STUDIES ON THE ROLE OF NEUROPEPTIDES: SUBSTANCE P AND NEUROTENSIN IN REPRODUCTIVE FUNCTION IN THE FEMALE RAT

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CERTIFICATE

This is to certify that Anahita Mistry has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph.D. ordinances of this University. I recommend her thesis entitled "STUDIES ON THE ROLE OF NEUROPEPTIDES: SUBSTANCE P AND NEUROTENSIN IN REPRODUCTIVE FUNCTION IN THE FEMALE RAT" for submission for the degree of Doctor of Philosophy in this University.

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DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me under the supervision of Dr. E. Vijayan. To the best of my knowledge no part of this thesis has been submitted for the award of research degree of any other University.

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ABBREVIATIONS

The following abbreviations are used in this thesis:

ACTH	acetylcholine
4-APP	amino pyrazolo (3, 4d) thymidine
ARGG	anti-rabbit gamma globulin
B	bound
BSA	bovine serum albumin
bwt	body weight
cAMP	3',5′-cyclic adenosine monophosphate
cGMP	3',5'-cyclic guanosine monophosphate
CPM	counts per minute
CRF	corticotropin releasing factor
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
EB	estradiol benzoate
EDTA	ethylene diamine tetra acetic acid
FSH	follicle stimulating hormone
GABA	gamma amino butyric acid
GnRH	gonadotropin releasing hormone
h/hrs	hour/hours
HCG	human chorionic gonadotropin
5-HT	1-5-hydroxytryptophan
^{125}I	125 ^^Iodine
ip	intraperitoneal
iu	international units

IV	intravenous
ivt	intraventricular
LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
MBH	medial basal hypothalamus
mCi	millicurie
ME	median eminence
MIF	melanocyte release inhibitory factor
min	minutes
a-MPT	a-methyl-p-tyrosine
MSH	melanocyte stimulating hormone
NIAMDD	National Institute of Arthritis, Metabolism and Digestive Diseases.
NIH	National Institute of Health
ng	nanograms
NRS	normal rabbit serum
NT	neurotensin
NTLI	neurotensin like immunoreactivity
OVX	ovariectomized
PBS	phosphosaline buffer
pmoles	picomoles
PMSG	pregnant mare serum gonadotropin
POA	preoptic area
POPOP	(1,4-bis[2-(4-methyl-D-phenyloxazolyl benzene)]
PPO	2,5-diphenyloxazole
FtIA	radioimmunoassay

RNA	ribonucleic acid
sc	subcutaneous
sec	seconds
SEM	standard error of the mean
SP	substance P
TC	total counts
TRH	thyrotropin releasing hormone
VIP	vasoactive intestinal peptide
Z	100% binding

CHAPTER I

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

The peptides constitute the newest class of molecules considered to play key roles as hormones, transmitters or modulators in the central and peripheral nervous systems. Certain neurons in the CMS, for instance neurosecretory neurons, synthesize and release small proteins (peptides) which act on peripheral target organs such as the anterior pituitary. During the past two decades there has been a remarkable progress in our understanding the processes of chemical signalling within the mammalian CNS. Ten years ago some eight or nine monoamine and amino acid neurotransmitters were known but to these must now be added thirty or more small peptides each with a potential chemical messenger function. Typically each messenger is discovered first as a factor, a substance of unknown chemical composition, that has a physiological effect such as dilation of arteries or contraction of muscle. Often it emerges that the factor is made up of amino acids. Then it emerges that the factor is active in the brain. Thus the factor is designated as a neuropeptide. This thesis focuses on two of these neuropeptides: Substance P (SP) and Neurotensin (NT).

The discovery of secretin (3ayliss and Starling, 1902) brought to physiology and medicine the idea of blood borne 'chemical messengers' (hormones) and ended the 19th century notion dominated by Pavlov that the system is solely under nervous control. Bayliss and Starling conceived the endocrine system as a complex of chemical messengers which coordinate the functions of different tissues of the body. Starling observed that these hormones, as he named them, have to be carried from the organ where they are produced to the organ which they affect by means of the blood stream and the continually recurrinq physiological needs of the organism must determine their repeated production and circulation through the body. Since then the field of endocrinology has come a long way.

It is not surprising that the two systems that coordinate and integrate the operation of the other systems of the body are related since their functions are related. Of the established endocrine organs the most important is the pituitary at the base of the brain connected to the hypothalamus by a stalk. One of the first demonstrations of the brain for normal endocrine function was that of Aschner who detected in 1912 that dogs with hypothalamic lesions developed atrophy of the genitalia. In 1920, Camus and Roussy discovered that lesions of the hypothalamus

were associated with diabetes insipidus. Subsequently changes in the estrous cycle following transection of the hypophyseal stalk were demonstrated (Richter, 1933). Grafe and Grunthal (1929) reported that hypothalamic lesions altered metabolic rates and Cahane and Cahane (1936) observed that destruction of the medial basal hypothalamic structures altered the histology of the thyroid.

The demonstration that nerve fibres secrete chemical agents was a major advance in neurophysiology. The hypothesis that autonomic nerve fiores secrete chemical agents that act as synaptic transmitters was originally advanced by Elliott (1905), Dixon (J907), Loewi (1921) and others but it was primarily the Scharrers (1919) who pioneered the concept that nerves can liberate hormones into the blood. Speidal (1919) noticed that giant neurons in the posterior spinal cord of elasmobranchs had morphological features in common with glandular cells and suggested that neurons might secrete hormonal products. Scharrer (1923) confirmed this suggestion by discovering similar neurosecretory cells initially in the hypothalami of fish, and later in other vertebrates. The above observations spurred Scharrer and Scharrer (1954) and Bargmann (1954, 1968) to postulate that certain hormones of the pituitary are of hypothalamic origin and propose

the concept of neurosecretion i.e., the ability of some hypothalamic neurons to secrete peptides related to the posterior pituitary. This was proved true when oxytocin and vasopressin were purified and characterized by du Vigneaud et al (1953a, b).

Although there is no direct connection between the hypothalamus and the anterior pituitary the two are linked. Harris (1955) provided the necessary anatomical evidence for the linkage of the hypothalamus to the pituitary and postulated the chemotransmitter hypothesis: the hypothalamic portion of the brain produced and released hormones into the portal vessels emanating in the ME and coursing down the pituitary stalk to the pituitary gland. These unique vessels had earlier been discovered by Popa and Fieldinq (1930). However, they had concluded that the flow was from the pituitary to the brain. That the blood flows downwards from brain to pituitary was first demonstrated by Houssay et al (1935) in amphibians. Wislocki and King (1936) showed that the blood in these vessels flowed from the hypothalamus towards the anterior pituitary in the rat. Green and Harris (1949) confirmed this later. These facts prompted the suggestion, apparently first made by Hinsey (1937) and later elaborated by Green and Harris (1949), that humoral agents secreted into the hypophyseal portal veins might control

Thyrotropin Releasing Hormone (TRH): Shibusawa et al (1956) and Schreiber et *al* (1961) proved the existence of a factor that stimulated the release of thyrotropin. This factor TRH has been sequenced as pyro-glutamyl-histidyl-prolineamide (Burgus et al, 1969; Boler et al, 1969). It was capable of stimulating Prl release also (Vale et al. 1973) and was the first hypothalamic hormone to be synthesized.

Luteinizing Hormone Releasing Hormone (LHRH): McCann et al (1960) showed that rat hypothalamic extract had LH-releasing activity. Luteinising hormone releasing hormone has been isolated from porcine (Baba et al., 1971) and ovine hypothalamic fragments (Amoss et al,1971) and its structural elucidation and synthesis was performed. It was found to be a decapeptide and is capable of inducing the release of both LH and FSH. Luteinising hormone releasing hormone release in turn is modified by a host of factors which include the monoamines of the hypothalamic neurons, particularly dopamine, norepinephrine and epinephrine (Schneider and McCann, 1970; Vijayan and McCann, 1978a, b; Negro-Vilar et al, 1979). Other substances which regulate LHRH and gonadotropin release include prostaglandins and GABA (Ojeda et al, 1977, 1980; Vijayan and McCann, 1978c).

Growth Hormone Release Inhibiting Factor (SRIF, Somatostatin): GH release-inhibitory hormone has been obtained in pure form (Brazeau *et* aj., 1973; Ling et al, 1973; Burgus ,et al. 1973) and has been shown to inhibit the release of GH and under certain circumstances thyrotropin and Prl from cells of the anterior pituitary (Vale ejb al, 1974).

Prolactin Releasing Factor (PRF): PRF activity was apparent in the hypothalami of mammals and birds (Bowers et al, 1971, Schally *et* al, 1973) part of which was attributed to TRH itself which itself has Prl releasing activity under certain conditions (Vale *et* al, 1977). Its chemistry is unelucidated.

Prolactin Release Inhibiting Factor (PIF): The presence of PIF activity was demonstrated several years ago in hypothalamic extracts (Bowers et, al^ 1971; Schally et aJL, 1973). Its nature is still dubious.

Other factors like MSH-releasing factor and MSH release inhibiting factor (MIF) have also been demonstrated in the brain (Schally et al, 1973; Vale et al, 1977).

In addition, by the application of radioimmunoassay (RIA) and immunocytochemical methods, evidence has

accrued for the presence of a host of small peptides in hypothalamic neurons. These peptides act as chemical neurotransmitters or other forms of chemical messengers but as yet there is only a fragmented understanding of their functional importance. Often they are found in parts of brain which have nothing to do with the realm in which the substance acts at the periphery of the body. They include Substance P (Chang and Leeman, 1970), Neurotensin (Carraway and Leeman, 1975a), Gastrin (Vander Haggen et al, 1975), Angiotensin I (Changaris et al. 1976), Angiotensin II (Mclean, 1975; Spinedi and Negro-Vilar, 1983) Cholecystokinin (Dockray, 1976), Vasoactive intestinal peptide (Said and Rosenberg, 1976), Secretin (Mutt et al, 1979), Bombesin (Dockray et al, 1979), Argininevasotocin (Milcu et al. 1963), Endorphins and Enkephalins (Nicoll et al, 1977). Besides there is also evidence for the presence of pituitary hormones such as MSH, GH, ACTH and Prl in the CNS (For reviews, see Bloom 1981; Iversen, 1983; McCann, 1983).

Table 1 gives a comprehensive list of neuro-peptides .

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TABLE 1. NEUROPEPTIDES

Pituitary peptides

- corticotropin (ACTH)
- growth hormone (GH)
- lipotropin
- α-melanocyte stimulating hormone (α-MSH)
- oxytocin
- vasopressin

Circulating hormones

- angiotensin
- calcitonin
- glucagon
- insulin

Gut hormones

- avian pancreatic polypeptide
- cholecystokinin (CCK)
- gastrin
- motilin
- pancreatic polypeptide (PP)
- secretin
- substance P
- neurotensin
- vasoactive intestinal polypeptide (VIP)

Opioid peptides

- dynorphin
- β-endorphin
- [Met]enkephalin
- [Leu]enkephalin
- kyotorphin

Hypothalamic releasing hormones

- luteinizing hormone releasing hormone (LHRH)
- somatostatin (SRIF)
- thyrotropin releasing hormone (TRH)
- Corticotropin releasing hormone (CRH)
- Growth hormone releasing hormone (GRH)

Miscellaneous peptides

- bombesin
- bradykinin
- carnosine
- neuropeptide Y
- proctolin

These 34 peptides have been described in neurons and nerve terminals within mammalian CNS other than those related to endocrine or neuroendocrine function (From Iversen, 1984),

SUBSTANCE P (SP)

Substance P is perhaps the most thoroughly studied to date of all the nonopioid neuropeptides. Several reviews (Nicoll et al, 1980; Pernow, 1983; Skrabanek and Powell, 1983) have appeared on its isolation, characterization and biological role. Substance P was first discovered by von Euler and Gaddum (1931) in extracts of brain and intestine but was not purified to homogenity until 1970 (Chang and Leeman, 1970). The isolation of SP was accomplished subsequent to the discovery of a sialogogic peptide in hypothalamic extracts (Leeman and Hammerschlag, 1967) which was shortly characterized as SP. The name substance P (for preparation) had been used in the laboratory of origin to designate the active agent in a particular preparation of tissue extracts. This nondescript term entered the literature in 1934 and has persisted (Gaddum and Schild, 1934). The amino acid sequence of SP was established in 1971 by Chang and Leeman as:

H_2N-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2.

It was prepared by Tregear et al, in 1971 permitting the development of precise methods of biochemical, histochemical, physiological and pharamacological studies of the peptide.

Distribution of Substance P

1, Central Nervous System: Substance P can be extracted from the brain of all vertebrate species from fish to mammals including man (Pernow, 1953; von Euler and Ostlund 1956; Laszlo, 1963). Substance P is present in numerous intrinsic neuronal pathways within the CNS. More than thirty different groups of SP containing neurons have been described in rat brain (Ljungdahl *ejt* al. 1978a, b). A particularly prominent projection originates from SP cells in the anterior striatum whose fibres descend to give rise to a dense terminal innervation of the substantia nigra. Several other SP containing neural pathways exist with particularly high densities of terminals in the hypothalamus and medial amygdala. Cerebral cortex and cerebellum contain very small amounts. Substance P is also present in amacrine cells of the vertebrate retina (Stell ,et al_, 1980).

2. Peripheral Nervous System: Immunocytochemistry and RIA have confirmed that SP is present in high concentration in certain primary sensory neurons and neurons intrinsic to the gastrointestinal tract (Jessell, 1982; Otsuka .et ^1, 1982). Numerous SP containing cell bodies are also present in spinal ganglia at all levels (Httkfelt et al. 1975a, b) as well as in the jugular ganglia (Httkfelt et al, 1977; Katz and Karten, 1980).

Within the gastrointestinal (gi) tract immunohistochemical studies have revealed the presence of SP in a scattered system of endothelial cells in the mucosal lining of the small and large intestine and also within intrinsic neurons of the enteric nervous system (Pearse and Polak, 1975; Schultzberg et al, 1980; Costa et, al, 1982).

3. Circulation: In some animals such as the guinea pig SP containing sensory fibres are found practically in every vascular bed. SP like immunoreactive nerve networks are present in the adventitia and at the border between the adventitia and blood vessels in the skin (Hokfelt et al, 1975b), dental pulp, gi tract (Schultzberg et al, 1980) and in the brain (Furness et al, 1982). High density of SP are found in the aorta and vena cava close to the heart (Furness et al. 1982).

4. Organs and Tissues: Substance P fibres are present in most peripheral tissues. Human skin (Dalsgaard et al, 1983), hind paw of cat (Hokfelt et al, 1975b; Cuello et al, 1977) contain SP immunoreactive fibres. Substance P positive fibres are also seen in the connective tissue of the tongue under the epithelium, particularly in the taste buds (Lundberg et alt 1979; Nishimoto et al, 1982). Substance P has also been partially

characterized from the rat pituitary gland (De Palatis et al, 1984).

Recent research has detected SP immunoreactive nerves in the male and female genitalia and reproductive organs of several mammalian species. SP like immunoreactivity was particularly concentrated in the glans penis and cervix. More SP-immunoreactive nerve fibres were found in the female gentalia than in the male. In the genital organs these nerves innervate the cervix, vagina, uterus and the fallopian tube of all the species studied and the ovary of the cat (Gu et al, 1983).

Biological Actions of Substance P

Central Effects

1. Neurochemical Effects: A number of observations indicate a functional interaction between SP and classical transmitters. Thus injection of SP into the rat cerebral ventricle stimulates the synthesis of dopamine, noradrenaline and 5-HT in various parts of the brain (Magnusson et. *al*, 1976). Substance P increases dopamine metabolites and ^{3}H-dopamine release from the ipsilateral striatum after infusion into the substantia nigra (Cheramy et al, 1978). SP also excites cells in the

noradrenergic locus ceruleus when applied locally (Davies and Dray, 1976; Guyenet and Aghajanian, 1979). These findings suggest that a possible function of SP is to control cerebral adrenergic systems.

2. Behavioural Effects: Intracerebroventricular injections of SP in rabbits and cats elicited various neurotropic effects such as inhibition of spontaneity, licking and stupor and a long lasting stimulation of respiration (von Euler and Pernow, 1956). Behavioural studies have shown that microinfusions of SP in the region of the dopaminergic neurons in the ventral segmentum of rat brain elicit an amphetamine-like behavioural response which appears to be due to activation of forebrain dopamine pathways (Stinus et al, 1978; Iversen et al, 1979; Kelley et al, 1979).

3. Antinociceptionj Cells which respond most vigorously to painful stimuli are also the ones which respond particularly sensitively to applied SP (Henry, 1982; Hall and Stewart 1983; Matsumura et al, 1985). From such findings the hypothesis has arisen that SP may be associated with sensory fibres that carry pain information.

4. Release of Anterior Pituitary Hormones: Substance P is involved in the control of hormone secretion from the anterior pituitary. It stimulates the release of growth

hormone when given both intraventricularly (Vijayan and McCann, 1980) and intravenously (Rivier et al, 1977) but has no effect on GH release from pituitary gland cells incubated in vitro (Rivier et al, 1977). Intraventricular SP stimulated the release of Prl and LH (Vijayan and McCann, 1979a).

Peripheral Effects

1. Effects on Vasculature: Substance P is one of the most potent hypotensive agents known. It produces hypotension due to peripheral vasodilation. This hypotensive effect of SP is not blocked by atropine, antihistamine or ganglionic blocking agents (Pernow 1953; Lofstrom et al, 1965).

2. Effect on Gastrointestinal tract: Substance P was found to effectively stimulate smooth muscle in all parts of the gi tract. The spasmogenic effect of SP was not blocked by atropine (Pernow, 1953) antihistamines (Douglas et al, 1982) or 5-HT antagonists (Fuxe et al, 1979) which suggests that SP acts directly on smooth muscles. Substance P also stimulates contraction of smooth muscles in the isolated uterus, ureter and urinary bladder of mammals, birds and amphibia (von Euler, and Pernow, 1956).

3. Secretory Activity: Systemic administration of SP in doses higher than 0.5 pmol/kg in the rat starts a secretory response within a few minutes. The effect is dose dependent and a fifty fold increase in salivary flow occurs (Liang and Cascieri, 1979). The salivary action appears to be a direct action of SP on the glands since it is not blocked by atropine, propranolol or phenoxybenzamine (Leeman and Hammerschlag, 1967).

Infusion of SP increases pancreatic juice secretion by 160% in the dog (Thulin and Holm, 1977). Substance P also affects the endocrine pancreatic secretion. In vitro (Effendic et al, 1977) and in vivo (Brown and Vale, 1976; Lundquist et al, 1978) studies have shown that SP inhibits the release of insulin induced by glucose and arginine, as well as the basal plasma insulin.

4. Hypocholesterolemic Effect: Intravenous pulse injection of SP reduces circulating cholesterol concentrations and also antagonizes the hypercholesterolemic action induced by NT (Raju and Vijayan, 1981).

Metabolism of SP: Substance P is synthesized initially as part of a larger polypeptide precursor from which the peptide is derived by proteolytic cleavage (Harmar et al, 1981). Several organs have a high capacity to inactivate SP.

Kidney homogenates seem to be the most effective, followed by spleen, liver and intestine (Eber and Lembeck, 1956, Ward and Johnson, 1978). It is rapidly inactivated in the brain, blood and other tissue by proteolytic enzymes particularly the so called SP-degrading enzyme (Lee et al, 1981).

NEUROTENSIN

In the course of purifying SP from bovine hypothalamic extract, Leeman and her colleagues detected a second bioactive principle that eluted before SP activity on ion-exchange chromatography. This fraction produced vasodilation, hypotension and increased vascular permeability but unlike SP did not produce salivation. It was named 'neurotensin' because of its localization in neural tissue and hypotensive properties. It was isolated and sequenced by Carraway and Leeman (1975a) as:
pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu.

DISTRIBUTION OF NEUROTENSIN

1. Central Nervous System: Using RIA and immunocytochemical techniques high density of NT containing cell bodies and nerve fibres are found in the hypothalamus with lesser amounts present in the limbic system and brain

stem nuclei (Uhl et al, 1977; Roberts et *al*, 1982). Neurotensin like immunoreactivity (NTLI) has also been detected in amacrine cells of the retina and in the anterior pituitary (Bredna et, *al*, 1981).

2. Peripheral Nervous System: In the spinal cord NT is distributed in a characteristic pattern (Gibson et al. 1981). Both nerve terminals and fibres are found almost exclusively in the upper laminae of the dorsal horn. Immunoreactive NT fibres are present in two distinct bands (Polak and Bloom, 1982).

Most of the body's NT occurs in the gi tract. Neurotensin like immunoreactivity has been detected in the gi tract of various animal species, ranging from deuterostomian invertebrates to mammals (Helmstaedter et al,. 1977; Polak et al, 1977). Enteric nerve fibres containing NTLI are found in the circular and longitudinal musculature of the stomach, caecum and in the myenteric plexuses of the oesophagus, stomach and duodenum
(
et; al, 1980). The major portion of gut NT however, occurs in endocrine cells of the gi mucosa (Helmstaedter et al, 1977).

3. Circulation: In the plasma too, NT can be detected at levels comparable to other hormones. Most of this NT

represents authentic peptide although other unde fined substances possessing carboxyl terminal homologies with NT are also present (Carraway et al, 1980).

Biological Actions of NT

Central Effects

1. Hypothermia: Centrally administered NT produces a dose dependent fall in the body temperature in rodents. This hypothermic effect of NT can be elicited by nanomolar concentrations of NT and appears to be mediated at sites within the CNS as it does not occur after peripheral administration of the peptide (Dorsa et al, 1979).

2. Antinociception: Neurotensin in various brain areas is thought to be involved in pain perception. Neurotensin decreases reactivity to painful stimuli when administered centrally (Clineschmidt and McGuffin, 1977).

3. Neurochemical Effects: Many of the centrally mediated effects of NT may be a consequence of its interaction with other brain neurotransmitter systems. Neurotensin has several pharmacological properties in common with a class of dopamine antagonists, the neuroleptics (Nemeroff et al, 1982). Microiontophoric application

of NT decreases the activity of dopamine sensitive neurons (McCarthy et al, 1979). Besides dopamine, noradrenaline and acetylcholine systems also respond to NT administration. Neurotensin enhances both the spontaneous and potassium stimulated release of noradrenaline from hypothalamic slices (Okuma and Osumi, 1982).

4. Hormone Release: Centrally administered NT has been shown to alter the release of hormones from the hypothalamus and pituitary gland. Neurotensin, injected into the third ventricle reduced basal and cold stimulated Prl and also LH release (Maeda and Frohman, 1978; Vijayan and McCann, 1979). Peripheral injection of NT on the ether hand increased both LH and Prl (Vijayan and McCann, 1979a). Neurotensin increases somatostatin while decreasing the levels of growth hormone (Maeda and Frohman, 1978; Abe et al, 1981). In contrast peripheral injection of NT increases plasma GH concentrations (Rivier et al, 1977).

Peripheral Effects

1. Smooth Muscle: Neurotensin is a potent vasodilator and hypotensive agent. It dilates both cutaneous and intestinal blood vessels (Carrawav and Leeman, 1973; Rosell et al, 1976). Peripheral administration of NT

reduces blood pressure in rats and rabbits (Carraway and Leeman, 1973). Pretreatment of animals with compound 48/80 which depletes mast cells of their amine content abolishes the hypotensive action of NT (Quirion et al, 1980). Thus NT may reduce blood pressure because of its ability to release histamine and other vasoactive substances from mast cells (Chahl and Walker, 1981).

Neurotensin exerts potent effects on smooth muscles of the gi tract. The peptide appears to have a biphasic role having components of both relaxation and contraction (Kitabgi and Freychet, 1978). A predominant effect of NT in the gi tract is the inhibition of peristaltic activity. For example, the peptide inhibits motor activity in the stomach, duodenum and colon (Thor et al, 1980).

2. Gastric and Pancreatic Secretion: Neurotensin inhibits the secretion of gastric acid upon peripheral administration. Intravenous infusions of neurotensin reduce gastric acid secretion elicited by pentagastrin (Andersson et al, 1980).

3. Hypercholesterolemia: Neurotensin raises circulating cholesterol level of the rat in a dose dependent fashion (Peric Golia et al, 1979; Raju, 1980; Raju and Vijayan,

1981). This hypercholesterolemic effect of NT is not altered by prior adrenalectomy or hypophysectomy (Peric Golia et al, 1979) but is diminished by 4-APP or SP (Raju, 1980).

4. Hyperglycemia: Another potent effect of NT is its ability to elevate blood glucose concentrations (Brown and Vale, 1976; Carraway et al, 1976; Nagai **and** Frohman 1976). It also raises glucagon levels in the blood (Nagai and Frohman, 1976).

Other peripheral effects of NT include transport of ions across the intestine (Mitchenere et al, 1981} Kachur et al, 1982) and interactions with mast cells to release histamine (Kurose and Saeki, 1981; Rossie and Miller, 1982).

Metabolism of NT: Neurotensin is hydrolyzed by enzymes present in brain and pituitary fractions including synaptosomes to give smaller fragments (McDermott et al, 1982; Checler et al, 1983). The (9-13) fragment is rapidly hydrolyzed and it has been shown that the C-terminal region of the peptide is essential for NT binding to central receptors (Quirion, 1983). The major route of degradation appears to be due to cleavage of the Arg-Arg bond (McDermott et al, 1983a). Plasma NT is very complex

being composed of NT as well as multiple variants and breakdown products (Carraway, 1982). Atleast 2 forms of NT occur in the blood, one of which is the N-terminal octapeptide (Aronin et al, 1982). Neurotensin can be biotransformed to 2 active N-terminal sequences by a specific trypsin like enzyme and the proline endopeptidase, respectively (McDermott et al, 1982, 1983a, b; Griffiths and McDermott 1984).

NEUROPEPTIDES AS NEUROTRANSMITTERS AND/OR MODULATORS

All the neuropeptides occur in particularly high concentrations in the terminals of the neurons which contain them. In vitro experiments have shown that these peptides can be released from nerve terminals in responses to depolarizing stimuli by a calcium dependent mechanism. This strongly suggests that the neuropeptides are normally secreted from neurons and that they act as transmitters or as some other form of chemical messengers within the CNS.

However, the utilization of a peptide by a nerve cell differs fundamentally from the utilization of an amino acid or a monoamine in the way the nerve cell synthesizes the substance and conserves it. A neuro-transmitter such as GABA, serotonin or dopamine is made

in a short series of steps from an amino acid in the diet by the action of enzymes. The need for the synthesis of the transmitter is somewhat reduced since the transmitter can be released and reabsorbed by the synaptic vesicles as and when the need arises. On the other hand, the peptide can be synthesized only by ribosomes. All the peptide neurotransmitters examined so far are synthesized first as a larger peptide. The active form of the molecule is produced through progressive cleavage by enzymes.

The release of a peptide neurotransmitter from an axon terminal is remote from the place where the peptide is made. The active form of the peptide which is stored in synaptic vesicles has to be transported to the site of release. Thus a cell releasing a peptide is unable to act on another cell repeatedly in a short span of time. In contrast, axon terminals of a monoamine or aminoacid releasing transmitter are capable of manufacturing their own transmitter. Therefore these terminals are lesser in their need for replenished supplies of fresh neurotransmitter from moment to moment.

Another way in which a peptide messenger differs from a monoamine neurotransmitter lies in the molecular details of how the peptide influences its target cells.

Classical neurotransmitters are excitatory or inhibitory. The neurotransmitter alters the permeability of the target cell membrane to ions of potassium, sodium or calcium. Consequently, the concentration of these ions inside and outside the target cell is different which gives rise to a voltage gradient across the membrane. The action of an excitatory neurotransmitter tends to diminish the voltage gradient i.e., it depolarizes the membrane. An inhibitory transmitter, on the other hand, increases the voltage gradient i.e., it 'hyperpolarizes' the membrane. A peptide, however, sometimes tends to make the target cell less likely to respond to other signals. This cannot be considered as inhibition. In some cases, the peptide keeps an excitatory transmitter from depolarizing the membrane and in other cases it prevents hyperpolarization by an inhibitory transmitter. The peptide itself produces no visible change in the voltage gradient across the membrane but it Modulates' the activity of the target cell.

EVIDENCE FOR SUBSTANCE P AND NEUROTENS IN AS A NEUROTRANSMITTER/NEUROMODULATOR

Substance P: Lembeck in 1953 first suggested that SP might act as a sensory neurotransmitter. Otsuka et al (1982) established this fact when they demonstrated a

high concentration of SP in dorsal roots and showed its release from spinal cord preparations in vitro on electrical stimulation of dorsal roots (Otsuka and Konishi, 1975). Substance P thus fulfils the criteria expected of a sensory transmitter: it is released from spinal cord in a calcium dependent manner in response to stimulation of dorsal roots and it excites firing in spinal cord neurons when applied to them (Jessell, 1982; Otsuka et al, 1982).

The ability of SP to cause transient changes in pain sensitivity when administered intrathecally (Jessell, 1982) suggests that SP may be 'the pain transmitter'. However this hypothesis remains to be fully established.

A neuromodulator role for SP was also established by Otsuka et a1 (1982) who studied the effect of SP on sympathetic ganglion cells in the inferior mesenteric ganglion. They found that the excitatory effects of SP are very slow in onset and prolonged in duration and by themselves cannot cause depolarization to excite the cells. The effect however, is to render ganglionic neurons more readily excited by other excitatory afferent inputs - a clear case of 'neuromodulation'•

Substance P provides another example of modulation in its action on nicotinic mechanisms (Ryall,1982). In

Renshaw cells and adrenal chromaffin cells SP has little effect when applied alone but it is able to inhibit selectively normal responses to stimulation of nicotinic sites.

Neurotensin: Recent evidence indicate that NT subserves a neurotransmitter role in the CNS. Firstly, NT is predominantly localized to synaptosomes free of brain homogenates (Uhl and Snyder, 1977). Secondly, NT is released from the hypothalamic fragments after depolarizing by potassium, this release being calcium dependent (Iversen et al, 1978; Maeda and Frohman, 1981). Thirdly, NT appears to interact with specific binding sites on neural elements. These high affinity binding sites exhibit a pattern of distribution in the brain and spinal cord similar to that of NT (Uhl and Snyder, 1977; Young and Kuhar, 1981). Fourthly, the application of nanomolar quantities of NT to single neurons of the cerebral cortex, bed nucleus of the stria terminals and spinal cord produces a depolarization (Phillis and Kirkpatrick, 1980; Suzue et al., 1981).

Neurotensin may also subserve a neurotransmitter role in the gi tract as it does in the CNS. However, because of the apparent paucity of NT containing neurons in the periphery the peptide may more likely function as a paracrine mediator of local cell activity or as. a hormone.

SCOPE OF THE PRESENT INVESTIGATION

Neuropeptides perform several different functions apart from the one for which they were originally discovered. A peptide may be used as a endocrine hormone, a paracrine factor, a neuroendocrine releasing factor, a neuromodulator or a neurotransmitter in different parts of the body or in different species (Iversen, 1983). In spite of considerable amount of data on the distribution and actions of SP and NT our knowledge of the functions of SP and NT in various organs remain incomplete. The multiplicity and overlap of different systems containing NT, SP and other peptides have made it difficult to elucidate the functional role of these peptides. They coexist with classical neurotransmitters and their integration with cholinergic and other putative neurotransmitters is still not clear. The functional relationships between the action of these two peptides that may operate in as yet undisclosed activities remain to be ascertained.

The ubiquitous but selective distribution of SP and NT in the brain reveals that they possibly participate in several neuroendocrine functions one of them being regulation of hormones from the anterior pituitary (Maeda and Frohman 1978; Vijayan and McCann, 1979a). Since there is some disagreement between different reports documented

on the role of these two peptides on hormone release from the anterior pituitary, it was sought in the first instance to monitor in vivo changes in gonadotropin levels after intravenous and intraventricular injections of SP and NT in OVX rats. In vitro studies were also performed by incubating hemipituitaries with varying doses of the peptide to determine specificity of the peptide action on hormone release.

The mechanism of action of SP and NT in the brain is obscure. Several peptide hormones elicit their biological actions by activation of the adenylate cyclase complex which results in increased endogenous cAMP production. Since cyclic nucleotides have been mediators for the action of several hormones it was considered worthwhile to study the alterations in cAMP and cGMP in the brain after intraventricular and intravenous administration of these peptides in OVX and OVX estradiol-primed rats.

The physiological consequences of anterior pituitary hormone release by these peptides on the reproductive system are unknown and by and large remain speculative. Studies also reveal that a given peptide may be present in multiple sites including the reproductive tract (Kriegar and Martin, 1981). Several peptides such as somatostatin (Lundberg et al, 1980), LHRH (Hsueh and Jones, 1981), ACTH

(Tsong et al, 1982), VIP (Goodnough et a1, 1979; Aim et al, 1980; Otte sen et al, 1982), p-endorphins (Tsong *et* al, 1982; Quigley et al, 1980) are present in the reproductive tract where their presence has proved a physiological role. Recent immunohistochemical investigations have indicated the presence of SP (Aim et al, 1978; Lundberg et al, 1980; Bucsics et al, 1983; Gu et al, 1983) in the reproductive organs of several species. More SP was present in the female reproductive tract than that in the male. In addition, age and sex related changes in anterior pituitary SP (De Palatis et al, 1982) have been reported. The SP content of the anterior pituitary was altered with changes in the estrous cycle (Antonowicz et al. 1982; Coslovsky et al, 1984). Binding sites of SP to the anterior pituitary varied during the estrous cycle (Kerdelhue et al, 1985). Substance P has also been shown to inhibit ovulation when infused into the cerebral ventricle (Naziemblo et al, 1983). Since NT and SP are closely related peptides it was contemplated that both these peptides may have a potential role in reproductive function. The effect of these peptides on some parameters of growth such as DNA, RNA and protein content and ^{3}H-thymidine incorporation into DNA was studied in immature rat uterus. Experiments were also performed to determine whether PMSG induced growth of immature rat uterus and ovaries is influenced by these peptides.

There have been some studies in recent times on the extra pituitary and extra hypothalamic action of a host of peptides, particularly GnRH (Hsueh and Erickson, 1979a,b; Sharpe, 1980; Lee and Brownstein 1981). It was therefore of interest to test if these peptides had any direct effect on the uterus after intrauterine administration. Studies were performed to test the potential effects of these peptides during pre and post implantation period.

Another fascinating feature, is the role these peptides play in regulating serum cholesterol, which is an obligate precursor for steroid hormone synthesis. Previous studies in our laboratory have shown that intravenour SP causes hypocholesterolemia while NT causes hypercholesterolemia (Raju and Vijayan, 1981). It has been suggested that NT plays a role in lipid homeostasis. It was therefore interesting to probe into this aspect further by studying the lipid profile in plasma and liver after intravenous and intraventricular injections of SP and NT in OVX-steroid primed rats. The entire lipid content, free and osterified cholesterol, triglycerides and fatty acids were evaluated. 4-APP a drug that selectively inhibits hepatic lipoprotein secretion of cholesterol (Shiff, 1977) was used to determine the specificity and possible site of action of these peptides in modifying circulating cholesterol levels.

CHAPTER II

EXPERIMENTAL PROCEDURES, MATERIALS AND METHODS

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ANIMALS

All the experiments were performed in colony bred rats, derived from Wistar strain, raised in our animal facility. Immature and adult female, ovariectomized (OVX), OVX-estrogen primed and pregnant rats were used. The rats were given water and standard rat pellet (Pragati Animal Feeds, Hyderabad) ad libitum. They were housed in an air conditioned room (25+2°C) and a regimen of 14 hrs light and 10 hrs dark cycle was maintained.

SURGICAL PROCEDURES

a. Ovariectomy: Adult female rats weighing 180-200 g were bilaterally ovariectomized under light ether anaesthesia using semi-sterile conditions and used 2-3 weeks after ovariectomy.

b. Implantation of third ventricular (ivt) cannula: Cannulae were prepared from 23 gauge stainless steel tubing (Small Parts Inc. Miami, Florida, USA). Each cannula was 17 mm in length and had a flat tip with a

beveled edge and was provided with a mandril to prevent its obstruction. Cannulation was performed as described by Antunes-Rodrigues and McCann (1970). The animals were anaesthetized with nembutal (Abbot Labs, USA 40 mg/kg ip) and the rat's head was fixed on a David-Kopf rat stereotaxic instrument. The following coordinates were used for the third ventricle: anterior-posterior = 1.3 mm behind bregma, lateral = just on the midline (above the superior longitudinal sinus) and vertical = 0.3 mm above the base of the skull. The cannula was mounted in the stereotaxic instrument with the aid of a stainless steel wire fitting exactly its inside diameter which served as the cannula guide. Two small holes were drilled in the adjacent parietal and frontal bones. Brass microscrews were screwed firmly into these holes to serve as an anchor for the dental cement. The cannula was introduced through a hole over the superior longitudinal sinus after the sinus was pulled gently to the left side with a hypodermic needle. This procedure prevented rupture of the sinus and consequent hemorrhage. The cannula was lowered to the skull base and then raised 0.3 mm. Its location in the 3rd ventricle was confirmed when cerebrospinal fluid (CSF) flowed continuously from the cannula. A small amount of dental cement was placed around the cannula and screws. Finally, after removing the guider mounted in the stereotaxic instrument an additional amount of cement was added around the cannula to cover the screws completely. A small amount of neosporin

ointment was mixed along with the dental cement to prevent bacterial infection. The cannula was now firmly fixed into position. The skin was left unsutured and the animals were returned to their own indivual cages until the day of the experiment. The mandril was removed every day rinsed in isotonic saline and returned to its original position so as to maintain the cannula clean and to get the animal acquainted with the experimental procedure to be followed (Vijayan and McCann, 1978a; 1979a,b).

c. Implantation of intravenous (iv) cannula: Indwelling silastic catheters were introduced into the external jugular vein using the technique of Harms and Ojeda (1974) as described by Vijayan and McCann (1979a,b). Cannula made of silastic sheets and tubing (Dow Corning, USA) was placed in the superior vena cava, adjacent to the heart, via the external jugular vein after the animal was anaesthetized with ether. The external end of the cannula was guided under the skin to emerge at the back of the head, and was then sutured in place.

d. Intraventricular Injection: Six to seven days after the cannulation, third ventricular injection of the peptide and/or control medium was performed according to the following procedure. The mandril was removed and the inner cannula of the same length was introduced. The

cannula was connected by a polyethylene plastic tube to a 10 µl Hamilton microsyringe filled with test material, freshly dissolved in saline, to be injected. After 60 sec with the inner cannula in its position in the animal's brain, the test solution was injected slowly in a volume of 2.5 μl while the animal was freely moving around. The inner cannula was removed approximately 60 sees later so that the entire test substance reached the third ventricle.

e. Intravenous injections: Intravenous injections were made twentyfour hours after implantation of the silastic catheter. On the day of the experiment an extension of polyethylene tubing (PE 50, 12" in length), filled with heparin in 0.9% NaCl, was attached to the distal end of the jugular cannula and the animals were left undisturbed for 30-60 min. During this time a preinjection blood sample (0.5-0.7 ml) was withdrawn over a period of 60 sec. Test substances dissolved in 0.9% saline were administered by iv pulse injection in a volume of 0.1 ml using a 1 ml syringe. Heparinized blood samples (0.5-0.7 ml) were collected from the external jugular vein cannula at varying intervals while the animal was freely moving in the cage. The volume of all samples was replaced immediately after each bleeding by an equal volume of saline. Plasma was separated by centrifugation at 4°C and stored frozen until the day of assay.

In some experiments, where sequential sampling of blood was not necessary, intravenous injection of test substances was performed directly into the external jugular vein which was exposed after the animal was lightly anaesthetized with ether.

f. Intrauterine injection: Rats were anaesthetized with ether, an incision was made in the peritoneum and uterine horns were exposed. Intraluminal injections of test substance and/or control medium were made near the utero-tubal junction in either horn using a Unimatrix syringe in a volume of 10 *l*. The incision was sutured and the skin closed by wound clips.

CHEMICALS

Kits for RIA of LH, FSH and Prl were obtained from the National Pituitary Agency, National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Bethesda, USA. Kits for the assay of cAMP and cGMP were purchased from Radiochemical Centre, Amersham, England, Neurotensin, Sephadex G-75, sodium azide, chloramine-T, heparin, bovine serum albumin, calf thymus deoxyribonucleic acid (DNA), yeast ribonucleic acid (RNA), rabbit liver glycogen, 2,5-diphenyloxazole (PPO), l-4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene (POPOP), hyamine hydroxide, triolein, compound 48/80, amino-pyrazolo (3,4d) thymidine

(4-APP), estradiol benzoate, pregnant mare serum gonodotropin (PMSG), prolactin (Prl), luteinising hormone (LH) and follicle stimulating hormone (FSH) were purchased from Sigma Chemical Co., St. Louis, USA. Substance P was procured from Sigma Chemical Company, St. Louis, USA and from Peninsula Laboratories, Belmont, USA. Anti Rabbit-Gamma -Globulin (ARGG) and Luteinising hormone-releasing-hormone were purchased from Calbiochem Laboratories, USA. Medium 199 was obtained from Difco Laboratories, USA. Pimozide was procured from Jenssen Pharmaceuticals, Belgium. [H]-thymidine (sp. activity 22, 600 mCi/mmol) was bought from Bhabha Atomic Research Centre, Trombay, India. All other chemicals were of analytical grade and were purchased locally.

ASSAY OF HORMONES

Plasma levels of LH, FSH and Prl were measured by radioimmunoassay (RIA) using a double antibody procedure as standardised in our laboratory (Babu, 1982). Radioimmunoassay kits for rat LH, FSH and Prl were obtained from the NIAMDD-NIH pituitary hormone distribution programme. Radioimmunoassay was performed according to the guidelines provided with the kit for each hormone.

RIA OF LH: The LH kit consisted of

1. Rat luteinising hormone antigen, highly purified for iodination NIAMDD-rLH-15.
2. Rat luteinising hormone antiserum (rabbit) NIAMDD-anti-r-LH-S-6.
3. Rat luteinising hormone reference preparation (NIAMDD-r-LH-RP-1).

Preparation of the Gel: Five grams of sephadex G-75, was added to 100 ml of phosphosaline buffer (PBS) (0.01 M $P0_4$, 0.05 M NaCl, 0.1% sodium azide, pH 7.6) and stirred for 30 minutes using a magnetic stirrer. It was(a) kept in a boiling water bath for 5 hours (b) allowed to stand 72 h at room temperature (c) stored in a refrigerator upto 4 months (d) placed at room temperature for 24 h before use.

Preparation of the Column:

I* Ten ml glass pipettes were used. The mouthpiece was cut off.

2. Tubes were scrupulously cleaned with chromic acid, hot water, tap water and double distilled water and dried.

3. A three way stopcock (Pharmaseal, Puerto Rico, USA) was attached to the tube by a 4 cm long latex tubing. Glass wool was placed in the tip of the tube.

4. The tube was washed twice with phosphosaline buffer and filled upto the 7 ml mark.

5. The gel was continuously stirred using a magnetic stirrer to keep the suspension homogeneous.

6. The gel was pipetted from the bottom of the flask as a well mixed slurry. When settling was under way, the outlet was opened and allowed to run freely. The slurry was continuously added as needed. The top was never allowed to settle before adding more slurry. The column was filled to a height of 15-20 cm. About 2 ml of buffer (PBS) was left at the top of the column. On the day of iodination (maximum 4 h before use) the column was equilibrated with 1 ml of *2yi* bovine serum albumin (BSA) in PBS and then washed with PBS. After a single use the column was discarded.

Iodination of Rat LH:

Reagents:

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l. ^{125}Iodine, carrier free, as sodium iodide with specific activity of 400 mCi/ml suitable for iodination of protein.

2. 0.5 M sodium phosphate buffer, pH 7.6.

3. Chloramine-T(5 mg/10 ml of 0.05 M $P0_4$, pH 7.6 buffer)

4. Sodium metabisulfite ($Na_2\$_20_5$) 25 mg/10 ml of 0.05 M $P0_4$, pH 7.6 buffer).

Chloramine-T and sodium metabisulfite were prepared freshly just prior to use.

1 mCi ^{125}I was added to a small disposable glass vial used as the reaction vessel. 25 ul of 0.5 M $P0_4$ buffer pH 7.6 was added. 2 ug of NIAMDD Rat LH-I-5 in 20 µl of $P0_4$ buffer was added next. 10 µl of chloramine T was then added. The vial was then agitated for 50 seconds after which 25 µl of sodium metabisulfite was added. The entire reaction mixture was applied to the sephadex G-75 column. The column was then eluted with phosphosaline (0.01 $P0_4$, 0.15 M NaCl buffer, pH 7.6). Fractions of 0.5 ml were collected in test tubes containing 50 microliters of 2% BSA in PBS buffer. These fractions were counted in a Packard autogamma scintillation spectrometer. Two peaks of radioactivity were detected. The first peak began at tubes 3-4 and trailed off by tube 6. A second peak containing free ^{125}I began at about tube 7. The iodinated rat LH was contained in the first peak (tubes 4-5). The fraction high on the trailing shoulder of this peak (tubes

4 and 5) contained the most immunoreactive and least damaged rat LH. This fraction was added to buffer in order to give 10,000 cpm per 100 µl, and stored at -20°C, until use.

Double antibody RIA procedure: The following steps were performed in sequence for the assay of plasma LH.

1) 10 x 75 mm disposable test tubes were used.

2) Buffer (1% BSA in 0.01 M $P0_4$, 0.15 M NaCl, 0.1% sodium azide, pH 7.6) was added to each tube in sufficient quantity to produce a final volume of 0.7 ml.

3) a. 25 *µ1* plasma to be assayed was added or
b. The reference preparation (NIAMDD -Rat-LH-RP-1) was dissolved in 1% BSA in phosphosaline and added in doses ranging from 1000 ng to 1 ng per tube, in sufficient detail (1000, 500, 250, 100, 50, 25, 10, 5, 2.5 and 1 ng) so that the entire curve can be constructed graphically.

4) Iodinated rat LH was added such that approximately 10,000 cpm were contained in 100 µl of 0.1% BSA-phosphosaline buffer.

5) 200 µl of the antiserum (NIAMDD-Rat-LH-S-6) in a final dilution of 1:40,000 in *3%* normal rabbit serum

(NRS)-0.05 M EDTA-PBS was added (at these dilutions, the antiserum was observed to bind 2b% of the labelled rat LH (B = 2/10x 100, see below),

In some tubes 200 µl buffer and 200 µ1 3% NRS-EDTA-PBS and 100 µ1 label were added to serve as background.

In a few tubes 200 µl buffer and 100 µl label and 200 µl antiserum were added to serve as zero (100% binding = Z).

In 2 or 3 tubes 100 µ1 label was taken to get the total counts (TC).

Tubes were agitated on vortex mixer.

Tubes were incubated for 24 hrs at room temperature.

At the end of this period, 200 µl of goat anti rabbit gamma globulin (ARGG) was added to precipitate maximally the antibody bound labelled rat LH.

Tubes were agitated on a vortex mixer.

Tubes were again incubated for 24 hrs at room temperature.

At the end of this incubation period all tubes were centrifuged at 1000 g for 30 min in a refrigerated centrifuge. The supernatant was discarded and the precipitate was counted in a gamma spectrometer.

The unknown samples were compared to the percentage of counts precipitated with the rat LH reference preparation, NIAMDD rat LH-RP-1. A curve was constructed

on semilogarithmic paper, and the unknown read directly from the curve obtained with LH-RP-1. Results are expressed as nanograms (ng) of rat LH-4-1 per ml of plas

RIA OF FSH: The following were provided with the FSH kit

1. Rat FSH antigen NIAMDD-r-FSH-I-5, highly purified for iodination.

2. Rat FSH antiserum (Rabbit) NIAMDD-Anti-r-FSH-S-11.

3. Rat FSH reference preparation NIAMDD-r-FSH-RP-1 (Biological potency = 150 x NIH-FSH-S1) (HCG augmentation assay).

Iodination of rat FSH: Iodination was performed as for LH except that 10 mg/ml of chloramine T was used.

Double antibody RIA procedures; Procedure was same as LH except reference preparation (FSH-RP-1) was dissolved in BSA phosphosaline in doses ranging from 2000ng to 10 ng (2000, 1000, 500, 250, 100, 50, 25, 10 ng). FSH antiserum was used at a dilution of 1:2500.

RIA OF Prl: The RIA kit for Prl consisted of

1. Rat prolactin antigen NIAMDD-r-Prl-1-5, highly purified for iodination.

2. Rat prolactin antiserum (rabbit) NIAMDD-anti-r-Prl-S-8.

3. Rat Prl reference preparation NIAMDD-r-Prl-RP-2. [(Biological potency = 30 iu/mg (pigeon local crop ay of Nicoll)].

Iodination: as for LH.

Double antibody procedure; As per LH and FSH except the reference preparation was diluted in PBS in a range of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 200 ng and antiserum was diluted so as to get a final dilution of 1:12,500.

As far as possible samples from a particular experiment were run in one assay, each in duplicate, to avoid interassy variation. In our laboratory the sensitivities of the assay were 5 ng LH, 10 ng FSH and 0.25 ng Prl. The inter and intrassay co-efficients of variation were 10 and 6% for LH, 9 and 5% for FSH and 10.4 and 5.5% for Prl respectively.

In vitro Hormone Assay: Various doses of neuropeptide were incubated with hemipituitaries in vitro. After decapitation anterior pituitaries were removed quickly, bisected longitudinally and kept in incubation flasks containing 2 ml of tissue culture medium 199, pH 7.4. Each flask contained two hemipituitaries. After a 30 min preincubation in a Dubnoff metabolic shaker at 37°C in an atmosphere of 5% C02 and 95% the medium was replaced with 2 ml of fresh medium containing the test substances and the incubation was continued for 3 hours. At the end

of the incubation period media were removed and stored frozen for later radioimmunoassay of gonadotropin and Prl as described above. Values are expressed as ng hormone released per ml of medium.

ASSAY OF CYCLIC AMP AND CYCLIC GMP

Preparation of tissue extract: The rats were killed by decapitation. Brains were removed quickly and frozen in liquid nitrogen. Hypothalami were dissected out according to the procedure of Glowinski and Iversen (1966) as a single block which included the pre-optic area and was limited laterally by the hypothalamic fissures and posteriorly by the mamillary bodies. The upper section of the block was cut 3-4 mm from the basal surface of the hypothalamus which included medial basal hypothalamus (MBH), pre-optic area (POA) and median eminence (ME) (Vijayan, 1974).

The frozen hypothalami were weighed and transferred to a precooled glass homogenizer with a teflon pestle (Potter-Elvehjem type). Two volumes of ice cold 10% trichloroacetic acid were added and the mixture was immediately homogenized. After it had stood for 15 min at 0 it was centrifuged for 30 minutes at 10,000 RPM at -4°C. The supernatant solution was transferred to a glass centrifuge tube and extracted 4 times with 3 volumes of

water saturated diethyl ether and twice with 3 volumes of Tris-HCl (pH. 7.5) saturated diethyl ether. Following the last ether extraction the solution was heated in a water bath at 100 C till the odour of ether disappeared. The volume of all the extracts was made up to 2 ml with Tris-EDTA buffer and the pH adjusted to 7.5. Cyclic AMP and cGMP in the neutralized extracts are stable indefinitely at -20°C.

Cyclic AMP Assay Procedure: The Radiochemical Centre, Amersham cAMP assay kit combines the high specificity and affinity for cAMP of a highly purified and stabilized binding protein with an improved charcoal separation step at 0 C. The assay was performed in small tubes suitable for centrifugation. All assay tubes were kept at 0°C in an ice bath. To the assay tubes containing the standard or the unknown, 50 μl of labelled [H]-cAMP (0.25 µCi) and 100 µl of the binding protein were added. Known amount of cAMP (1 pmol -16 pmol) and aliquots of unknown samples were added to the assay tubes. Typically, a volume of 50 µ1 was reserved for these components. To determine blank counts 150 µ1 of 0.05 M Tris-EDTA buffer pH 7.5, containing 4mM EDTA was taken in duplicate tubes containing labelled cAMP. All the tubes were vortex mixed for about 5 seconds and then the ice bath containing the tubes was left in a cold room at 2-4°C for 2 hours. At the end of this period

100 μl of charcoal suspension was added, mixed briefly and the tubes were replaced in ice bath. The tubes were centrifuged for about 5 minutes in a refrigerated centrifuge to sediment the charcoal. The tubes were centrifuged not less than one minute nor more than six minutes after addition of charcoal. Without disturbing the sediment a 200 μl sample was removed and placed in scintillation vials containing Bray's mixture (4 g PPO, 0.2 g POPOP, 60 g napthalene, 20 ml ethylene glycol, 100 ml of methanol made up to 1 litre with dioxane). The vials were counted in a Beckman liquid scintillation spectrometer. The ratio of cpm bound in the absence of unlabelled cAMP (C_0) to the cpm bound in the presence of standard or unknown unlabelled cyclic AMP (C) was calculated. From the C_0/C value for an unknown sample, the number of pmoles of cAMP were calculated using the standard curve. The cAMP activity is expressed as pmoles/mg of tissue.

Assay of Cyclic GMP: The Radiochemical Centre, Amersham, cGMP RIA kit combines the use of a specific antibody with tritiated cGMP at high specific activity and provides a simple, sensitive and specific assay for cGMP. The assay is based on the competition between unlabelled cGMP and a fixed quantity of the tritium labelled compound for binding to an antiserum which has a high specificity and affinity for cGMP. The amount of labelled cGMP bound to

the antiserum is inversely related to the amount of cGMP present in the assay sample. Measurements of the antibody bound radioactivity enables to determine the amount of unlabelled cGMP in the unknown sample. Separation of the antibody bound cGMP from the unbound nucleotide is achieved by ammonium sulphate precipitation, followed by centrifugation. The precipitate is dissolved in water and its activity determined by liquid scintillation counting.

The assay was performed in small tubes which were suitable for centrifugation. The tubes were kept in an ice bath. 50 µ1 of tritiated cGMP were added to each tube. To two tubes 100 µl of Tris-EDTA buffer was added to determine zero-dose binding. To two other tubes 100 µl of blank reagent provided with the kit were added to determine assay blank. To the rest of the tubes 100 µ1 of standard (0.5 pmol to 8.0 pmol) or unknowns were added. To each tube 50 µl of antiserum was added. The tubes were vortexed for a few seconds and the ice bath containing the tubes was placed in a cold room at 2-4°C for one and a half hours. At the *end* of this period, one ml of ice-cold $(NH4)_2S0_4$ (60% saturated solution with water) was added to each of the tubes. The tubes were vortexed again and returned to the ice bath. Five minutes after the addition of (NH_4) $S0_4$ the tubes were centrifuged at 0°C. The supernatant liquid was decanted and the tubes were left upside down on a tissue to drain.

Excess liquid remaining on the walls of the tube was wiped with tissue and dissolved in 1.0 ml of distilled water and 100 µl of hyamine hydroxide. The tubes were capped and vortexed until all the precipitate was dissolved. The sample was then transferred to scintillation vials containing Bray's fluid and counted in a liquid scintillation spectrometer. Blank counts per minute were determined by averaging the CPM for the blank tubes. Co (CPM bound in the absence of unlabelled cGMP) was determined by averaging the CPM in the 'Zero-dose' tubes and subtracting the blank CPM. Cx (CPM bound in the presence of standard or unknown cyclic GMP) was determined by averaging the CPM for the remaining pairs of tubes and subtracting the blank CPM. C o/Cx was calculated for each level of standard cyclic GMP and for the unknown. A graph was plotted for C0/Cx against pmol of standard cGMP on linear graph paper. From the Co/Cx value for the unknown samples, the number of pmol of inactive cyclic GMP from the standard curve was read. Results are expressed as pmol of cGMP/mg tissue.

EXTRACTION AND ESTIMATION OF NUCLEIC ACIDS

The nucleic acids (RNA and DNA) were extracted according to the procedure of Schmidt and Thannhauser (1945) as per the modifications of Munro (1966). Two ml of 10%

homogenate (w/v) of the tissue was mixed with 2.5 ml of ice cold 10% TCA and centrifuged to remove acid soluble compounds. The sediment was washed once with 2.5 ml of ice cold 10% TCA. The final sediment remaining after removal of the acid soluble components was extracted twice with 5 ml of 95% ethanol and the extract was removed by centrifugation. An alcohol ether (3:1) wash was given to the sediment to remove any traces of lipids. The lipid free tissue was suspended in 2 ml of IN KOH and incubated for 2 hrs at 37°C. DNA and protein were then precipitated by the addition of 0.4 ml of 6 N HC1 and 2.6 ml of 5% TCA and allowed to stand in ice for 10 minutes and centrifuged. The supernatant fraction was collected separately to estimate RNA content. The sediment was suspended in 2.5 ml of 5% TCA and boiled at 90°C for 15 minutes with occasional shaking. The mixture was centrifuged and the supernatant was collected in a test tube. The sediment was washed with 1.5 ml of 5% TCA and the combined supernatants were taken for the estimation of DNA.

Estimation of DNA and RNA: DNA was estimated by the diphenylamine colour reaction and RNA by the orcinol reaction (Burton, 1956; Schneider 1957). For estimation of DNA one ml of the DNA extract was mixed with 2 ml of diphenylamine reagent (diphenylamine reagent was prepared by dissolving one gram of purified diphenylamine in 100 ml

of glacial acetic acid and 2.75 ml of concentrated H_2S0_4) and heated for 10 minutes in boiling water. The intensity of blue colour was read at 600 nm in a ECIL spectrophotometer. The amount of DNA present in a sample was determined from a standard curve using calf thymus DNA as the standard. For the estimation of RNA one ml of RNA extract was diluted to 1.5 ml with 5% TCA and heated for 30 minutes after adding 1.5 ml of orcinol reagent (One gram of purified orcinol was dissolved immediately before use in 100 ml of concentrated HCl containing 0.5 g of $FeCl_3$) in a boiling water bath. The intensity of the green colour was then read at 700 nm. A standard curve was prepared using purified yeast RNA as the standard.

Estimation of Protein; Protein content was estimated by the method of Lowry et al (1951) using crystalline bovine serum albumin as standard.

Incorporation of [H] - Thymidine into uterine DNA

For incorporation of [Me- H] thymidine into uterine DNA, 1 µCi of [Me- H] thymidine per uterine horn was injected intraluminally into the uterus. The rats were autopsied at varying time intervals for time course experiment and 1 hrs after ^{3}H-thymidine administration in all other experiments. Uteri were dissected out,

weighed on a torsion balance and homogenized in 2.0 ml of distilled water. The DNA fraction was separated as described above. An aliquot of the DNA supernatant was used for counting with Bray's scintillation fluid in a Beckman liquid scintillation counter. DNA was estimated as described above.

Determination of Glycogen; Glycogen was determined by the phenol-H_2SO4 acid technique of Lo et al (1970), The tissue was dissected out, excised *of fat* weighed and transferred to the bottom of a capped test tube kept in ice. One-half ml of 30% KOH saturated with Na_2S0_4 was added to the samples and the tubes were placed on a.poiling water bath for 20-30 min till a homogeneous solution was obtained. The tubes were then cooled in ice and *95%.* ethanol (1.1-1.2 volumes) was added to precipitate the glycogen from the alkaline digestate. The samples were placed in ice for 30 min and were then centrifuged at 340 xg for 20-30 min. The super-natants were carefully aspirated. The glycogen precipitates were dissolved in 3 ml of distilled water. An appropriate aliquot of the above glycoqen solution was pipetted into another test *tbc* and brought to a samie volume 1 ml by addition of distilled water. *One* mi of 5%. phenol was added to the above followed by five ml of 96-98% $H2S0_4$. The HoSO. was added rapidly to ensure good mixing.

The tubes were allowed to stand in ice for 10 min. after which they were shaken and placed for 10-20 min in a water bath at 25-30°C before readings were taken. Blanks were prepared by Using 1.0 ml distilled water instead of glycogen solution. Samples of standard glycogen (from rabbit muscle) containing 5 to 100 µg glycogen were also subjected to similar phenol-H_2S0_4 treatment. The absorbance was read on an ECIL spectrophotometer at 490 mµ. The tissue content of glycogen was calculated and expressed as mg of glycogen/100 g wet wt tissue.

EXTRACTION OF TOTAL LIPID

1) From Plasma: Total lipids were extracted from the plasma by Folch washing (Folch, 1957). Two ml of plasma was taken in a 50 ml volumetric flask containing 32 ml chloroform: methanol (1:1). The mixture was brought momentarily to boil by placing the flask for 1 min in a water bath. The flask was allowed to cool and more chloroform was added to the mark. The mixture was filtered into a 50 ml measuring cylinder through a chloroform ethanol washed filter paper (Whatman no. 41). The washings were collected to make the volume 25 ml. Ten ml of saline (0.85%) was added, mixed well and the cylinder was stoppered and left overnight at 4^0 C to allow the layers to separate. The upper aqueous methanol layer was removed by suction.

The lower chloroform layer was washed 3 to 4 times with pure solvent upper phase (chloroform: methanol: water, 3:48:47 with 0.29% NaCl). Rinsings were collected in pre-weighed weighing bottles. The washed extracts were evaporated to dryness and dried in a vacuum dessicator over anhydrous calcium chloride and the weight of lipid was recorded. The lipid extract was dissolved in 5 ml of chloroform: methanol (2:1).

2) From Liver: 1 gm of liver tissue was homogenized in 20 ml of chloroform: methanol (2:1) in a Potter-Elvehjem type homogenizer. The homogenate was filtered through a fat free filter paper into a glass stoppered vessel. The crude extract was mixed to 0.2 its volume of an 0.85% NaCl solution. The mixture was allowed to separate into 2 phases by standing and further extracted as described above. The final lipid extract was dissolved in 5 ml of chloroform: methanol and used for the estimation of total and free cholesterol, triglycerides and free fatty acids.

Estimation of Triglycerides: Triglycerides were estimated according to the spectrophotometric method of Van Handel and Zilversmit (1957) as modified by Foster and Dunn (1973) using triolein as a standard.

Determination of free fatty Acids: Free fatty acids were determined by a colorimctric ultranicromethod of Novac (1965) using palmitic acid as standard.

ESTIMATION OF CHOLESTEROL

Serum cholesterol was estimated by the Zak method as modified by Martinek (1965). The following reagents were prepared.

Ferric chloride reagent for standardization: In a 100 ml volumetric flask, 150 mg of powdered FeCl3 6H2O was dissolved and diluted to the mark in glacial acetic acid.

Stock standard cholesterol: In a 100 ml volumetric flask 100 mg of cholesterol was dissolved in 100 ml of glacial acetic acid.

Working standard cholesterol: In a 100 ml volumetric flask, 150 mg of powdered FeCl3.6H2O was dissolved in about 50 ml of glacial acetic acid. 10 ml of stock standard cholesterol was added and made up to the mark with glacial acetic acid. This was freshly prepared.

Precipitating and colour reagent: In a 1 1 volumetric flask, 1.8 g of powdered FeCi3.6H2o and 100 mg of Cittir

acid monohydrate was dissolved in glacial acetic acid. Two ml of concentrated HC1 was added and it was diluted to the mark with glacial acetic acid.

Acetone Methanol Mixture: 50 ml of acetone was mixed with 50 ml of absolute methyl alcohol.

Digitonin: 1 gm of digitonin was dissolved in 50% (v/v) ethyl alcohol by heating to 60°G. It was allowed to cool to room temperature, made up to 100 ml with ethyl alcohol (50% and filtered through Whatman no. 42 filter paper.

Acetic acid 10% (v/v): 5 ml of glacial acetic acid was diluted to 50 ml with distilled water.

Acetic acid 95% (v/v): 50 ml of distilled water was diluted to 1 litre with glacial acetic acid.

Total cholesterol assay: 6.0 ml of precipitating and colour reagent was pipetted into conical centrifuge tubes. 0.1 ml of plasma was added into each tube and vortexed. The tubes were allowed to stand for 2-3 minutes and centrifuged with stoppers inserted for 5 minutes. 3 ml of the supernatant fluid from the centrifuge tubes were pipetted into Erlenmeyer flasks. 3.0 ml of precipitating and colour reagent were

pipetted into another flask which served as blank. To each flask 2.0 ml of concentrated H_2S0_4 was added down the sides of the flask. The solution was mixed well and allowed to stand for 5 minutes after which it was mixed again a second time. The flasks were left to cool at room temperature for at least 10 minutes before readings were taken in a spectrophotometer at 560 nm against the blank. A calibration curve was constructed by using different concentrations of working standard cholesterol instead of serum and processed as above. The concentration of plasma cholesterol was read from the calibration curve.

Free-cholesterol Assay: To 1.9 ml of acetone-methanol mixture in a conical centrifuge tube, 0.1 ml plasma was added dropwise with constant shaking to obtain a finely dispersed precipitate. The tubes were stoppered and subjected to vigorous shaking. They were allowed to stand 2-3 minutes and centrifuged for 5 minutes. 1.0 ml of the clear supernatant was pipetted into another set of centrifuge tubes. 1 ml of clear *lyi* (w/v) digitonin and 1 drop of 10% (v/v) acetic acid was added. The tubes were vortexed well and allowed to stand at room temperature for 10 minutes after which they were centrifuged for 10 minutes. The supernatant was discarded and the precipitate was washed twice with 4 ml of acetone. The tubes were dried. To the dried digitonide in the centrifuge tube 2.0 ml of precipitating

and colour reagent was added and 1.0 ml of 95% (v/v) acetic acid. After mixing well 2.0 ml of concentrated H_2S0_4 was added. Further procedure is as for total cholesterol.

Cholesterol Esters; Cholesterol esters are calculated as the difference between the total and free cholesterol assays.

Estimation of total Cholesterol in liver: Total lipids were dissolved in 5 ml of chloroform: methanol. 0.1 ml aliquot was taken for the estimation of total cholesterol according to the method Zak et al (1954).

Estimation of Free Cholesterol in liver: Free cholesterol in liver was estimated by the method of Sperry and Web (1950).

STATISTICAL EVALUATION

Significance of differences in sequential changes in the same group and between control and experimental groups were determined by Student's 't' test.

CHAPTER III

EFFECT OF SUBSTANCE P AND NEUROTENSIN ON GONADOTROPIN AND PROLACTIN RELEASE

CHAPTER III

EFFECT OF SUBSTANCE P AND NEUROTENSIN ON GONADOTROPIN AND PROLACTIN RELEASE

A variety of neurotransmitters have been shown to influence the release of hormones from the anterior pituitary. Several reviews have appeared on this subject (Vale et al, 1977; Weiner and Ganong, 1978; McCann, 1980, 1982; Negro-Vilar et al, 1980; Meites and Sontag, 1981; Vijayan, 1985a). Dopamine, norepinephrine and serotonin, each of which is highly concentrated in the hypothalamus have been extensively studied for their roles in controlling anterior pituitary function (McCann et al, 1977, 1981; Vijayan and McCann, 1978a,b; Negro-Vilar et al, 1979: Ojeda et al, 1982). Evidence also accrued indicating the involvement of a number of putative neurotransmitters and/or neuropeptides notably vasoactive-intestinal peptide (Vijayan et al, 1979b), gastrin (Vijayan et .al, 1978), cholecystokinin (Vijayan at al, 1979a), bombesin (Walsh et al, 1979; Babu and Vijayan 1983a), secretin (Babu and Vijayan, 1983a) angiotensin (Steele et al, 1981, 1982), neurotensin and substance P (Maeda and Frohman, 1978; Vijayan and McCann, 1979a, 1980) in modulating anterior pituitary hormone release. These neurotransmitters are believed to act on the receptors of neurons carrying the hypothalamic

peptidergic hormones to stimulate or inhibit their release into the portal vessels through which they reach the anterior pituitary. They can also be released directly into the portal vessels and act on the pituitary.

Substance P (O'Connor, 1982) and neurotensin (Uhl and Snyder, 1976) are present in high concentrations in the hypothalamus and hence it is very likely that they participate in neuroendocrine events. Leeman's group (Makino et al. 1973) demonstrated an increase in the secretion of LH and FSH following the intravenous administration of NT. Rivier et *al* (1977) and Maeda and Frohman (1978) also reported an increase in LH secretion following the intravenous injection of NT. However, using OVX rats Vijayan and McCann (1979a) could elicit no change in plasma LH levels following the intravenous administration of NT. In contrast, intraventricular injections of NT significantly lowered plasma LH levels (Maeda and Frohman, 1978; Yijayan and McCann, 1979a; Tache et al, 1979).

In view of the discordant effects of SP and NT on gonadotropin and prolactin release reported by different authors it was of interest to re-evaluate the effect of SP and NT on hormone release in OVX conscious rats. Additional in vitro experiments were performed by incubation

of hemipituitaries with the peptide in order to localize the effects of the peptide.

EXPERIMENTAL PROCEDURE

Implantation of stainless steel cannula into the third ventricle and introduction of silastic jugular vein cannula in OVX rats were carried out as described in Chapter II). On the day of the experiment, an extension of polyethylene tubing (PE 50, 12" in length) filled with 0.9% heparin-saline was attached to the distal end of the jugular cannula and animals were left undisturbed for 30-60 minutes. During this period a preinjection blood sample (0.6-0.8 ml) was withdrawn over a period of 60 seconds.

Substance P and NT were freshly prepared in 0.9% saline. Systemic administration by iv pulse injection was performed in a volume of 100 *µ1* using a tuberculin syringe whereas third ventricular injections were made in a volume of 2 µ1 using a 10 µl Hamilton microsyringe as described previously (Chapter II). Only one each of the effective doses of the peptide used earlier (Vijayan and McCann, 1979a) were chosen for both ivt and iv injections. Hepari-nized blood samples (0.6-0.8 ml) were collected from the external jugular vein at 5, 15, 30 and 60 min while the

animals were freely moving in the cage. The volume of all samples was replaced by 0.9% saline. Plasma was separated by centrifugation and stored frozen until the day of the assay.

In vitro experiments:

Ovariectomized rats were killed by decapitation, anterior pituitaries were quickly removed, bisected longitudinally and placed randomly in incubation flasks containing 2 ml of tissue culture medium 199 adjusted to pH 7.4. Each flask thus contained 2 hemipituitaries. After a 30 min preincubation in a Dubnoff metabolic shaker at 37°C in an atmosphere of 5% $C0_2$ and 95% 0_2 the medium was replaced with 2 ml of fresh medium containing different doses of the peptide and the incubation was continued for three hrs. Control flasks contained medium alone. At the end of the incubation, the media were removed and frozen for later RIA of gonadotropins and Prl. Values were expressed as ng hormone released/ml of medium.

Radioimmuno assay of LH, FSH and Prl was performed as detailed in Chapter II.

RESULTS

ln vivo Experiments: Plasma LH and FSH levels were elevated in OVX animals when compared to basal levels in normal female rats. Plasma Prl levels were relatively low in the OVX rats. Plasma hormone concentrations were not altered after third ventricular injections of 2 μl or iv injections of 100 µ1 of 0.9/ saline. Since there were no significant changes in the hormone values in ivt or iv saline injected rats, both values were pooled to serve as control and represented as mean + SEM.

Effect of SP on Plasma LH Levels:

Intravenous injection of 1 μg of SP produced a significant decline in plasma LH levels at 15 and 30 min ($P<0.05$). Third ventricular injections of 2.0 ^g of SP however, induced a significant ($P<0.05$) elevation in plasma LH levels within 5 min of injection. The values remained significantly ($P<0.001$) elevated for 30 min and returned to preinjection level by 60 min (Table 1).

Effect of NT on plasma LH levels:

Intravenous injection of 1 µg of NT had no effect on plasma LH levels. However, third ventricular injections

of 2.0 µg of NT significantly (P<0.05) lowered plasma LH levels within 5 min of injection. The suppressive effect of intraventricular NT (P<0.001) persisted till 60 min duration of the experiments (Table 2).

Effect of SP on plasma Prl levels:

Intravenous injection of 1 µg of SP produced significant elevations (P<0.001 at 5 and 15 min, P<0.05 at 30 min) of plasma Prl. A2 µg dose of SP administered intraventricularly also elevated plasma Prl levels significantly (P<0.001) within 5 min of injection. The rise in Prl after iv SP was greater than that produced by a 2 µg dose injected into the third ventricle. Prolactin titers remained elevated for 30 min after iv or ivt injection but declined to preinjection values by 60 min (Table 3).

Effect of NT on Plasma Prl levels:

Plasma Prl was significantly (P<0.001) increased within 5 min after iv administration of 1 μg of NT. Prolactin titers remained elevated for 30 min and returned to preinjection levels at the end of 60 mins. In contrast,

•third ve

ficant decrease in plasma prolactin levels within 5 min of

injection ($P<0.001$). Prolactin levels remained significantly ($P<0.001$) low for the sixty min duration of the experiment (Table *A*).

Plasma FSH levels were not modified by iv or ivt injections of SP or NT (Results not shown).

IS vitro Studies: Incubation of SP or NT (doses ranging from 10-1000 ng/flask) with hemipituitaries had no effect on gonadotropin release into the medium. However, both peptides at higher doses of 100 and 1000 ng/per flask were able to enhance release of Prl into the medium. The low dose of 10 ng had no effect (Tables 5 and 6).

DISCUSSION

Intravenous administration of SP caused a decrease in plasma LH levels whereas intraventricular administration of SP caused an increase in LH (Vijayan and McCann, 1979a). Using an antiserum to SP, Kerdelhue et a1 (1978) evoked a significant increase in plasma LH and FSH and postulated an inhibitory role for fSP in gonadotropin regulation.

Kato et al. (1976) and Rivier et al (1977) first reported that systemic administration of SP and NT elevated Prl levels and attributed the actions to an effect on the

CNS. Maeda and Frohman (1978) and Rivier et al (1977) did not see any effect of NT on Prl release from dispersed pituitary cells in vitro. On the other hand, Vijayan and McCann (1979a) and Enjalbert et al (1982) found a stimulation of Prl secretion from hemipitutitaries incubated with SP and NT. A stimulation of Prl secretion was also reported following systemic administration of NT (Vijayan and McCann, 1979a). Intraventricular injections of SP produced an increase in plasma Prl whereas ivt NT caused a lowering of plasma Prl levels (Vijayan and McCann, 1979a). Blackburn et al (1980) infused NT at physiologically relevant concentrations in human subjects and found no changes in plasma levels of Prl, LH or GH. The present results obtained with two minimal effective doses of SP and NT from earlier studies (Vijayan and McCann, 1979a) confirm the earlier observation that these peptides can modify LH and Prl release in OVX rats. Substance P injected intravenously at 1 μg dose produced a slight decline of plasma LH at 15 and 30 mins. In contrast to the lowering obtained after iv injection, intraventricular SP produced a significant elevation in plasma LH titers within 5 min of injection. Substance P failed to alter LH release in vitro. The results thus indicate a stimulatory role for SP at the hypothalamic level. Substance P probably acts via increased release of LHRH since systemic injection of SP suppressed rather than elevated LH.

Intraventricular as well as iv SP elevated plasma Prl within 5 min of injection. Plasma Prl was elevated more by the iv injection of 1 µg dose than by the 2 μg ivt dose. Incubation of pituitaries in, vitro with SP also resulted in increased Prl secretion into the medium at the low dose of 100 ng/flask. The elevation in Prl after SP seems to be due to a direct action on the lactotrophs since SP released Prl on incubation with hemipituitaries. A direct action on the lactotrophs is also supported by the results of Kato et al (1976) who found that plasma Prl was elevated by large doses of SP given iv in rats with large hypothalamic lesions which presumably removed the gland from hypothalamic influences. Increase of Prl with systemic administration of SP could be due to stress evoked by inducing changes in blood pressure. Stress is known to elevate prolactin (Krulich et al 1978). Hence the increase in Prl after iv injection could either be a stress response or mediated by direct stimulation of lactotrophs. The elevation of Prl following ivt injection of SP may be due to a direct stimulation of the pituitary after its uptake by portal vessels and delivery to the gland since it was active in vitro.

In the case of NT, iv injection had no effect on plasma LH levels whereas third ventricular injections were effective in inducing a decrease in plasma LH levels.

It is likely that the intraventricularly administered peptide acted to suppress release of LHRH since the peptide failed to alter LH release in vitro. FSH probably did not decline, inspite of a lowering of LHRH since it has a relatively longerhalf life in the incubation.

Intravenous NT elevated plasma Prl titers whereas ivt NT reduced Prl levels. Incubation of pituitaries in vitro released Prl into the medium. The likelihood of ivt NT acting directly on the pituitary is rather remote because if NT had a direct effect on the lactotrophs it was of a stimulatory nature. The intraventricularly administered NT presumably suppressed Prl release either via a stimulation of Prl-inhibiting factor (PIF) or by an inhibition of PRL-releasing factor PRF or by a combination of both actions. NT may have exerted its actions directly on the releasing factor producing neurons or on neurons which in turn synapsed with these neurons. For example, it might have stimulated the dopaminergic tuberoinfundioular neurons which in turn could stimulate PIF release or the dopamine released itself could act as PIF. Andrade and Aghajanian (1981) and Haubrich et al (1982) have suggested an activation of the dopaminergic neurons by NT. Blockade of dopamine transmission either by inhibition of synthesis of catecholamines with α-MT or by blockade of its action by receptor blockers, such as spiroperidol blocked the action of NT (McCann et al, 1

As in the case of SP, the elevation of Prl release following iv injection of NT could have been the result of a non-specific stress which is known to stimulate Prl release. Intravenous administration of NT could probably also activate the lactotrophs since in vitro studies indicated a remarkable release of Prl by low doses of NT. Neurotensin thus seems to have a dual role on Prl release- an inhibitory effect at the level of the hypothalamus and a stimulatory effect at the level of pituitary. Dual hormonal effects for the modulation of Prl secretion by another putative neurotransmitter GABA have already been documented (Schally et al, 1977; Enjalbert et al, 1978; Vijayan and McCann, 1978c; Locatelli et al. 1979). Hence opposite effects of NT administered either systemically or into the cerebral ventricle on hormone release is a feasible proposition.

Rivier et al (1977) had reported a stimulation of Prl follwoing systemic administration of SP and NT and attributed it to an effect on the CNS. The present results confirm these observations but emphasises the fact that this response may be due to stress or to a direct action on the pituitary. Present results thus reconfirm the suggestion that SP and NT present in the hypothalamus are capable of regulating the release of hormones from the anterior pituitary.

TABLE 1. EFFECT OF SP ON PLASMA LH LEVELS IN OVX RATS

	Plasma LH (ng/ml)				
Treatment	Preinjection sample	5 min	15 min	30 min	60 min
Saline (8)	15.37±2.32	14.98±1.8	14.475±1.52	13.75±1.59	14.7±1.51
SP iv 1.0 µg (4)	16.0 ±1.6	13.75±2.0	8.2 ±1.3*	8.2±1.2*	11.4±1.4
SP ivt 2.0 µg (4)	13.33±1.98	21.5 ±1.05*	23.3 ±2.3**	26.27±1.8**	17.26±0.99

Plasma LH levels (ng/ml) 5, 15, 30 and 60 min after administration of 0.9% NaCl or SP in OVX concious rats. In this and subsequent tables results are mean ± SEM. The number in parentheses indicates the number of animals in each group. Since there was no significant difference in hormone levels following iv or ivt injection of saline at any of the time intervals studied, these values were pooled.

*P<0.05
**P<0.001 vs preinjection level

TABLE 2. EFFECT OF NT ON PLASMA LH LEVELS IN OVX RATS

Treatment	Plasma LH (ng/ml)				
	Preinjection sample	5 min	15 min	30 min	60 min
Saline (8)	15.37±2.32	14.98±1.8	14.475±1.52	13.75±1.59	14.7 ±1.51
NT iv 1 µg (5)	15.2 ±1.8	15.0 ±1.2	14.4 ±0.8	13.6 ±1.4	13.2 ±1.7
NT ivt 2 µg (5)	14.4 ±0.66	10.99±0.33*	7.36 ±0.33**	6.37±0.679**	4.819±1.38**

Effect of iv (1 µg) or ivt (2 µg) injections of NT on plasma LH levels (ng/ml) in OVX conscious rats 5, 15, 30 and 60 min after peptide administration.

*P<0.05
**P<0.001 vs. preinjection level

TABLE 3. EFFECT OF SP ON PLASMA Prl LEVELS IN OVX RATS

	Plasma Prl (ng/ml)				
Treatment	Preinjection sample	5 min	15 min	30 min	60 min
Saline (8)	27.93 ±3.325	27.99 ± 4.16	26.98 ± 3.13	26.98 ±2.32	24.99±3.99
SP iv 1 µg (4)	27.315±1.995	95.345±13.9**	84.655±12.6**	61.995±9.97*	29.31±5.32
SP ivt 2 µg (4)	21.995±1.99	54.015± 5.32**	53.35 ± 4.655**	45.985±5.3**	22.66±1.33

Plasma Prl levels (ng/ml) in OVX conscious rats following the administration of iv (1 µg) or ivt (2 µg) SP at various time intervals.

*P<0.05
**P<0.001 vs. preinjection level.

TABLE 4. EFFECT OF NT ON PLASMA Prl LEVELS IN OVX RATS

Treatment	Plasma Prl ng/ml				
	Preinjection sample	5 min	15 min	30 min	60 min
Saline (8)	27.93 ±3.325	27.99 ±4.16	26.98 ±3.13	26.987±2.32	24.99 ±3.992
NT iv 1 µg (5)	27.15 ±3.90	112.635±15.015**	145.985±6.65*	58.67±4.655*	29.975±1.995
NT ivt 2 µg (5)	25.985±2.66	9.31±3.325**	7.98±0.65**	5.985±1.33**	5.32 ±1.995**

Plasma Prl levels (ng/ml) in OVX conscious rats following the administration of iv (1 µg) or ivt (2 µg) NT at various time intervals.

*P<0.05
vs. preinjection level.
**P<0.001

TABLE 5. EFFECT OF SP (10-1000 ng) ON GONADOTROPIN AND Prl RELEASE *IN VITRO*.

	Hormone released (ng/ml medium)		
Dose (ng/flask)	LH	FSH	PRL
SP 10	345.2 ± 35.0	2456.6 ± 335.0	2543.1 ± 199.4
100	392.0 ± 54.1	2224.0 ± 294.3	4343.5 ± 243.5**
1000	355.3 ± 34.2	2536.4 ± 148.6	4473.5 ± 107.8**
Control	343.0 ± 9.8	2216.4 ± 248.4	2751.5 ± 446.8

Effect of different doses of SP on gonadotropin and Prl release from hemipituitaries incubated *in vitro* for 3hrs. Each incubation flask contained 2 ml of culture medium. Control flasks contained medium alone.

**$P<0.001$ vs control.

TABLE 6. EFFECT OF NT (10-1000 ng) ON GONADOTROPIN AND Prl RELEASE *IN VITRO*.

	Hormone released (ng/ml medium)		
Dose (ng/flask)	LH	FSH	PRL
NT 10	462.1 ± 54.3	2636.2 ± 268.2	3595.3 ± 548.4
100	413.0 ± 41.4	2416.8 ± 262.6	5117.6 ± 129.0**
1000	396.4 ± 35.3	2446.6 ± 364.6	4888.8 ± 356.4**
Control	428.7 ± 34.2	2328.6 ± 212.6	2647.2 ± 436.3

Effect of different doses of NT on gonadotropin and Prl release from hemipituitaries incubated *in vitro* for 3hrs. Each incubation flask contained 2 ml of culture medium. Control flasks contained medium alone.

**P<0.001 vs. control.

CHAPTER IV

EFFECT OF SUBSTANCE P AND NEUROTENSIN ON HYPOTHALAMIC cAMP AND cGMP IN OVX AND OVX EB-PRIMED RATS

CHAPTER IV

EFFECT OF SUBSTANCE P AND NEUROTENSIN ON HYPOTHALAMIC CAMP AND cGMP LEVELS IN OVX, AND OVX EB-PRIMED RATS

Substance P and NT are present in the CNS. Immunohistochemical and RIA studies have revealed the presence of SP in most parts of the CNS of all mammals studied so far including the human brain. The highest concentrations are in the mesencephalon, hypothalamus and preoptic area with insignificant amounts in the cerebellum (Brownstein et al, 1976; Kanazawa and Jessell, 1976; Gale et al, 1978; Cooper et al, 1981). A high density of immuno-reactive fibres has been found in the preoptic nucleus and SP fibres and terminals are abundant in the median eminence in close connection with the portal vessels supplying the anterior pituitary gland (Hokfelt et al, 1978). Subcellu-lar distribution studies reveal that in the hypothalamus SP is localized in synaptosome particles (Duffy et al, 1975). A significant feature of the distribution of SP in the brain is the uneven distriDution with very large accumulation in some areas and other areas being completely devoid of the peptide (Brownstein et al, 1976; Cuello et al, 1982). This selective distribution of the peptide in the brain attributes specific functional activity in regions of high concentration.

Neurotensin is localized in both human and rat brain in a complex network of pathways. It is especially concentrated in the hypothalamus, central amygdaloid nucleus and the bed nucleus of the stria terminalis (Cooper et al, 1981; Polak and Bloom, 1982). Radioimmunoassay by Carraway and Leeman (1976) and by Kobayaishi et al, (1977) have shown relatively large concentrations in the median eminence, hypothalamus, pituitary, thalamus and brain stem. There is a particularly high concentration of NT and its receptors in the hypothalamus. The hypothalamus appears to be a likely site for several of NT's neuro-endocrine functions (Uhl and Snyder, 1981).

The abundance of SP and NT in the hypothalamus and other evidence suggests that they play a role as synaptic transmitters or modulators of synaptic function. Among the broad spectrum of pharamacological actions is the 'ability of these peptides to affect several functions of the CNS including release of hormones from the anterior pituitary (Chapter III). However, the mechanism of its action in the CNS remains unclear.

Cyclic nucleotides play an important role as media-tors of hormonal and drug effects in the CNS (Rall and Sattin, 1970). Several lines of evidence suggest that cAMP may be important in brain function. In vitro the

activities of the enzymes responsible for its synthesis (adenyl cyclase) and degradation (phosphodiesterases) are higher in the brain than in any other tissue (Sutherland et al, 1962) and begin to develop at a time when the brain is becoming functionally mature (Schmidt et al, 1970). Adenyl cyclase, cAMP phosphodiesterase and cAMP itself are concentrated in the synaptosomal fraction of brain tissue (De Robertis et al, 1967; Johnson et al, 1972) where the neurohormones are found (Molinoff and Axelrod, 1971).

Although levels of cGMP in the brain are markedly lower than that of cAMP, together with cAMP it has been implicated in a number of neural functions (Nathanson, 1977). High levels of guanylate cyclase, the enzyme responsible for its synthesis are present in synaptosomes.

Earlier studies have indicated that the secretagogue effect of SP in tissues other than the brain like the exocrine pancreas is mediated through guanylate cyclase (Albano et *al*, 1978; Sjodin et *ad*, 1980). Duffy and Powell (1975) have reported a stimulation of brain cAMP on incubation with SP. In addition, the release of NT like immunoreactivity (NTLI) from hypothalamic fragmants by dibutryl cAMP suggests the involvement of an adenylate cyclase system (Maeda and Frohman, 1981). Therefore it is

likely that the effects of these neuropeptides in the hypothalamus are mediated through cyclic nucleotides. The present experiments were designed to evaluate levels of hypothalamic cAMP and cGMP after ivt and iv injections of the peptide.

EXPERIMEMENTAL PROCEDURE

Adult rats were OVX while lightly anaesthetized with ether and were used 2-3 weeks after surgery. Estradiol benzoate (EB) was dissolved in sesame oil and administered subcutaneously in doses of 1, 5, 10 and 100 µg per rat. Animals were sacrificed 24 hours later by decapitation, the brain was excised immediately and frozen in liquid nitrogen. The hypothalamus was dissected out by the procedure of Glowinski and Iversen (1966) and assayed for cAMP and cGMP as detailed in Chapter II.

Both OVX and OVX rats primed with 5ug of EB for 24 hours were injected with freshly prepared Substance P or Neurotensin. 1 or 5 µg of peptide in 50 µl of 0.9% saline was administered iv through the jugular vein while the rat was lightly anaesthetized with ether. Third ventricular injections of the peptide were made in a volume of 2.5 µl of physiological saline using a 10 µ1 Hamilton syringe. Controls received equal volume of saline.

Animals were sacrificed at 5, 15, 30 and 60 min after peptide administration and brain tissue was processed for cAMP and cGMP as described in Chapter II,

In a subsequent experiment 2.5 µg dose of SP was injected into the third ventricle immediately followed by 2.5 µg dose of neurotensin. Animals were sacrificed and levels of cAMP and cGMP were determined as before.

RESULTS

Hypothalamic cAMP and cGMP in intact adult, OVX and OVX, EB primed rats:

Hypothalamic cAMP levels decreased significantly ($P<0.05$) in OVX rats compared to that in intact controls. In contrast, cGMP increased significantly in OVX animals. The administration of EB, however, reversed these effects. A dose of 1 µg EB was marginally effective but doses of 5, 10 and 100 µg significantly elevated cAMP and decreased cGMP levels (Table 1). Since the 5 µg dose was effective in restoring cAMP levels in OVX animals to that of intact controls, a 5 ug dose of EB was used for priming OVX ratss in subsequent experiments.

Hypothalamic cAMP levels in OVX rats after SP:

Table 2 shows cAMP levels in the hypothalamus of OVX rats after 1 and 5 µg of iv pulse injection and 2.5 µg of ivt injection of SP. Since there was no significant difference in cAMP values after the iv or ivt administration of saline, values were pooled together to represent control values. There is an overall reduction in cAMP level after both doses of iv SP. The maximum ($P<0.01$) difference was at thirty minutes after injection of the peptide. On the other hand, there is an increase ($P<0.02$) within 5 min after ivt injection which persisted for 30 min (Table 2).

Hypothalamic cAMP levels in OVX, EB-primed rats after SP:

Intravenous or ivt injection of saline did not alter hypothalamic cAMP levels in OVX, EB-primed rats. Hence values for controls obtained from both experiments were pooled and represented as mean + SEM. Intravenous SP at doses of 1 and 5 µg significantly ($P<0.05$) lowered cAMP levels. The decrease was greater with 5 µg dose than with the 1 μg dose. Lowering of cAMP levels was evident within 5 min after injection and persisted for 60 min duration of the experiment. On the other hand, there is a significant ($P<0.02$) increase within 5 min after ivt injection (Table 3).

Hypothalamic cAMP levels in OVX rats after NT:

Intravenous administration of either 1 or 5 µg doses of NT in OVX rats had no effect on cAMP levels. However, ivt injection of 2.5 µg NT significantly ($P<0.02$) lowered cAMP concentration within 5 min of administration (Table 4).

cAMP levels in OVX, EB-primed rats after NT:

Cyclic AMP levels did not change after iv pulse injection of NT in OVX, EB-primed rats. Third ventricular NT decreased cAMP levels significantly within 5 min after injection, a pattern comparable to that seen in OVX rats (Table 5).

Hypothalamic cGMP level in OVX rats after SP:

Intravenous administration of SP 1 or 5 μg, enhanced the levels of cGMP in OVX rats within 15 min. The 5 μg dose was more effective than the 1 µg dose. However, ivt administration of the peptide reduced cGMP levels with respect to controls or injected animals (Table 6).

Hypothalamic cGMP levels in OVX EB-primed rats after SP:

Intravenously administered SP, 1 or 5 µg, in OVX, EB-primed rats significantly ($P<0.001$) stimulated cGMP accumulation within 15 mins. Intraventricular injection of 2.5 µg of peptide however lowered cGMP levels significantly ($P<0.001$) at 5 min after peptide injection which remained low until 60 min duration of the experiment (Table 7).

Hypothalamic cGMP levels in OVX rats after NT:

Intravenous pulse injection of 1 or 5 µg NT in OVX rats failed to modify hypothalamic cGMP levels at any of the time invervals studied. In contrast ivt injections of 2.5 µg NT induced a marked increase ($P<0.001$) in cGMP levels within 5 min after the peptide administration. This increase persisted throughout the 60 min duration of the experiment (Table 8).

cGMP levels in OVX, EB primed rats after NT:

Intravenous administration of neurotensin in OVX, EB-primed rats also failed to alter hypothalamic cGMP levels except for a slight decrease at 60 minutes after the 5 µg dose. On the other hand ivt injection of NT significantly elevated cGMP levels ($P<0.001$) within 5 min of injection (Table 9).

cAMP and cGMP levels after ivt SP followed by NT in OVX, EB-primed rats:

Concomitant administration of 2.5 µg of SP and NT intraventricularly in OVX, EB-primed rats failed to modify the levels of cAMP or cGMP (Table 10).

DISCUSSION

Cyclic AMP and cyclic GMP are naturally occurring constituents of brain tissue which are thought to be mediators of hormone action. In the absence of stimulation by exogenous hormones the concentration of intracellular nucleotides is relatively constant. Ovariectomy resulted in a decrease in cAMP and an increase in cGMP levels in the hypothalamus. These changes were reversed by exogenous administration of EB. A dose of 1 µg/rat for 24 hours was only marginally effective but doses of 5, 10 and 100 µg/rat significantly elevated cAMP while reducing cGMP levels. Although there is no available data, linking EB with cGMP in the hypothalamus, it has been reported that *in* vivo EB increased cAMP levels in whole hypothalami from young female rats (Gunaga et al, 1974). It was also reported that diethylstilbestrol and 17-β estradiol, but not less active estrogens, elicited two fold accumulation of cyclic AMP in hypothalami from immature female rats incubated

in vitro (Weissman and Skolnick, 1975; Weissman, et al, 1975). Goldberg et al (1973) have postulated that the intracellular concentrations of cAMP vary inversely with the intracellular concentrations of cGMP with respect to certain regulatory events. An agonist-antagonist hypothesis for interaction of the two cyclic nucleotides has been suggested (Goldberg et al, 1973). The present results supports this line of argument since OVX decreased cAMP while increasing cGMP. In the above experiments OVX and OVX, EB-primed rats were preferred to intact adult females in order to avoid fluctuations in nucleotide levels during the estrous cycle. Cyclic fluctuation in nucleotide levels have been previously established (Kimura et al 1980). It is interesting to note that estradiol 17-β is able to sustain the increase in cAMP for 24 hours and that there is an opposite pattern being followed in the case of cGMP.

There have been numerous reports on the action of different agents on the cAMP system in brain homogenates, brain slices, brain cell cultures or on postmortal increase. Thus for example, depending on age and/or species of animals catecholamines, depolarising agents, adenosine and and electric stimulation cause an alteration in cAMP in brain slices in vitro (Kakiuchi and Rall 1968; Kakiuchi

et al, 1969; Sattin and Rail, 1970; Palmer *et* al,, 1973). Parallel experiments in vivo have been hampered by (1) very rapid postmortem changes in cerebral cAMP content with the consequent difficulty in establishing in vivo control levels of the nucleotide (Breckenridge, 1964; Steiner et al, 1972; Nahorski and Rogers, 1973) and (2) by the inability of most systemically administered biogenic amines and peptides to cross the blood-brain barrier (Weil Malherbe et al., 1961). In the above experiments, these technical difficulties have at least been partially circumvented by using experimental conditions which minimize the postmortem increase in nucleotides by rapidly freezing the brain by immersing in liquid nitrogen. Control values obtained in the above experiments fall well within the range observed by others (Steiner et al, 1972; Ferrendelli et al., 1972; Wellmann and Schwabe, 1973; Folbergrova, 1977). There are conflicting reports as to whether SP and NT can cross the blood brain barrier and reach the brain after systemic injection. Substance P may (Stern et al, 1973) or may not (von Euler and Pernow, 1975) cross the blood brain barrier. Hence experiments were performed after both intravenous and intraventricular injections.

Intraventricular injections of SP increased the level of cAMP while decreasing cGMP levels. Duffy and

Powell (1975) had observed a stimulation in adenylate cyclase in particulate preparations from rat and human brains. This increased cyclic nucleotide formation was regulated by changes in Ca^{2+} concentrations. Glucagon, ACTH and insulin did not affect cAMP concentrations (Williams et *al* 1969; von Hungen and Roberts, 1973). Intravenous administration of SP on the other hand, decreased cAMP and increased cGMP levels. However, iv administration of NT failed to alter nucleotide levels. There are very few observations that relate in a direct way a given effect of a substance be it a peptide or a monoamine to changes in hypothalamic nucleotides. The present results point to an opposing relationship both between cAMP and cGMP and also between SP and NT. Earlier findings have indicated that many substances produce diverse effects on cAMP and cGMP in tissues (Ferrendelli et al, 1970; George et al 1970; Hadden et al, 1972). From the above observations it is noticed that cAMP and cGMP have opposing effects on cellular metabolic mechanisms. Intraventricular administration of SP produced marked elevation, evident within 5 minutes, while iv injection resulted in reduction of cAMP which was evident by 15 min. Intravenous administration of NT, however, failed to modify nucleotide levels probably because it does not cross the blood-brain barrier (Oldendorf, 1981).

Intraventricular administration of SP immediately followed by an equivalent dose of NT did not modify nucleotide levels. This shows that the increase in cAMP and decrease in cGMP caused by SP can be reversed by NT and vice-versa.

The present results thus point to the hypothalamus as being a site of action for SP and NT. However, results do not conclude whether the alterations in nucleotide levels is a direct effect of the peptides or an indirect one due to stimulation of some other metabolic parameter which in turn alters nucleotide levels. A striking parallel was obtained previously in control of LH release from the anterior pituitary by SP and NT (Vijayan and McCann, 1979a). Intraventricular injection of SP induced a significant elevation in plasms LH levels whereas NT produced a significant reduction of the hormone. Reevaluation of the effects of SP and NT on plasma LH levels in OVX rats supported this observation (Chapter III). The present results thus implicate a possible mediatory role for cyclic nucleotides in the action of SP and NT in the brain, particularly in the hypothalamus.

TABLE 1. HYPOTHALAMIC cAMP AND cGMP (pmoles/mg wet wt) IN ADULT FEMALES, OVX AND OVX-PRIMED RATS.

Treatment	cAMP pmole/mg tissue	cGMP pmole/mg tissue
Control (7)	1.741±0.35	0.057±0.01
OVX (6)	0.856±0.21*	0.120±0.02***
EB 1 µg (4)	1.07 ±0.29	0.091±0.01*
EB 5 µg (4)	1.73 ±0.72	0.044±0.01****
EB 10 µg (4)	2.835±0.87***	0.022±0.01****
EB 100 µg (5)	3.285±0.41***	0.0198±0.01*

cAMP and cGMP (pmole/mg tissue) levels in adult, OVX and OVX-primed rats with different doses of EB for 24 h. Adult untreated females represent controls. Values in this and subsequent tables are mean ± SEM. The no. in parentheses indicates the no. of animals used per group.

*P<.05
***P<.01
****P<.001
} < vs control.

TABLE 2. HYPOTHALAMIC cAMP IN OVX RATS AFTER iv OR ivt INJECTIONS OF SP

	cAMP (pmole/mg wet wt)			
Treatment	5 min	15 min	30 min	60 min
Saline control	0.85 ±0.03	0.828±0.19	0.849±0.03	0.853±0.12
SP iv 1 µg	0.610±0.16	0.340±0.24	0.280±0.18***	0.630±0.33
SP iv 5 µg	0.513±0.09***	0.230±0.18	0.193±0.05***	0.240±0.18*
SP ivt 2.5 µg	1.699±0.01**	1.980±0.29**	1.480±0.53*	0.957±0.23

Hypothalamic cAMP (pmole/mg wet wt) levels after iv (1 or 5 µg) or ivt (2.5 µg) of SP in OVX rats at different time intervals. Each value is a determination of 3-4 animals. Since there was no difference in cAMP values after iv or ivt administration of saline, values were pooled together to represent control values in this and subsequent tables.

*P<.05
**P<.02
***P<.01
vs control.

TABLE 3. HYPOTHALAMIC cAMP LEVELS IN OVX, EB-PRIMED RATS AFTER SP.

	cAMP (pmole/mg wet wt)			
Treatment	5 min	15 min	30 min	60 min
Saline control	1.74 ±0.21	1.728±0.39	1.73 ±0.42	1.694±0.13
SP iv 1 µg	0.405±0.07*	0.467±0.14	0.365±0.12*	0.503±0.21
SP iv 5 µg	0.365±0.04*	0.373±0.09*	0.218±0.03*	0.361±0.07*
SP ivt 2.5 µg	2.491±0.05*	3.217±0.12**	2.96 ±0.04	1.650±0.08

Hypothalamic cAMP (pmole/mg wet wt) levels in OVX rats primed with 5 µg of EB for 24 h and treated with iv (1 or 5 µg) or ivt (2.5 µg) SP. Each value represents mean ± SEM of 3-4 determinations.

*P< .05

**P< .02

TABLE 4. HYPOTHALAMIC cAMP IN OVX RATS AFTER NT.

Treatment	cAMP pmoles/mg wet wt			
	5 min	15 min	30 min	60 min
Saline	0.85 ±0.03	0.828±0.19	0.849±0.03	0.853±0.12
NT iv 1 µg	0.77 ±0.18	0.992±0.21	0.793±0.41	1.053±0.26
NT iv 5 µg	0.691±0.42	0.610±0.06	0.891±0.18	0.873±0.32
NT ivt 2.5 µg	0.136±0.12**	0.153±0.11*	0.340±0.05***	0.036±0.28

Hypothalamic cAMP (pmoles/mg wet wt) levels in OVX rats treated with iv (1 or 5 µg) or ivt (2.5 µg) NT. Each value represents mean ± SEM of 3-4 determinations.

*P<.05
**P<.02
***P<.01
vs control.

TABLE 5. HYPOTHALAMIC cAMP IN OVX, EB-PRIMED RATS TREATED WITH NT.

	cAMP (pmoles/mg wet wt)			
Treatment	**5 min**	**15 min**	**30 min**	**60 min**
Saline	**1.74 +0.21**	**1.728+0.39**	**1.73 +0.42**	**1.694+0.13**
NT iv 1 \xg	**2.108+0.52**	**1.820+0.26**	**1.783+0.40**	**1.981+0.23**
NT iv 5 \ig	**1.740+0.81**	**1.65 +0.28**	**2.284+0.60**	**1.312+0.15**
NT ivt 2.5 ^ig	**0.218+0.15*** **	**0.489+0.06***	**0.819+0.12**	**1.080+0.21**

Hypothalamic cAMP (pmoles/mg tissue) levels in OVX rats primed with 5 ug EB for 24 h and treated with iv (1 or 5 ^g) or ivt (2.5 *\ig)* NT. Each value represents mean + SEM of 3-4 determinations.

*P<.05 j
***P<.01 I
I vs control.

TABLE 6. HYPOTHALAMIC cGMP LEVELS IN OVX RATS TREA1ED WITH SP.

	cGMP (pmoles/mg wet wt)			
Treatment	5 min	15 min	30 min	60 min
Saline control	0.130+0.02	0.118+0.01	0.121+0.01	0.132+0.01
SP iv 1 \xg	0.093+0.04	0.171+0.01*	0.173+0.01***	0.151+0.01
SP iv 5 ng	0.141+0.01	0.272+0.01***	0.279+0.01***	0.196+0.01***
SP ivt 2.5 \ig	0.031+0.01***	0.049+0.02*	0.072+0.01**	0.038+0.02***

Hypothalamic cGMP (pmole/mg wet wt) after iv (1 or 5 ^ig) or ivt (2.5 \xg) of SP in OVX rats at different time intervals. Each value is a determination of 3-4 animals. Since there was no difference in cGMP values after iv or ivt administration of saline, values were pooled together to represent control values in this and subsequent tables.

*P<.05
**P< .01 vs control.
***P<.001

TABLE 7. HYPOTHALAMIC cGMP LEVELS IN OVX EB-PRIMED RATS TREATED WITH SP.

	cGMP (pmoles/mg wet wt)			
Treatment	5 min	15 min	30 min	60 min
Saline control	0.092±0.01	0.089±0.01	0.918±0.01	0.093±0.01
SP iv 1 µg	0.093±0.02	0.171±0.02**	0.173±0.01*	0.196±0.03**
SP iv 5 µg	0.091±0.01	0.238±0.01***	0.361±0.01***	0.130±0.01
SP ivt 2.5 µg	0.031±0.01***	0.021±0.06***	0.035±0.01	0.038±0.02

Hypothalamic cGMP levels (pmoles/mg wet wt) in OVX rats primed with 5 µg of EB for 24 h and treated with iv (1 or 5µg) or ivt (2.5 µg) SP. Each value represents mean ± SEM of 3-4 determinations.

*P<.02
**P<.01
***P<.001
vs control.

TABLE 8. HYPOTHALAMIC cGMP LEVELS (pmole/mg wet wt) IN OVX RATS AFTER NT.

	cGMP (pmoles/mg wet wt)			
Treatment	5 min	15 min	30 min	60 min
Saline control	0.130±0.02	0.188±0.01	0.121±0.01	0.132±0.01
NT iv 1 µg	0.135±0.01	0.083±0.03	0.093±0.01	0.108±0.01
NT iv 5 µg	0.099±0.01	0.089±0.01	0.125±0.05	0.098±0.01
NT ivt 2.5 µg	0.302±0.01***	0.401±0.01***	0.518±0.01***	0.372±0.01***

Hypothalamic cGMP levels (pmole/mg wet wt) in OVX rats treated with iv (1 or 5 µg) or ivt (2.5 µg) NT. Each value represents mean ± SEM of 3-4 determinations.

***P<.001 vs control.

TABLE 9. HYPOTHALAMIC cGMP LEVELS (pmole/mg wet wt) IN OVX EB-PRIMED RATS TREATED WITH NT

	cGMP (pmoles/mg wet wt)			
Treatment	5 min	15 min	30 min	60 ming
Saline control	0.092±0.01	0.089±0.01	0 091±0.018	0.093±0.01
NT iv 1 µg	0.089±0.01	0.083±0.01	0.093±0.06	0.068±0.01
NT iv 5 µg	0.097±0.01	0.086±0.01	0.096±0.04	0.043±0.01*
NT ivt 2.5 µg	0.318±0.02**	0.563±0.01**	0.310±0.01**	0.121±0.01

Hypothalamic cGMP levels (pmole/mg wet wt) in OVX rats primed with EB(5 µg/rat) for 24 h and treated with iv (1 or 5 µg) or ivt (2.5 µg) NT for different time intervals. Each value represents mean ± SEM of 3-4 determinations.

*P<.05
**P<.001
vs control.

TABLE 10. cAMP AND cGMP AFTER ivt ADMINISTRATION OF SP FOLLOWED BY NT IN OVX, EB-PRIMED RATS.

Treatment	cAMP pmoles/mg wet wt	cGMP pmoles/mg wet wt
Saline	1.728±0.39	0.089±0.01
SP ivt 2.5 µg	3.217±0.12	0.021±0.06
NT ivt 2.5 µg	0.489±0.06	0.581±0.01
SP+NT ivt 2.5 µg each	1.698±0.07	0.073±0.01

cAMP (pmoles/mg) and cGMP (pmoles/mg) after ivt administration of 2.5 µg of SP immediately followed by 2.5 µg of NT in OVX rats primed with EB (5 µg/rat for 24 h). Rats were decapitated 15 min after peptide administration.

CHAPTER V

EFFECT OF SUBSTANCE P AND NEUROTENSIN ON THE IMMATURE RAT UTERUS AND OVARY

CHAPTER V

EFFECT OF SUBSTANCE P AND NEUROTENSIN ON THE IMMATURE RAT UTERUS AND OVARY

Substance P and NT have been shown to alter hypothalamic and pituitary hormone release (Chapter III). However, the consequences of such an effect on the physiology of the reproductive system remain speculative. Age and sex related changes in anterior pituitary SP have been documented (De Palatis et al, 1982). The SP content of the hypothalamus varies with the estrous cycle (Antonowicz et al, 1982; Coslovsky et al, 1984) and also shows a circadian variation (Kerdelhue et al, 1981). Evidence has accrued for a role of SP during ovulation. Infusion of SP into the third ventricle at proestrous retarded the number of rats ovulating (Naziemblo et al, 1983). In addition, recent evidence indicates the presence of SP in peripheral tissues (Bucsics et al, 1983) including the genitalia of the rat, guinea pig, cat, mouse and man. Substance P immunoreactive nerve terminals were found to innervate the cervix, vagina, uterus, fallopian tube and ovary (Gu et al, 1983). Since SP and NT are closely related it was contemplated that both these peptides have a potential role on the reproductive system. The effects of these two peptides in immature female rats was the object of this study.

The present study aimed to evaluate uterine and ovarian responsiveness to SP and NT. This was accomplished using the EB and PMSG induced ovarian and uterine growth as a model system. Exogenous SP and NT were administered concomitantly with EB and PMSG. Alterations in uterine and ovarian wts, DNA, RNA, protein content and ^{3}H-thymidine incorporation into DNA were used as parameters to study growth.

EXPERIMENTAL PROCEDURE

A. 20 day old female rats were injected with different doses of (0.5, 1 and 5 µg) of EB in sesame oil, sc and sacrificed 24 hrs later by cervical dislocation and the rate of ^{3}H-thymidine incorporation into uterine DNA was determined. In subsequent experiments, 20 day old rats were primed with 1 µg/rat of EB in sesame oil, sc or treated with vehicle alone and sacrificed 24 hrs later. Both primed and unprimed rats were treated with different doses of freshly prepared SP or NT in 0.9% NaCl, ip, for different time intervals as shown in the results. For incorporation of ^{3}H-thymidine into uterine DNA, 1 µCi of ^{3}H-thymidine per uterine horn was injected intraluminally into the uterus for varying time intervals for time course experiments and one hour before sacrifice in all other experiments. All rats were sacrificed 24 hrs after

injection of EB or vehicle. Uteri were dissected out and weighed to the nearest 0.2 mg. The DNA, RNA and protein fractions were separated and estimated according to the procedures described in Chapter II. An aliquot of the DNA supernatant was used for counting with Bray's scintillation fluid in a Beckman spectrometer.

Ovine LH, FSH, LHRH, compound 48/80 (condensation product of formaldehyde and P-methoxyphenethyl benzhydryl ether hydrochloride) were dissolved in 0.9% saline. Ovine Prl was dissolved in 0.9% NaCl adjusted to pH 8.0 with 0.1 N NaOH. Dopamine hydrochloride was dissolved in 1% ascorbic acid whereas pimozide was dissolved in 0.1 M tartaric acid. 50 µg/rat of LH or FSH or Prl or dopamine, 10 µg/rat of LHRH or compound 48/80, 0.63 mg/kg bw of pimozide were administered ip in a volume of 100 µl immediately before the administration of NT (2 µg/rat) in EB primed rats and the rate of ^{3}H-thymidine incorporation into uterine DNA was measured as described previously.

B. In a preliminary experiment 25 day old female rats were injected with 5, 10 or 20 iu/rat, of PMSG dissolved in 0.9% NaCl, sc, or SP or NT (1 or 2 µg/rat, sc) for 3 days. Controls received saline alone. On the 4th day the body weight was recorded and ovaries and uteri were removed, weighed and assayed for DNA, RNA and protein.

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B. In a preliminary experiment 25 day old female rats were injected with 5, 10 or 20 iu/rat, of PMSG dissolved in 0.9% NaCl, sc, or SP or NT (1 or 2 µg/rat, sc) for 3 days. Controls received saline alone. On the 4th day the body weight was recorded and ovaries and uteri were removed, weighed and assayed for DNA, RNA and protein.

In a subsequent experiment, rats were injected with 20 iu of PMSG, sc, followed by 2 µg SP or NT, sc daily for 3 days. On the fourth day after recording the body, ovarian and uterine weights, DNA, RNA and protein content of the ovaries and uteri were measured.

RESULTS

Effect of NT/SP on ^{3}H-thymidine incorporation, protein, DNA and RNA content of 21 day old female EB primed rat:

Intrauterine administration of ^{3}H-thymidine leads to a very efficient and reproducible uptake of the label. Considerable amount of ^{3}H-thymidine (1537.6 $\pm$ 597.03 CPM/ 100 µg DNA) was incorporated per h in immature rats and the incorporation was found to be linear atleast up to 4 hrs (Table-1).

^{3}H-thymidine incorporation into uterine DNA was significantly enhanced by EB. Table-2 shows the increase ^{3}H-thymidine incorporation after different doses of EB. Since 1 µg dose significantly ($P<.01$) increased the incorporation of ^{3}H-thymidine into DNA, in subsequent experiments a 1 µg dose of EB was used for priming rats.

Peripheral administration by ip injection of 2 µg NT significantly ($P<.001$) augmented the stimulatory effect of EB with a maximal effect at 2 hrs after the peptide

injection. There was no stimulation in estradiol induced DNA synthesis by NT at later times (Table-3). On the other hand, SP showed no significant effect on incorporation of ^{3}H-thymidine into uterine DNA (Table-4). In a dose response study with NT a 2 µg dose was nearly equally effective to that of a 10 µg dose (Table-5).

Neurotensin or SP in unprimed rats did not alter ^{3}H-thymidine incorporation into uterine DNA. There was no significant change in DNA, RNA or protein content in NT or SP treated rats 2hrsafter peptide administration. However, uterine DNA synthesis, as reflected by an increase in ^{3}H-thymidine incorporation showed an increase in EB primed rats (Table-6).

Table-7 demonstrates that the increase in ^{3}H-thymidine incorporation into uterine DNA is specific to NT action. LHRH (10 µg/rat ip) alone had no effect on ^{3}H-thymidine incorporation in EB primed rat. LH or FSH (50 µg/rat ip) administered concomitantly with NT failed to alter NT induced ^{3}H-thymidine incorporation whereas Prl (50 µg/rat ip) administered along with NT significantly ($P<.05$) increased ^{3}H-thymidine incorporation into uterine DNA compared to NT treated rats (Table-7).

Dopamine hydrochloride (50 µg/rat ip) or compound 48/80 (10 µg/rat ip) administered concomitantly with NT significantly ($P<.05$) reduced NT induced ^{3}H-thymidine incorporation into uterine DNA. On the other hand, simultaneous administration of pimozide (0.63 mg/kg body wt ip), a dopamine receptor blocker, significantly ($P<.05$) increased ^{3}H-thymidine incorporation into uterine DNA (Table-8).

Effect of SP/NT on PMSG induced uterine growth in 27 day old rat:

There was a dose-related increase in uterine wt in rats treated with 5, 10 and 20 iu PMSG for 3 days starting on day 25 of age. Both doses of SP or NT significantly ($P<.01$ for 1 µg dose and $P<.001$ for 2 µg dose) suppressed the uterine wts. When administered concomitantly with 20 iu of PMSG a 2 µg dose of SP significantly ($P<.01$) suppressed uterine wt when compared to that of rats treated with PMSG alone. There was a slight suppression in uterine wt in rats treated with NT (2 µg/rat/daily for 3 days sc) along with PMSG (Fig. 1).

Fig 2. shows that ovarian wet wt was significantly ($P<.001$) increased with all doses of PMSG. There was a

reduction in ovarian wt in rats treated with SP or NT alone. Substance P administered along with PMSG for 3 days significantly ($P<.01$) reduced the ovarian wet wt when compared to rats treated with 20 iu of PMSG alone. However, this suppression of ovarian weight was not to that of control level (Fig. 2).

Doses of 10 iu or 20 iu of PMSG administered for 3 days significantly ($P<.001$) increased uterine DNA content in rats whereas a 2 µg dose of SP or NT significantly ($P<.001$ for SP and $P<.05$ for NT) decreased uterine DNA content. Administration of SP + PMSG, significantly ($P<.05$) reduced uterine DNA content compared to rats treated with PMSG alone. On the other hand, NT + PMSG did not induce any alteration in uterine DNA content when compared to rats treated with 20 iu of PMSG alone (Fig. 3).

Fig. 4 demonstrates dramatic increase in the DNA content of ovaries with all doses of PMSG. A dose of 2 µg SP significantly ($P<.05$) reduced ovarian DNA content whereas NT administered alone was with out any effect. Neurotensin + PMSG significantly ($P<.05$) increased the ovarian DNA content.

The protein content of uteri treated with PMSG (20 iu/rat daily for 3 days) was significantly enhanced ($P<.01$). However, uterine protein content was significantly ($P<.001$) suppressed in rats treated with SP or NT. Concomitant administration of SP and PMSG reduced the protein content of the uterus to that of control level while NT administered along with PMSG was without effect (Fig. 5).

The ovarian protein content increased sharply with PMSG treatment. Both doses of SP or NT failed to modify the protein content of the ovaries. Administration of SP or NT along with PMSG caused significant ($P<.05$) decrease in the ovarian protein content (Fig.6).

The RNA/DNA ratio in control rat uterus was 0.41. Administration of PMSG did not alter the ratio of RNA/DNA significantly since RNA and DNA content of the uterus increased concomitantly. However, 1 µg SP or NT administered for three days significantly ($P<.01$) reduced the RNA/DNA ratio. The RNA/DNA ratio in uterus of rats treated with PMSG + SP or NT also reduced significantly ($P<.05$) when compared to rats treated with PMSG alone (Fig.7).

Fig.8 indicates that the ratio of ovarian RNA/DNA increases with PMSG administration. Administration of SP

or NT did not change the ovarian RNA/DNA ratio. Administration of SP and NT concomitantly with PMSG produced a slight reduction in the ovarian RNA/DNA ratio when compared to PMSG alone treated rats (Fig.8).

DISCUSSION

Estrogens initiate a complex set of biochemical responses in the immature rat uterus culminating in uterine growth at 24-36hrs(Clark et al, 1973; Katzellenbogen and Gorski, 1975; Mukku et al, 1982). Many factors have been investigated in relation to uterine growth in EB treated immature rats notably uptake of water and small molecules, protein, DNA, RNA content and incorporation of radioactive precursors into nucleic acids. ^{3}H-thymidine incorporation into DNA serves as sensitive indicator of estrogen action (Kaye et al, 1972). The present results confirm earlier findings (Stormshak et al, 1976) that a single dose of EB causes maximal stimulation of ^{3}H-thymidine incorporation 24 hrs later. This 24hrs response is associated with increase in mitotic activity (Kaye et al, 1972; Quarmby and Korach, 1984). Substance P did not exert any action whereas NT augmented this effect of EB with a maximal effect 2 hrs after peptide administration. None of the actions of NT have been related to the reproductive system. Nevertheless, NT is known to act on the intestinal smooth muscle

(Kitabgi, 1982). The uterine smooth muscle is very similar to that of the gi tract (Kuriyama, 1968). Peripheral administration of NT does not have central effects as it does after ivt injection (Vijayan and McCann, 1979a, 1980, Chapter III). Therefore, it is plausible that NT can exert some action on the uterus. The maximal effect of NT on uterine DNA synthesis in EB primed rats was at 2 hrs. The peptide probably binds to uterine receptors setting off a train of events that persists long after it is degraded. Alternately, the physiological effect of the peptide could be due to cross reaction with receptors meant for substances akin to NT which are physiological mediators of its action. Its action may indicate some secondary phenomenon resulting from the initial stimulation of some essential biochemical processes.

The stimulation of EB induced ^{3}H-thymidine incorporation by NT was unaltered by concomitant administration of LH and FSH whose trophic effects on the reproductive system are well known. Luteinising hormone releasing hormone (LHRH) too, which is similar to NT by having its NH_2 terminus a pyroglutamyl moiety, failed to alter ^{3}H-thymidine incorporation when administered alone. Luteinising hormone releasing hormone had no effect on the NT induced stimulation in ^{3}H-thymidine incorporation. However,

concomitant administration of Prl with NT further increased the stimulatory effect of NT on ^{3}H-thymidine incorporation. The involvement of Prl on cell proliferation in the reproductive system (Keenan et al, 1981) as well as the pituitary (Kalberman et al, 1980; Jacobi and Lloyd, 1981; Burdman et al, 1983) have been reported. However, present data does not indicate whether NT and Prl increase ^{3}H-thymidine incorporation into uterine DNA independently. Administration of dopamine which inhibits Prl release, along with NT resulted in a decrease in DNA synthesis whereas pimozide, a dopamine receptor blocker, caused an opposite effect suggesting that the dopaminergic system may be involved in the stimulatory effect of NT on uterine ^{3}H-thymidine uptake.

The estrogen induced release of histamine (Szego, 1965) which appears to be involved in the rapid onset of hyperemia and water imbibition is difficult to correlate with findings regarding the genome activation. The hypotensive effect of neurotensin is thought to be mediated via histamine (Quirion et al, 1980; Rioux et al, 1982). Compound 48/80, which depletes mast cells of its histamine content and is a well known histamine liberator (Feldberg and Talesnik, 1953; Lipner, 1971) when administered along with NT decreased the NT induced ^{3}H-thymidine incorporation

in EB-primed rats. However, SP is also known to release histamine (Johnson and Erdos, 1973) and the fact that it failed to modify ^{3}H-thymidine influx into the cell is difficult to interpret.

In contrast to the effect of a single injections of NT in the 21 day old rat, three injections of NT or SP, in 2 day old rat seemed to have an inhibitory role in the reproductive system. Administration of 1 and 2 µg of NT or SP reduced uterine and ovarian wts in rats unchallenged with exogenous PMSG. In the PMSG treated rats too SP significantly lowered the uterine and ovarian weights as well as the macromolecular content as reflected by a decrease in the content of DNA, RNA and protein. These represent an alteration in the gross metabolism of the body since body wts were unaltered by peptide treatment.

Suppression of PMSG and human choronic gonadotropin (HCG) induced uterine and ovarian growth by brain peptides is not entirely a novel phenomenon. Arginine vasotocin and melatonin extracted from the pineal has been shown to consistently lower ovarian and uterine wts in rats (Vaughan et al, 1976a,b; Johnson et al, 1978). In this regard it is interesting to note that SP is present in exceptionally high quantities in the pineal gland (Marks, 1977). Recent

studies also indicate inhibition of the action of steroid hormones by LHRH and its agonists (Sundaram et al, 1981).

The patterns of change in ovarian wts in this study treated with PMSG alone is consistent with that found in literature (Sasamota and Johke, 1975). The pattern of ovarian growth changes with peptide may possibly reflect inhibition of follicular growth by interfering with the action of pituitary hormones at the level of the ovary. The apparent suppression in uterine growth possibly reflects decreased estrogen levels in rats treated with peptide or direct effects at the level of the uterus to inhibit growth. The mechanism of the antisteroid action of the peptide remains to be established.

The present results thus indicate that SP and NT possibly exert some influence on the process of growth and differentiation. Clearly, the effects of SP and NT on the 21 day old rats and 28 day old rats are different. Either the sensitivity to these peptides is altered or the different hormone regimen is responsible for these varied effects. The inhibitory role of SP is consistent with that found earlier (Naziemblo et al, 1983) which was antiovulatory.

TABLE 1. ^{3}[H]-THYMIDINE INCORPORATION INTO UTERINE DNA AFTER INTRAUTERINE ADMINISTRATION IN IMMATURE RATS

Time after ^{3}H-thymidine administration (h)	^{3}H-thymidine incorporation CPM/100 µg DNA
1	1537.0 ± 597.0
2	3095.2 ± 852.1
3	5042.0 ± 170.7
4	8295.6 ± 440.0

Rats were injected with ^{3}H-thymidine (1 µCi/ uterine horn) and sacrificed at the times indicated. The rate of ^{3}H-thymidine incorporation into DNA was determined as in Materials and Methods. Values are mean ± SEM.

TABLE 2. ^{3}H-THYMIDINE INCORPORATION INTO UTERINE DNA AFTER PRIMING WITH DIFFERENT DOSES OF EB.

Dose of Estradiol Benzoate	^{3}H-thymidine incorporation CPM/100 µg DNA/h
Control	1537.0 ± 597.0
0.5 µg	4031.8 ± 541.2
1 µg	9050.0 ± 935.1**
5 µg	20290.8 ± 494.2***

^{3}H-thymidine incorporation into uterine DNA after priming with different doses of EB (Sc for 24 hrs) dissolved in sesame oil in 21 day old rat. Controls received vehicle alone. ^{3}H-thymidine was administered intraluminally for 1 h. Values are mean ± SEM.

**P<0.01
***P<.001
vs control

TABLE 3. ^{3}[H]-THYMIDINE INCORPORATION INTO UTERINE DNA/h IN ESTRADIOL PRIMED IMMATURE RATS AFTER 2 µg OF NT FOR DIFFERENT TIME INTERVALS.

Time after NT administration (h) before sacrifice	% change over control	E/C value
1	54.9	1.6
2	70.3	2.1
4	10.9	1.1
8	-1.3	0.9
12	-17.9	0.8
24	21.2	1.2

Rats were primed with 1 µg EB and sacrificed 24 hrs later. The rate of ^{3}H-thymidine incorporation was determined as in Materials and Methods 1 h after the administration of ^{3}H-thymidine. Neurotensin 2 µg/rat ip was injected at the times indicated.

E/C = Experimental/Control

TABLE 4. ^{3}H-THYMIDINE INCORPORATION INTO UTERINE DNA/h IN EB PRIMED IMMATURE RATS AFTER 2 µg OF SP FOR DIFFERENT TIME INTERVALS.

Time after SP administration (h) before sacrifice	% change over control	E/C value
1	-3.75	0.96
2	5.3	1.05
4	11.4	1.12
8	17.46	1.2
12	34.9	1.53
24	10.8	1.08

Rats were primed with 1 µg EB and sacrificed 24 hrs later. The rate of ^{3}H-thymidine incorporation was determined 1 h after the administration of ^{3}H-thymidine. Substance P 2 µg/rat was injected ip at the times indicated.

E/C = Experimental/Control

TABLE 5. ^{3}H-THYMIDINE INCORPORATION INTO UTERINE DNA, 2 h AFTER DIFFERENT DOSES OF NT IN EB PRIMED RATS.

Dose of NT	^{3}H-thymidine incorporation CPM/100 µg DNA/h
Control	9050.0 ± 935.1
100 ng	10163.7 ± 1063.4
2 µg	18801.7 ± 1848.2***
10 µg	21202.0 ± 905.3***
50 µg	26873.0 ± 1212.6***

Rats were primed with 1 µg EB and sacrificed 24 hrs later. The rate of ^{3}H-thymidine incorporation was determined 1 h after the administration of ^{3}H-thymidine. Different doses of NT were administered 2 hrs before autopsy. Values are mean ± SEM.

***P<.001 vs control.

TABLE 6. EFFECT OF NT/SP (2 μg/RAT FOR 2 HOURS) ON UTERINE WEIGHT, DNA, RNA, PROTEIN AND ^{3}H-THYMIDINE INCORPORATION INTO DNA IN IMMATURE RATS.

Treatment	Uterine wet wt (mg)	DNA content μg/uterus	RNA content μg/uterus	Protein content μg/uterus	^{3}H-thymidine incorporation CPM/100 μg DNA/h
C	22.4±0.9(11)	188.1±18.0	85.0± 4.4	352.5±42.4	1537.6± 597.0
EB	39.5±1.0(66)[a]	293.7±25.0[b]	133.3±13.3[b]	591.6±35.6[b]	9050.0± 935.1
NT	21.0±1.1(6)	190.3±17.3	78.3±10.9	363.0±24.0	1223.0± 429.3
EB+NT	44.3±1.6(28)[a]	276.0±21.6[b]	185.0±34.6[b]	626.0±71.0[b]	18801.7±1848.2[c]
SP	20.7±1.1(6)	192.1±20.6	82.3±11.2	358.1±28.2	1397.1± 832.4
EB+SP	42.0±2.4(11)[a]	295.6±23.48[b]	144.1±12.4[b]	602.4±26.8[b]	9563.6±1794.3

NT or SP = treated with 2 μg of the peptide/rat ip for 2 hrs, EB+NT or SP = treated with NT or SP in rats previously primed with EB. Values are mean ± SEM. Numbers in parentheses indicate the number of animals used. C = Control, EB = primed with 1 μg Estradiol benzoate for 24 h.

a = P<.001 compared to controls
b = P<.05 compared to controls
c = P<.001 compared to controls and/or EB treated animals.

TABLE 7. EFFECT OF LHRH, LH, FSH AND Prl ON NT INDUCED UTERINE ^{3}H-THYMIDINE INCORPORATION IN EB PRIMED IMMATURE RAT.

Treatment	^{3}H-thymidine incorporation CPM/100 µg DNA/h
Control	9050.0 ± 935.1
NT	18801.7 ± 1848.2
LHRH	10373.1 ± 567.8
LHRH + NT	17648.6 ± 609.3[a]
LH + NT	17,762.0± 518.9[a]
FSH + NT	20,664.0± 837.2[a]
Prl + NT	24,588.0± 721.3[c]

^{3}H-thymidine incorporation into uterine DNA, 2hrs after the administration of NT (2 µg/rat ip), in combination with LHRH (10 µg/rat ip), LH (50 µg/rat ip), FSH (50 µg/rat) or Prl (50 µg/rat ip) in EB primed (1 µg/rat/24hrs) 21 day old rat.

a $P < .001$ compared to control

c $P < .001$ compared to control

c $P < .05$ compared to NT treated rats.

TABLE 8. EFFECT OF DOPAMINE, COMPOUND 48/80 AND PIMOZIDE ON NT INDUCED ^{3}H-THYMIDINE INCORPORATION IN EB PRIMED IMMATURE RAT.

Treatment	^{3}H-thymidine incorporation CPM/100 µg DNA/h
NT	18,801.7 ± 1848.2
NT + dopamine	12,787.3 ± 477.6*
NT + compound 48/80	12,964.9 ± 1339.2*
NT + pimozide	26,832.0 ± 2328.4*

^{3}H-thymidine incorporation into uterine DNA after 2 µg (ip) of NT for 2hrs in combination with dopamine, compound 48/80 or pimozide in EB primed (1 µg/rat for 24hrs) 21 day old rat.

*P<.05 vs NT treated rats.

Fig. 1 Uterine wts (mg) of 25 day old rats treated with different doses of PMSG(sc) and/or SP/NT(sc) daily for 3 days. On day 28 rats were autopsied 24 hrs after they received the last injection. Controls received saline alone. In this and subsequent figures results are mean ± SEM. The numbers in parentheses indicate the number of animals used per group.

*P< .05
**P< .01
***P< .001
vs control

++ P < .05 vs PMSG (20 IU)

UTERINE WET WT.(mg)
100
80
60
40
20
CONTROL
(7)
PMSG 5 I.U
(4)
PMSG 10 I.U
(7)
PMSG 20 I.U
(6)
SP 1μg
(7)
SP 2μg
(4)
NT 1μg
(4)
NT 2μg
(5)
PMSG 20 I.U
SP 2μg
(5)
PMSG 20 I.U
NT 2μg
(5)

Fig. 2 Ovarian wts (mg) of 25 day old rats treated with different doses of PMSG(sc) and/or SP/NT(sc) daily for 3 days. On day 28 rats were autopsied 24 hrs after they received the last injection.

*P<.05
**P<.01 vs control
***P<.001

++ P<.01 vs PMSG(20 iu)

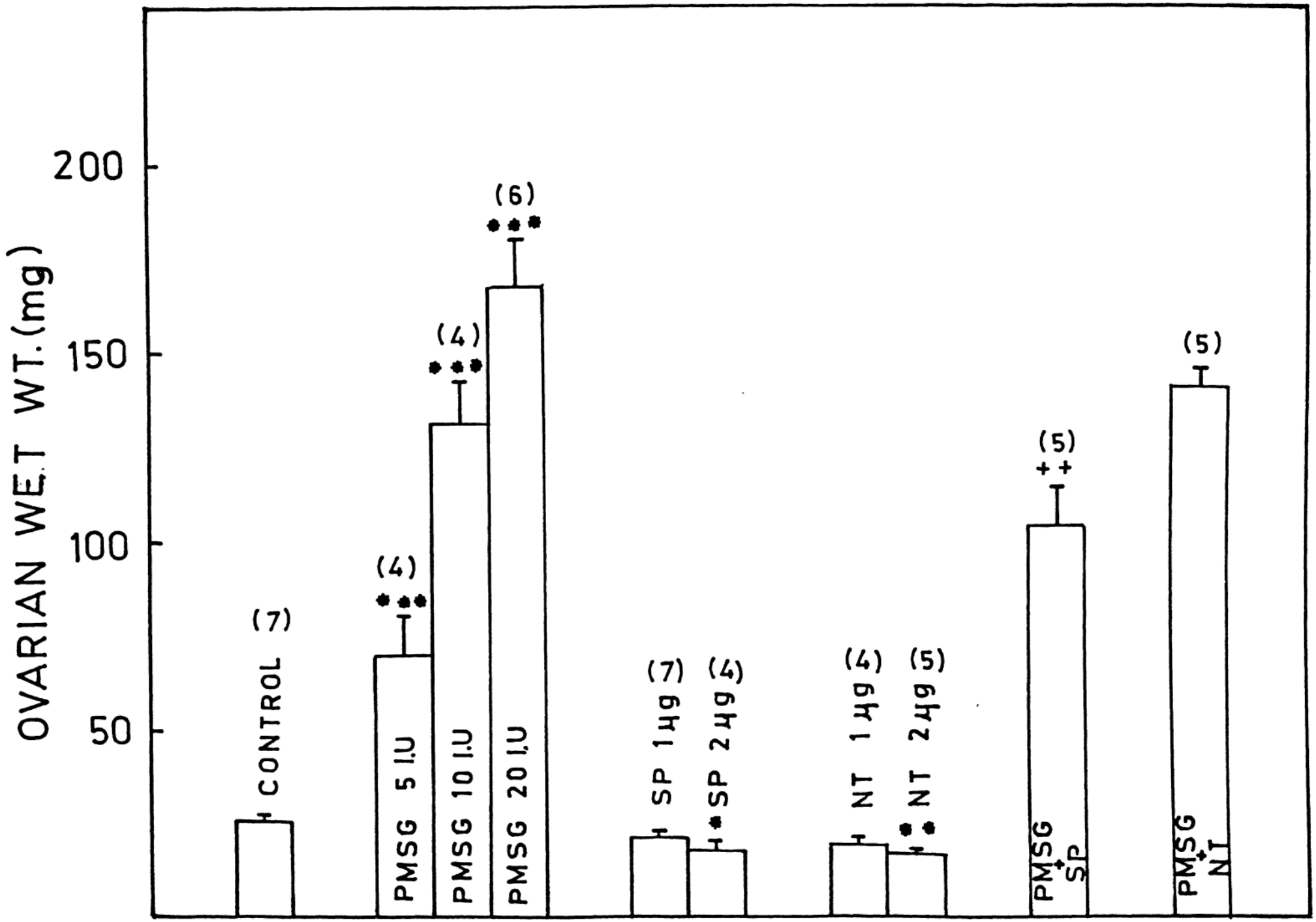

OVARIAN WET WT.(mg)
200
150
100
50
(7)
CONTROL
(4)
PMSG 5 I.U
(4)
PMSG 10 I.U
(6)
PMSG 20 I.U
(7)
SP 1μg
(4)
SP 2μg
(4)
NT 1μg
(5)
NT 2μg
(5)
PMSG + SP
(5)
PMSG + NT

Fig. 3 Uterine DNA content (µg) of 25 day old rats treated with different doses of PMSG(sc) and/or SP/NT(sc) daily for 3 days. On day 28 rats were autopsied 24 hrs after they received the last injection.

*P<.05
***P<.001 } vs control

+ P<.05 vs PMSG(20 iu)

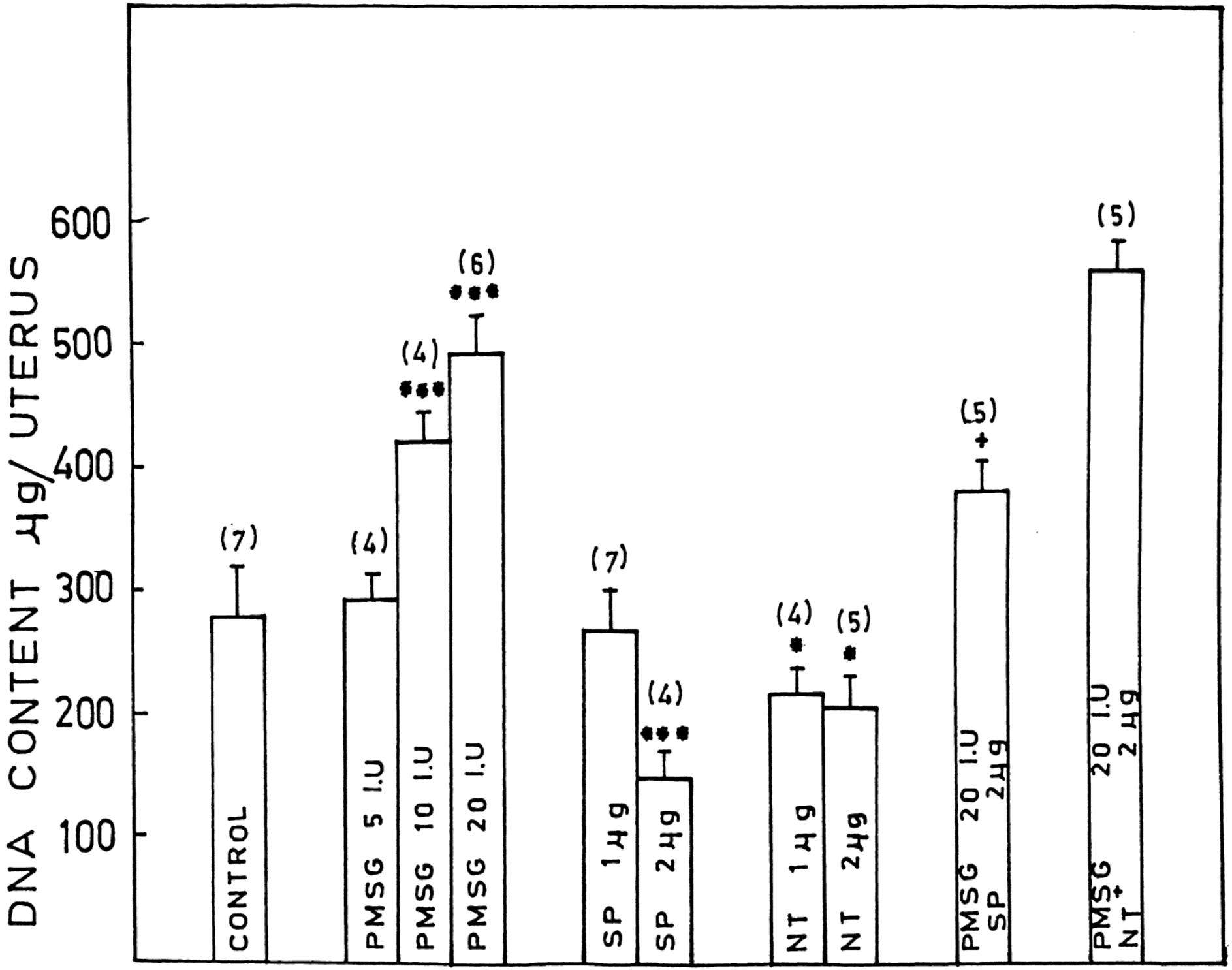

DNA CONTENT µg/ UTERUS
600
500
400
300
200
100
(7)
(4)
(4)
(6)
(7)
(4)
(4)
(5)
(5)
(5)
CONTROL
PMSG 5 I.U
PMSG 10 I.U
PMSG 20 I.U
SP 1µg
SP 2µg
NT 1µg
NT 2µg
PMSG 20 I.U SP 2µg
PMSG 20 I.U NT+ 2µg

Fig. 4 Ovarian DNA content (µg) of 25 day old rats treated with different doses of PMSG(sc) and/or SP/NT(sc) daily for 3 days. On day 28 rats were autopsied 24 hrs after they received the last injection.

*P< .05
***P< .001 } vs control

+ P< .05 vs PMSG(20 iu)

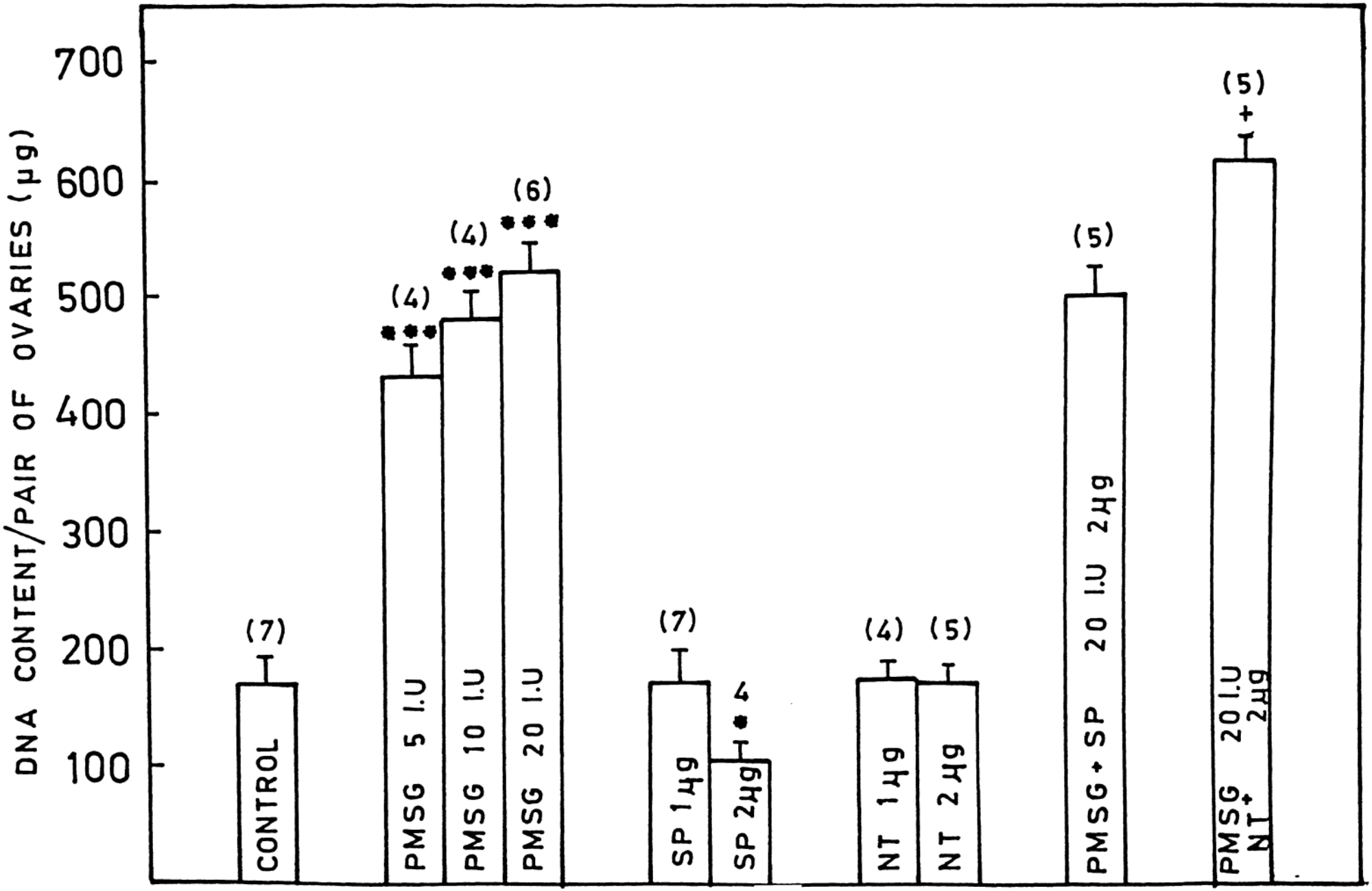

DNA CONTENT/PAIR OF OVARIES (µg)
700
600
500
400
300
200
100
(7)
CONTROL
(4)
PMSG 5 I.U
(4)
PMSG 10 I.U
(6)
PMSG 20 I.U
(7)
SP 1µg
SP 2µg
(4)
NT 1µg
(5)
NT 2µg
(5)
PMSG + SP 20 I.U 2µg
(5)
PMSG 20 I.U + NT 2µg

Fig. 5 Uterine protein content (mg) of rats injected with PMSG and/or SP/NT sc for 3 days starting on day 25. Rats were autopsied on day 28 24 hrs after the last injection.

**P<.01
***P<.001 } vs control

++ P<.01 vs PMSG(20 iu)

PROTEIN CONTENT/UTERUS (mg)
1
2
3
4
CONTROL
(7)
PMSG 5 I.U
(7)
PMSG 10 I.U
(4)
PMSG 20 I.U
(9)
SP 1μg (7)
SP 2μg (4)
NT 1μg (7)
NT 2μg (5)
PMSG 20 IU
SP 2μg
(5)
++
PMSG 20 IU
NT 2μg
(5)

Fig. 6 Ovarian protein content (mg) of rats injected with PMSG and/or SP/NT for 3 days starting on day 25. Rats were autopsied on day 28 24 hrs after the last injection.

***P< .001 vs control

+ P< .05 vs PMSG(20 iu)

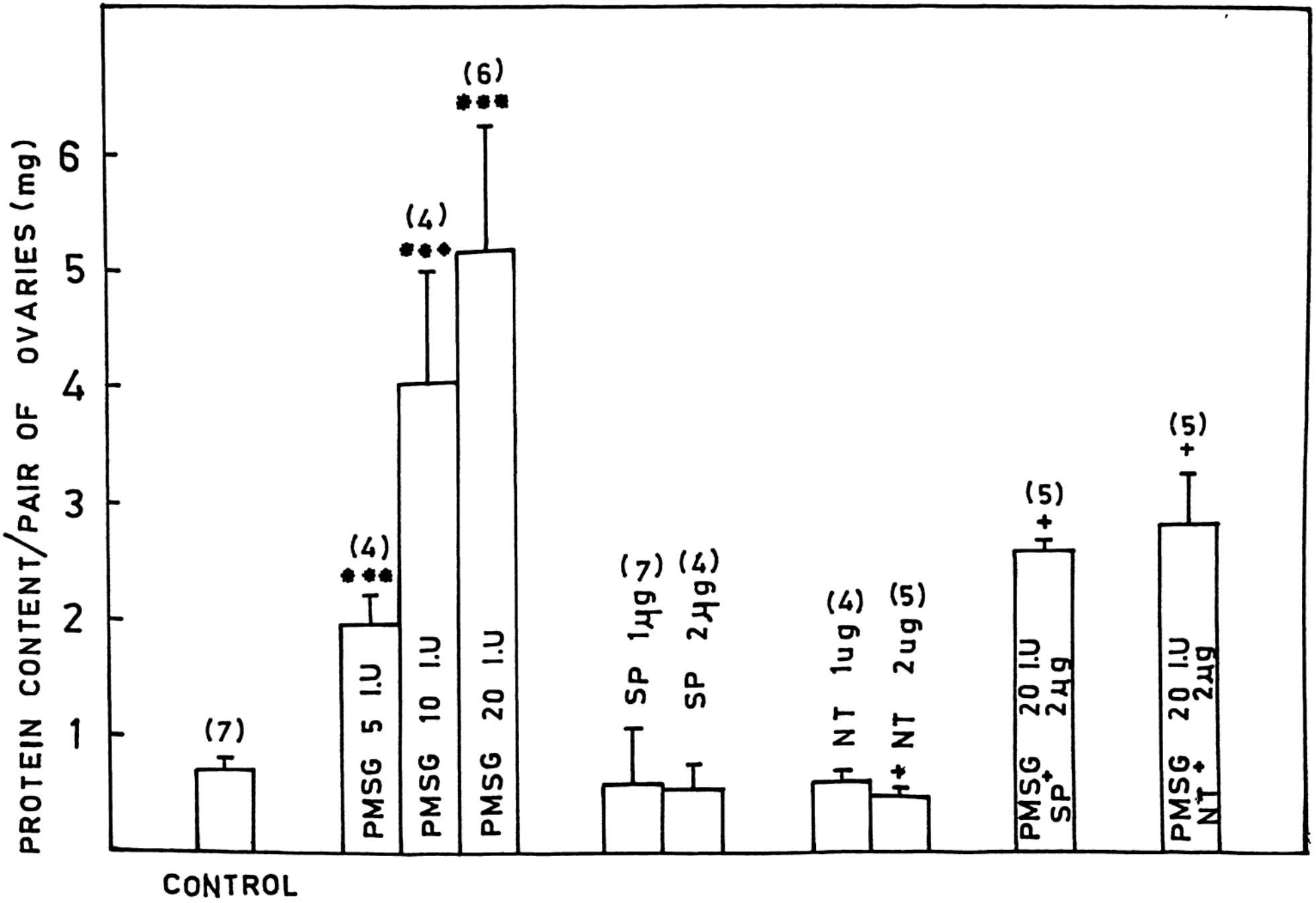

PROTEIN CONTENT/PAIR OF OVARIES (mg)
1
2
3
4
5
6
(7)
(4)
(4)
(6)
(7)
(4)
(4)
(5)
(5)
(5)
CONTROL
PMSG 5 I.U
PMSG 10 I.U
PMSG 20 I.U
SP 1μg
SP 2μg
NT 1ug
+ NT 2ug
PMSG 20 I.U SP + 2μg
PMSG 20 I.U NT + 2μg

Fig. 7 Uterine RNA/DNA ratio of 25 day old rats treated with different doses of PMSG(sc) and/or SP/NT(sc) for 3 days. On day 28 rats were autopsied 24 hrs after they received the last injection.

**P<.01 vs control

+ P<.05 vs PMSG(20 iu)

UTERINE RNA/DNA RATIO
0.5
0.25
CONTROL
(7)
PMSG 5 I.U
(7)
PMSG 10 I.U
(4)
PMSG 20 I.U
(6)
SP 14g
(7)
SP24g
(7)
NT 14g
(7)
NT 2ug
(5)
PMSG 20 IU
SP 24g
(5)
PMSG 20 IU
NT 24g
(5)

Fig. 8 Ovarian RNA/DNA ratio of 25 day old rats treated with different doses of PMSG(sc) and/or SP/NT(sc) for 3 days. On day 28 rats were autopsied 24 hrs after they received the last injection.

***P<.001 vs control

+ P<.05 vs PMSG(20 iu)

OVARIAN RNA/DNA RATIO
0.5
0.25
CONTROL
(7)
PMSG 5 I.U
(7)
***
PMSG 10 I.U
(7)
***
PMSG 20 I.U
(6)
***
SP 1μg
(7)
SP 2μg
(4)
NT 1μg
(7)
NT 2μg
(5)
PMSG 20 I.U
SP 2μg
(5)
+
PMSG 20 I.U
NT 2μg
(5)
+

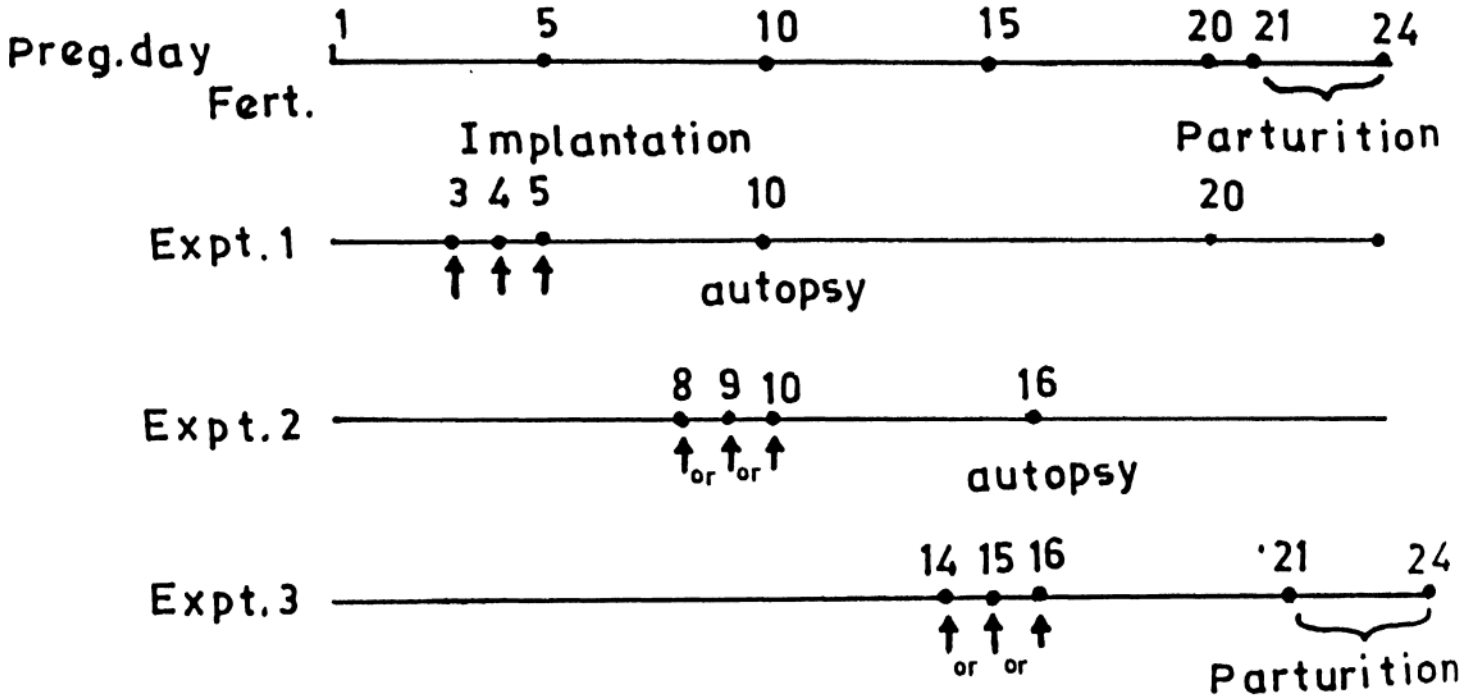
CHAPTER VI

EFFECT OF SUBSTANCE P AND NEUROTENSIN ON IMPLANTATION AND GESTATION IN THE PREGNANT RAT

A number of antifertility agents have been investigated for the development of post-coital contraception during the last decade. Most of these are formulations containing estrogenic or progestational compounds or both (Yuzpe, 1979; Porter and Jones, 1981). However, other compounds notably bromocryptine (Muller et al, 1980), serotonin (Aliverti et al, 1982; Mitchell and Hammer, 1983; Mitchell et al, 1983), histamine (Hoos and Hoffman, 1983; Wordinger et al, 1985), prostaglandins (Batta and Martini, 1975; Biggers et al, 1981), HCG (Hahn et al, 1980; Kato et al, 1983) and LHRH have been demonstrated to be successful to varied extent. Luteinising hormone releasing hormone, its agonists and antagonists can disrupt the normal pattern of hormone secretion before or after implantation and interfere with pregnancy (Corbin et al, 1976, 1979; Humphrey et al, 1976, 1977; Beattie and Corbin 1977; Rivier et al, 1979; 1981; Nillius, 1983; Babu and Vijayan, 1983b). In addition, LHRH also exerts a local extrapituitary antifertility effect (Jones, 1979; Bex and Corbin, 1981; Vijayan, 1985b).

The pregnant rat has been frequently used for testing various substances applied directly into the uterus to determine their effects on implantation and fetal viability. One approach is to inject various test substances directly into the uterine horn and to observe the subsequent effect on reproductive function (Batta and Martini, 1975; Bo et al,1976; Connor et al, 1976; Jones, 1979; Wu and Gu, 1981; Vilar-Rojas et al, 1982). Substance P is prevalent in the genitourinary tract (Alm et al, 1978; Gu et al, 1983). Neurotensin is similar to LHRH in having its N-terminus a pyroglutamyl moiety. Furthermore, the results of the previous chapter have indicated a role for SP and NT in peripheral reproductive processes. Hence it seemed pertinent to define the role, if any, of SP and NT during pregnancy. The present study describes the effect of intrauterine micro injection of SP and NT on implantation and fetal survival. Uterine glycogen, an important energy source for both embryonic development (Demers et al, 1972) and parturition (Chew and Rinard, 1979) serves as a convenient marker for the development and maintainence of pregnancy (Vasilenko et al, 1981). In addition to recording the number of viable fetuses uterine glycogen levels were monitored.

Treatment schedule for testing the anti implantation effect of SP/NT



The top line in the figure shows events in a normal pregnancy. In the different experiments arrows indicate intrauterine injections of saline or SP/NT

EXPERIMENTAL PROCEDURE

Adult female rats displaying regular 4 day cycles were allowed to cohabit with males of proven fertility on the evening of proestrous and the presence of sperm in the vaginal smear on the following morning was considered day 1 (D1) of pregnancy. Three different experimental schedules were designed to test the effect of SP or NT at various stages of pregnancy as outlined in Fig. 1. 100 µg of SP or NT were dissolved in 2.5 µl of 0.9% NaCl and injected into the right horn of the uterus below the utero-tubal junction on days 3,4 or 5 or on days 8,9,10. The contralateral horn served as a control. Two groups of control animals received an intrauterine injection of 2.5 µl of saline in the right uterine horn on either day 4 or day 9. Animals were sacrificed on day 10 or on day 16 and the uterine horns were divided and weighed individually. The number of viable fetuses in each horn were recorded. Fetuses from both horns in control and experimental groups were examined and weighed individually. The distinction between alive or dead was clear because of size and colour difference of the implantation sites. The glycogen content of the uterus was determined by the method of Lo et al, (1970) as described in chapter-II. Groups of rats were injected intraluminally on D14 or D15 or D16 with 100 µg of SP or NT in 2.5 µl saline into both uterine horns. One

group was injected with 2.5 µl saline into both uterine horns. These rats were allowed to continue for term and allowed to parturate. The length of gestation and the number of pups born were recorded.

RESULTS

The injected horn (right) of the control group was unaffected by the administration of 2.5 µl saline on D 4. There was no alteration in the number of viable fetuses in the right horn injected with SP or NT on D 3 of pregnancy. However, there was a significant reduction in the number of viable fetuses in the right horn of rats injected with SP or NT on D 4 or D 5 of pregnancy. The weight of the right horn and the glycogen content also decreased in SP or NT treated rats on D 4 or D 5 of pregnancy. There was no significant difference between the left horn of the treated and control group with regard to the number of viable fetuses, weight of the uterus or glycogen content (Table. 1 and 2).

Table 3 and 4 demonstrate that there is no significant difference between the right horn treated with SP or NT on D8, D9 or D10 of pregnancy and the untreated contralateral horn with regard to the number of viable fetuses, weight of the uterus or glycogen content. There

was also no difference between saline injected right horn and SP/NT injected horns on D9 of pregnancy.

Intrauterine administration of 100 µg SP or NT on D14, D15 or D16 of pregnancy did not alter gestation length or the number of pups born as compared to rats injected with saline (Table 5).

DISCUSSION

The present study demonstrates that intrauterine administration of 100 µg of SP or NT on D4 or D5 of pregnancy disrupts implantation. Results indicate a local claudogenic effect of SP/NT when administered intraluminally. This local effect is reflected in the decreased weight of the injected horn and its contents and the decreased glycogen content. However, the contralateral uninjected horn is unaffected with regard to the number of fetuses, weight of the uterus and glycogen concentration. The effect of SP/NT therefore, seems to be a direct one. The peptide, injected intraluminally in the right uterine horn is not being absorbed into the systemic circulation in sufficient quantities to produce an effect in the contralateral horn. The antifertility effect of SP/NT prior to implantation occurs through a mechanism other than that involving the hypophyseal - ovarian axis.

However, the mechanism by which SP/NT exert their antifertility effect remains unelucidated. Normal endometrial function particularly during periods of enhanced metabolic activity requires abundant supply of O_2 and metabolic substrates (Yochim, 1975; Heald, 1976). In response to an implanting blastocyst the uterus in many mammals exhibits a localized increase in vascular permeability at the implantation sites (Psychoyos, 1973) which is thought to be elicited by vasoactive mediators. The process of implantation begins when mammalian zygote reaches the stage of blastocyst during its development (Hicks and Gil-Recaseus, 1980) and makes contact with the endometrial surface epithelium which is differentiated into specific regions called implantation sites. Both SP and NT are potent vasoactive substances. The inhibition of implantation by SP/NT appears to be effective only during the periimplantation period on day 4 or 5 - the day when the blastocyst loses its zona and interactions between the blastocyst and uterus are initiated. Substance P is capable of altering vascular permeability directly on blood vessels and indirectly through mast cell histamine (Foreman and Jordan, 1983). Vascular alterations in the uterus impairs implantation (Franklin and Brent, 1964) and fetal development (Bruce, 1977) as has been demonstrated for serotonin (Mitchell and Hammer, 1983). The antiimplantation effect of SP/NT may be due to alterations in vascular permeability caused by the peptides.

No effect on implantation was seen when SP/NT was injected on day 3, possibly because the ova have not yet entered the uterus. It is also interesting to note, no dramatic deleterious effects on pregnancy and gestation when SP/NT were injected during the latter half of pregnancy after the implantation period. The present data thus suggests that SP and NT are capable of exerting a direct, local antifertility effect on the process of implantation.

TABLE 1. POST-COITAL CONTRACEPTIVE EFFECT OF A SINGLE INTRAUTERINE (RIGHT HORN) INJECTION OF SP ON D3, D4 OR D5 OF PREGNANCY

Treatment	No. Viable Fetuses (mean ± SEM)		uterine Weight (gm) (mean ± SEM)		Glycogen content (mg/100 g) (mean ± SEM)	
	Right	Left	Right	Left	Right	Left
Saline D4(6)	5.9 ±0.87	5.72±0.67	2.0±0.20	1.8±0.31	220.4± 5.2	218.0±6.8
SP D3(4)	6.11±0.92	5.93±0.62	1.9±0.11	1.9±0.12	232.1±8.6	216.4±9.3
SP D4(5)	1.98±0.87*	6.01±0.73	0.4±0.21**	2.1±0.11	141.0±10.6**	210.5±6.9
SP D5(4)	2.01±0.58*	5.81±0.71	0.6±0.3*	1.9±0.20	162.9±8.1*	206.4±8.4

Figures in parentheses indicate the number of animals used per group.

*P<.05
**P<.01
} vs contralateral horn

TABLE 2. POST COITAL CONTRACEPTIVE EFFECT OF A SINGLE INTRAUTERINE (RIGHT HORN) INJECTION OF NT ON D3, D4 OR D5 OF PREGNANCY

Treatment	No. Viable fetuses (mean ± SEM)		uterine weight (gm) (mean ± SEM)		Glycogen content (mg/100 g) (mean ± SEM)	
	Right	Left	Right	Left	Right	Left
Saline D4(4)	5.9 ±0.87	5.72±0.67	2.0±0.20	1.8±0.31	220.4±5.2	218.0±6.8
NT D3(4)	5.16±0.67	5.91±0.81	1.9±0.33	1.9±0.41	224.6±6.8	230.8±5.2
NT D4(4)	2.42±0.55*	6.1 ±0.58	0.6±0.32**	2.0±0.30	185.4±8.4*	222.4±3.8
NT D5(4)	1.52±0.68*	5.73±0.72	0.5±0.11*	1.8±0.12	176.8±6.6*	215.3±5.6

*P<.05
**P<.01
} vs contralateral horn

TABLE 3. EFFECT OF A SINGLE INTRAUTERINE INJECTION OF SP (RIGHT HORN) ON D8, D9 OR D10 OF PREGNANCY

Treatment	No. of Viable fetuses (mean ± SEM)		uterine Weight (gm) (mean ±SEM)		Glycogen content (mg/100 g) (mean ± SEM)	
	Right	Left	Right	Left	Right	Left
Saline D9(4)	5.92±0.56	6.18±0.70	6.4±0.8	7.1±0.5	230.4±18.3	235.4±8.4
SP D8(4)	6.12±0.23	5.9 ±0.29	7.8±0.5	6.8±0.4	218.4±9.8	241.4±8.1
SP D9(4)	6.05±0.41	4.99±0.81	6.2±0.3	6.9±0.8	244.6±10.2	228.6±7.6
SP D10(4)	5.86±0.56	5.91±0.34	5.9±0.6	6.3±0.5	230.1±6.9	229.1±7.2

TABLE 4. EFFECT OF A SINGLE INTRAUTERINE INJECTION OF NT (RIGHT HORN) ON D8, D9 OR D10 OF PREGNANCY

Treatment	No. Viable fetuses (mean ± SEM)		uterine Weight (gm) (mean ± SEM)		Glycogen content (mg/100 g) (mean ± SEN)	
	Right	Left	Right	Left	Right	Left
Saline D9(4)	5.92±0.56	6.18±0.70	6.4±0.8	7.1±0.5	230.4±18.3	235.4±8.4
NT D8(4)	6.01±0.49	6.2 ±0.56	6.9±0.2	6.3±0.6	236.8± 9.3	232.1±5.6
NT D9(4)	7.00±0.51	6.3 ±0.82	5.9±0.6	6.1±0.3	230.4±5.2	222.1±6.8
NT D10(4)	5.32±0.45	6.2 ±0.91	6.0±0.2	6.0±0.4	214.8±9.8	231.6±6.8

TABLE 5. EFFECT OF SP/NT ON GESTATION AND SIZE OF LITTER

Treatment	Day of Delivery	No. of Pups/ litter surviving
Saline D15(5)	22.4 ± 0.5	9.4 ± 0.7
SP-D14 or D15 or D16(11)	23.1 ± 0.2	8.6 ± 0.9
NT-D14 or D15 or D16(12)	22.4 ± 0.6	8.5 ± 0.7

Effect of intrauterine injection of saline or SP/NT on either D14, D15 or D16 of pregnancy. Since there was no significant difference in the values on either days, the results for each peptide are pooled and represented as mean ± SEM.

CHAPTER VII

EFFECT OF SUBSTANCE P AND NEUROTENSIN ON PLASMA AND HEPATIC TOTAL LIPID, CHOLESTEROL, TRIGLYCERIDE AND FREE FATTY ACID LEVELS

CHAPTER VII

EFFECT OF SUBSTANCE P AND NEUROTENSIN ON PLASMA AND HEPATIC TOTAL LIPID, CHOLESTEROL, TRIGLYCERIDE AND FREE FATTY ACID LEVELS

Few recent studies have revealed that SP and NT cause transient alterations in circulating cholesterol (Raju, 1980; Peric Golia et al, 1980, 1984). Cholesterol is an obligatory precursor for steroid hormone synthesis (Bransome, 1968). It is also a necessary component of virtually all cellular membranes of higher animals. A possible role for NT in cholesterol transport has been speculated (Carraway and Leeman, 1975b). Preliminary experiments by Peric Golia et al, (1979) demonstrated a significant hypercholesterolemic effect of NT not modified by adrenalectomy or hypophysectomy. Subsequently, studies in our laboratory confirmed the hypercholesterolemic effect of NT which was antagonized by SP (Raju, 1980; Raju and Vijayan 1981). Neurotensin is abundantly present in the small intestine in co-existance with synthesis and assembly of some cholesterol transporting lipoproteins (Mahley et al, 1971; Fernstrom et al, 1980) suggesting a regulatory role in the intenstinal transport of cholesterol.

Neurotensin like immunoreactivity (NTLI) increased after food intake (Besterman et al, 1978; Mashford et al, 1978) and fat seemed to be the most important constituent of food in this respect (Rosell and Rőkaeus, 1979; Ferris et al, 1981, 1982). Alcohol and fatty acid stimulate release of NT from the small intestine (Ferris et al, 1985). It is however not clearly known which component of dietary lipid is the most effective in stimulating release of NTLI. A suggestion that NT may be involved in lipid homeostasis was made (Miller and Hendricks, 1981). To evaluate this hypothesis further the total lipid content, cholesterol, triglyceride and free fatty acid levels of the plasma were monitored after NT administration. Experiments were also performed with SP for comparison. Since the liver plays a major role in the overall metabolism of cholesterol, hepatic lipid levels were also measured after peptide administration.

Experiments were carried out in unanaesthetized, OVX rats primed with estrogen bearing chronically implanted indwelling jugular venous cannulae. To test whether the hypocholesterolemia or hypercholesterolemia caused by SP and NT, respectively, is peripheral or mediated by CNS, the peptides were injected into the third ventricle in rats bearing third ventricular stainless steel cannulae. 4-aminopyrazolo (3,4-d) pyrimidine (4-APP), a drug which

selectively inhibits hepatic secretion of cholesterol (Shiff et al, 1971), was also used to determine the possible site of action of the peptides in modifying circulating cholesterol levels.

EXPERIMENTAL PROCEDURE

Adult female rats, were OVX and used for experiments 3-4 weeks after the surgery. Different doses of estradiol benzoate (EB) in 0.1 ml sesame oil were administered sc for 24 hrs. Subsequently, a dose of 5 µg of EB/rat was used for priming the rats 24hrs before the experiment. Four to seven days before the experiments rats were implanted with third ventricular stainless steel cannulae and on the day prior to the experiment, indwelling silastic catheters were introduced into the external jugular vein as described previously (Chapter II). On the day of the experiment, an extension of polyethylene tubing (PE 50, 12" in length) filled with heparin 0.9% NaCl was attached to the distal end of the jugular cannula and the animals were left undisturbed for 30-60 min. During this time, a preinjection blood sample (0.4-0.6 ml) was withdrawn.

The peptides were freshly prepared in 0.9% saline. Systemic administration by iv pulse injection of test substance

was made in volume of 100 µl through the intrajugular cannula in a dose of 1 or 5 µg. An intermediate dose of 2.5 µg was injected into the third ventricle in a volume of 2.5 µl using a Hamilton microsyringe as described previously (Chapter II). In all cases, injection time was about 60 sec and all experiments were performed in the morning between 0900 and 1100 hrs. Heparinized blood samples (0.4-0.6 ml) were collected from the external jugular vein at 5, 15, 30 and 60 min while the animal was freely moving in the cage. The volume of all samples was replaced immediately after each bleeding by an equivalent volume of saline. Plasma was separated by centrifugation and stored frozen until assayed. At the end of 60 min the rats were sacrificed by decapitation and the liver was dissected out. It was cleared of adhering fat, weighed and homogenized in 20 volumes of chloroform: methanol, 2:1 and processed further for determination of the total lipid content as outlined in Chapter II. Standard procedures were used for the estimation of total and free cholesterol, triglycerides and free fatty acids (See Chapter II). Esterified cholesterol was calculated as the difference between total and free cholesterol.

To determine if availability of circulating lipoproteins influence the effects of the peptide, the adenine analogue, 4-amino pyrazolo (3,4-d) pyrimidine (4-APP) was

administered. Intraperitioneal injections of 4-APP (10 mg/Kg bw) were given daily for 3 days in 10 mM sodium phosphate buffer.

RESULTS

Total plasma lipid, cholesterol, triglyceride and fatty acid in adult OVX and OVX EB primed rats:

Total plasma lipid content, free cholesterol and free fatty acids were not modified by OVX. However, the total cholesterol level was significantly ($P<.05$) increased after OVX. The bulk of the cholesterol form which increased after OVX was the esterified form. A single injection of estradiol was effective in reversing the changes induced by OVX. Since 5 µg dose of EB was effective in bringing cholesterol levels to that of adult control levels, a 5 µg dose was used for priming rats in subsequent experiments. Plasma triglyceride levels were reduced significantly ($P<.01$) after OVX. Administration of 5 or 10 µg EB in OVX rats significantly ($P<.01$) increased plasma triglyceride levels (Table 1).

Effect of SP and NT on total plasma lipid:

The total plasma lipid content was unaltered by either iv or ivt injections of SP or NT at any time intervals studied in OVX estradiol primed rats (Table 2).

Effect of SP and NT on plasma cholesterol levels:

Intravenous pulse injection of 1 μg of SP or NT or third ventricular injections of 2.5 μg of peptide failed to modify circulating cholesterol levels. However, a 5 μg dose of SP administered iv produced a significant decrease in cholesterol levels ($P<.05$) at 30 and 60 mins. In contrast, 5 μg NT, iv caused a significant increase ($P<.05$ at 30 and 60 min) in plasma cholesterol levels (Fig. 1 and 2). Similarly free cholesterol levels were also significantly elevated by 5 μg dose of NT. However, there was no change in the esterified cholesterol levels (Fig. 3 and 4). A 5 μg dose of SP produced a significant decline in both the esterified and free cholesterol levels at 30 and 60 min after iv pulse injection resulting in a net decrease in total circulating cholesterol level (Figs. 5 and 6).

Effect of SP and NT on plasma triglyceride levels:

Third ventricular injection of 2.5 μg dose of SP or NT failed to modify circulating triglyceride levels. Intravenous SP, 5 μg dose however, significantly reduced circulating triglyceride levels 30 and 60 min after administration. On the contrary, plasma triglyceride levels were significantly ($P<.05$) elevated by iv pulse injection of 5 μg NT (Figs. 7 and 8).

_ffect of SP and NT on plasma free fatty acid levels:

Third ventricular injection of 2.5 µg dose of SP or NT significantly ($P<.001$) reduced plasma free fatty acid levels within 5 min of injection. The decrease persisted till 60 min duration of the experiment. Intravenous pulse injection of 1 µg SP caused no change in the plasma free fatty acid levels. However, there was a singificant ($P<.001$) reduction within 15 min after the 5 µg dose of SP. In contrast, iv pulse injections of 1 and 5 µg of NT significantly ($P<.01$) elevated plasma free fatty acid levels. Free fatty acid levels remained elevated for the 60 min duration of the experiment (Figs. 9 and 10).

Effect of SP and NT on total hepatic lipid content:

Total hepatic lipid content was not altered one hour after iv or ivt administration of either peptide (Table 3).

Effect of SP and NT on hepatic cholesterol:

Intraventricular injections of 2.5 µg of SP or NT failed to modify hepatic cholesterol. Hepatic cholesterol was also unaltered by 1 µg of iv SP or NT. However, 5 µg dose of SP, iv, significantly ($P<.05$) reduced hepatic

cholesterol. Similar dose of NT, however, significantly ($P<.01$) increased hepatic cholesterol concentration. The fraction of the total cholesterol that was reduced by SP and increased by NT was the free cholesterol fraction (Fig.11).

Effect of SP and NT on hepatic triglyceride:

Hepatic triglyceride levels were unaltered by 2.5 µg ivt or by 1 µg iv pulse injection of SP and NT. 5 µg of SP, iv, significantly ($P<.05$) decreased whereas 5 µg of NT, iv, significantly ($P<.05$) increased hepatic triglyceride levels (Fig. 12).

Effect of 4-APP and NT on circulating and tissue Cholesterol:

Administration of 4-APP or SP significantly reduced plasma and hepatic cholesterol levels ($P<.001$ for 4-APP; $P<.01$ for SP) in the estradiol primed rats. Administration of 5 µg NT in 4-APP or SP pretreated rats blocked hypocholesterolemic actions of SP or 4-APP whereas NT alone produced significant elevations in both circulating and hepatic cholesterol levels (Fig. 13 and 14).

DISCUSSION

Ovariectomy induced on elevation in plasma cholesterol levels while reducing plasma triglyceride levels. A single injection of 5 µg EB was effective in restoring circulating cholesterol levels to that of control. The OVX, estradiol-primed rat was used in these experiments to simulate the natural physiological state of the animal. Previous work in our laboratory (Raju and Vijayan 1981; Vijayan and Raju, 1982) as well as that of others (Peric Golia et al, 1979, 1984) has implied a role for NT and SP in modifying circulating cholesterol levels. A proposal that NT may be involved in lipid homeostasis was made (Miller and Hendricks, 1981) on the basis of localization of NT-like immunoreactivity in the gi tract of the rat. Present studies therefore evaluated the total plasma lipid content and also the cholesterol, triglyceride and free fatty acid levels. The results however, indicate that the figure for total lipid is of little value in itself as there was no change in the total plasma lipid after SP or NT. To assess the significance of variation one needs to know what changes are there in the constituents composing it. Present findings indicate that peripheral administration by iv pulse injection of SP and NT have opposite effects on circulating cholesterol levels. Substance P caused a transient hypo-cholesterolemia whereas NT produced a hypercholesterolemic

effect. However, both the peptides were ineffective at a dose of 1 µg/rat. A minimum dose of 5 µg/rat was required to produce an effect on circulating cholesterol levels. The bulk of the plasma cholesterol which increased after NT administration was the esterified cholesterol whereas SP induced a reduction in the free cholesterol. Description of the levels of plasma cholesterol is complicated by the fact that all the cholesterol in the plasma forms part of an exchangeable pool that includes a substantial fraction of the total tissue cholesterol. This exchangeable pool undergoes turnover owing to its enterohepatic circulation with net loss in the faeces and replacement by endogenous synthesis and absorption of cholesterol from food. A further complication arises from the presence and turnover within the plasma owing to the conversion of free and esterified cholesterol. It is necessary therefore to consider the interchange of cholesterol molecules within the plasma as well as the net flux caused by continuous replacement of cholesterol in the whole exchanged pool. Peric-Golia et al (1980) reported that NT had no effect on the 7 α-hydroxylation of cholesterol. They also examined the effect of NT on the concentration of cholesterol and bile acids in gal bladder (Peric-Golie et al, 1984). They observed a decrease of cholesterol concentrations in the ileum after NT administration suggesting that additional circulating cholesterol may be brought into the plasma pool from the

intestine. Considerable concentration of NT and SP has been demonstrated in the rat intestine (Carraway and Leeman, 1976). Some lipoproteins which are active in cholesterol transport are also synthesized in this segment of the gut (Mahley et al, 1971; Fernstrom et al, 1980) and rapidly transported via the mesenteric lymph (Green et al, 1978) into the plasma. The stimulatory effect of NT on the plasma cholesterol was attributed to either intestinal overproduction of NT or hypothalamic hypersecretion followed by an increased delivery of NT to the gut and other tissues active in cholesterol transport. A possible physiological role for both these peptides as humoral agents responsible for neurogenic hyper- and hypocholesterolemia (Friedman et al, 1969) was also suggested since they are present in the hypothalamus. However, failure of any alteration in circulating cholesterol levels after third ventricular injections of these peptides questions this possibility.

Neurotensin and SP not only affect cholesterol levels but also circulating triglyceride levels. Neurotensin causes a transient increase in triglyceride levels whereas SP produced a lowering of triglyceride levels after iv pulse injection. The plasma free fatty acid levels were also reduced with intravenous SP whereas iv NT, increased the plasma free fatty acid levels. In contrast no effect on circulating cholesterol and triglyceride levels after

third ventricular injections of SP and NT was noticed. The free fatty acids is now known to be the most active form of the plasma lipids. Intracerebroventricular administration of dopaminergic and cholinergic agents have been known to control peripheral lipid mobilization by altering serum free fatty acid levels (Uchida and Nomoto, 1982; 1983). Substance P and NT have been shown to alter cholinergic and dopaminergic pathways in the central nervous system (Ryall, 1982; Reches et al, 1982, 1983). Present results do not point out whether the decrease brought about by NT and SP are mediated by the cholinergic or dopaminergic system. The mobilization of free fatty acids in the periphery could perhaps account for the hypothermia caused by these peptides.

4-APP has been frequently used to study sterol metabolism in rats (Christie et al, 1979). It lowers the hepatic secretion of lipoproteins. The reversal of the inhibitory effects of 4-APP on hepatic secretion of cholesterol by NT in earlier (Raju and Vijayan, 1981) and present studies favour the liver as a possible site of action for the peptide in modulating cholesterol levels. Nagai and Frohman (1976) had earlier pointed out the liver as one of the sites of neurotensin action. The liver plays a major role in the overall metabolism of cholesterol. Firstly under normal conditions the liver is involved in

the biosynthesis of cholesterol. Secondly, the liver is responsible for the only quantitatively important pathway of cholesterol catabolism namely the formation of biles acids. Third, the liver is critically involved in cholesterol absorption and participates in an enterohepatic circulation of cholesterol of considerable magnitude. Finally, the liver plays a central role in the regulation of the plasma concentrations of cholesterol and cholesterol ester via the formation and secretion of plasma lipoproteins.

The present results demonstrate that the dose of NT which increases circulating cholesterol levels, also increase hepatic concentration of cholesterol. Substance P produced an opposite effect, by reducing circulating cholesterol as well as hepatic cholesterol concentration. Hepatic triglyceride levels were reduced by SP and increased by NT. 4-APP a drug that lower hepatic secretion of lipoproteins, lowered circulating and hepatic concentrations of cholesterol. Administration of NT to 4-APP treated rats reversed these effects. The triglyceride content was also increased in NT treated rats. There exists a temporal relationship between circulating and hepatic levels of cholesterol and triglyceride. Reduction of plasma free fatty acids by SP and its increase by NT possibly offer a basis for their effects on cholesterol. It has been established that the primary source of lipoprotein-triglyceride

fatty acids is free fatty acids which are released from adipose stores transported to the liver to be esterified and incorporated as plasma lipoproteins (Havel, 1961). It has been hypothesized, therefore, that the marked plasma free fatty acid depression induced by SP would reduce the availability of free fatty acid for esterification sufficiently to limit the amount of triglyceride formed and thus decrease the concentration of plasma low density lipoproteins. Reduction of cholesterol would thus ensue as a result of reduced level of plasma low density lipoproteins since these are the primary form in which cholesterol is removed from the liver by the blood. However, the reduction of plasma free fatty acids after ivt administration does not result in any changes in plasma or hepatic cholesterol or triglyceride content. Probably there is some feedback mechanism which regulates the hypothalamic secretion of the peptide. These results indicate that the liver is one of the likely sites of action of SP and NT which act to inhibit and stimulate the hepatic secretion of lipoproteins respectively.

TABLE 1. EFFECT OF OVX AND EB ON LEVELS OF PLASMA TOTAL LIPID, CHOLESTEROL, TRIGLYCERIDE AND FREE FATTY ACID.

	Total lipid mg/100 ml	Cholesterol			Triglycerides mg/100 ml	Free fatty acids meq/l
		Total mg/100 ml	Esterified mg/100 ml	Free mg/100 ml		
Control(6)	293.8±5.8	86.20± 2.23	63.91± 2.9	22.28±3.5	116.7±13.4	0.61±.071
OVX(5)	284.5±9.2	99.07± 4.3[a]	77.07± 4.0[a]	22.0 ±0.89	84.6±18.4[c]	0.54±.003
OVX+1 µg EB(6)	291.4±6.1	90.3 ± 5.8	68.04± 3.2	23.2 ±0.91	90.8±15.2	0.56±.010
OVX+5 µg EB(6)	289.1±3.2	84.3 ± 6.29	59.3 ± 4.08[b]	25.0 ±1.9	119.8±20.6[b]	0.55±.003
OVX+10 µg EB(5)	295.6±6.2	67.4 ±10.1[b]	43.4 ± 7.7[d]	24.0 ±7.7	128.4±19.4[b]	0.57±.04

Total plasma lipid, cholesterol, triglyceride and free fatty acid in OVX and OVX, EB (1, 5 and 10 µg/rat SC in sesame oil for 24 h) primed adult rats. Mature, untreated rats served as controls. Values are mean ± SEM. The numbers in parentheses indicate the number of animals used per group.

a $P<.05$ vs control

b $P<.05$ vs OVX

c $P<.01$ vs control

d $P<.01$ vs OVX

TABLE 2. EFFECT OF SP AND NT ON PLASMA TOTAL LIPID LEVELS IN OVX EB PRIMED RATS.

Treatment	Plasma Lipid (mg/100 ml)
Control (Preinjection)	283.8 ± 5.8
SP iv	286.4 ± 2.3
SP ivt	292.5 ± 6.4
NT iv	288.4 ± 8.1
NT ivt	280.3 ± 4.5

Plasma total lipid levels in OVX, EB (5 µg/rat sc for 24 h) primed rats after iv (1 and 5 µg) and ivt (2.5 µg) 5, 15, 30 and 60 min. after injection of SP/NT. Values for different time intervals and for 1 and 5 µg iv dose of the peptide have been pooled and represented as mean ± SEM.

TABLE 3. EFFECT OF SP AND NT ON HEPATIC TOTAL LIPID LEVELS IN OVX EB PRIMED RATS.

Treatment	Hepatic total Lipid (mg/g)
Control	43.6 ± 5.1
SP iv	41.8 ± 3.2
SP ivt	42.5 ± 3.1
NT iv	43.1 ± 6.1
NT ivt	40.8 ± 5.6

Plasma hepatic levels (mg/g) in OVX, EB (5 µg/rat sc for 24 h) primed rats after iv (1 and 5 µg) and ivt (2.5 µg) 1 h after administration of peptide. Values for 1 and 5 µg iv doses of the peptide have been pooled since there was no significant difference between them. Results are mean ± SEM.

Fig. 1 Effect of iv and ivt administration of SP on total plasma cholesterol levels in OVX, EB-primed rats (5 μg EB/rat sc) at different time intervals. Results in this and subsequent figures are mean ± SEM. The numbers in parentheses indicate the number of animals used per group. Preinjection values from all groups are pooled since there was no difference between them.

*P<.05 vs preinjection

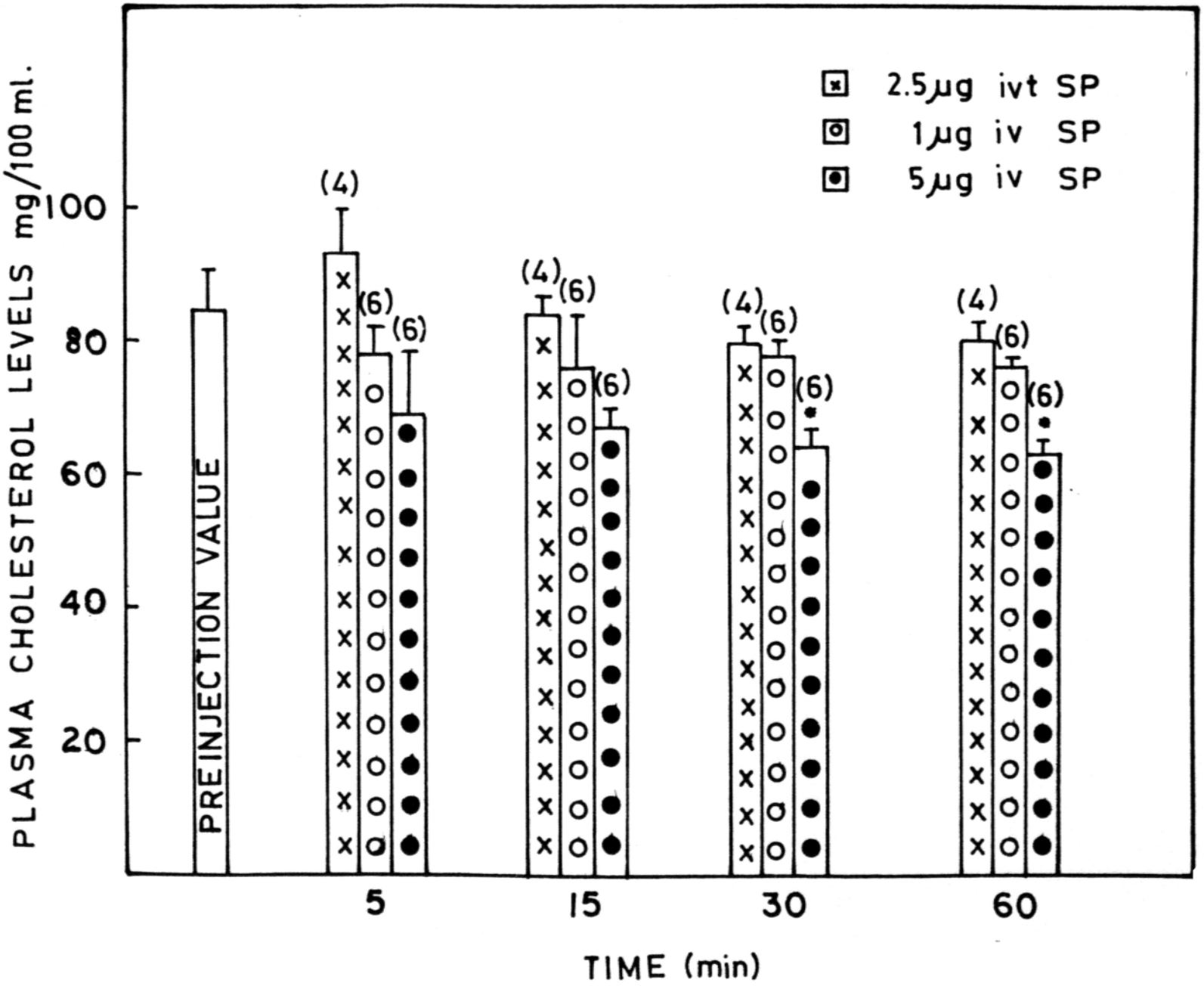

PLASMA CHOLESTEROL LEVELS mg/100 ml.
100
80
60
40
20
PREINJECTION VALUE
2.5μg ivt SP
1μg iv SP
5μg iv SP
(4)
(6)
(6)
(4)
(6)
(6)
(4)
(6)
(6)
(4)
(6)
(6)
5
15
30
60
TIME (min)

Fig. 2 Effect of iv and ivt administration of NT on total plasma cholesterol in OVX, EB-primed rats at different time intervals.

*P< .05 vs preinjection

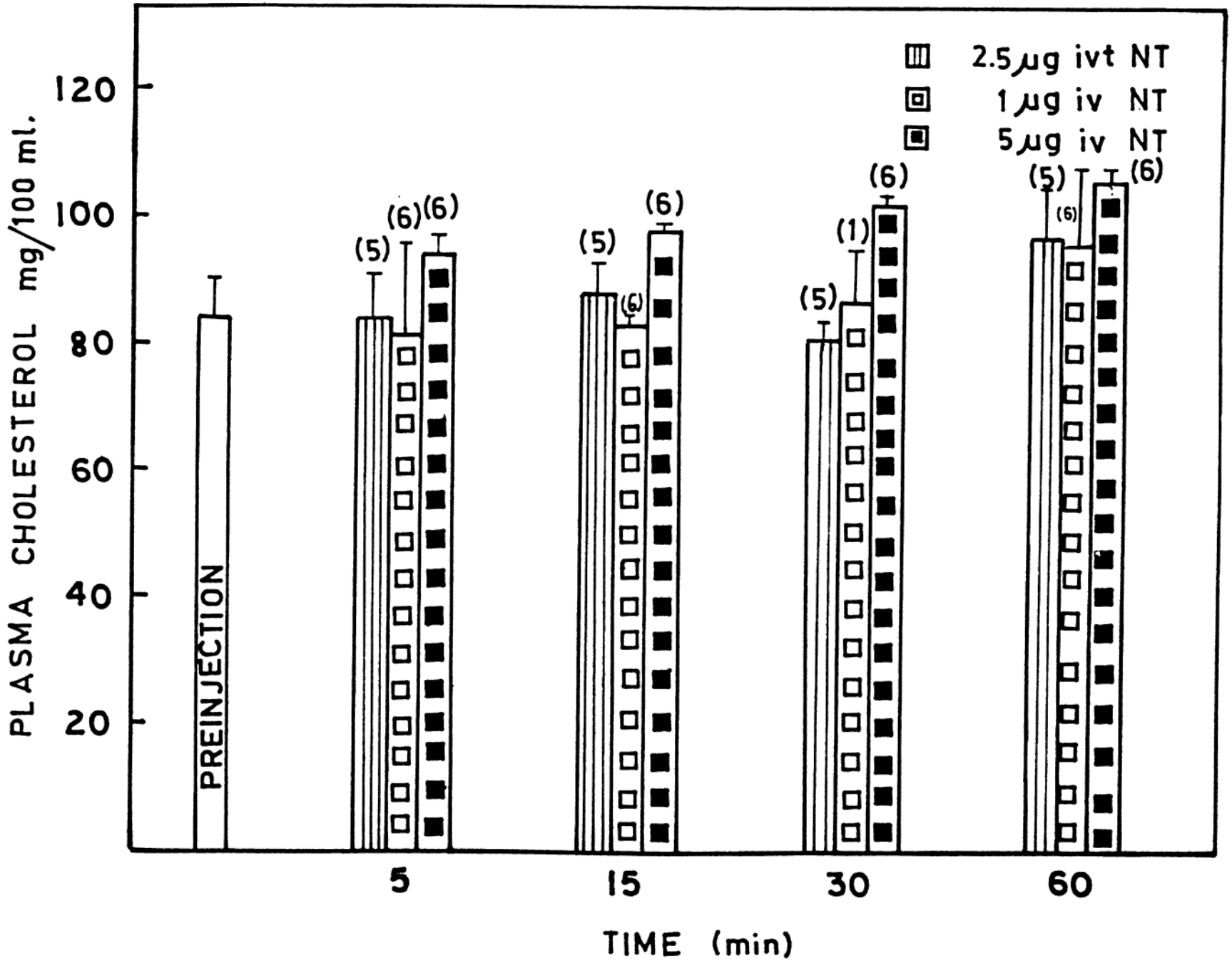

PLASMA CHOLESTEROL mg/100 ml.
120
100
80
60
40
20
PREINJECTION
2.5μg ivt NT
1μg iv NT
5μg iv NT
(5)
(6)
(6)
(5)
(6)
(6)
(5)
(1)
(6)
(5)
(6)
(6)
5
15
30
60
TIME (min)

Fig. 3 Effect of iv and ivt administration of NT on plasma free cholesterol levels in OVX, EB-primed rats at different time intervals.

*P< .05
**P< .01
} vs preinjection

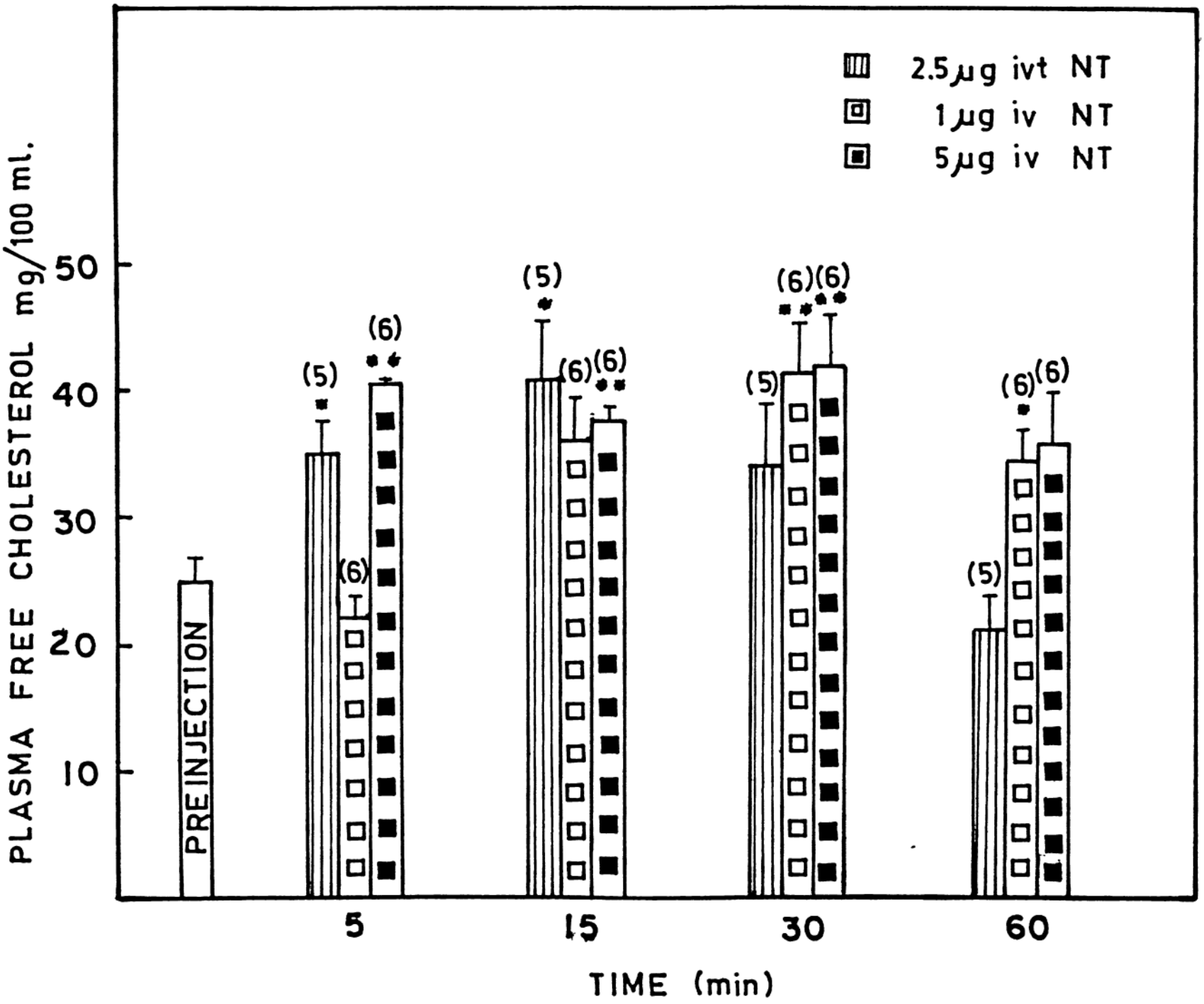

2.5μg ivt NT
1μg iv NT
5μg iv NT
PLASMA FREE CHOLESTEROL mg/100 ml.
10
20
30
40
50
PREINJECTION
(5)
(6)
(6)
(5)
(6)
(6)
(5)
(6)(6)
(5)
(6)
(6)
5
15
30
60
TIME (min)

Fig. 4 Effect of iv and ivt administration of NT on plasma esterified cholesterol levels in OVX, EB-primed rats at different time intervals.

*P< .05 vs preinjection

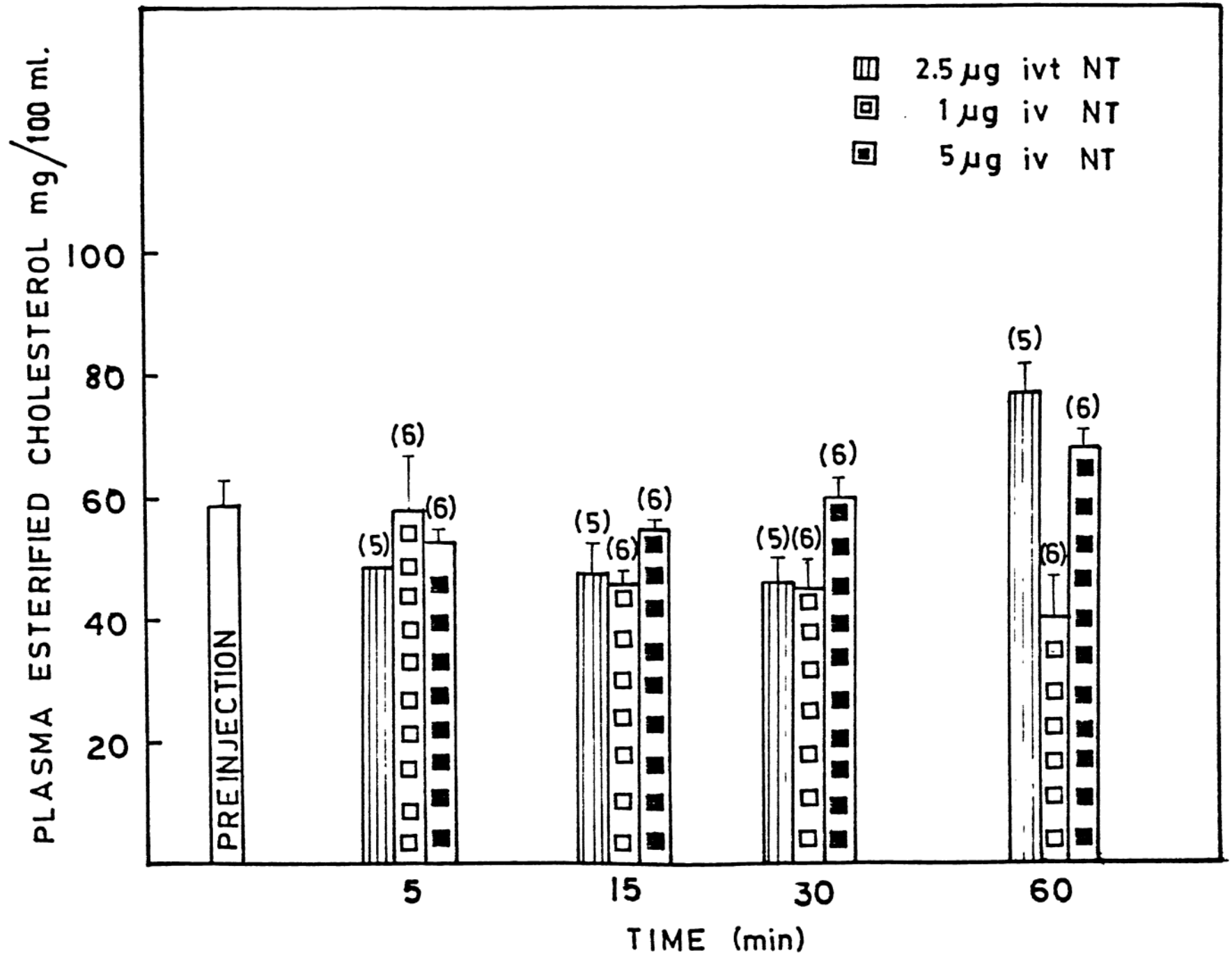

PLASMA ESTERIFIED CHOLESTEROL mg/100 ml.
100
80
60
40
20
PREINJECTION
2.5 µg ivt NT
1 µg iv NT
5 µg iv NT
(5)
(6)
(6)
(5)
(6)
(6)
(5)
(6)
(6)
(5)
(6)
(6)
5
15
30
60
TIME (min)

Fig. 5 Effect of iv and ivt administration of SP on plasma free cholesterol levels in OVX, EB-primed rats at different time intervals.

*P< .05 vs preinjection

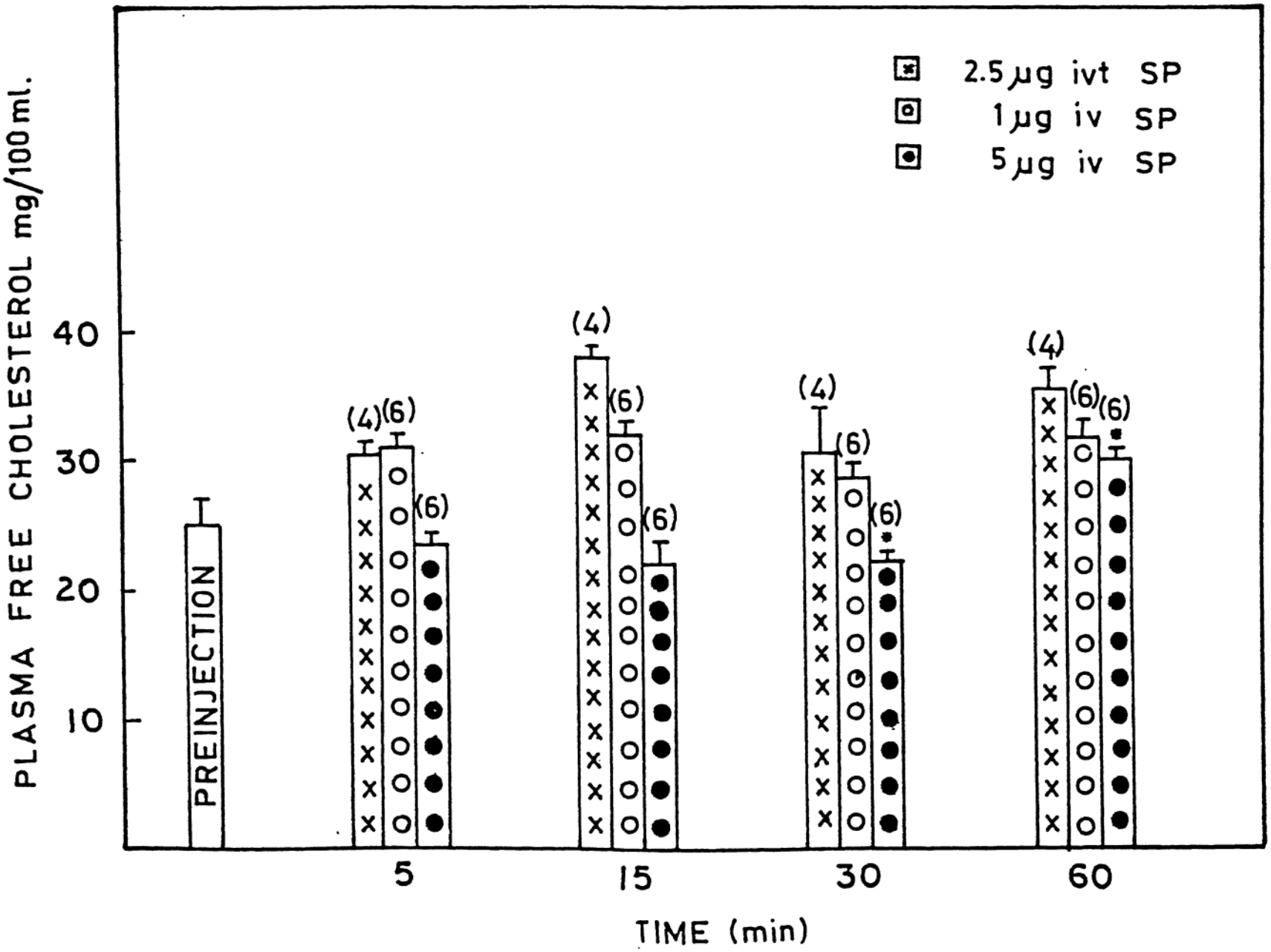

PLASMA FREE CHOLESTEROL mg/100 ml.
40
30
20
10
PREINJECTION
(4)
(6)
(6)
(4)
(6)
(6)
(4)
(6)
(6)*
(4)
(6)
(6)*
5
15
30
60
TIME (min)
2.5 µg ivt SP
1 µg iv SP
5 µg iv SP

Fig. 6 Effect of iv and ivt administration of SP on plasma esterified cholesterol levels in OVX, EB-primed rats at different time intervals.

*P< .05
**P< .01
} vs preinjection

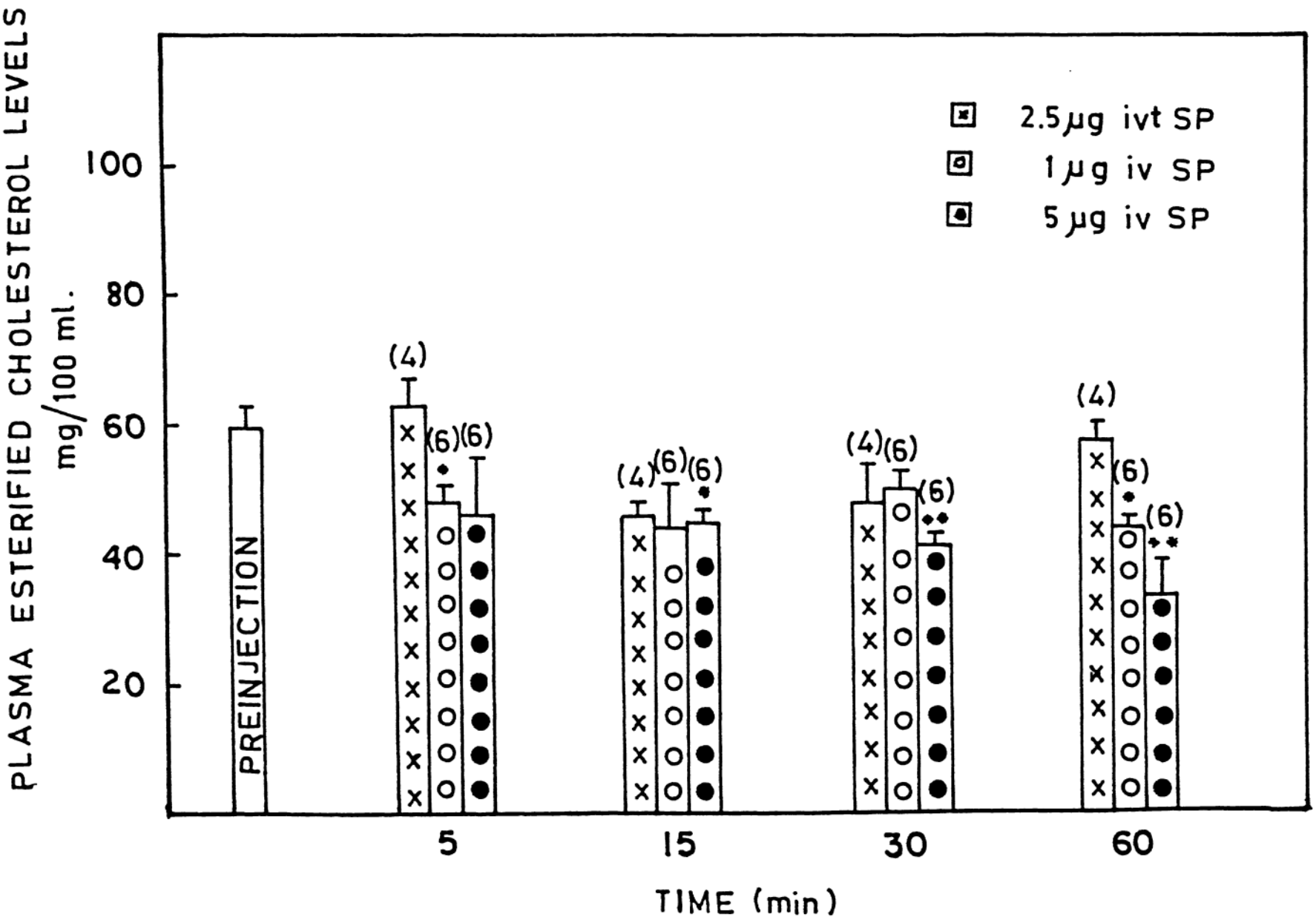

PLASMA ESTERIFIED CHOLESTEROL LEVELS
mg/100 ml.
100
80
60
40
20
PREINJECTION
(4)
(6)(6)
(4)(6)(6)
(4)(6)
(6)
(4)
(6)
(6)
2.5μg ivt SP
1μg iv SP
5μg iv SP
5
15
30
60
TIME (min)

Fig. 7 Effect of iv and ivt administration of SP on plasma triglyceride levels in OVX, EB-primed rats at different time intervals after peptide administration. Preinjection values from all groups have been pooled and represented as mean ± SEM.

*P< .05 vs preinjection

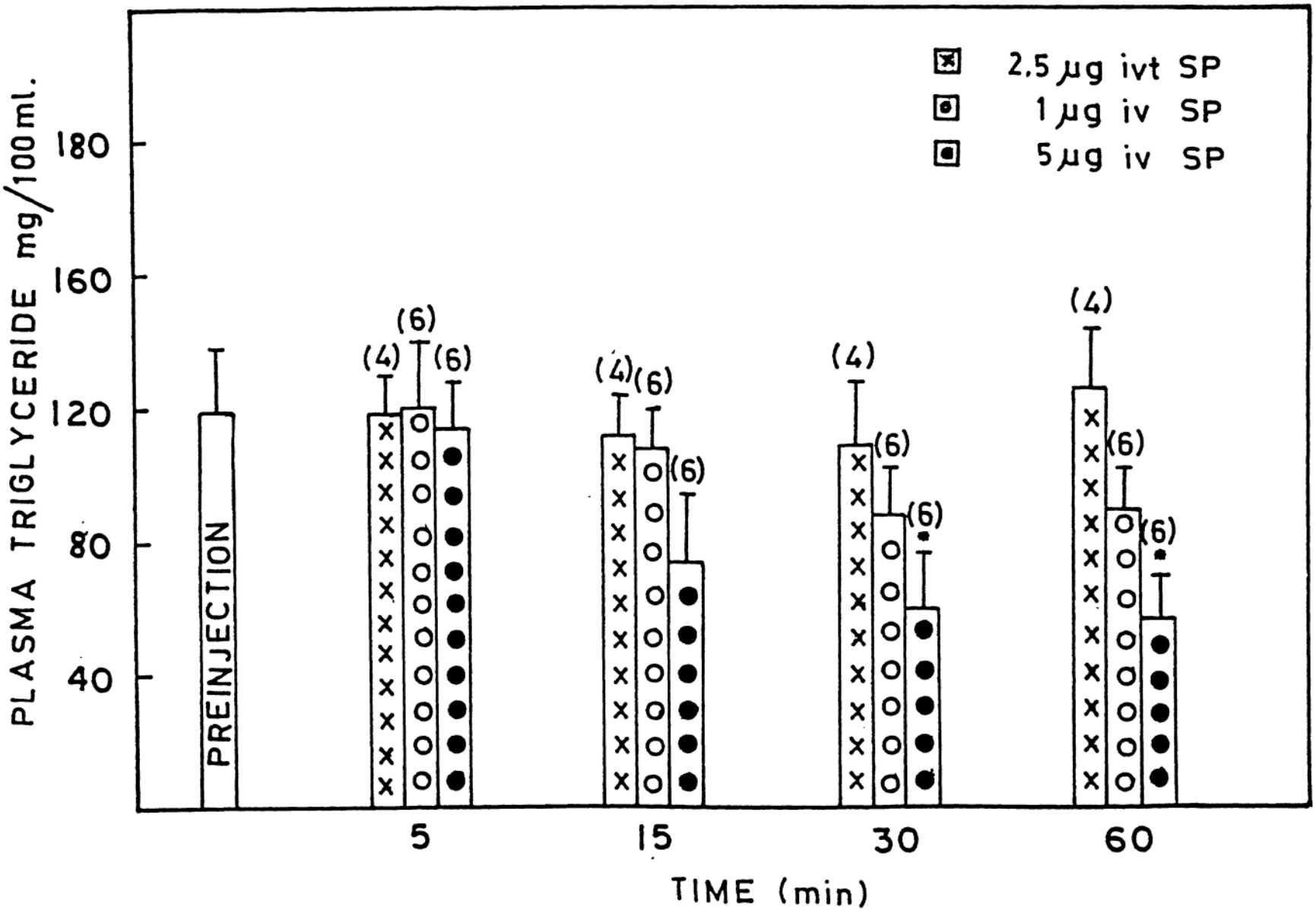

PLASMA TRIGLYCERIDE mg/100ml.
180
160
120
80
40
PREINJECTION
2.5 µg ivt SP
1 µg iv SP
5 µg iv SP
(4)
(6)
(4)
(6)
(6)
(4)
(6)
(6)
(4)
(6)
(6)
(4)
(6)
(6)
5
15
30
60
TIME (min)

Fig. 8 Effect of iv and ivt administration of NT on plasma triglyceride levels in OVX, EB-primed rats at different time intervals after peptide administration.

*P< .05 vs preinjection

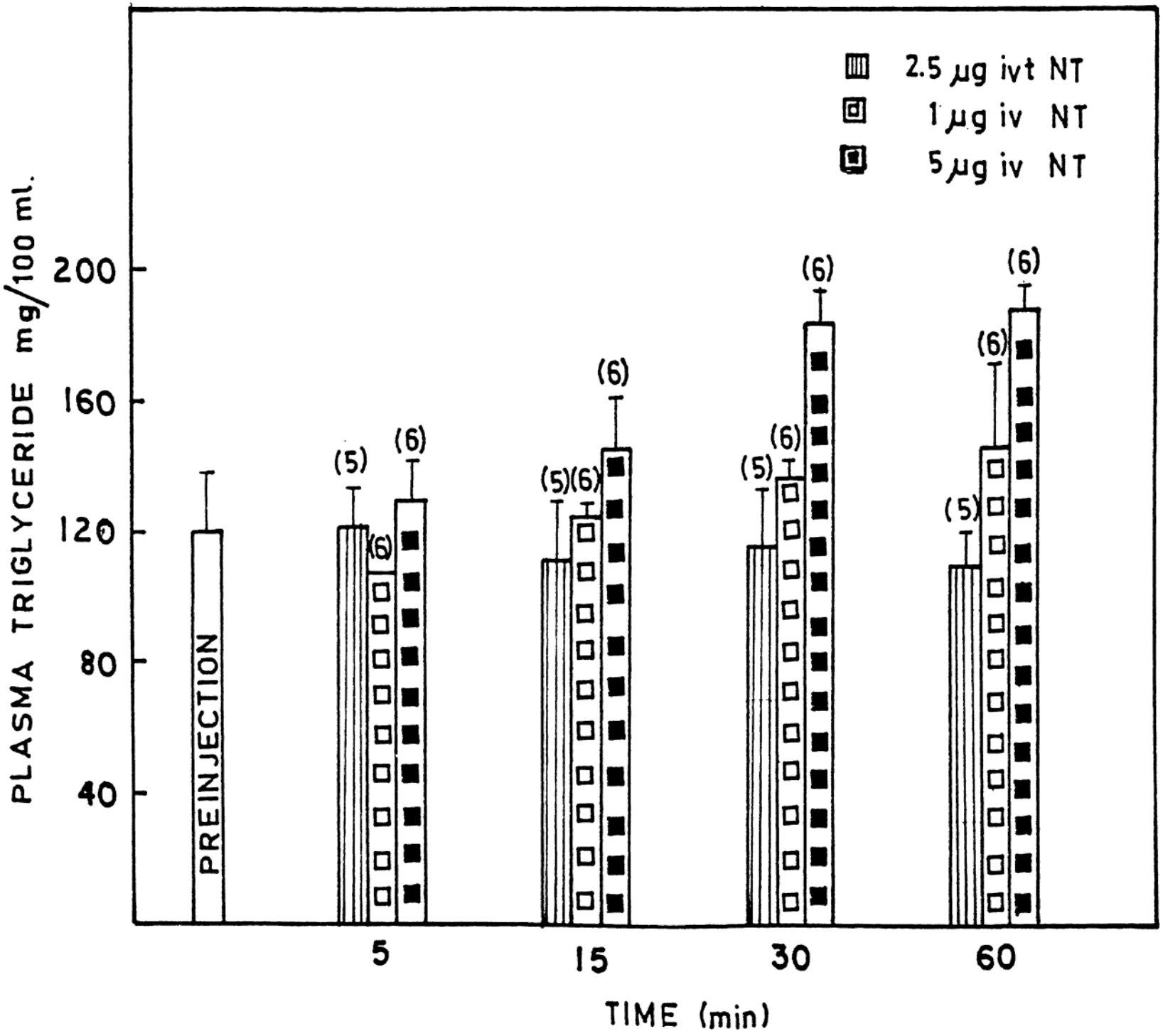

2.5 µg ivt NT
1 µg iv NT
5 µg iv NT
PLASMA TRIGLYCERIDE mg/100 ml.
200
160
120
80
40
PREINJECTION
(5)
(6)
5
15
30
60
TIME (min)

Fig. 9 Effect of iv and ivt administration of SP on plasma free fatty acid levels in OVX, EB-primed rats at different time intervals.

**P<.01 vs preinjection

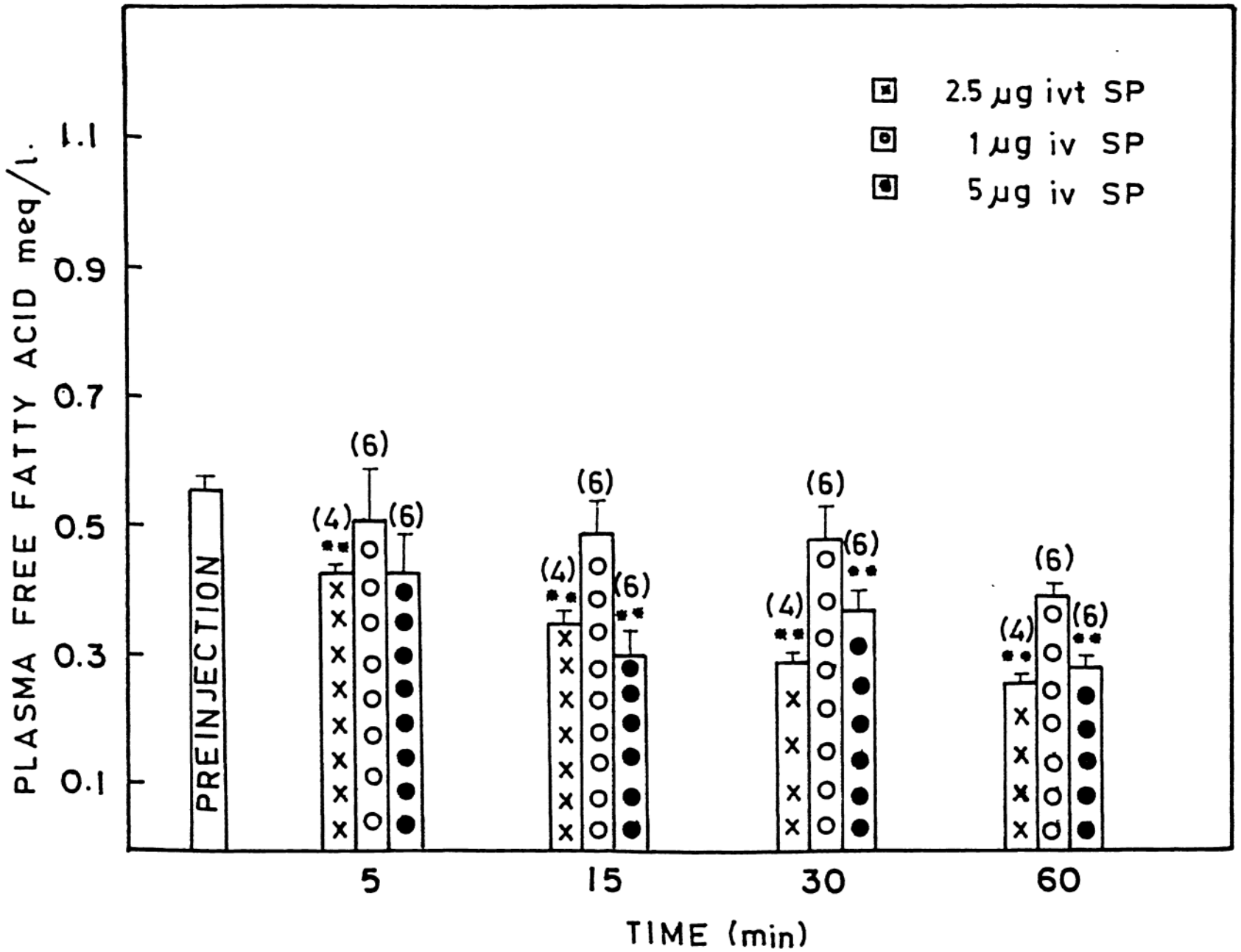

PLASMA FREE FATTY ACID meq/l.
1.1
0.9
0.7
0.5
0.3
0.1
PREINJECTION
2.5 µg ivt SP
1 µg iv SP
5 µg iv SP
(6)
(4)
(6)
(6)
(4)
(6)
(6)
(4)
(6)
(6)
(4)
(6)
5
15
30
60
TIME (min)

Fig. 10 Effect of iv and ivt administration of NT on plasma free fatty acid levels in OVX, EB-primed rats at different time intervals.

**P< .01 vs preinjection

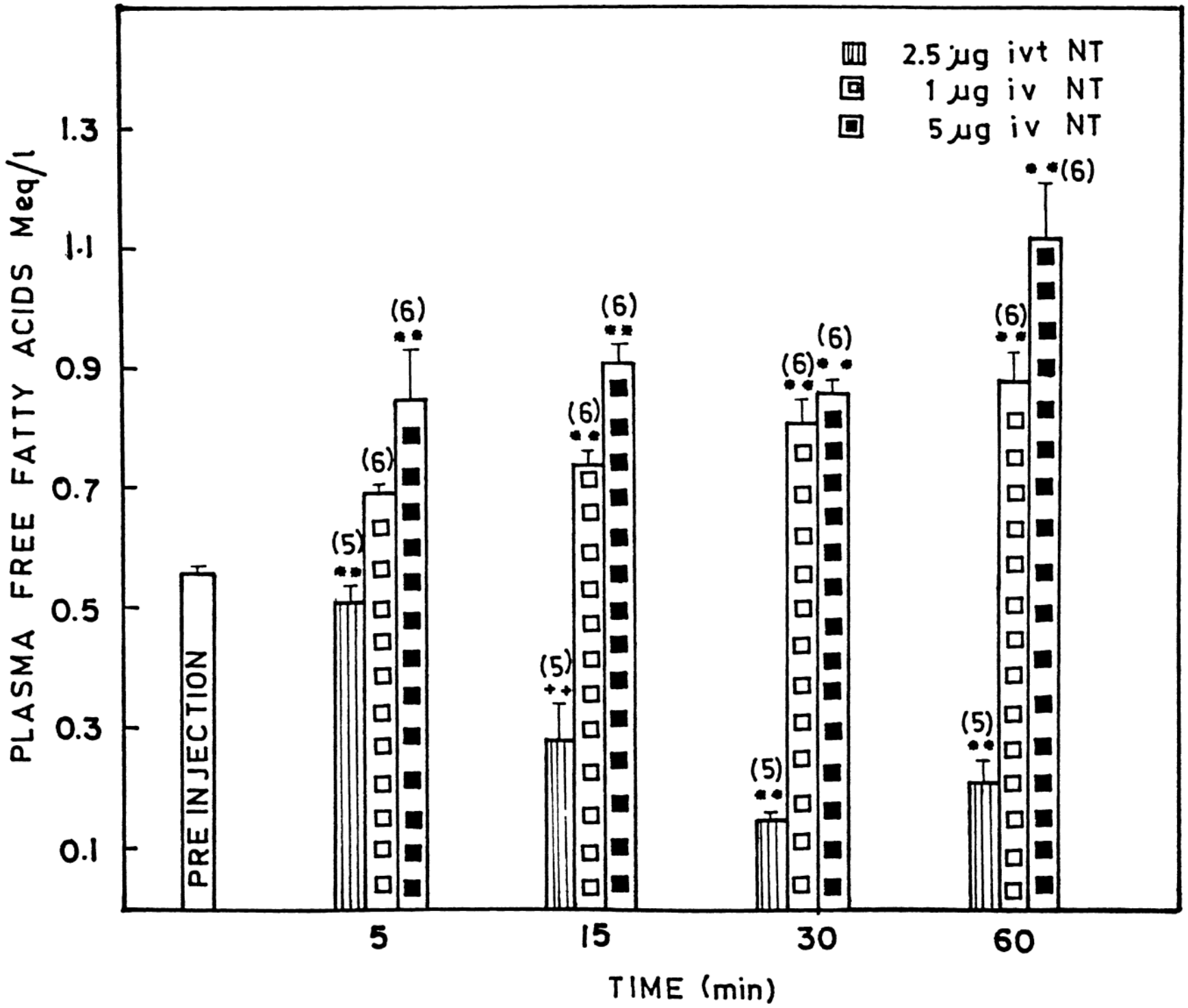

2.5 µg ivt NT
1 µg iv NT
5 µg iv NT
PLASMA FREE FATTY ACIDS Meq/l
1.3
1.1
0.9
0.7
0.5
0.3
0.1
PRE INJECTION
(5)
(6)
5
15
30
60
TIME (min)

Fig. 11 Effect of iv and ivt administration of SP and NT on hepatic total and free cholesterol levels in OVX, EB-primed rats, 1 h after administration of peptide. Controls received saline alone.

*P< .05
**P< .01
vs control

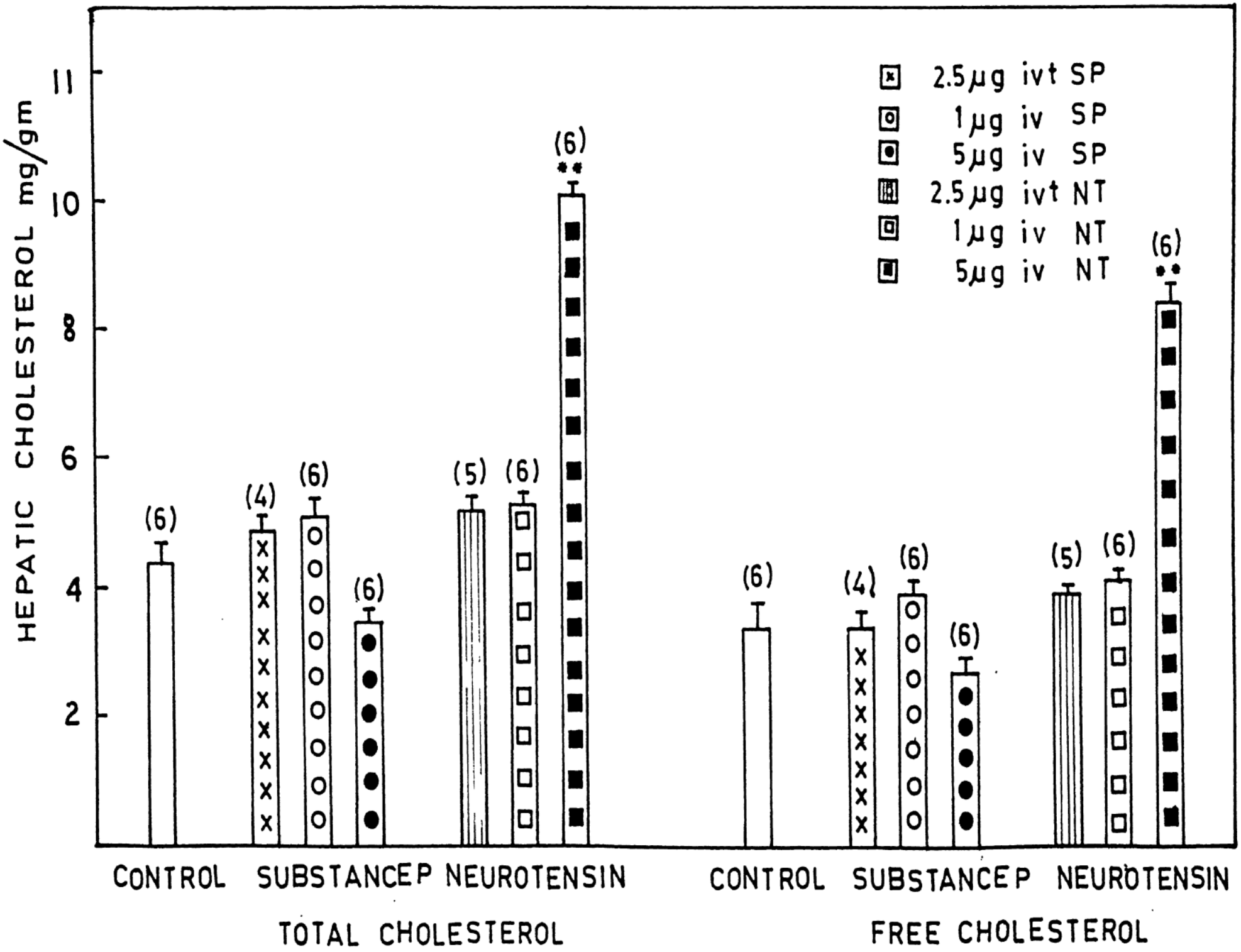

HEPATIC CHOLESTEROL mg/gm
11
10
8
6
4
2
2.5µg ivt SP
1µg iv SP
5µg iv SP
2.5µg ivt NT
1µg iv NT
5µg iv NT
(6)
(4)
(6)
(6)
(5)
(6)
(6)
**
(6)
(4)
(6)
(6)
(5)
(6)
(6)
**
CONTROL
SUBSTANCE P
NEUROTENSIN
CONTROL
SUBSTANCE P
NEUROTENSIN
TOTAL CHOLESTEROL
FREE CHOLESTEROL

Fig. 12 Effect of iv and ivt administration of SP and NT on hepatic triglyceride levels in OVX, EB-primed rats, 1 h after administration of peptide.

*P< .05 vs control

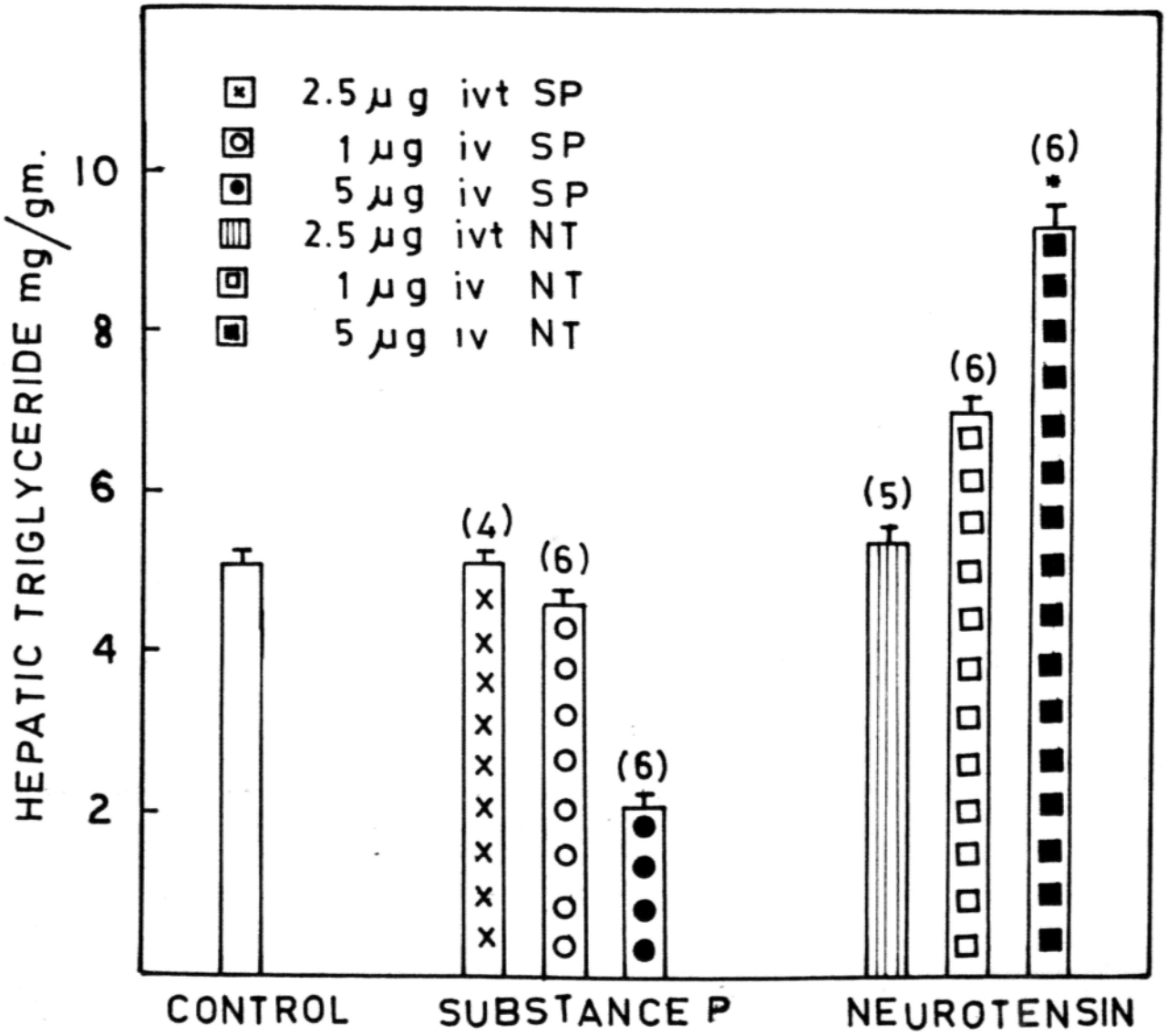

2.5 μg ivt SP
1 μg iv SP
5 μg iv SP
2.5 μg ivt NT
1 μg iv NT
5 μg iv NT
HEPATIC TRIGLYCERIDE mg/gm.
10
8
6
4
2
(4)
(6)
(6)
(5)
(6)
(6)
*
CONTROL
SUBSTANCE P
NEUROTENSIN

Fig. 13 Plasma cholesterol levels in OVX, EB-primed rats after 4-APP(10 mg/Kg bwt for 3 days, ip) or SP/NT (5 μg iv for 1 h) alone and 4-APP or SP followed by NT for 1 h. Controls received saline alone.

*P<.05
**P<.01 } vs control

a P<.05 vs NT

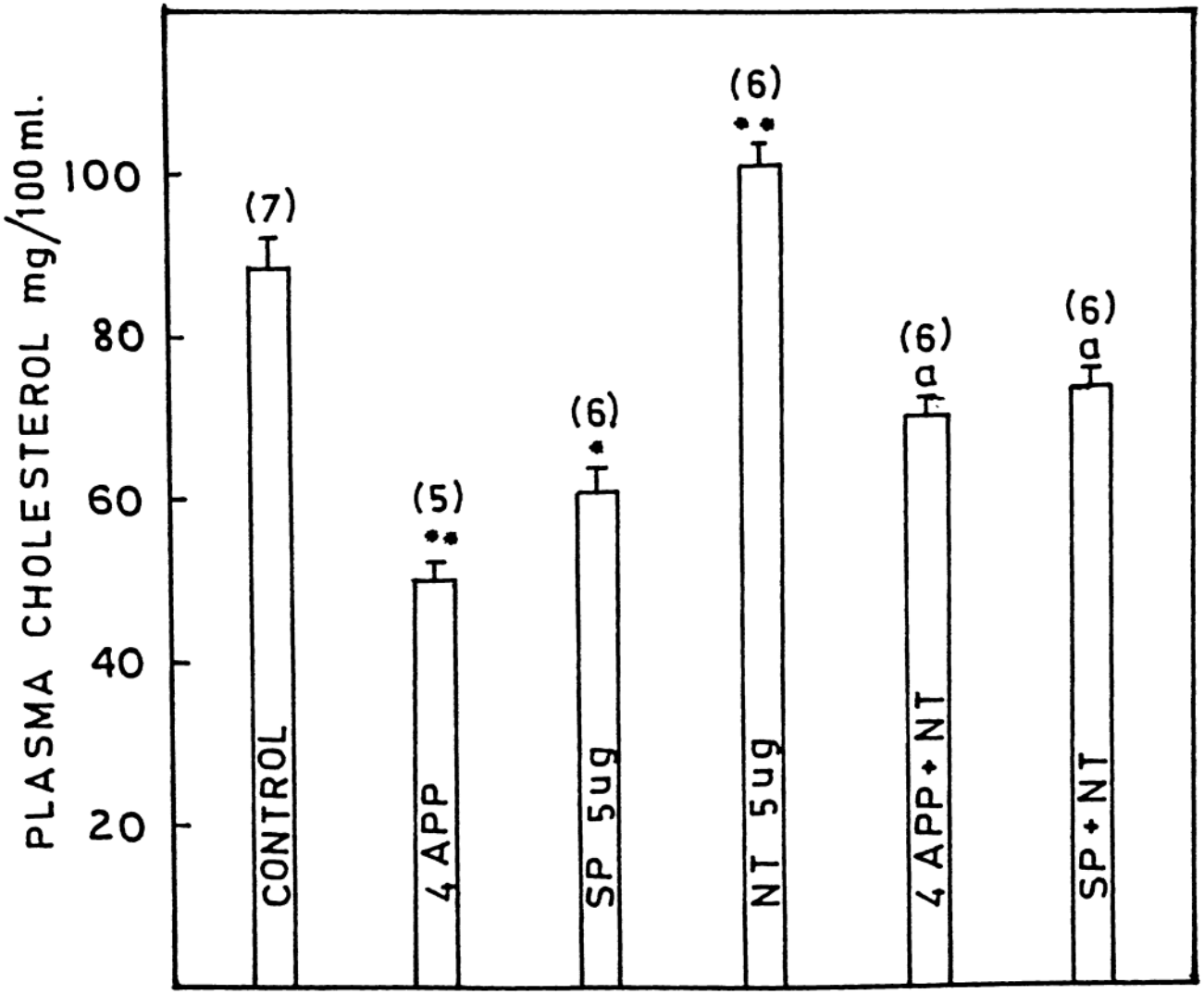
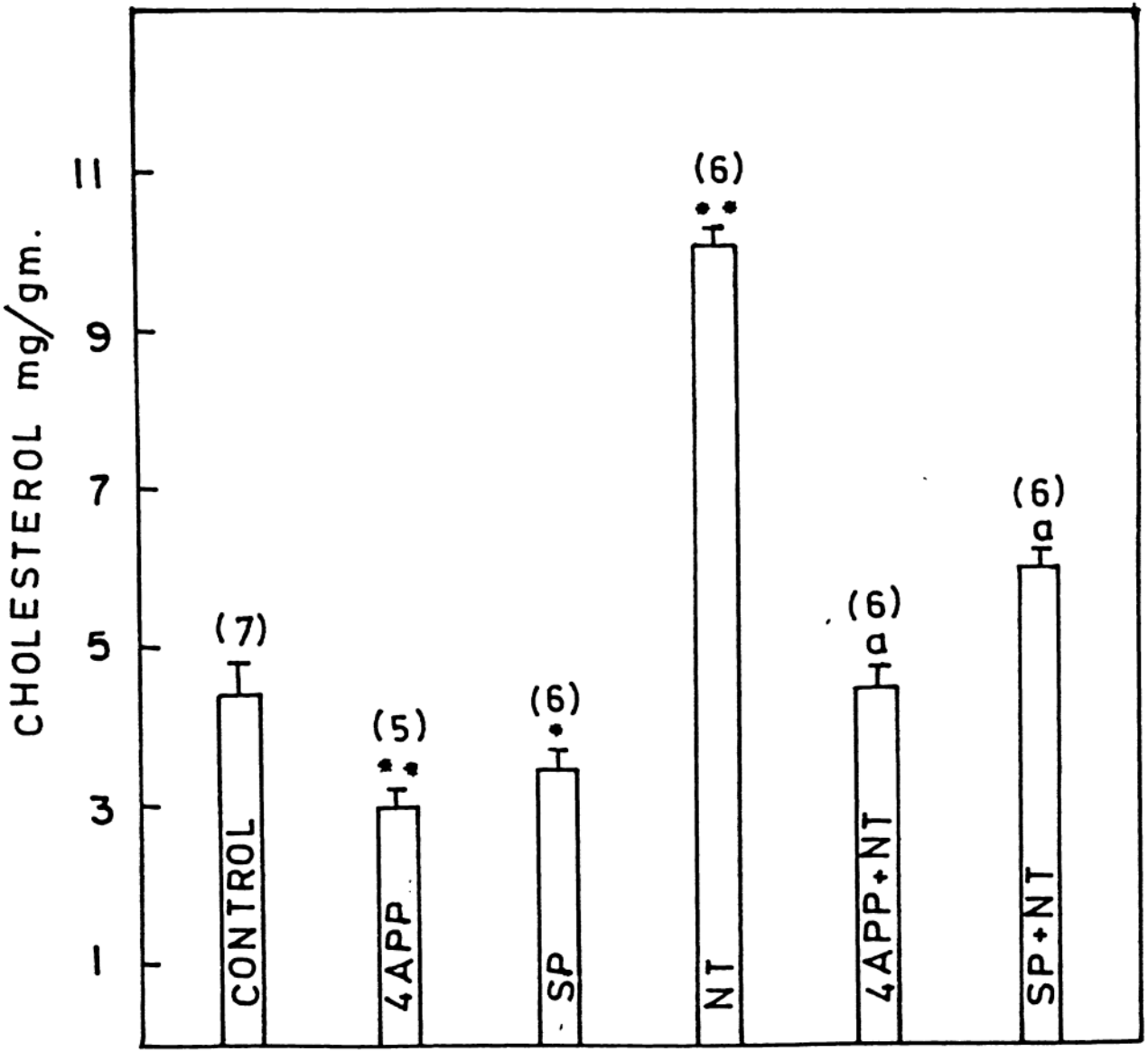

PLASMA CHOLESTEROL mg/100ml.
100
80
60
40
20
(7)
(5)
(6)
(6)
(6)
(6)
CONTROL
4 APP
SP 5ug
NT 5ug
4 APP + NT
SP + NT

Fig. 14 Hepatic cholesterol levels in OVX, EB-primed rats after 4-APP(10 mg/Kg bwt for 3 days ip) or SP/NT (5 µg·iv for 1 h) alone and 4-APP or SP followed by NT. Controls received saline alone. Animals were sacrificed 1 h after NT administration.

*P<.05
**P<.01
vs control

a P<.05 vs NT


CHOLESTEROL mg/gm.
11
9
7
5
3
1
(7)
CONTROL
(5)
4APP
(6)
SP
(6)
NT
(6)a
4APP+NT
(6)a
SP+NT

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSION

Just about a decade has elapsed since the isolation and characterization of SP and NT. In this period an impressive amount of information has accumulated concerning the distribution, localization and biological functions of these 'messenger' peptides. The present studies have served to amplify knowledge about the broad spectrum of biological effects of these peptides. It is apparent that these peptides exert diverse central as well as peripheral effects depending on the dose, the method of administration and the hormonal state of the animal.

Substance P and NT participate in a number of endocrine as well as non-endocrine functions in the body. One of the most striking features is the ability of these peptides to alter the release of hormones from the anterior pituitary. Results from the present study unequivocally support a stimulatory role for SP at the hypothalamic level on LH release. Microgram doses of SP injected through the ivt cannula elevated plasma LH levels whereas equivalent doses administered by iv pulse injection lowered LH titers. Substance P failed to alter LH or FSH release in <u>in vitro</u> incubation studies. Intraventricular as well as iv injections of SP elevated prolactin levels. Prolactin

was also elevated on incubation of hemipituitaries with SP. Systemic administration of NT on the other hand had no effect on plasma LH but increased plasma Prl. Intraventricular injection of NT reduced both LH and Prl. Incubation of hemipituitaries *in vitro* with SP or NT released Prl into the medium. Substance P probably acts via increased release of LHRH since systemic injection of SP suppressed rather than elevated LH. Elevation of Prl following intraventricular injection of SP may be due to a direct stimulatory effect on the pituitary after its uptake by portal vessels and delivery to the gland since it was active *in vitro*. Neurotensin, probably acted via decreased release of LHRH after intraventricular administration. It must be remembered however, that SP and NT form part of a family of peptides besides the hypothalamic releasing hormones that participate in the release of hormones from the anterior pituitary (McCann *et al*, 1980, 1981; Vijayan, 1985a). Thus, opiod peptides, vasoactive intestinal peptide, bombesin, secretin, cholecystokinin, gastrin are all known to alter the release of anterior pituitary hormones. A host of these peptides may act individually or in conjunction with one another to alter the levels of monoamines or hypothalmic releasing factors which in turn may influence pituitary hormone release. In this connection it is pertinent to note that SP and NT interact with cholinergic and dopaminergic systems in the

brain (Reches et al, 1982, 1983; Ryall, 1982; Quirion, 1983). Acetylcholine which stimulates LH release is implicated in the physiological regulation of LH secretion (Vijayan and McCann, 1980b). In mammals dopamine is the major Prl inhibitory factor released from the terminals of tuberoinfundibular dopaminergic neurons in the median eminence (Ojede et al, 1974; Macleod and Lehmeyer, 1974; Macleod, 1976). Alterations in the release of anterior pituitary hormones by SP and NT could be through cholinergic and dopaminergic interactions. Furthermore, in terms of potency SP and NT are active only in microgram doses in modifying anterior pituitary hormone release. On the other hand, nanogram doses of vasoactive intestinal peptide and cholecystokinin applied intraventricularly are capable of elevating and reducing plasma LH, respectively (McCann et al , 1980; Vijayan, 1985a). The release of hormones from the anterior pituitary is thus a complex process. Substance P and NT play a small, albeit, significant role in modifying the release of anterior pituitary hormones.

Substance P and NT are present in high concentrations in the hypothalamus (Brownstein et al, 1976; Kanazawa and Jessell, 1976; Uhl and Snyder, 1977, 1979). Going by the dictum that peptides are found in high concentrations in those regions that subserve the functions affected by administration of the peptide, the hypothalamus is one of

the recognized sites of action of these peptides. Intraventricular injections of SP elevates cAMP while reducing cGMP levels. In contrast, intraventricular injections of NT decrease cAMP and increase cGMP levels. Intravenous administration of SP decrease cAMP levels whereas intravenous NT is without effect. A survey of the literature indicates a study by Duffy and Powell (1975) where an increase in cAMP levels on incubation of brain slices with SP *in vitro* has been demonstrated. Neuropeptides are supposed to elicit responses in target cells either by activation or inhibition of adenylate cyclase (Greengard, 1978) or by stimulation of the breakdown of phosphatidyl inositol containing phospholipids in the membrane of the target cells (Michell *et al*, 1981; Farese, 1983). Present results demonstrate that at least in the hypothalamus where the presence of SP and NT receptors have been demonstrated (Brownstein *et al*, 1976; Uhl, 1982), the peptides cause fluctuations in the cAMP and cGMP levels. It has been shown previously that vasoactive intestinal peptide (Quik *et al*, 1978), melatonin (Vacas *et al*, 1981), dopamine (Gunaga and Menon, 1973), histamine-H_2 receptors, alter levels of adenyl cyclase to elicit their response.

The recent finding of SP and NT like immunoreactive material in tissues outside the brain-gut axis (Bucsics

et al, 1983; Polak and Bloom, 1982) allows the inclusion of these potently acting peptides in the newly recognized group of peripheral regulatory peptides (Polak and Bloom, 1980) known to exert key roles in a number of bodily functions. The fact that they are present in the hypothalamus and pituitary, physiological regulating centres and exert an effect on hormone release, suggests that they may play a physiological role. A single injection of NT given intraperitoneally evoked an increase in the estradiol induced uterine DNA synthesis. Histamine, dopamine and Prl altered this response. While the modulatory roles of dopamine and Prl in reproductive (Keenan et al, 1981) and non-reproductive tissue (Jacobi and Lloyd, 1981; Burdman et al, 1983) is documented, the modulatory role of histamine is difficult to interpret. The effect of estradiol on histamine release is classified as one of the early actions of estradiol whereas maximal DNA synthesis occurs 24hrsafter the administration of estradiol. NT is a potent hypotensive agent. Whether its action on estradiol induced DNA synthesis is secondary to alteration in uterine blood flow remains to be established. On the other hand, administration of 2 µg SP or NT once daily for a period of 3 days evoked a marked antiestrogenic effect and reduced PMSG induced uterine growth. Such an effect has been demonstrated for arginine vasotocin

(Vaughan et al, 1976). In this connection, it is pertinent that SP is also present in remarkably high concentrations in the pineal (Marks, 1977). It could be one of the active pineal antigonadotrophs in conjunction with other peptides.

Studies on the pregnant rat indicate that these peptides given postcoitally exert a direct effect on the uterus and its environment for the growing conceptus. Luteinising hormone releasing hormone is known to exert such direct antifertility effects on the uterus (Humphrey et al, 1977; Bex and Corbin, 1981; Corbin et al, 1979; Babu and Vijayan, 1983b). Whether the antiimplantation effect of these peptides is due to cross reaction with LHRH like peptides is not known. Neurotensin resembles LHRH and also TRH in having its NH_2 terminus a pyroglutamyl moiety. Administration of SP and NT after implantation of the zygote produced no deleterious effects on pregnancy and gestation.

Cholesterol is the obligate precursor of steroid hormone synthesis and is also an essential component of membranes. Any alteration in cholesterol levels is likely to affect steroid hormone synthesis. Substance P and NT cause hypo- and hypercholesterolemia, respectively, when administered by iv pulse injection as reported previously

(Peric-Golia et al; 1979, Raju and Vijayan, 1981). A minimum dose of 5 µg was required for this response. The cholesterolemia was not centrally mediated but rather a peripheral response to these peptides. Not only a hypocholesterolemia but also a hypotriglyceridemia was evident with iv SP with a reverse effect with NT. Intraventricular administration of SP or NT failed to modify circulating or hepatic cholesterol or triglyceride levels. However, ivt administration of either peptide was effective in reducing plasma levels of free fatty acids. 4-APP a drug that lowers hepatic secretion of lipoproteins, reduced circulating as well as hepatic cholesterol concentrations. Administration of NT to 4-APP treated rats, reversed these effects. Neurotensin was also able to overcome the decrease in cholesterol induced by SP.

It must be borne in mind that the nature of the SP and NT receptor is not fully known. There probably exists a number of sub-receptors for these peptides (Watson et al, 1983; Harmar, 1984; Quirion, 1985). It may respond to SP/NT, a fragment of SP/NT or it could be that SP and NT release something which in turn causes the biological effect. In this regard, SP belongs to a family of related peptides, the tachykinins which share several properties. Neurotensin on the other hand, resembles TRH and LHRH as

mentioned previously. Hence, when administered concomitantly with SP, NT is able to prevent the rise in cAMP caused by SP. It also circumvents the hypocholesterolemic effect of SP. It is likely that a peptide exerts a spectrum of effects and that the spectrum of any given peptide overlaps to some extent with that of certain others, although the potency may vary.

Caution must be followed in interpreting these results. These are the effects of exogenously administered peptide. The next issue is whether endogenous peptide participates in any of these actions. Although the presence of SP and NT in the circulation has been demonstrated it is unclear whether the peptide operates in a paracrine fashion. The very short half life in the blood suggest that its effect might be confined to areas near the site of release. The source(s) and target(s) for circulating SP/NT have yet to be precisely defined.

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APPENDIX

The following paper was published from this thesis.

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