sex of the rat from which the AP cells were prepared.
3. The effect of SP is dependent on the age of the rat from which the AP cells were prepared.
4. There are specific binding sites for SP on AP membranes.
5. Certain characteristics of SP binding correlate with the bioactivity of SP.

There is no doubt that a great number of questions have been raised as a result of this research. These studies will serve as a stimulus to embark on an entirely new avenue of research on the role of SP in the regulation of LH secretion. The direction that future studies will take will be to investigate further the underlying basis for the sex differences in the stimulation of LH secretion from AP cells in culture by SP, differences that are due to maturational processes and to various other endocrine changes.
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