FACTORS AFFECTING LUTEINIZING HORMONE RELEASING HORMONE AND SOMATOSTATIN RELEASE FROM RAT HYPOTHALAMI IN VITRO

ELIZABETH GODDARD

A thesis submitted for the degree of Master of Science in the faculty of Medicine, University of Cape Town.

March 1981

CONTENTS

contd...

ACKNOWLEDGEMENTS

I would like to express my appreciation to the following people:

Professor R.P. Millar for his guidance and advice during my period of study under his supervision.

Carolyn Kewley for her encouragement and helpful suggestions in the preparation of this thesis.

Chris Tobler for technical advice and supply of iodinated LHRH.

John Morta for his expert assistance in the handling of the large number of rats required for this project.

My colleagues in the Department of Chemical Pathology for helpful discussions and suggestions.

Liz Thornewill for her patience in the many tedious hours spent typing this manuscript.

My parents for their support and encouragement during my studies.

Professor M.C. Berman for financial assistance.

Financial assistance was also obtained from the South African Medical Research Council.

SUMMARY

Conditions for the viable maintenance of the isolated rat hypothalamus in culture, have been investigated. Hank's and Earle's media were suitable for incubation but tissue necrosis occurred rapidly in Eagle's Minimal Essential medium. Hypothalami were suspended in Hank's physiological buffer and factors affecting the release of luteinizing hormone releasing hormone (LHRH) and immunoreactive somatostatin (IRS), investigated. The viability of the tissue was assessed by oxygen consumption and by the rate of protein synthesis. Protein synthesis continued for up to 60 min but declined rapidly during the next 60 min of incubation. Amino acid uptake, however, remained active for 90 min. The rate of LHRH release was constant for the first 60 min of incubation (140 pg/h), thereafter increasing to very high levels (250 pg/h) concomitant with the decline in protein synthesis, suggestive of loss of cellular integrity and peptide leakage. Release of LHRH from the incubated hypothalamus was found to be a temperature dependent process.

LHRH degrading peptidases were secreted by the hypothalamus. The peptidase inhibitor, bacitracin (10^{-3}M), completely inhibited LHRH degradation and was thus included in the incubation medium in all studies.

In viable hypothalami release of LHRH and IRS was less than 5% of the total tissue content. There was no nett increase in tissue content of LHRH or IRS during incubation.

In the presence of 1mM Ca^{2+}, 60 mM K^{+} stimulated both LHRH and IRS release. There was no stimulation of release in the absence of Ca^{2+} (Ca^{2+} free medium + 0.5 mM EGTA) or in the presence of veraparmil (20μ), a Ca^{2+} channel blocker.

The ionophore X537A (1μM) increased the release of LHRH and IRS. Verapamil completely inhibited X537A induced release of LHRH but only partially inhibited the release of IRS. The ionophore A23187 (1μM) increased IRS release but decreased LHRH release. Verapamil did not affect the ionophore - induced response of the two neuropeptides. These findings point to differences in the mode of action of the two ionophores and suggest a difference in the mechanism of release of LHRH and IRS from the incubated hypothalamus.

Although extensive data are available on the effect of neurotransmitters on the secretion of anterior pituitary hormones in vivo little is known about the physiological and biochemical mechanism of their action in this regard. Many neurotransmitter effects may be mediated via their influences on the secretory activity of the hypothalamic peptidergic neurons. In the present study it was found that certain biogenic amines (10^{-5}M to 10^{-7}M) influence the release of LHRH and IRS and that these effects differ for the two peptides. LHRH release was decreased by GABA and increased by melatonin, serotonin, noradrenalin, and adrenalin. L-DOPA, dopamine, acetylcholine and histamine were without effect on LHRH release from rat hypothalami. IRS release was decreased by histamine and increased by serotonin, melatonin and adrenalin. Noradrenalin, L-Dopa, dopamine, acetylcholine and GABA were without effect on IRS release from the incubated hypothalamus.

The effect of a number of neuropeptides (50 - 500 ng/ml) on LHRH and IRS release from the incubated rat hypothalami were investigated. The basal release of LHRH was decreased when hypothalami were incubated in the presence of oxytocin and vasopressin. TRH, somatostatin-14, neurotensin, substance P, met-enkephalin and leu-enkephalin did not alter the basal

release of LHRH. The effect of β-endorphin could not be determined since the peptide was found to interfere with the LHRH radioimmunoassay. The basal release of IRS was increased by neurotensin , substance P, LHRH and TRH. Met-enkephalin, leu-enkephalin, vasopressin, oxytocin and β-endorphin did not alter the basal release of IRS from the incubated hypothalami.

The feedback regulation of LHRH release by anterior pituitary gonadotrophic hormones and gonadal hormones may in part be mediated by alteration in LHRH secretion.

LHRH release was unaffected by *in vitro* addition to testosterone proprionate (20 - 50 ng/ml), β-oestradiol (20 - 50 pg/ml), LH (luteinizing hormone) (1 - 100 ng/ml) and the LHRH agonist des - Gly^{10}- $D\text{-}leu^{6}$ - LHRH ethylamide (10 - 50 ng/ml). IRS release was markedly increased by the *in vitro* addition of β-oestradiol (20 - 50 pg/ml). When rats were castrated LHRH release from their isolated hypothalami was significantly less than that from hypothalami isolated from intact rats. This release was unaltered by *in vivo* pretreatment of castrated rats with testosterone proprionate (100 µg/day) or β-oestradiol (1µg/day). There was an increase in hypothalamic LHRH release when intact rats were pretreated *in vivo* with β-oestradiol (1µg/day). IRS release from hypothalami of castrated rats was increased by *in vivo* pretreatment with β-oestradiol.

Our investigations have established that functionally active, excised hypothalami can be maintained *in vitro* for 60 min as indicated by oxygen consumption, protein synthesis and the ability to stimulate neuropeptide release. This sytem has been shown to be suitable for

investigating the biochemistry of neurosecretion, the role of neurotransmitters and neuropeptides in neurosecretion and the mode of action of hormones in feedback regulation of hypothalamic LHRH and IRS release.

ABBREVIATIONS

Ab	antibody
ACTH	adrenocorticotrophic hormone
ADH	antidiuretic hormone
AMP	adenosine 5' - monophosphate
Arg	arginine
Asp	aspartate
Asn	asparagine
ATP	adenosine 5' - triphosphate
BSA	bovine serum albumin
C	centigrade
Ca^{2+}	calcium
C or COOH terminus	carboxy terminal end
CCK	cholecystokinin
Ci	curie(s)
cm	centimetre
CNS	central nervous system
CO_2	carbon dioxide
cpm	counts per minute
CRF	corticotrophin releasing factor
CSF	cerebrospinal fluid
Cys	cysteine
EGTA	ethyleneglycol-bis n'n' tetra acetic acid
FSH	follicle stimulating hormone
gm	gram
g	gravity
GABA	γ amino-butyric acid

Glu	glutamic acid
Gly	glycine
h	hour(s)
His	histamine
Ile	isoleucine
IRS	immunoreactive somatostatin
K^+	potassium
K	daltons
L	litre
Leu	leucine
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
Lys	lysine
M	molar (mole per litre)
MBH	medial basal hypothalamus
MEM	minimal essential medium
Met	methionine
mg	milligram
MIF	melanocyte inhibiting factor
min	minute(s)
ml	millilitre
mm	millimetre
mM	millimolar
mol	mole(s)
MRF	melanocyte releasing factor
MSH	melanocyte stimulating factor
mV	millivolt

µ	micro
N	normal
N terminal	amino terminal end
Na^+	sodium
ng	nanogram(s)
$-NH_2$	amide
O_2	oxygen
OH	hydroxy
OVLT	organum vasculosum of the lamina terminalis
%	percentage
PBS	phosphate buffered saline
pg	picogram
p-Glu	pyro-glutamic acid
Phe	phenylalanine
PRL	prolactin
Pro	proline
Ser	serine
(S)ME	(stalk) median eminence
$t_{\frac{1}{2}}$	half life
TRF	thyrotrophin releasing factor
TRH	thyrotrophin releasing hormone
Trp	tryptophan
TSH	thrytrophin stimulating hormone
Tyr	tyrosine
VIP	vasoactive intestinal polypeptide

1. INTRODUCTION

INTRODUCTION

In 1849, Berthold demonstrated that if testes were transplanted into castrated cocks, normal male development was maintained in the birds. This was the first paper of any importance in the field of endocrinology as it established that if a particular organ is an endocrine gland it will maintain its normal function if transplanted at a distant site in the body (Harris, 1955). However, it was not until the 1930's that physiological evidence for the regulation by the CNS of gonadotrophin secretion from the pituitary was postulated. In 1928 working on the diencephalon of teleost fish Ernest Scharrer proposed the classical neurosecretory cell which possessed both neuronal and endocrine properties. This neurosecretory concept was further extended by the discovery of a hypothalamic - hypophyseal portal vascular system in mammals (Popa and Fielding, 1930 ; Wislocki and King, 1936).

Initially, the hypothalamus was thought to activate the posterior lobe of the pituitary which in turn activated the anterior lobe of the pituitary by humoral transmission (Hinsey and Markee, 1933). Direct humoral impulses to the adenohypophysis were proposed by Friedgood (1936), Harris (1937) and Brooks (1938). Taubenhaus and Saskin (1941) suggested from their work on the rat hypophysial portal vessels that an acetylcholine-like substance was carried from the hypothalamus to the anterior pituitary. A more detailed study on humoral transmission by Harris (1944) and Green and Harris (1947) established that in mammals there was no major tract of nerve fibres connecting the hypothalamus and anterior pituitary. They proposed that pituitary

function was controlled by humoral substances which were secreted by the hypothalamus into the portal system. These early ideas of neurohumoral regulation of the function of the anterior pituitary gland were based on experiments involving electrical stimulation or lesioning of the hypothalamus, transplantation of the gland or sectioning of the pituitary stalk and thus interrupting the blood supply between the hypothalamus and the pituitary. Studies on the functional activity of the target organ of the anterior pituitary hormones (ovary, testis, adrenal cortex and thyroid) after the treatments revealed that regulation of pituitary hormone secretion was dependent on the integrity of its hypophysial portal blood supply. It was concluded that these portal vessels which arise in the median eminence as capillaries of the primary plexus, carry humoral agents (the releasing and inhibiting factors) from the hypothalamus to the pituitary. The blood borne releasing factors translate neural impulses in the CNS into biochemical responses by increasing or decreasing the secretion of the appropriate trophic hormone from the anterior pituitary.

Initially, the blood flow in the portal system was thought to be from the pituitary to the hypothalamus (Popa and Fielding, 1930; Wislocki and King, 1936). However, Green and Harris (1949) established that the blood flow in the portal vessels was from the tuber cinereum to the adenohypophysis. It was on the basis of these conclusions that studies were initiated to isolate the releasing and inhibiting factors from the hypothalamus and it led to their successful isolation,characterisation and synthesis by Schally and Guillemin. It is therefore significant that Oliver et al (1977) and more recently, Bergland and Page (1979)

have provided evidence that there is retrograde blood flow away from the pituitary towards the hypothalamus and other brain areas. This offers potential for short loop feed-back regulation and the possibility that the hypothalamus can metabolize pituitary peptides eg. β-lipotrophin to β-endorphin (Fig. 1a).

A large number of physiological studies established that the majority of physiological processes are ultimately regulated by release and release-inhibiting factors secreted by the hypothalamus. These factors were found to control the pituitary hormones involved in regulating reproduction (FSH and LH), growth (GH), carbohydrate metabolism (ACTH), thryoid function (TSH), milk production (PRL), pigmentation (MSH), water balance (ADH or vasopressin) and uterine contraction (oxytocin) (Blackwell and Guillemin, 1973; Schally et al, 1973; Petersen and Guillemin, 1974).

The hypothalamus is an area of the diencephalon lying at the base of the brain, ventral to the thalamus and forming the floor and part of the lateral walls of the third ventricle. It is bounded anteriorly by the optic chiasma and posteriorly by the mamillary bodies (Fig. 1b). The median eminence of the tuber cinereum, in the floor of the third ventricle, is connected to the pituitary by means of a stalk (Schally et al, 1973).

The first of these hypothalamic hypophysiotrophic factors (Table 1.1) to be isolated was a tripeptide, thyrotrophin - releasing factor (TRF, TRH) by Burgus et al (1969, 1970), who purified 300,000 sheep hypothalami to isolate 1 mg TRH. Concurrently, Schally's group

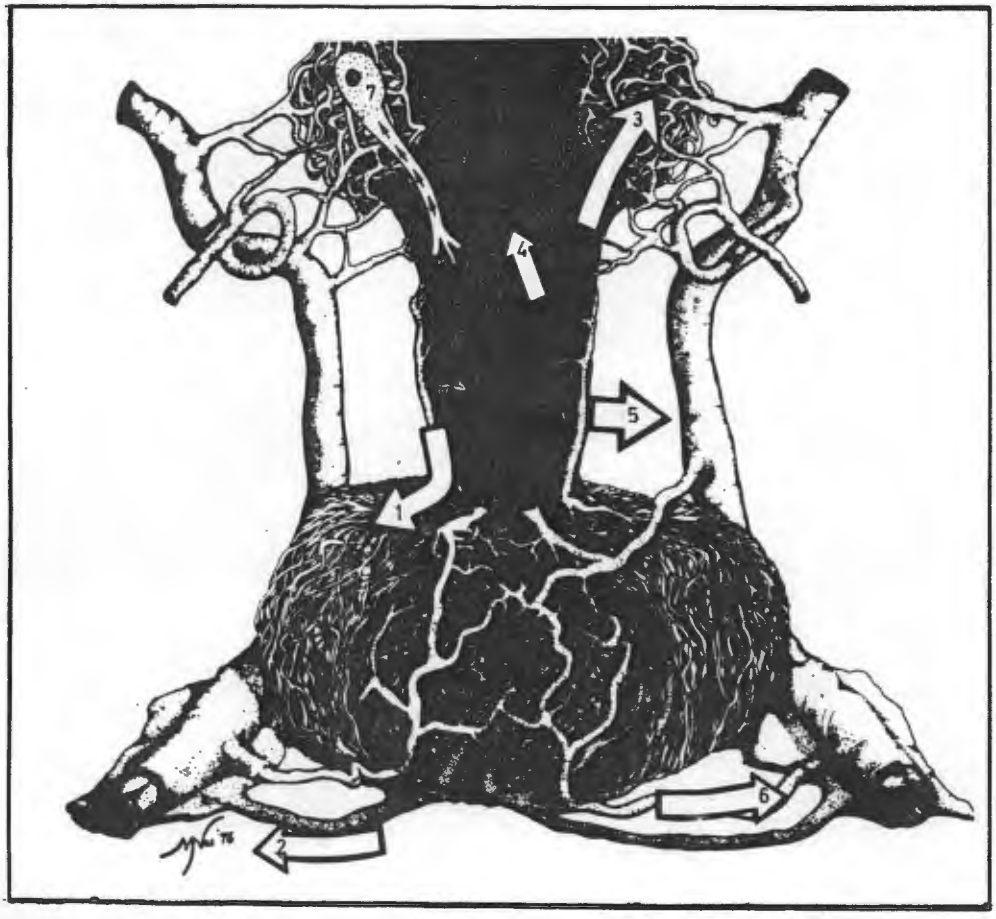


FIGURE 1.1(a) Diagram of the vasculature of the rhesus monkey pituitary; posterior view. The posterior portion of the infundibulum has been removed and the arrows indicate the potential routes from the neurohypophysis since adenohypophyseal secretions are conveyed to the capillary bed of the neurohypophysis where they may leave via any of 7 potential routes. 1) portal vessels to adenohypophysis 2) to cavernous sinus 3) infundibulum to hypothalamus 4) tanycyte transport into the ventricles 5) leakage through fenestrations of the portal vessels 6) arteries may serve as efferent vascular channels 7) retrograde axonal flow to the hypothalamus. Five of these routes are directed to the brain (from Bergland and Page, 1978).

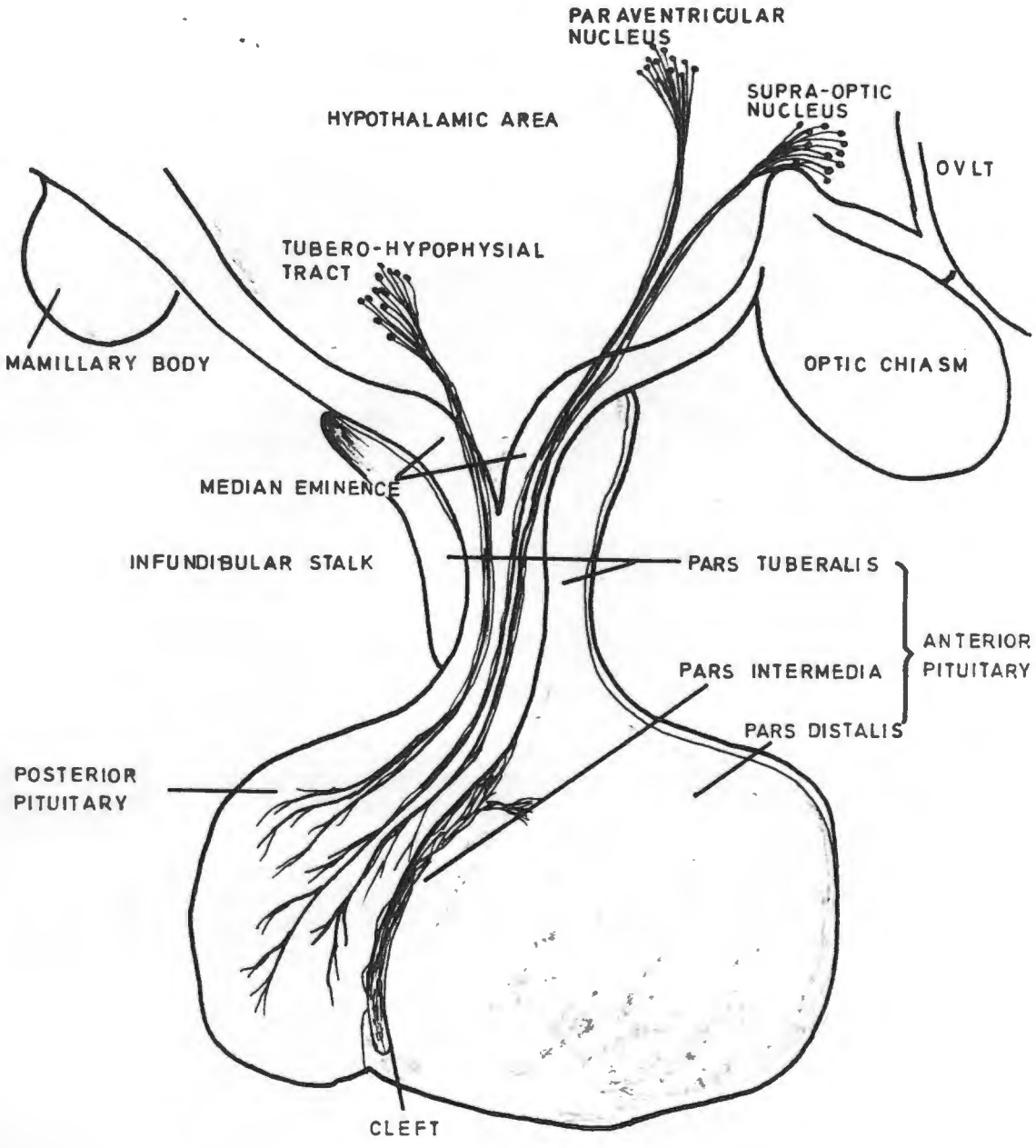


FIGURE 1.1 (b) Diagram of the hypothalamopituitary region.

(modified from Netter, 1965).

TABLE 1.1.

Sequences and principal actions of LHRH, somatostatin-14 and TRH on the anterior pituitary (modified from Vale et al, 1977)

NAME	SEQUENCE	PRINCIPAL HYPOPHYSIOTROPIC ACTIONS
LHRH, LRF, LRH GnRH (gonadoliberine)	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	↑ LH ↑ FSH
GHRIF, SRIF SOMATOSTATIN-14	H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp HO-Cys-Ser-Thr-Phe-Thr-Lys	↓ GH ↓ TSH
TRH, TRF (thyroliberine)	pGlu-His-Pro-NH_2	↑ TSH ↑ PRL

LRF luteinizing hormone-releasing factor
LRH luteinizing hormone-releasing hormone
GnRH gonadotrophin releasing hormone
GHRIF growth hormone release-inhibiting hormone
SRIF somatomedin release-inhibiting factor

↑ increase ↓ decrease

reported the structure of porcine TRH to be identical to that of ovine TRH (Nair et al, 1970).

In 1971 Schally's group characterized the structure of the decapeptide luteinizing hormone - releasing hormone (LHRH) which stimulated the release of both LH and FSH from pituitary cells in culture. Later that year Guillemin's group confirmed the structure from material of ovine origin (Burgus et al, 1972). Two years later, Guillemin's group (Vale et al, 1972) characterized a cyclic tetradecapeptide somatostatin (SS) which possessed potent GH release-inhibiting properties. Schally's group found the structure of porcine somatostatin to be identical (Coy et al, 1973). A number of higher molecular weight immunoreactive species of somatostatin have since been described in various tissues and shown to have biological activity. (Schally et al, 1975; Zingg and Patel, 1979; Vale et al, 1976; Millar, 1978; Spiess and Vale, 1978, 1980; Rorstad et al, 1979). Recently, a twenty eight amino acid form of somatostatin was isolated from porcine intestine by Pradayrol et al (1980) and was subsequently isolated in extracts of ovine hypothalami (Brazeau et al, 1980) and porcine hypothalami (Schally et al, 1980). It has been shown to have GH inhibiting activity equal to or fourteen times greater than that of the tetradecapeptide somatostatin. Other proposed putative hypothalamic factors which have not as yet been isolated are CRF (ACTH), PIF and PRF (PRL), MIF and MRF (MSH) and GRF (GH). Morphine is known to release GH, thus it seemed possible that an endogenous opiate like substance may be involved in the release of GH as well as other pituitary hormones. Hughes et al (1975) isolated the opioid pentapeptides leu- and met-enkephalin from brain extracts and in 1976, Li et al, isolated the untriakontapeptide, β-endorphin, from the camel pituitary gland.

Subsequently, the enkephalins were localized to neurons and cell bodies within the hypothalamus (Hökfelt et al, 1977). Of the endogenous opiate peptides isolated to date, only these three have any claim to a physiological function (Beaumont and Hughes, 1979). It is clear that there is a very close and complex relationship between opioid peptides and hypothalamic mechanisms which may explain some of their effects on GH, PRL, LH, FSH, and ADH secretion from the pituitary gland. However, enkephalins do not appear to be releasing and inhibiting factors because they do not have a direct effect on pituitary hormones. They probably operate in a similar way to neurotransmitters in stimulating or inhibiting peptidergic neurons which secrete releasing and inhibiting factors.

Once LHRH, somatostatin and TRH had been synthesized it was possible to render these peptides immunogenic by linkage to hapten carriers. Thus specific antibodies were produced and used in radioimmunoassays (RIA) which allowed for quantitation and localization of the peptides in tissues and biological fluids.

DISTRIBUTION AND BIOLOGICAL ACTION OF LHRH

DISTRIBUTION

Studies based on the direct determination of LHRH by RIA of extracts from either thin hypothalamic slices (Wheaton et al, 1975) or discrete hypothalamic nuclei, removed by "punch techniques" (Palkovits et al, 1974), showed a similar distribution pattern for the neuropeptide. LHRH is found over the optic chiasma and in the region extending caudally along the base of the brain to the ME (Fig. 1a). The bulk of LHRH is concentrated in the ME and arcuate nucleus region (85% of total hypothalamic content). These findings are in good agreement with earlier work (McCann, 1962; Crighton et al, 1970; Quijada et al, 1971). A large part of the preoptic LHRH is concentrated extrahypothalamically in the OVLT (Wheaton et al, 1975). It was first detected in this region by immunohistological techniques (Zimmerman et al, 1974) and later confirmed by RIA of extracts of OVLT (Kizer et al, 1976). Smaller, but significant quantities of LHRH have been found in the remaining periventricular organs (subfornical, subcomisural and area postrema), the amygdala, the para-olfactory cortex (Hökfelt et al, 1976), and in the pineal gland (Kizer et al, 1976; Jonas et al, 1975; White et al, 1974; Gross, 1976).

BIOLOGICAL ACTION

LHRH is known to stimulate the release of LH and FSH, induce ovulation and increase sperm counts and the production of gonadal steroids in a variety of species (Vale et al, 1977). Immunisation of experimental animals against LHRH results in a decrease in serum LH and FSH levels, infertility, and gonadal atrophy (Arimura et al, 1973, 1974). To date no substance has been isolated which selectively

modulates FSH secretion, although clinical and experimental circumstances do exist in which secretion of LH and FSH can be dissociated. A possible explanation is that LHRH is the only hypothalamic stimulator of LH and FSH release but peripheral, and in particular gonadal factors, may have differential feedback effects on the release of individual hormones from the anterior pituitary. Inhibin, an aqueous testicular and ovarian extract has been reported to selectively block the spontaneous and LHRH-induced secretion of FSH but not LH (Franchimont et al, 1975 ; Setchell and Jacks, 1974). The effect of inhibin is probably exerted synergistically with these of the gonadal steroids (Labrie et al, 1979). Androgens have also been shown to have not only specific but also opposite effects at the pituitary level on the control of LH and FSH secretion, while estrogens stimulate LH and FSH secretion and progesterone stimulates FSH but has a biphasic effect on LH secretion. (Drouin and Labrie, 1976; Labrie et al , 1979;(Fig. 1.2)

The existence of LHRH in extrahypothalamic nerve fibres, taken together with behavioural studies support a possible direct action of LHRH on the CNS that could modulate sexual behaviour independently of the gonadal steroids or the pituitary gonadotropin status of the animal. Thus delivery of LHRH to appropriate neural cells mediating this response could occur either by direct projections of the LHRH neurons or by transport through the CSF (Vale et al, 1977).

Immunocytochemical evidence suggests that LH and FSH are contained within the same pituitary cell type (Herbert, 1976). A prerequisite for secretion of the pituitary hormones appears to involve the binding of LHRH to a specific receptor on the plasma membrane of the

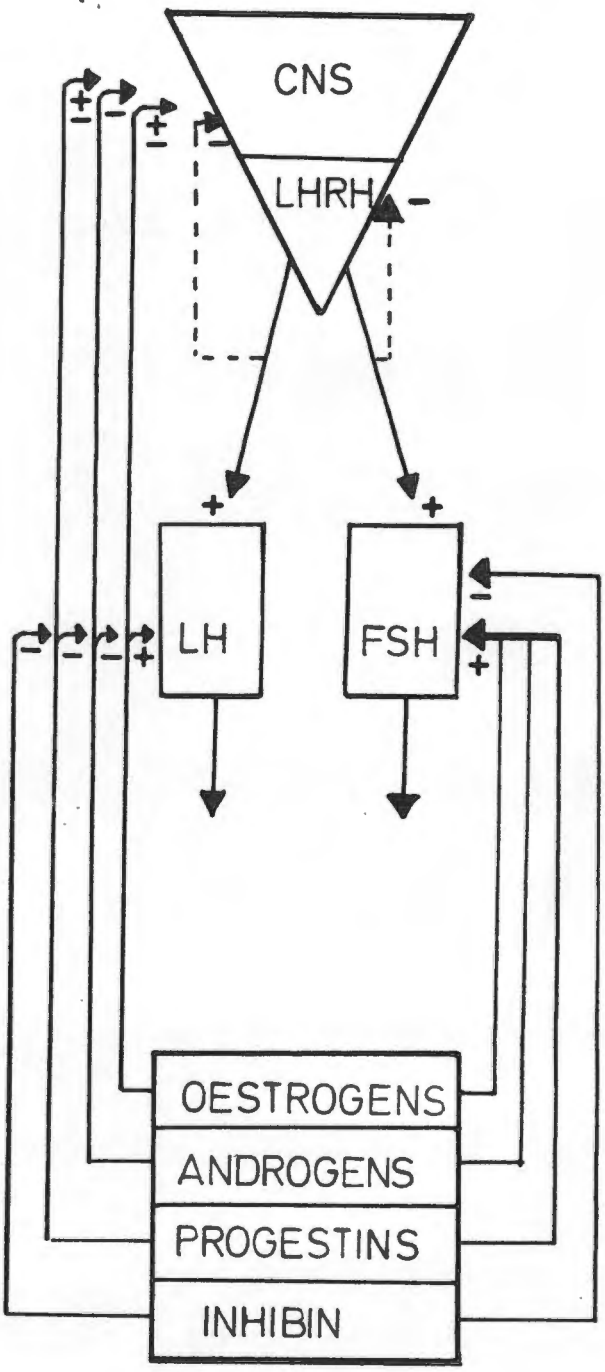


FIGURE 1.2 Schematic representation of the feedback regulation of LHRH secretion.

(modified from Labrie *et al*, 1979)

pituitary cell. Studies with analogues have established that nearly the complete decapeptide sequence of LHRH is required for biological activity. This implies that strict structural features are implicit to the receptor binding LHRH. The mechanism of action of the neuropeptide at the level of the pituitary cell is unknown although a redistribution of Ca^{2+} in the gonadotropin cell (Hopkins and Walker, 1978) and the adenyl cyclase system (Labrie et al, 1979) have been implicated.

DISTRIBUTION AND BIOLOGICAL ACTION OF SOMATOSTATIN

DISTRIBUTION

Growth hormone release-inhibiting activitiy was first detected by Krulich et al, (1971) in the ME and to a lesser extent in the suprachiasmatic area of rat brain. Subsequently somatostatin was found in many other sites in the CNS. Other areas of the hypothalamus with high concentration of somatostatin are the arcuate, ventromedial, ventral premaxillary and the periventricular nuclei. Somatostatin is found in much lower concentrations in the periventricular organs and pineal gland (Brownstein et al, 1975; Kizer et al, 1976). The distrubition of somatostatin is not limited to the CNS structure as large amounts are found in the gastrointestinal tract, the pancreas and the thyroid gland (Hökfelt et al, 1975; Arimura et al, 1975(a); Leclerc et al, 1976; Parsons et al, 1976).

BIOLOGICAL ACTION

Somatostatin inhibits GH release *in vivo* and *in vitro* in response to virtually every known stimulus in man and animals. It also inhibits the release of TSH, ACTH, insulin, glucagon, pancreatic polypeptide, gastrin, cholecystokinin, pancreozymin, secretin, VIP, renin, PTH and calcitonin to name some of its endocrine actions. Somatostatin has also been shown to inhibit or decrease non-endocrine phenomena such as platelet aggregation and acetylcholine release in addition to effects on the CNS. (Sheppard, 1979, a comprehensive review).

Somatostatin does not inhibit indiscrimantly as it has been shown to be without effect on LH and FSH secretion, and the release of steroids from the adrenal cortex and the gonads in normal animals (Vale et al, 1977).

Since somatostatin has a short biological half life in the peripheral circulation it is unlikely that any effect of the neuropeptide on the endocrine pancreas is due to somatostatin of hypothalamic origin. The D cells of the pancreas have been shown to have long cytoplasmic processes terminating on other cell types where local release of somatostatin could control their function. This suggests paracrine regulation by somatostatin of glucagon, insulin and gastrin secretion from the pancreas (Larsson et al, 1979).

The mechanism by which somatostatin exerts the inhibitory action is not understood. Interference with Ca^{2+} transport at the nerve terminals and in the pancreas as well as an inhibitory effect on pituitary cyclic AMP and Ca^{2+} accumulation have been described (Tan et al, 1977; Curry and Benett, 1976; Ishibashi et al, 1979; Borgeot et al, 1974; Bicknell and Schofield, 1976).

DEGRADATION OF LHRH AND SOMATOSTATIN

Degradative enzymes may play a part in controlling hormone action by:
(i) regulating the amount of hormone available for release at the site of production
(ii) regulating the amount of hormone actually reaching site of action
(iii) controlling the duration of action through hormonal inactivation at the receptor sites in a particular target tissue.
(iv) the removal of the hormone from the general circulation so that too large a response to the hormone is prevented.

Proteolytic enzymes are responsible for the inactivation of the active peptide into inactive metabolites which may be rapidly excreted or recirculated and reutilised by the body (Marks, 1977; Griffiths and Kelly, 1979).

DEGRADATION OF LHRH

Inactivation of LHRH by hypothalamic extracts were first reported by Griffiths et al (1973) and Sandow et al (1973). The peptidase activity inactivating LHRH changes with castration and gonadal steroid injection (Griffiths et al, 1975), with neonatal androgen treatment (Griffiths et al, 1976), at a time of puberty (Griffiths et al, 1978) and under the influence of dopamine (Marcano de Cotte et al, 1980). This suggests that the enzymes may have a functional significance in controlling the amount of LHRH available for release from the hypothalamus. It has also been suggested that the peptidases in the hypothalamus may be responsible for activation of

LHRH from the parent pre prohormone (Millar et al, 1978(b)).

The hypothalamic peptidases inactivating LHRH are known to interfere with in vitro studies of LHRH biosynthesis and release (McKelvy et al, 1976). Thus the polypeptide antibiotic, bacitracin was used to inhibit degradation of LHRH released. Bacitracin completely inhibits the peptidases at a concentration of 10^{-4}M and has a Ki of 1.9×10^{-5}M (See Chapter 2).

Subcellularly, the enzymes are present in soluble/cytoplasmic and particulate fractions and have an optimum pH of 7.4. Part of the specificity is naturally inferred on LHRH by nature of its blocked C and N terminal ends. For the degradation of LHRH, Loudes et al (1978), have determined that the peptidase has a Km of 2.1×10^{-8}M, although Sundberg and Knigge (1978), have calculated a Km of 2.1×10^{-8}M and V max of 1.7 pg/min/µg protein.

Several sites of enzymic cleavage of the decapeptide have been indicated. Marks (1977) has suggested that cleavage of the Gly-NH_2 ^{10} from the C terminus may occur. Subsequently a pyroglutamate aminopeptidase (Bauer et al, 1979) and a post-proline cleaving enzyme (Knisatschek and Bauer, 1979; Hersch and McKelvy, 1979) have been isolated from brain and pituitary extracts, which cleave the pyro-Glu^{1}-His^{2} bond and the Pro^{9}-Gly-NH_2 ^{10} bond. A more controversial issue is the reported cleavage of the Tyr^{5}-Gly^{6} bond (Hudson et al, 1976) and/or the Gly^{6}-leu^{7} bond (Koch et al, 1974; Fridkin et al, 1977; Akapyon et al, 1979) by brain, hypothalamus, pituitary and liver extracts. Koch et al (1974) and Kelly et al (1978) have found that the 1-6 sequence of LHRH is the only product of enzyme cleavage detectable using extracts from hypothalamic and

pituitary tissue. More recently a non-chymotrypsin- like endopeptidase has been partially purified and characterized from anterior pituitary extracts, which cleaves LHRH (Km=180μM) at the Tyr^5-Gly^6 and His^2-Trp^3 bonds (Horsthemke and Bauer, 1980) (Fig. 1.3). Thus it is apparent that there must be several LHRH degrading enzymes and also amino and carboxy peptidases in tissue extracts which may subject the initial cleavage products to further degradation.

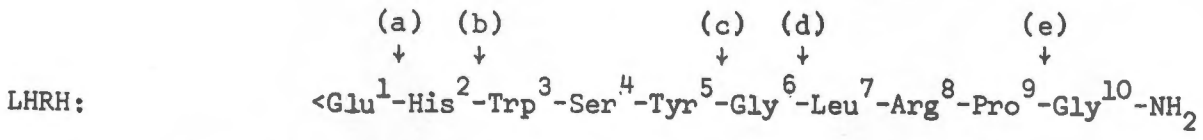
DEGRADATION OF SOMATOSTATIN

Enzymes degrading somatostatin-14 have been extracted from the thalamus, cerebellum, cortex and hypothalamus. Little is known about the subcellular distribution of these peptidases. It has shown that soluble and particulate subcellular fractions prepared from the hypothalamus rapidly degrade somatostatin. The peptidases involved have an optimum pH at 7.4 and a second peak at pH 9.0 and are specific for somastostatin (Griffiths et al, 1977; Dupont et al, 1978). Some specificity is probably due to the cyclic nature of somastostatin and in agreement with the studies of Marks (1976), the hypothalamic neutral peptidases appear to cleave the Trp-Lys bond initially although other sites of action are possible (Kelly et al, 1978). A non-specific neutral peptidase (pH 7.0) purified from bovine hypothalamic extracts (Akopyan et al, 1978) is thought to cleave at the Asp^5-Phe^6, Phe^6-Phe^7 or The^{10}-Phe^{11} bonds. An acid peptidase (pH 3.2) isolated from hypothalamic extracts may also be involved (Akopyan et al, 1978).

Recently, a neutral peptidase in hypothalamic extracts has been shown to convert a high molecular weight immunoreactive species (25K) of somastatin to an intermediate immunoreactive species (4K) and somatostatin-

FIGURE 1.3

PROPOSED SITES OF ENZYMIC CLEAVAGE OF LHRH AND SOMATOSTATIN-14

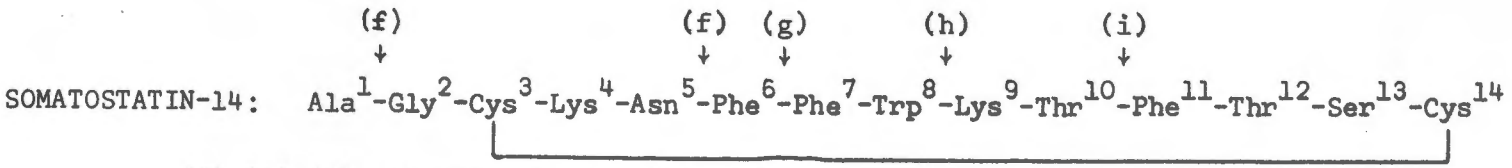


(a) Bauer *et al* 1979; (b) Horsthemke and Bauer 1980.

(c) Hudson 1976; Horsthemke and Bauer 1980;

(d) Koch *et al* 1974; Fridkin *et al* 1977; Kelly *et al* 1978; Akopyan *et al* 1979.

(e) Marks 1977; Knisatschek and Bauer 1979; Hersh and McKelvy 1979.



(f),(g),(i) Akopyan *et al*, 1978, 1979.

(h) Marks 1976, Akopyan *et al*, 1979.

14 (Zingg and Patel, 1980). Thus it appears that peptidases are important in the cleavage and activation of prohormones and thus may have a regulatory role in controlling somatostatin levels in the hypothalamus (Fig.1.3).

NEUROSECRETORY MECHANISMS

In the last decade it has become apparent that many of the polypeptide hormones are synthesized by way of a prohormone. The prohormone may be derived from precursor molecule, the preprohormone, which has an additional sequence of hydrophobic residues at the amino terminus. Blobel and Dobberstein (1975) predicted such prepeptides in the biosynthetic pathway for secretory polypeptides as part of the "signal hypothesis" to explain translocation of newly synthesized polypeptides into the lumen of the endoplasmic reticulum.

Supportive evidence for the existence of a precursor of LHRH was the isolation of a putative high molecular weight immunoreactive form of LHRH (Millar et al, 1977, 1978). Although little is known of the biosynthesis of somatostatin a number of high molecular weight immunoreactive species (25K-5K) have been described. Spiess and Vale (1980) isolated 12K, 4K and 2K immunoreactive species of somatostatin and proposed that the larger immunoreactive species are prosomatostatin fragments possibly intermediates in the precursor product conversion. Similarly Pradayrol et al (1980) and Schally et al (1980) proposed that somatostatin-28 may be a prohormonal form of somatostatin-14. However, Guillemin's group (Brazeau et al, 1980) proposed that somatostatin-14, as originally isolated, may be a biologically active fragment of a larger molecule of greater specific activity. In view of their biological activity and the fact that the 3K and somatostatin-14 species are contained in and isolated from SME synaptosomes (Kewley, 1981) it is conceivable that they are both products of a larger prohormonal form.

Most of the work on peptide neurosecretion has been done on the release of oxytocin, vasopressin and the neurophysins from the posterior

pituitary (Gainer et al, 1979). This system may be regarded as a classical model for neurosecretion and may be analogous to the biosynthesis and secretion of LHRH and somatostatin from the hypothalamus into the hypophyseal portal system. Thus it hypothesized that a high molecular weight precursor form of the neuropeptide is synthesized ribosomally in the cell bodies (Millar et al, 1978). Once synthesized the high molecular weight precursor is sequestered into vesicles in the Golgi and transported along the axon to the nerve terminal where the vesicles are stored prior to secretion. The vesicles may contain the enzymes necessary for the post-translational processing of the prohormones to smaller peptide products, although to date none have been isolated.

Some neuropeptides, including LHRH, and many neurotransmitters have been shown to be localised in two storage pools, viz. a small "readily releasable pool" and a larger storage pool whose contents are more difficult to mobilize. (Dreifuss, 1975; Barnea et al, 1977; Israel et al, 1979). It has been suggested (Sachs and Haller, 1968) that the readily releasable pool consists of granules located in close proximity to the plasma membrane which discharge their contents following membrane depolarization. Newly synthesized hormones may first enter the readily releasable pool before equilibrating with the storage pool.

Although peptidergic neurons are not morphologically different from the classical neurotransmitter there appears to be a difference in the mode of replenishing the released substance (Hökfelt et al, 1980). There is apparently no synthesis of peptides in the nerve terminal nor has any reuptake of released peptide been demonstrated. In contrast,

neurotransmitter concentrations in the nerve terminal are kept fairly constant by efficient replacement of released transmitter by (i) enzymatic synthesis in the nerve terminal (ii) reuptake from the synaptic cleft (iii) supply of neurotransmitter (or precursors) in secretory vesicles from the cell body via axonal tranaport (Iversen, 1978: Hökfelt et al, 1980).

The final stages of the neurosecretory process can be divided into at least 4 stages:
(i) Depolarization (ii) Ca^{2+} transport across the plasma membrane (iii) transport of the secretory vesicles to the plasma membrane (iv) fusion and exocytosis.

Depolarization and the role of Ca^{2+} in neurosecretion will be discussed in detail.

(i) DEPOLARIZATION

There is a high concentration of K^+ ions within nerve cells and a low concentration of Na^+ ions, relative to the extracellular concentration of these ions. This distribution of cations results from Na^+ ions being pumped out and K^+ ions into the cell by the Na^+/K^+ ATPase pump. Also, the plasma membrane of the nerve cell at rest is much more permeable to K^+ ions than it is to Na^+ ions. Thus, more positive ions are pumped out of the cell than into it. Since most of the intracellular anions (phosphate, sulphates and protein ions) are nondiffusible the negative charges remain within the cell which becomes electronegative relative to the outside (which is electropositive). Thus a potential difference of - 70 mV is formed accross the plasma membrane. Nerve cells are unique in that they can propogate an action potential. The first event is an increase in the

Na^+ permeability, causing Na^+ ions to move into the cell neutralizing the negative charge. This leads to further opening of adjacent Na^+ channels and propagation of a wave of depolarization. This is followed by an increase in K^+ permeability as the Na^+ permeability returns to normal. The efflux of K^+ ions re-establishes the charge difference across the membrane. Then the K^+ permeability decreases back to normal and there is active outward transport of Na^+ ions across the cell membrane (Guyton, 1976).

Depolarization of nerve cells in vitro can be effected via several mechanisms:

(i) Electrical stimulation

(ii) Ouabain is known to inhibit the Na^+/K^+ dependent ATPase and thereby cause membrane depolarization (Skou,1965). However, if this were so, intracellular Na^+ would be expected to increase and intracellular K^+ decrease. There is evidence that the intracellular Na^+ remains unchanged and that the effect of ouabain is via changes in the K^+ ion concentration (Verity et al, 1979).

(iii) Veratridine and associated alkaloid compounds induce depolarization by opening Na^+ channels and allowing an influx of Na^+ (Ohta et al, 1973).

(iv) Since the plasma membrane is relatively permeable to K^+ the presence of high extracellular K^+ perturbs the transmembrane potential, K^+ enters the neuron, neutralizing the negative charge and thus inducing depolarization. This effect is not blocked by tetrodotoxin (a specific Na^+ channel blocker) indicating that the mechanism of action of K^+ induced depolarization is not mediated via the opening of Na^+ channels. It is usually assumed that the depolarizing action of high K^+ ions simulates neuronal excitation. However, at the neuromuscular junction, raised extracellular K^+ concentrations

or an electric current have been shown to depolarize nerve terminals. It has been demonstrated while the depolarizing current actually reduced the amount of transmitter released from the nerve terminal, the increased K^+ concentration had the opposite effect. This difference suggests that K^+ ions may influence transmitter release via a mechanism distinct from depolarization (Gage and Quastel, 1965; Cooke and Quastel, 1973). Moreover, incubation in a constant level of high K^+ ions does not produce pulsatile action potentials as electrochemical gradients cannot be re-established (Somjen, 1979).

(v) Ionophores have been shown to depolarize membranes. The ionophore A23187 transports Ca^{2+} and Mg^{2+} across the plasma membrane (Reed and Lardy, 1972). This rapid Ca^{2+} influx mimics, in part, the events preceeding the neurosecretory event. Ionophore X537A binds mono and divalent cations. In the absence of extracellular Ca^{2+}, X537A stimulates vasopressin release from the isolated neurohypophysis while A23187 is without effect (Nakazato and Douglas, 1974). The greater effectiveness of the ionophore X537A is attributed to its ability to transport monovalent cations, thereby causing depolarization (Cochrane and Douglas, 1975).

(ii) ROLE OF CA^{2+} IN NEUROSECRETION

Ca^{2+} entry into the nerve terminal is thought to be essential for neurosecretion. Resting nerve cells are impermeable to Ca^{2+}. The arrival of the action potential at the nerve terminal leads to an increased permeability and consequent influx of Ca^{2+} (Rahamimoff and Abramovitz, 1978) which is a function of the membrane potential (Douglas, 1974). The intracellular concentration of Ca^{2+} rises from a resting level of 10^{-8}M to 10^{-5}M free Ca^{2+}. Calcium is known to cross the plasma membrane via at least 2 channels (Baker, 1972):

(i) the initial entry via a Na^+ related channel and

(ii) the subsequent entry via a voltage dependent Ca^{2+} channel.

The rise in intracellular Ca^{2+} is the predominant trigger for the initiation of fusion of the synaptic vesicles with the synaptic plasma membrane and the subsequent release of neuropeptides. This process has been termed stimulus-secretion-coupling (Douglas 1975). Ca^{2+} is thought to surround the synaptic vesicles near the synaptic plasma membrane, causing a rearrangment of the water soluble components of the plasma membrane resulting in a spontaneous membrane fusion (Llinás, 1977). Exocytotic release from the vesicles may or may not involve the loss of the entire vesicle contents. Acher (1979) postulated that the entire vesicle contents are released in neurohypophyseal secretion.

A number of mechanisms are involved in lowering the elevated intracellular Ca^{2+} concentration (Rahamimoff and Abramovitz, 1978).

(i) at Ca^{2+} concentrations of 10^{-6}M or greater, intraterminal mitochondria will take up calcium.

(ii) Ca^{2+} can be buffered by binding to cytoplasmic proteins eg. calmodulin (Chung, 1980).

(iii) Ca^{2+} extrusion from the nerve cell in a Na^+ dependent manner.

(iv) diffusion of Ca^{2+} from the nerve terminus.

THE ROLE OF NEUROTRANSMITTERS IN CHEMICAL TRANSMISSION

Chemical transmission across the synapse involves transmitter synthesis, storage, release, reaction with the receptor and termination of transmitter actions.

The first step in chemical transmission is the synthesis of the transmitter molecules in the nerve terminals. Each neuron can usually only synthesize one specific transmitter. The transmitter molecules are manufactured de novo as a precursor molecule which then undergo a series of enzymatic reactions. The transmitter molecules are stored in the axon terminal in synaptic vesicles. There may be thousands of synaptic vesicles in each nerve terminal, each containing tens of thousands molecules of transmitter.

Release of the transmitter is initiated by the nerve impulse increasing the permeability of the nerve terminal to calcium ions which activates the release mechanism. The actual mechanism of release is still controversial. Some groups believe that the synaptic vesicles fuse directly with the presynaptic membrane and discharge their contents into the synaptic space, while others believe that there is a mobile pool of transmitter molecules liberated through special channels.

The released transmitter molecules interact with specific receptor sites in the post synaptic membrane. Many transmitter receptors have two functional components: a binding site for the transmitter and a pore passing through the membrane that is selectively permeable to certain ions. The binding of the transmitter to the receptor causes

a conformational change of the binding site which opens the pore in the membrane allowing ions to flow down their concentration gradients into or out of the cell. This results in an increase or decrease of the neuron firing rate.

Some transmitters are inactivated by enzymes in the synaptic space eg. acetylcholinesterase destroys acetylcholine. Others, such as noradrenalin are taken up and destroyed by enzymes in the nerve terminal while others are recycled and stored in the synaptic vesicles.

PURPOSE OF STUDY

The hypothalamus is an important tissue regulating a wide spectrum of physiological processes in vertebrates. Nevertheless, many aspects of the basic biochemistry and physiology of secretion of the hypothalamic peptide hormones remains unknown. One of the most important aspects of the regulation of hypothalamic peptide hormone secretion is the so called negative feedback by end organ hormones.

The purpose of this thesis was to establish indices which could be used to monitor the viability of the incubated hypothalamus. Once established, this provided a system to study the factors affecting LHRH and IRS release from the hypothalamus. Studies on the nature of the feedback mechanisms of steroids on LHRH release were initiated. To date it has been difficult to assess factors controlling LHRH release from the hypothalamus *in vivo* as it is secreted in nanomolar amounts and once secreted, is rapidly degraded ($t_{\frac{1}{2}}$ = 6 minutes in blood). However, taken in conjunction with *in vivo* pretreatment of animals, the incubated hypothalamus provides an *in vitro* system for studying the physiological and biochemical end organ feedback regulation as well as short loop feedback and/or autoregulation of the hypothalamus.

2. MATERIALS AND METHODS

2.1. Materials

The sources of peptides used are as follows:

LHRH and D-leu^{6}-desGly^{10} LHRH ethylamide, Ayerst Laboratories; ovine LH, NIH (Bethesda , USA); Tyr^{1}-somatostatin, a gift from Dr. D. Coy (New Orleans, USA); somatostatin-14, neurotensin and substance P, U.C.B. bioproducts peptide department (Bruxelles); β-endorphin, leu-and met-enkephalin, Beckman; TRH, Calbiochem; vasopressin, Parke-davies Laboratories; oxytocin, Sandoz Laboratories.

The following reagents were obtained from Sigma Chemical Co. (St. Louis): L-DOPA, dopamine, noradrenalin, adrenalin, acetylchloline, histamine, serotonin, melatonin, GABA(γ-amino butyric acid), 17β-oestradiol, testosterone, EGTA, TPCM,* TLCM,* ouabain, cytochalasin cyclohexamide and actinomycin D. The sources of other reagents are as follows: ^{125}I NaI (IMS 30) and [^{3}H] amino acid hydrolysate (TRK 440), Radio chemical centre (Amersham UK); carboxymethyl cellulose (CM 32), Whatman (Kent, U.K.); verapamil (Isoptin), Knoll AG (Ludwigshafen); X537A, Hoffman le Roche Inc. (Nutley N.J.); A23187, vincristine and vinblastine Eli Lilley laboratories (Indianapolis, Indiana).

* TPCM: N-α-tosyl-phenylalanyl chloromethane; TLCM: N-α-tosyl-L-lysyl chloromethane.

Antiserum 774 used for the radioimmunoassay of immunoreactive somatostatin was a gift from Professor. A. Arimura (New Orleans, U.S.A.)

2.2. METHODS

2.2.1 ANIMALS

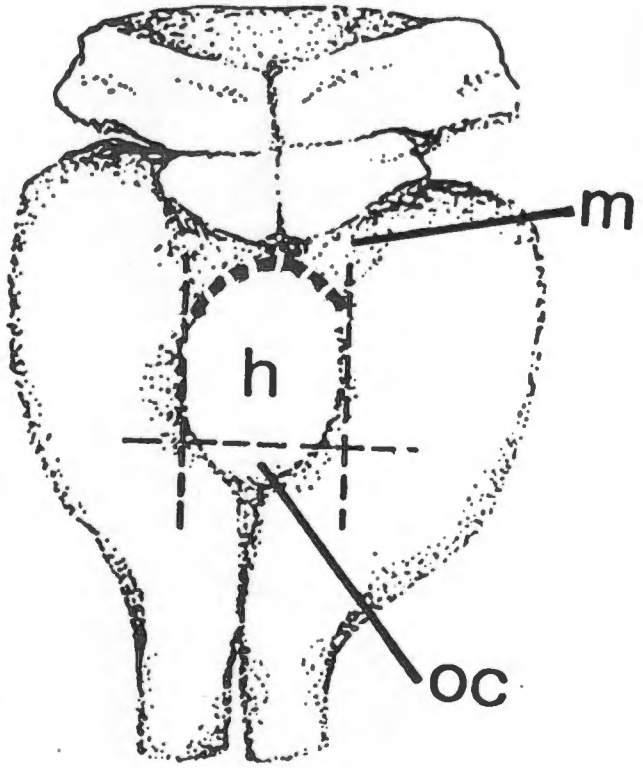
Male Long-Evans rats (250-350g) were kept on a 14:10 light : dark cycle in a humidity and temperature controlled room with food and water available ad libitum. Each experiment was commenced at 9.00 am to avoid possible differences in diurnal hypothalamic content and secretion of LHRH and somatostatin (Kerdelhue et al, 1978).

2.2.2 ISOLATION OF THE HYPOTHALAMUS

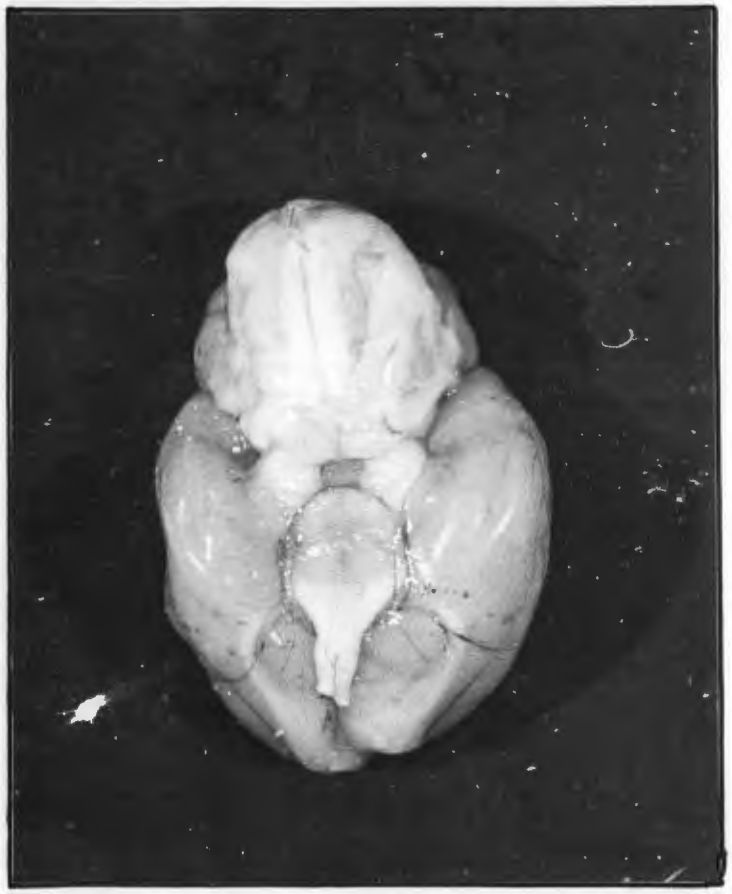
Rats were decapitated and the hypothalamus (including the median eminence) removed within 1-2 mins. The area dissected out extended from the anterior edge of the optic chiasma to the mammillary body, 1.5 - 2.0 mm from the midline and approximately 2-3 mm in depth. The dissection was carried out with a razor blade to ensure the cleanest cut possible with the least damage to the tissue (Fig. 2.1).

2.2.3 INCUBATION OF THE HYPOTHALAMUS

The hypothalami were pre-incubated in Wasserman tubes in 1.0 ml of Hank's physiological medium (pH 7.4) which contained 10^{-3}M bacitracin to prevent peptidase degradation of LHRH and somatostatin (McKelvy et al, 1976). For storage purposes the incubation medium contained the antibiotics penicillin, neomycin and streptomycin (PNS) each at a concentration of 5 ml/L. After 45 min of preincubation the hypothalami were washed twice, transferred, and the incubation continued for 60 min in Wasserman tubes containing 0.5 ml medium (pH 7.4) which contained 10^{-3}M bacitracin, 10 uCi of tritiated amino acid ($[^3H]$ amino acid hydrolysate) and the appropriate substance(s) to be tested. The incorporation of $[^3H]$ amino acids into tissue reflected the ability of the tissue to synthesize proteins which was then used as an index of tissue viability. Both the pre-incubation and the incubation were carried out with continuous shaking at 37°C in an atmosphere of 95% O_2 and 5% CO_2.



A



B

FIG 2.1

Ventral surface of the rat brain

A. Schematic representation of the area dissected out in the isolation of the rat hypothalamus from the ventral surface of the rat brain as seen in B.

H : hypothalamus M : mamillary body

OC: optic chiasma

Five hypothalami were used in each treatment group and all experiments were repeated more than once for confirmation of results.

After the 60 min incubation period, the hypothalami were removed and the incubation medium stored at 4^{o} for $\pm$ 2 h prior to measurement of LHRH and somatostatin immunoreactivity.

2.2.4 ESTIMATION OF PROTEIN SYNTHESIS

The hypothalami were washed once to remove the radioactivity from the outside of the tissue, and homogenized in 2 ml of cold distilled water in a Potter-Elvehjem homogenizer (clearance .135 mm). 0.2 ml of this homogenate was transferred to a Wasserman tube containing 0.2 ml of cold 1M perchloric acid (PCA) and the protein precipitated by centrifugation at 2000g for 5 min at 4^{o}C. The precipitate was washed three times with cold 0.5M PCA and the supernatants thereof, pooled. Scintillant (5 ml Dimulene) was added to the precipitate dissolved in 200 µl H_2O and to the pooled supernatants, and the radioactivity was assessed (Beckman β scintillation counter). The degree of incorporation of amino acids into the hypothalamic tissue was taken as an index of protein synthesis and tissue viability. Within any treatment group non-viability was clearly indicated by an obvious discrepancy in [^{3}H]-amino acid incorporation.

2.2.5 TISSUE EXTRACTION

To determine the tissue content of LHRH and somatostatin, the remaining hypothalamic homogenate (1.8 ml) was extracted in 2N acetic acid and the suspension was centrifuged at 12500 x g for 30 min at 4^{o}C in a Sorvall RC2B centrifuge. The supernatant was decanted, lyophilized and stored under vacuum in a dessicator. Lyophilised extracts were reconstituted in phosphate buffered saline containing gelatin (0.04M phosphate, 0.15 M sodium chloride, 0.01 M disodium ethylene diaminotetra-acetic acid, 0.1% sodium azide, pH 7.0 and 0.1% gelatin (BDH)) (PBS gelatin) for assay of LHRH and somatostatin immunoreactivity.

2.2.6 ESTIMATION OF OXYGEN CONSUMPTION

Oxygen uptake by the hypothalamus was measured at 37°C with a Clarke-type electrode (YSI Model 53 oxygen monitor, Yellow Springs Instrument Company, Ohio). The hypothalami were incubated, with continuous agitation, in 4 ml Hank's medium and the O_2 uptake recorded. The medium was replaced every 15 min over a period of 3h to ensure an adequate supply of O_2 and glucose to the tissue.

O_2 uptake was calculated according to the following equation:

$$\text{Oxygen uptake: } \mu \text{ atoms/min} = \frac{\text{units/min}}{x_{100}} \times O_2 \times \frac{2 \times V}{x_{100}}$$

where units/min = slope of the curve read from the recorder chart paper which was marked 0 to 100 units.

x_{100} = x-y where x is the pen deflection when the electrode was equilibrated with air saturated buffer.

y is the pen deflection when oxygen tension in the buffer = 0mm Hg (anaerobic)

Oxygen concentration (x_{100}) in the medium at 37°C is taken as 235 μ moles/L

μ atoms O_2/ml = 0,235 x 2 = 0,470

V = volume of the incubation medium.

2.3 RADIOIMMUNOASSAY OF LHRH

The method of radioimmunoassay (RIA) used was a modification of that described by Hendricks et al (1975).

LHRH ANTISERUM

Antiserum 1076 was produced in this laboratory by conjugation of LHRH (Ayerst Laboratories) to keyhole limpet haemocyanin by carbodi-imide condensation (Hendricks et al, 1975). Since there are no free COOH groups in the LHRH molecule, condensation must take place between a NH_2 group of the decapeptide (e.g. Gly-NH_2) and a COOH on the haemocyanin molecule. The specificity of the antiserum 1076 is affected by changes in the middle of the peptide (residues Ser^4 to Leu^7) and differences in the NH_2 and COOH termini of the molecule are not affected. It therefore seems likely that the site of conjugation is at His^2, Arg^8, Gly^{10}. This antiserum (1076) showed no cross reaction with any of the neurotransmitters, hypothalamic or pituitary peptides tested except for β endorphin which was shown to interfere in the radioimmunoassay.

The antiserum 422 (gift from A.Arimura) was produced to LHRH adsorbed on polyvinylpyrrolidone. This antiserum is directed toward both the NH_2 and COOH termini and it is anticipated that it will not detect conformational changes in the middle portion of the decapeptide.

RADIOIODINATION OF LHRH

Synthetic LHRH (Ayerst Laboratories) was labelled with ^{125}I using a modification of chloramine T method of Greenwood and Hunter (1963).

LHRH ASSAY PROTOCOL

Standards were prepared in solutions identical in composition to that in which peptide release was assayed. 100µl of standard

(in double dilution from 625 - 1 pg) or 100 µl of sample was added to glass Wasserman tubes containing 200 µl PBS - gelatin buffer, followed by the addition of 100 µl of diluted antiserum with mixing (initial dilutions 1076, 1:20,000; 422,1:1,000). Gelatin was added to the buffer to lessen the number of counts adsorbing to the walls of the glass tubes. The assay was preincubated at room temperature for 20 min, 100 µl ^{125}I LHRH (~ 10,000 cpm) added and the assay incubated overnight at 4°C. Separation of free LHRH from antibody bound ^{125}I - LHRH was achieved by the addition of 750 µl dextran (0,05%) coated charcoal (Sigma Chemical Company). The assay was incubated for 15 min at 4°C, centrifuged at 1000 x g for 15 min at 4°C, the supernatant decanted and the bound ^{125}I-LHRH counted in a Beckman auto-gamma counter. A typical standard curve using antiserum 1076 is shown in Fig. 2.2. The intra-assay co-efficient of variation was 7.3% and the inter-assay co-efficient of a variation was 4.4%.

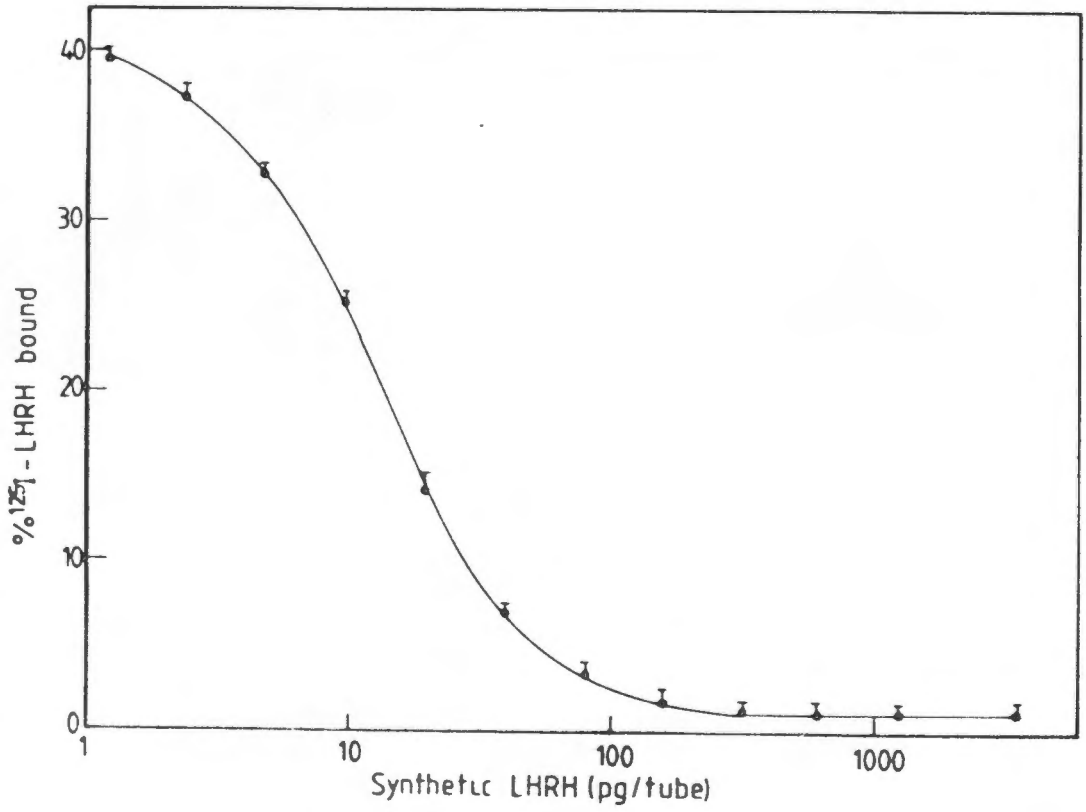


FIG 2.2

Displacement of ^{125}I-LHRH bound to antiserum 1076 by increasing amounts of synthetic LHRH. Binding of ^{125}I-LHRH to antiserum (B) is expressed as a percentage of the total ^{125}I-LHRH (T) added ($^{B}/_{T}$ x 100). Each point represents the mean of two determinations + 1S.D.

2.4 RADIOIMMUNOASSAY OF SOMATOSTATIN

A radioimmunoassay for somatostatin (based on the method of Arimura et al (1975(b)) was developed in this laboratory.

SOMATOSTATIN ANTISERUM

Antiserum 774 (gift from Arimura) was produced by immunizing sheep with cyclic somatostatin-14 conjugated to human serum gamma globulin. The antiserum showed no cross reaction (<0.01%) with any of the neurotransmitters, hypothalamic and/or pituitary peptides tested. Cross reaction with somatostatin analogues was tested and it was demonstrated that while the antiserum can tolerate changes at the N-terminal end of the peptide the cyclic conformation of the peptide is essential for antibody binding indicating that the antigenic site is directed towards the Phe^{6}-Trp^{8} portion of the molecule. Although the specificity of antiserum 774 has been demonstrated, in view of the recent findings that somatostatin-14 and a 3K immunoreactive species are released from stalk median eminence synaptosomes (Kewley,1981) it seems unlikely that only somatostatin-14 is released from the incubated hypothalamus. To embrace this possibility the term immunoreactive somatostatin has been used throughout this thesis.

RADIOIODINATION OF SOMATOSTATIN

Somatostatin does not contain a tyrosine residue and thus cannot be iodinated. Therefore, an analogue of somatostatin with tyrosine substituted for alanine at the 1 position (NH_2 terminus) was used for iodination (tyrosylated somatostatin, a gift from D.Coy) (Coy et al 1973).

Initially, radioactive somatostatin was produced using the chloramine T method of Greenwood and Hunter (1963), with purification of ^{125}I – tyrosylated somatostatin on CM -32. However, radioactive somatostatin produced by this method showed poor immunoreactivity and rapid deterioration. A solid phase lactoperoxidase method for somatostatin iodination was developed which produced a stable

radioactive somatostatin. To 5µg of tyrosylated somatostatin in 10ul of 0.01M HCL in a glass vial (0.5 x 1.5 cm) were added: 20 µl of 0.5M phosphate, 3 µl of solid phase lactoperoxidase (prepared according to Karonen et al (1975)) and 1.5mCi of ^{125}I (IMS30, Radiochemical Centre, Amersham). The reaction was initiated by the addition of 300 ng of hydrogen peroxide in 5 µl of water. After 15 min with occasional finger flicking a further 300 ng of hydrogen peroxide was added. After 30 min, 100 µl of 0.01M sodium phosphate pH 7.4 was added and the reaction mixture was centrifuged at 1000g for 3 min to precipitate the solid phase lactoperoxidase. The supernatant was applied to a CM32 cation exchange column equilibrated with 0.002M ammonium acetate, pH4.6. Iodide damaged peptide eluted with 0.2M ammonium acetate, pH 4.6 (approximately 50 mls) and iodinated somatostatin was eluted with 0.5 M ammonium acetate, pH 4.6 (Fig. 2.3). Fractions containing the main peak of ^{125}I – tyrosylated somatostatin were pooled, aliquoted into vials coated with 1.0% BSA to minimise adsorption of label on the walls of the vials and stored at $-20^{o}C$.

PROTOCOL FOR SOMATOSTATIN ASSAY

Standards were prepared in solutions identical in composition to that in which peptide release was assayed. 100 µl of standard (double dilutions from 5000-1.2 pg) or 100 µl of sample was added to Wasserman tubes containing 200 µl of PBS-gelatin buffer followed by 100 µl of Ab 774 (initial dilution 1:16000). The tubes were shaken and preincubated at room temperature for 2 hr, 100 µl of ^{125}I-tyrosylated somastatin (~10000 cpm) was added and the tubes incubated overnight at $4^{o}C$. Separation of the free ^{125}I - tyrosylated somatostatin from antibody bound ^{125}I – tyrosylated somatostin was achieved with dextran-coated charcoal as described in the LHRH assay. The bound ^{125}I – tyrosylated somatostatin was counted in a Beckman auto-gamma counter. A typical standard curve using antiserum 774 is shown in (Fig. 2.4). The intra-assay coefficient of variation was 9.2% and inter-assay coefficient of variation was 15.7%.

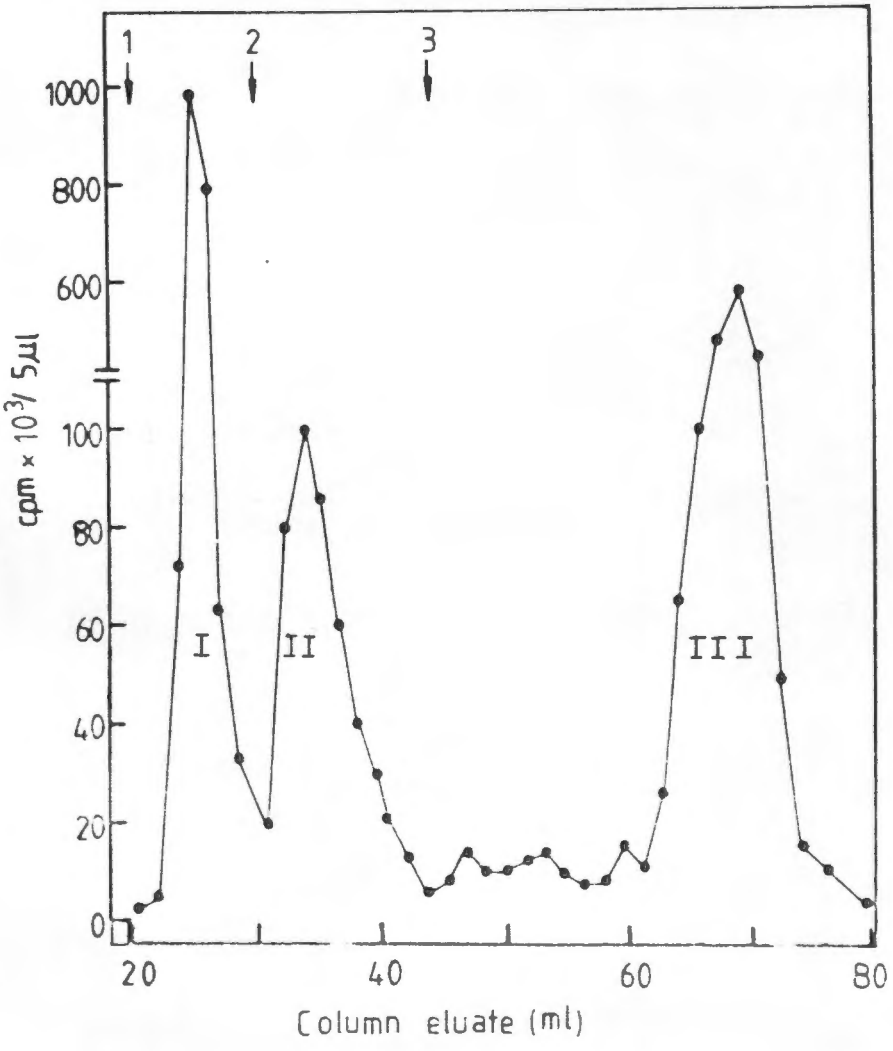


FIG 2.3

Elution profile of ^{125}I- Tyr^{1} - somatostatin - 14. Sequential elutions with 0.002 (1), 0.02 (2) and 0,5M (3) ammonium acetate buffers pH 4.6 are indicated. Peak I represents free Na^{125}I , peak II showed poor immunoreactivity and is assumed to represent intermediate products, peak III showed good immunoreactivity and represents ^{125}I- Tyr^{1} - somatostatin - 14.

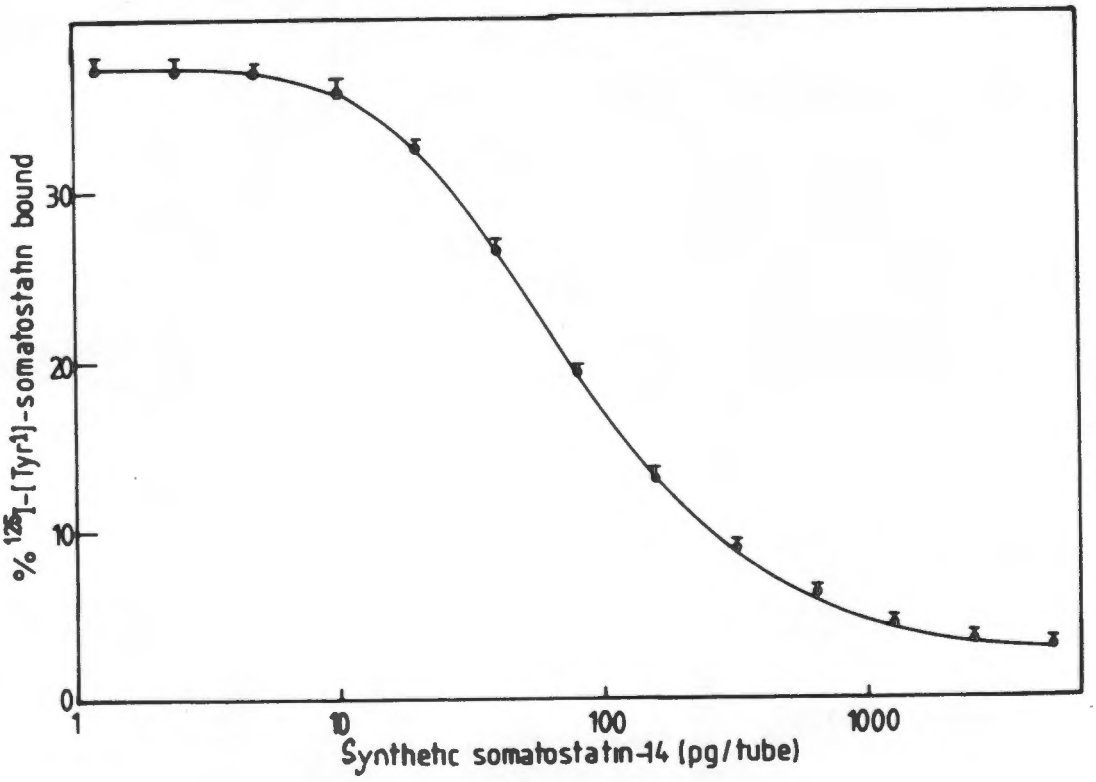


FIG 2.4

Displacement of ^{125}I - [Tyr^1] - somatostatin bound to antiserum 774 by increasing amounts of synthetic somatostatin-14. Binding of ^{125}I - Tyr^1 - somatostatin -14 (B) to antiserum is expressed as a percentage of the total ^{125}I - Tyr^1 - somatostatin-14 (T) added ($^{B}/_{T}$ x 100). Each point represents the mean of two determinations + 1 S.D.

2.5 STATISTICAL METHODS

The following statistical analyses of data were performed using a Hewett Packard HP 32E calculator:

2.5.1 Mean

$$\bar{x} = \frac{1}{n} \sum x$$

where x = the observations

$\sum$ = the sum of

n = number of observations

2.5.2 Standard deviation

$$S.D. = \sqrt{\frac{(x - \bar{x})^2}{n - 1}}$$

where $n < 30$

2.5.3 Standard error of the mean

$$S\ E\ M = \frac{S.D}{\sqrt{n}}$$

2.5.4 Coefficient of variation

$$= \frac{S.D.}{\bar{x}} \times 100$$

2.5.5 Levels of significance were calculated according to the students t-test (Finney, 1964). Values of p (probability) were determined from appropriate tables (in Giba-Geigy). When $p \geqslant 0.05$ the difference between sample and control were regarded as not significant.

2.6 COUNTING OF RADIOACTIVITY

GAMMA EMITTERS

^{125}I-radioactivity was determined in an autogamma spectrometer. A Beckman Gamma 310 system (efficiency ~75%) was used, in which samples were counted for 2 min or to a preset error of 5%.

BETA EMITTERS

[^{3}H]radioactivity was determined in a Beckman liquid scintillation system LS-233 (counting efficiency [^{3}H] ~32%). Samples were dissolved in 5 ml Dimilume - 30 (Packard) and counted for 10 min or to a preset error of 5%.

3. INVESTIGATIONS INTO THE OPTIMUM CONDITIONS FOR INCUBATION OF THE HYPOTHALAMUS IN VITRO.

INTRODUCTION

In order to study the control of release of neuropeptides from incubated rat hypothalami it was first necessary to establish conditions for the viable maintenance of isolated hypothalami in culture.

Several reports of incubation systems for isolated hypothalamic tissue have been described (Bennett et al, 1975; McKelvy et al 1976; Rotsztejn et al, 1976; Berelowitz et al, 1978; Bigdelli and Snyder, 1978; Sundberg and Knigge, 1978; Negro-Vilar et al, 1978; Sheppard et al,1979).

In these studies the conditions necessary for the maintenance of biochemically functional hypothalami in vitro were not thoroughly investigated.

We had previously observed that on prolonged incubation, LHRH release increased markedly presumably due to tissue necrosis and leakage of the peptide from neurons. It was therefore important to select an incubation period which maintained the viability of the tissue and yet allowed adequate time for test substances to take effect and for sufficient neuropeptide release to occur.

In this section an examination of the effects of medium, temperature and time on hypothalamic release of LHRH has been undertaken. A number of indices of functional viability have been employed:

1. Protein snythesis is an index of hypothalamic viability as it is dependent on a wide spectrum of biochemical pathways as well as the maintenance of subcellular integrity.

2. The ability of the hypothalamus to utilise oxygen.

3. The ability of the hypothalamus to release LHRH and somatostatin in the presence of depolarizing concentrations of K^+ (60mM).

In initial experiments, LHRH was undetectable in all incubation media used. This could be accounted for by the concomitant release of degrading peptidases known to be present in the hypothalamus (Griffiths and Kelly, 1979). Bacitracin (an antibiotic), has been shown to inhibit LHRH degradation by peptidases extracted from the hypothalamus (McKelvy et al, 1976). Thus the ability of bacitracin to prevent the breakdown of hypothalamic LHRH released into the incubation medium was tested.

3.1. METHODS

The first studies were directed at determining the most suitable medium, temperature and time for the incubations.

3.1.1. Hypothalami were dissected from rats within 1-2 mins of death as described in Chapter 2

In initial studies the hypothalami were immediately placed in Hank's Earle's or Eagle's Minimal Essential Medium (MEM) (Table 3.1). LHRH release from and LHRH content of the hypothalami were monitored at intervals over a period of 3h.

3.1.2. To determine the appropriate concentration of bacitracin which adequately inhibited LHRH degradation, hypothalami were incubated in Hank's medium for 3h at 37°C. Aliquots (100 µl) of this medium were added to tubes containing ~10 000 cpm ^{125}I- LHRH and increasing concentrations of bacitracin (10^{-7}M to 10^{-2}M). After a further incubation at 37°C for 3h the peptidases were inactivated by boiling and the amount of undegraded ^{125}I- LHRH determined by binding to excess LHRH antiserum (# 1076).

3.1.3. To establish the most suitable temperature of incubation for the system, the release of LHRH from and the LHRH content of, the isolated hypothalamus at 11°, 27° and 37°C was measured. Hypothalami were incubated in Hank's medium containing bacitracin.

TABLE 3.1 COMPOSITION OF INCUBATION MEDIA

	HANK'S	EARLE'S
	g/L	g/L
NaCL	8.00	6.80
KCL	0,40	0,40
$CaCl_2$	0,14	0.20
$MgSO_4.7H_2O$	0.10	0.10
$MgCl26H_2O$	0.10	-
$Na_2HPO_4.2H_2O$	0.06	-
$NaH_2PO_4H_2O$	-	0.125
KH_2PO_4	0.06	-
Glucose	1.00	1.00
Phenol Red	0.02	0.05
$NaHCO_3$	0.35	2.20
Gas Phase	Air	Air/CO_2
PNS	5ml/L	5ml/L
Bacitracin	$10^{-3}M$	$10^{-3}M$

EAGLE'S MEM

Amino Acids	mg/L	Vitamin	mg/L
L- Arginine HCL	126.4	D-Câ-Pantothenate	1.0
L-Cysteine	24.0	Choline Chloride	1.0
L-Glutamine	292.0	Folic Acid	1.0
L-Histidine $HCl.2H_2O$	41.9	i-inositol	2.0
L-Isoleucine	52.4	Nicotinamide	1.0
L-lysine HCl	73.1	Pyridoxal HCl	1.0
L-Methionine	14.9	Riboflavin	0.1
L-Phenylalanine	33.0	Thiamine HCl	1.0
L-Threonine	47.6		
L-Tryptophan	10.2		
L-Tyrosine	36.2		
L-Valine	46.8		

Eagle's MEM was made up in Hank's medium containing 5ml calf serum and 0.5ml PNS. The pH of Hank's and Earle's media was pH 7. Eagle's MEM was made up in Hank's medium (without $NaHCO_3$) containing 5ml calf serum and 5ml PNS The pH was adjusted to 7,6 with bicarbonate.

3.1.4. To select a suitable incubation period at 37°C, hypothalami were preincubated for 45 mins in 1 ml of Hank's medium. The hypothalami were washed twice and incubated for up to 150 min in Hank's medium containing [^{3}H] amino acid hydrolysate (Radiochemical Cente, Amersham). At 30 min intervals during the incubation, LHRH release from and amino acid uptake by hypothalamic tissue and amino acid incorporation into protein was recorded.

3.1.5. Oxygen utilisation by the hypothalamic tissue incubated in Hank's medium at 37°C was measured with a Clarke Electrode.

3.1.6. In experiments where K^+ stimulation of LHRH release was used as a criterion of functional viability, hypothalami were preincubated (see Chapter 2) and then placed in Hank's medium with and without 60 mMK^+.

The incubation was continued for 60 min and the release of LHRH into the medium and protein synthesis by the tissue was recorded.

3.2. RESULTS

3.2.1. The LHRH content of the hypothalami remained unchanged during a 3h incubation in Hank's and Earle's media (Fig 3.1). In Eagle's MEM hypothalamic LHRH content decreased to undetectable levels during the incubation period (Fig. 3.1). Consequently Hank's medium was used in all subsequent studies.

In the absence of bacitracin, release of LHRH from the isolated hypothalamus was undetectable in all of the incubation media (results not shown).

3.2.2. At a concentration of 10^{-5}M, bacitracin gave 60% inhibition of catabolic peptidase activity. Concentrations of 10^{-4}M bacitracin, and greater, gave complete inhibition of LHRH degradation (Fig. 3.2). In all subsequent experiments in which the incubation medium contained 10^{-3}M bacitracin, the released LHRH and IRS could be detected in the medium.

3.2.3. During a 3h incubation period the basal release of LHRH from hypothalami in culture was low at 11°C (25.8 ± 4.7 pg/hypoth.) and increased at 27°C (272 ± 32 pg/hypoth.) and 37°C (310 ± 52pg/hypoth.) (Fig. 3.3). In all further experiments, incubations were carried out 37°C.

3.2.4. The rate of LHRH release from and [^{3}H]amino acid uptake by the hypothalami remained constant during the first 90 min of incubation (± 47pg LHRH/h and 600000 cpm respectively). However protein synthesis declined after 60 min (Fig. 3.4). During the final 60 min of incubation, loss of cell viability and peptide leakage was suggested by the reduction in both protein sythesis and amino acid uptake and the large increase in LHRH release. In view of these findings in all subsequent experiments the incubation period was limited to 60 min following a 45 min preincubation period.

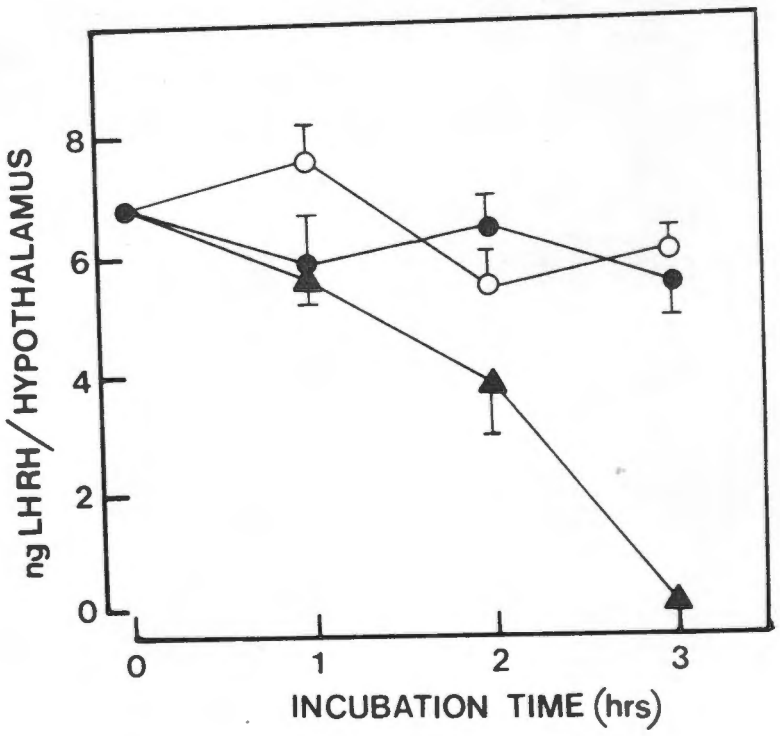


FIG 3.1

Effect of incubation medium on hypothalamic LHRH content. Hypothalami were incubated for 3 h in Hank's (●——●), Earle's (o——o) and Eagle's minimal essential medium (MEM) (▲——▲) LHRH content was significantly less in MEM at 2h ($p < 0.05$) and 3h ($p < 0.0001$). Values are means ± SEM, n=4.

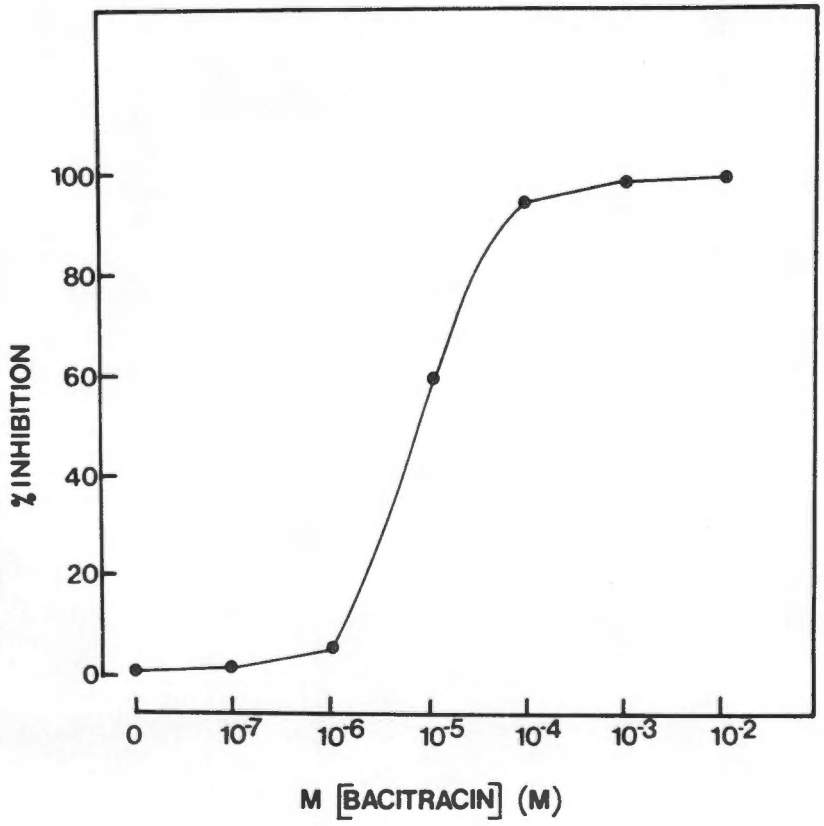


FIG 3.2

Inhibition of degradation of exogenous LHRH by bacitracin.

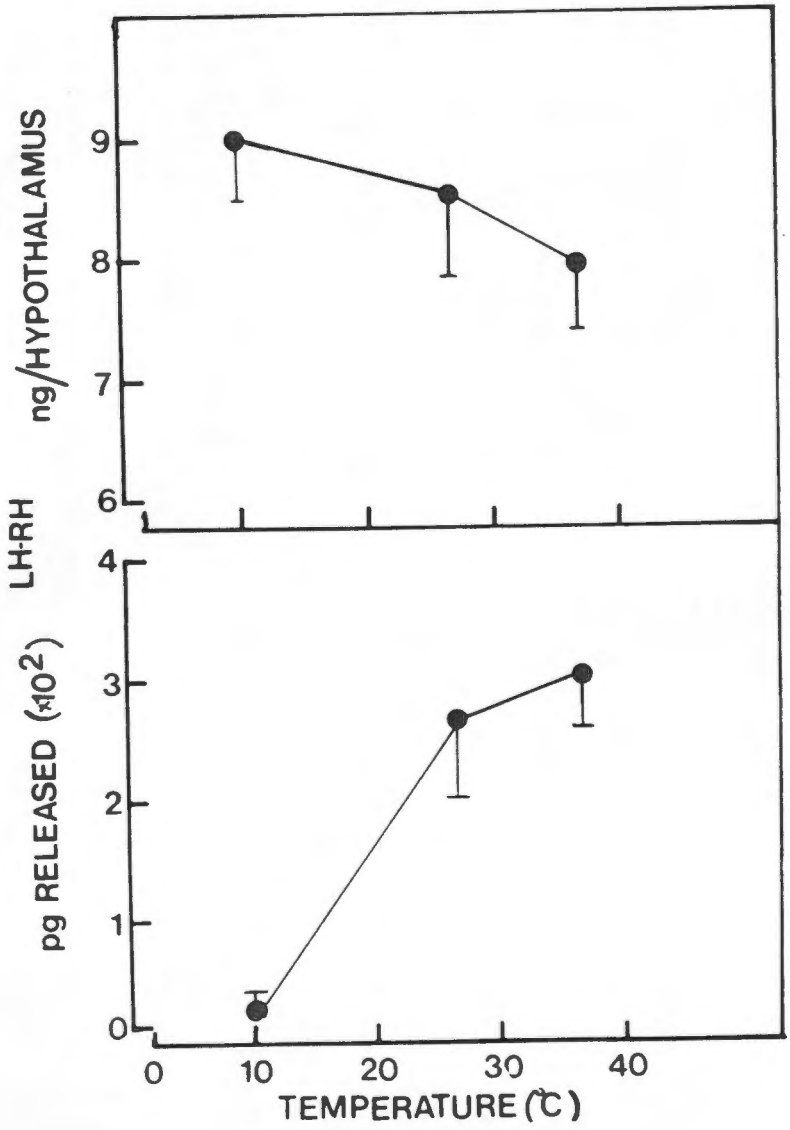


FIG 3.3

Effect of temperature on LHRH release (pg) and LHRH content (ng) in the rat hypothalamus.

Hypothalami were incubated in Hank's medium for 3h. Values are means ± SEM, n=4.

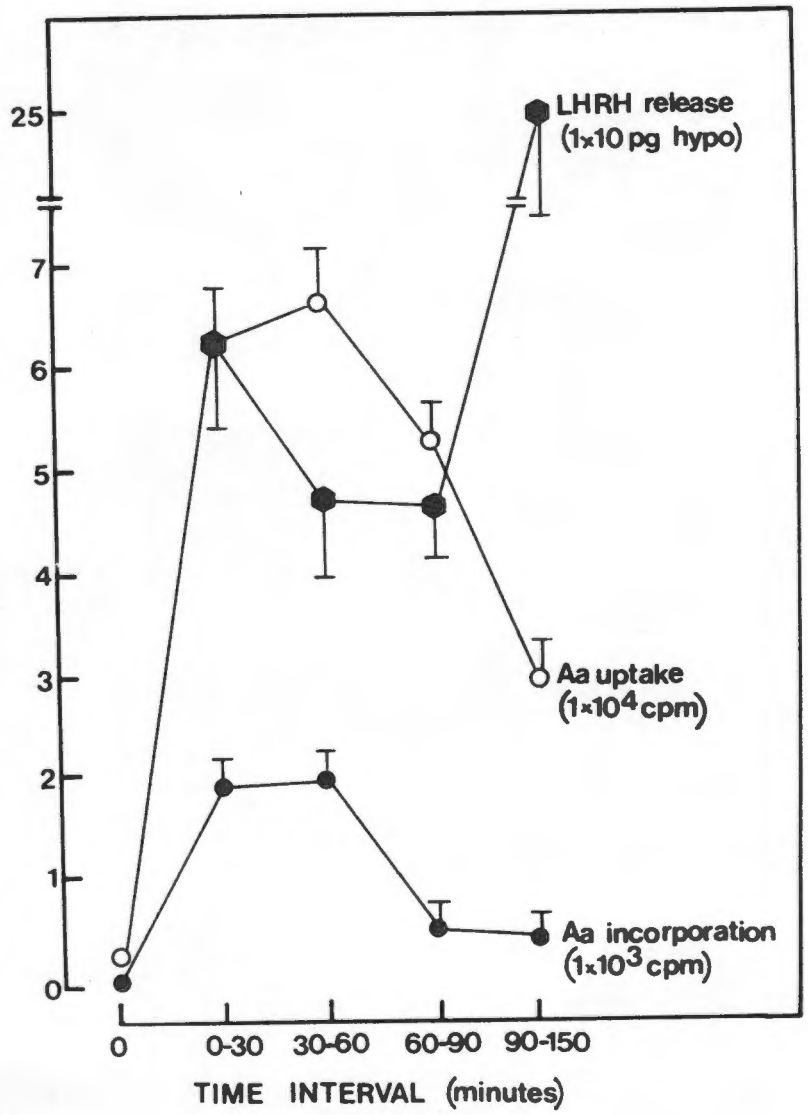


FIG 3.4

Effect of time on LHRH release from and amino acid incorporation and uptake into, incubated hypothalami.

3.2.5 The rate of oxygen consumption stabilised during the preincubation period and remained constant for the duration of the 60 min incubation period (Fig 3.5). Thereafter oxygen consumption declined concomitantly with the decline in protein synthesis and increased LHRH release.

The third criterion of viability was the K^+-stimulated release of LHRH and IRS from hypothalami. The Ca^{2+} dependence of the K^+- stimulated release of both neuropeptides was demonstrated (See Results: Chapter 4)

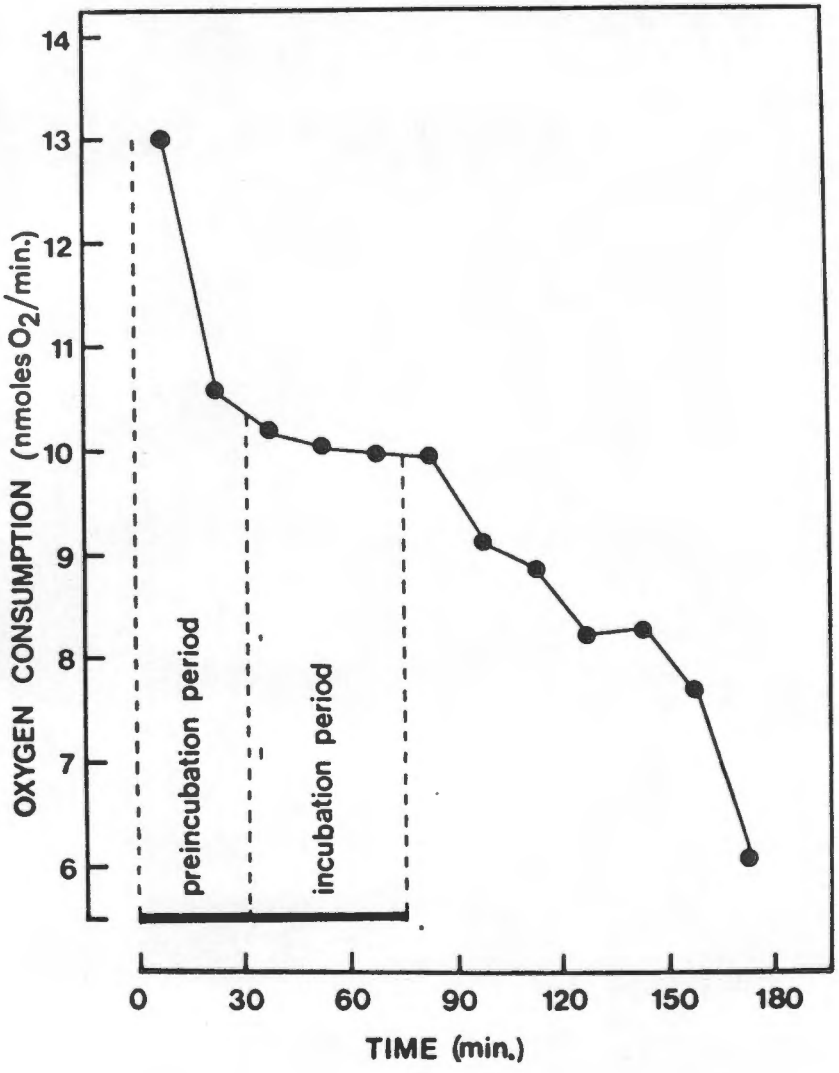


FIG 3.5

Hypothalamic oxygen consumption in Hank's medium. Hypothalami were incubated in Hank's medium (4 ml) at 37°C. The incubation medium was replaced at 15min intervals.

3.3 DISCUSSION

In the present study, conditions were established for the maintenance of isolated rat hypothalami *in vitro* for a period of 60 min. Protein synthesis, amino acid uptake, oxygen consumption, low basal release of neuropeptide and the ability to K^+-stimulate neuropeptide release from the hypothalami during this period were taken as indices of the functional viability of the system.

However, other studies of release from isolated hypothalami have not established optimal conditions for viability. It has been reported that fragments of hypothalamus remained viable for 7-13 days *in vitro* under carefully controlled conditions (Hirooka et al, 1978). Bradbury et al, (1974) has found that during an incubation period of 3 h the incubated hypothalami showed considerable metabolic activity and maintained a reasonable electrolyte balance. However there was evidence of histological damage to the tissue at the end of this incubation period. This agrees with the present observation of a decrease in protein synthesis, oxygen consumption and amino acid uptake and a great increase in LHRH release after incubation for periods longer than 60-90 min.

The reason for the inability to detect any LHRH in the hypothalami incubated in Eagle's MEM is not known. Degradation of the neuropeptide within the intact subcellular compartments was not expected. Hypothalami incubated in Hank's medium or Earle's medium were able to maintain the hypothalamic LHRH content during the incubation period.

Release of LHRH from incubated hypothalami was found to be a temperature dependent process. Moreover, the K^+- stimulated release of the neuropeptides was Ca^{2+} dependent. This is taken as indirect evidence that the basic functional properties of the hypothalamus are preserved under the present experimental conditions.

In the absence of bacitracin, LHRH release could not be demonstrated. This could be due to:

(1) degradation by peptidases which are known to be in the hypothalamus and may be secreted concomitantly with the neuropeptide.

(2) reuptake of the neuropeptide.

(3) inhibition of release of the neuropeptide by released neurotransmitters and the same or other neuropeptides.

(4) no release occurring.

Bacitracin inhibited the degradation of ^{125}I -LHRH at $10^{-4}M$ and greater concentrations. Although the inhibition of degradation of non-iodinated LHRH was not studied, the ^{125}I -LHRH was prepared by a method shown to produce biologically active peptide (Marshall and Odell, 1975). Moreover, after incubating hypothalami in medium containing $10^{-4}M$ bacitracin, LHRH release could be demonstrated. This suggests that bacitracin acts by inhibiting peptidase degradation of LHRH rather that bacitracin itself stimulating LHRH release from the hypothalamus in a non-specific manner. Surprisingly in other studies in which bacitracin was not included in the incubation medium (Rudenstein et al, 1979) LHRH release was apparently even higher than in the present study. These high estimates may, in part, be due to peptidase degradation of ^{125}I -LHRH in the radioimmunoassay, thus causing a reduced binding to antibody and a consequent overestimate of LHRH release.

In all incubations hypothalamic LHRH content remained unchanged, suggesting that although protein synthesis occurs, no net detectable biosynthesis of LHRH is taking place. These observations contradict those of Sundberg and Knigge, (1978). who used a similar incubation system but noted an increase in hypothalamic LHRH content. These differences could have been due to the specificity of the antisera employed in the radioimmunoassays and their relative interaction with LHRH, LHRH fragments and putative prohormonal LHRH. Antiserum 1076, used in this study, requires residues Ser^4 to Leu^7 of LHRH for effective binding and recognises both putative prohormonal LHRH and decapeptide LHRH (Millar et al, 1977). Thus a conversion of prohormonal LHRH to LHRH might not show much increase in immunoreactivity using antiserum 1076. If the antiserum used by Sundberg and Knigge was specific for decapeptide LHRH, the net increase in immunoreactivity might reflect conversion of prohormonal LHRH to LHRH without necessitating de novo biosynthesis. To resolve this issue,

we reassayed the LHRH content of incubated hypothalami using antiserum 422 which interacts poorly with prohormonal LHRH since it requires both N and C terminus of LHRH for binding (see Chapter 2). Again no net increase in LHRH immunoreactivity was noted (results not shown).

In view of the present findings in subsequent experiments hypothalami were first preincubated for 45 min and then incubated for 60 min in Hank's medium containing 10^{-3}M bacitracin, in a 95% O_2, 5% CO_2 atmosphere at 37^{o}C.

4. THE Ca^{2+} REQUIREMENTS OF LHRH AND IRS RELEASE FROM THE INCUBATED RAT HYPOTHALAMUS.

INTRODUCTION

High concentrations of K^+ depolarize excitable plasma membranes and lead to the secretion of neurotransmitters (McIlwain, 1952) (Jones and McIlwain, 1979). The secretory process is dependent on the extracellular calcium, which apparently enters the nerve terminus after the depolarising event (Blaustein et al, 1977). The present section describes studies on the release of LHRH and IRS from incubated rat hypothalami in the presence of depolarising agents and the role of Ca^{2+} in the secretory event.

Depolarization of peptidergic nerve terminals is thought to result in an increase in the passive permeability to Na^+ prior to or in conjunction with an increase in the Ca^{2+} entry. (Thorn et al, 1978). Na^+ is required for the generation and propagation of action potentials but it is not essential for the release process in vitro (Dreifuss et al, 1971 ; Dreifuss, 1975; Hartter and Ramirez, 1980). The release of hormones can occur in Na^+ free solutions and in the presence of tetrodotoxin which blocks Na^+ channels (Douglas, 1964).

In the presence of 60mM KCL, K^+ ions flow freely through the membrane and neutralise the intracellular net negative charges thus stimulating the depolarization event. A calcium dependent, K^+- stimulated release of LHRH and IRS from hypothalami has been shown (Rotsztejn et al, 1976; 1977, 1978; Berelowitz et al, 1978; Bigdeli and Snyder, 1978; Negro-Vilar et al, 1978, 1979; Sheppard et al, 1979; Drouva et al, 1980; Maeda and Frohman, 1980; Richardson et al, 1980; Hartter and Ramirez, 1980).

Ouabain has been used as an alternative method of inducing membrane depolarization (Bigdeli and Snyder, 1978; Hartter and Ramirez, 1980). This may provide a more physiological approach to investigate depolarization as it is effective without the addition of non-physiological concentrations of K^+.

To investigate the role of Ca^{2+} in the neurosecretion of peptides, the effect of the carboxylic ionophores X537A and A23187 on the release of LHRH and IRS from incubated hypothalami was studied. In addition to their known depolarizing action both ionophores transfer Ca^{2+} across the cell membrane (Cochrane and Douglas, 1975). The less specific Ca^{2+} ionophore, X537A, is a more effective depolarizing agent due to its ability to transfer monovalent cations, particularly Na^{+} and K^{+}, across the cell membrane down their concentration gradients.

Verapamil specifically blocks Ca^{2+} channels (Malaisse et al, 1977). Thus, incubation of the isolated hypothalamus with this substance, in the presence of either A23187 or X537A may assist in differentiating the direct role of Ca^{2+} from that of a depolarizing effect of cations in ionophore-induced, neuropeptide release. In the present investigations K^{+}, ouabain and the ionophores X537A and A23187 were used to induce Ca^{2+} release of neuropeptides from the hypothalamus. The calcium requirements for and the effect of verapamil on the induced release of LHRH and IRS was studied.

4.1. METHODS

Hypothalami, dissected as previously described, were preincubated in 1 ml of Hank's medium for 45 min. Thereafter they were incubated in 0.5 ml of Hank's medium for 60 min. All test substances were added at the beginning of the 60 min incubation period. In depolarization experiments 60 mM KCL or 10 µM ouabain was present in the incubation medium. In other experiments Ca^{2+} was omitted from the medium and 0.5mM EGTA added to chelate any free Ca^{2+}. The ionophore X537A or A23187 was present at a concentration of 1µM or 10µM. When present, the concentration of verapamil was 20µM. At the end of the incubation period the release of LHRH and IRS into the medium was assayed and uptake and incorporation of [^{3}H]· amino acid hydrolysate by the hypothalamic tissue were monitored (See Chapter 2).

4.2 RESULTS

In the presence of Ca^{2+}, addition of 60 mM KCL or 10 µM ouabain to the incubation medium stimulated LHRH and IRS release from the incubated hypothalami (Fig. 4.1, 4.2). However, in the absence of Ca^{2+} (Ca^{2+}-free medium and 0.5 mM EGTA), 60mM KCL failed to stimulate the release of LHRH and IRS from the incubated rat hypothalami (Fig. 4.1, 4.2). Basal release of both neuropeptides was also decreased in the absence of Ca^{2+}. Verapamil (20 µM) completely inhibited K^{+}-stimulation of LHRH release but only partly inhibited IRS release (Fig. 4.1, 4.2).

The ionophores X537A and A23187 were added to the incubation medium at a final concentration of 1µM since higher levels (10µM) inhibited protein synthesis (results not shown). Ionophore X537A increased LHRH and IRS release from incubated rat hypothalami (Fig. 4.3, 4.4).

This ionophore-induced release of LHRH was completely blocked by verapamil while IRS release was only partially reduced (Fig. 4.3, 4.4). Ionophore A23187 reduced LHRH release under all conditions (Fig. 4.3). IRS release was enhanced in the presence of this ionophore and Ca^{2+}. This effect was not blocked by verapamil (Fig. 4.4). However A23187 did not induce IRS release from hypothalami incubated in Ca^{2+} free medium.

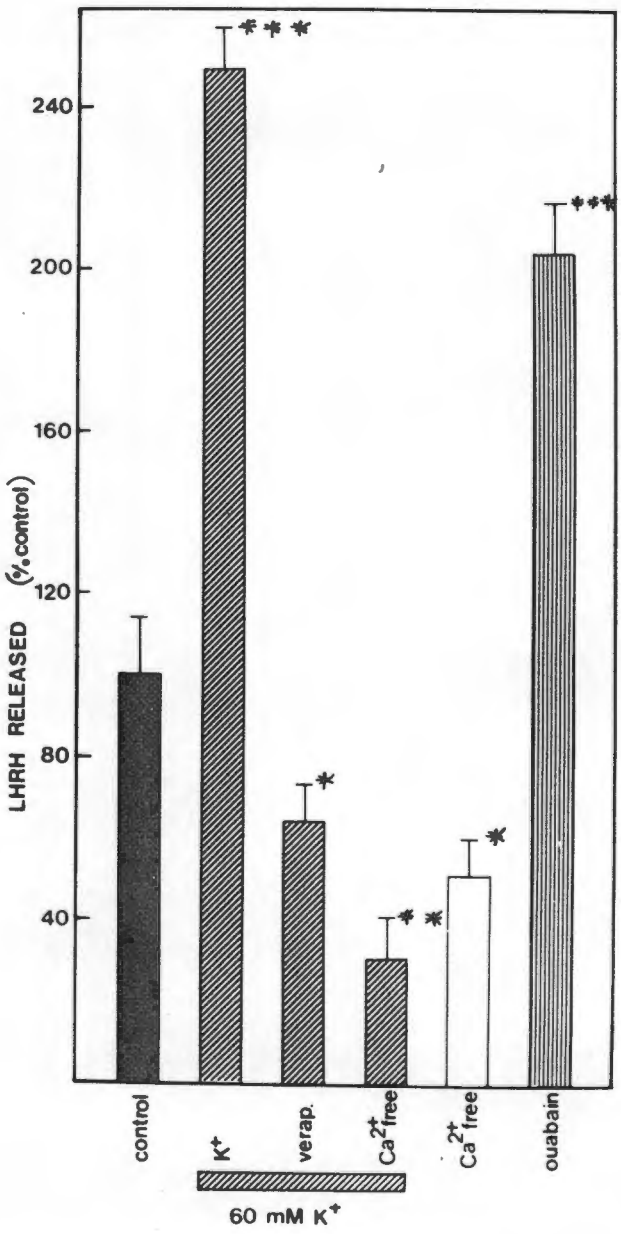


FIG 4.1

Ca^{2+} requirements for the K^+-stimulated release of LHRH from rat hypothalami in vitro.

Values are means ± SEM (n=5) expressed as a percentage of control values with no additions.

Ca^{2+} free: calcium free Hank's with 0.5 mM EGTA.

Significant differences: * $p < 0.1$, * * $p < 0.025$, * * * $p < 0.0005$.

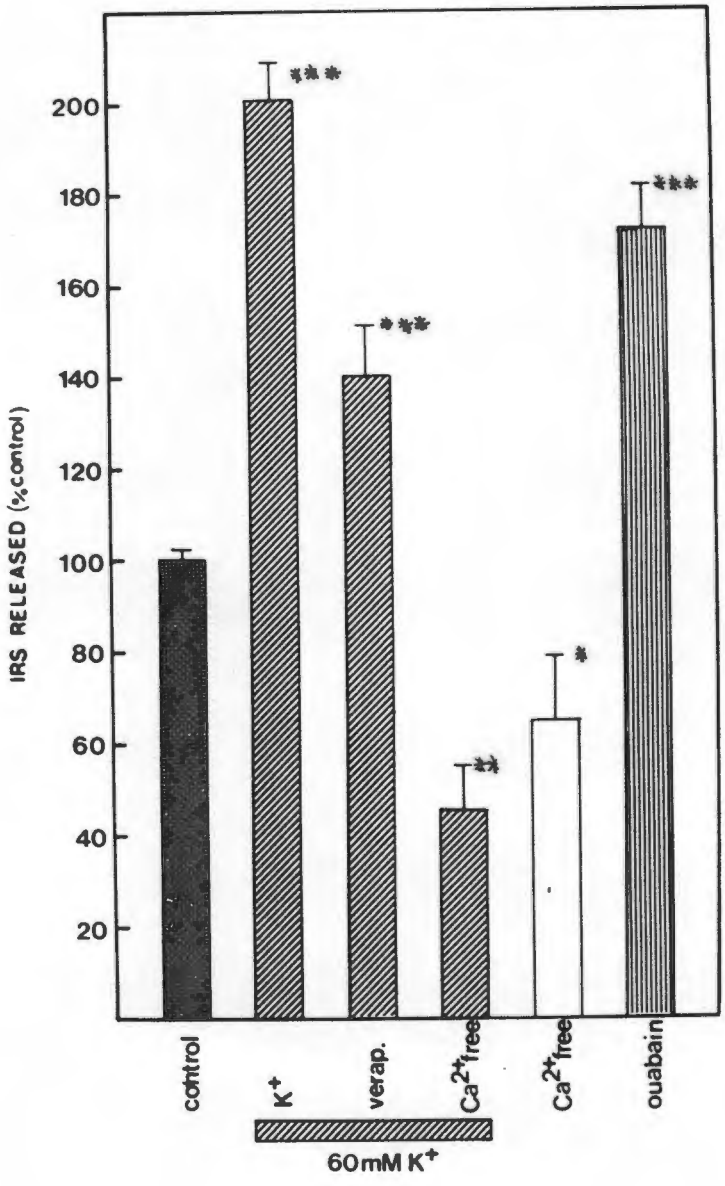


FIG 4.2

Ca^{2+} requirements for the K^{+}-stimulated release of IRS from rat hypothalami in vitro .

Values are means ± S.E.M. (n=5) expressed as a percentage of control values with no additions.
Ca^{2+} free: calcium free Hank's with 0.5mM EGTA.
Significant differences : * $p < 0.1$, ** $p < 0.025$, *** $p < 0.0005$.

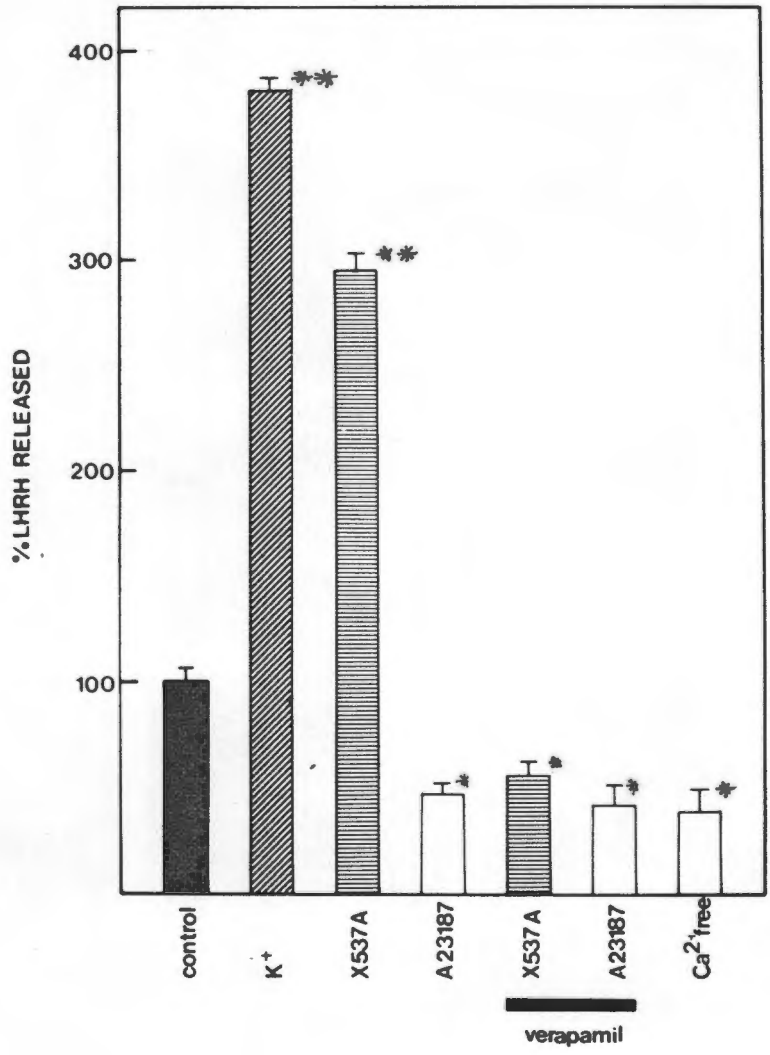


FIG 4.3

The effect of ionophores X537A and A23187 on LHRH release from rat hypothalami in vitro .

Values are means ± SEM (n=5). Ca^{2+} free : calcium free Hank's with 0.5 mM EGTA.

Significant differences * $p < 0.025$, ** $p < 0.0005$.

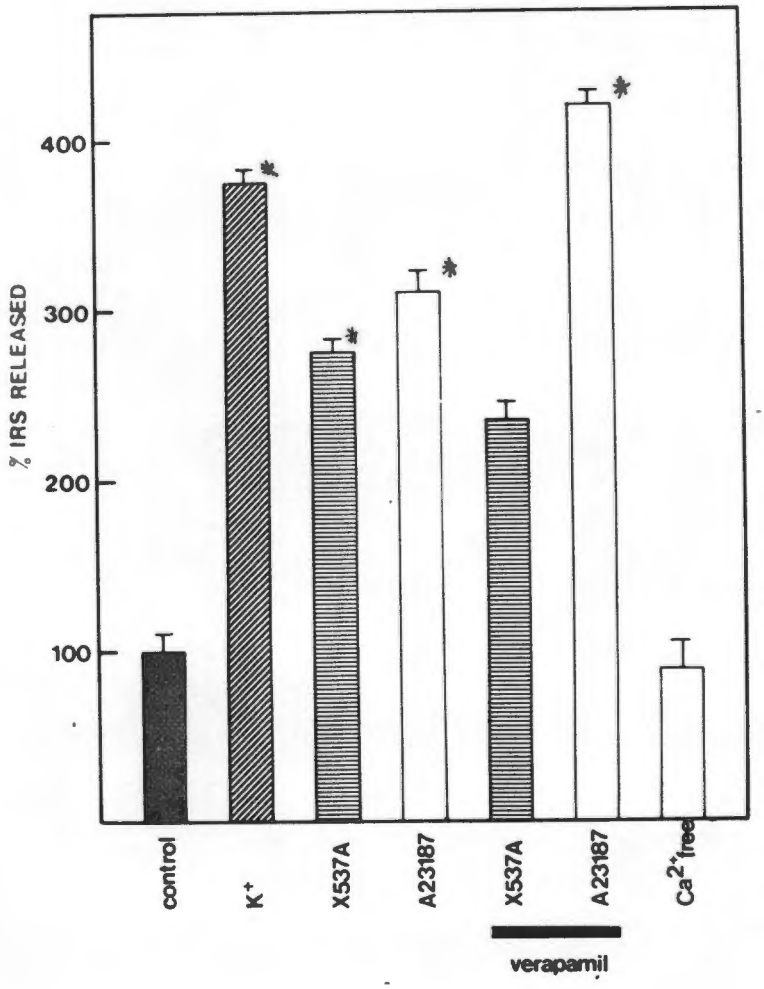


FIG 4.4

Effect of ionophores X537A and A23187 on IRS release from rat hypothalami in vitro.

Values are means ± SEM (n=5). Ca^{2+} free: calcium free Hank's with 0.5mM EGTA.
Significant differences. * $p < 0.0005$.

4.3. DISCUSSION

A K^+ (60mM) - stimulated release of IRS was consistently demonstrated. It was not always possible to produce K^+-stimulated release of LHRH from the incubated hypothalamus. However, when K^+-stimulated release of both hormones was demonstrated it was invariably greater than that shown with ouabain. This is in agreement with the results of Bigdeli and Snyder, (1978).

In the absence of Ca^{2+} (Ca^{2+}-free medium and 0.5 mM EGTA) K^+-stimulation was not effective and there was a decrease in the basal release of both hormones. Sheppard (1980) demonstrated that the addition of 0.5mM EGTA to the medium decreased basal IRS release and prevented K^+-stimulated release of this neuropeptide. However, as the author still retained 2.5mM Ca^{2+} in the medium, this would mean that 1 5mM free Ca^{2+} was still present. Since this concentration is similar to the physiological concentrations of free Ca^{2+}, these findings are somewhat perplexing.

The present results suggest that there are differences in the Ca^{2+}-dependence of the release of the two peptides. Verapamil completely inhibited the K^+-stimulated release of LHRH but only partially inhibited IRS release. This suggests that IRS release is, in part, independent of Ca^{2+} entry into the nerve terminal or that different Ca^{2+} channels are involved which are insensitive to verapamil. The results clearly point to differences in the release mechanism for the two neuropeptides. Differential release of LHRH and IRS has been replicated in synaptosomes (Kewley, 1981).

In the presence of 1µm ionophore the viability of the incubated hypothalamus was maintained as indicated by protein synthesis and $[^3H]$-amino acid uptake.

The release of LHRH from the incubated rat hypothalamus induced by X537A may be analagous to that produced by depolarization. The inhibition of this release by verapamil implicates the involvement of extracellular calcium which enter the nerve terminal in the presence of ionophore.

A23187 was unable to stimulate LHRH release. It is possible that:

(1) the two ionophores differ in their mode of action at the nerve terminal.

(2) A23187 may promote Mg^{2+} entry (Reed and Lardy, 1972). Mg^{2+} is known to inhibit the neurosecretory process (Thorn et al, 1978).

(3) the Ca^{2+} influx induced by A23187 may be a continuous rather than a transient effect. This could resemble a pathological condition in which there is disruption of the ion permeability barriers (Schlaepfer, 1977).

(4) A23187 may enter the peptide neuron and mobilise intracellular Ca^{2+} (Brown et al, 1980).

Differences in the mechanism of action of the two ionophores is exemplified by the IRS results. Verapamil did not inhibit X537A-induced release of IRS and in fact, enhanced A23187-induced release. However, there was inhibition of IRS release in Ca^{2+}-free medium in the presence of A23187. This implies that extracellular Ca^{2+} is essential for the ionophore-induced release. The ionophore may, nevertheless, induce the formation of Ca^{2+} channels which are insensitive to the action of verapamil.

The differential effects of K^{+}-stimulation, the ionophores X537A and A23187 and verapamil, on the release of LHRH and IRS from the incubated rat hypothalamus point to differences in the Ca^{2+} requirements for the release of the two neuropeptides.

5. THE ROLE OF PROTEIN SYNTHESIS, PROTEOLYTIC ENZYMES AND THE MICROTUBULAR AND MICROFILAMENTOUS SYSTEM IN THE RELEASE OF LHRH FROM THE INCUBATED RAT HYPOTHALAMUS.

INTRODUCTION

In the classical model of neuronal peptide biosynthesis and secretion it has been postulated that the peptide is first synthesized as a precursor molecule via ribosomal mechanisms in the cell body and then packaged into secretory granules where post-translational cleavage to the peptide products occur. The secretory granule is transported down the axon and is stored at the nerve terminal prior to release (Gainer and Brownstein, 1978). It is debatable whether the microtubular and microfilamentous systems play a role in neuropeptide release. There is evidence that colchicine, vinblastine and vincristine do not play a direct or functionally important role in release (Rufener et al, 1972; Russell and Thorn, 1973). Cytochalasin B, however, has been shown to decrease release of hormones (Douglas and Sorimachi, 1972), but since it is also known to interfere with glucose uptake and utilisation it is thus debatable whether its action involves microfilaments in the release process or whether exocytosis is dependent on glycolysis in the conditions studied (Dreifuss, 1975). Nevertheless, there are reports that colchicine, vinblastine and vincristine decrease release of several hormones by interfering with the microtubule system (Williams and Wolff, 1971; Labrie et al, 1973).

In order to determine whether LHRH has similar biochemical and subcellular processing, the effects of inhibitors of protein synthesis, proteolytic cleaving enzymes and the microtubular and microfilamentous system on its release were investigated.

5.1. METHODS

These experiments were conducted before optimization of the system. Rats were decapitated and the hypothalami were rapidly dissected as previously described. The hypothalami were incubated for 3h at 37°C in Hank's medium containing either [^{3}H]-amino acid hydrolysate as an index of protein synthesis or [^{3}H]-uridine as an index of RNA synthesis. To differentiate the effect of inhibiting protein synthesis at either the transcription or translation stage, the incubations were carried out in the presence of actinomycin D and cycloheximide (200 µg/ml or 1000 µg/ml). To decrease protein synthesis indirectly, potassium cyanide (5mM) and sodium azide (5mM) were added to the medium.

The trypsin inhibitor, N-α-tosyl-L-lysyl chloromethane (TLCM) and the chymotrypsin inhibitor N-α-tosyl-phenylalanyl chloromethane (TPCM), were added at a concentration of 200 µg/ml.

In a separate study inhibitors of the microtubular and microfilamentous system, colchicine (100 µg/ml), vinblastine (100 µg/ml), vincristine (100 µg/ml) or cytochalasin B (100 µg/ml) were added to the medium.

In each experiment the release of LHRH into the medium, the LHRH hypothalamic content and the uptake and incorporation of [^{3}H] amino acid hydrolysate or [^{3}H] uridine by the hypothalamic tissue were determined, at the end of the incubation period. (See Chapter 2).

5.2 RESULTS

Cycloheximide and Actinomycin D decreased protein synthesis of the incubated rat hypothalamus. Actinomycin D caused a greater decrease and was effective at lower concentrations (Table 5.1). RNA synthesis, as monitored by incorporation of [^{3}H]-uridine into PCA precipitable RNA was surprisingly impaired with both treatments (Goddard, 1978). LHRH release from the incubated hypothalamus was decreased by inhibitors of protein synthesis in a dose dependent manner (Table 5.1).

Moreover, there was a close correlation between the degree of inhibition of protein synthesis and the degree of decrease in basal release of LHRH. The hypothalamiccontent of LHRH and the uptake of [^{3}H]-amino acid hydrolysate (Table 5.1)and [^{3}H]-uridine (Goddard, 1978.) were unaffected by inhibitors of protein synthesis. KCN and NaN_3 decreased protein synthesis concomitant with a marked increase in LHRH release from the hypothalami.TLCM did not effect the release or hypothalamic content of LHRH. TPCM was also without effect (Table 5.2).However both inhibitors caused a marked decrease in protein synthesis in the tissue but neither affected the uptake of the [^{3}H]-amino acid hydrolysate by the hypothalamic tissue (Table 5.2).

None of the tested inhibitors of the cytoskeletal system inhibited LHRH release (Table 5.3)

TABLE 5.1

Effect of Inhibitors of Protein Synthesis on LHRH Release from the rat hypothalamus in vitro

Additions	LHRH release (%)	LHRH hypothalamic content (%)	Incorporation PCA ppt. (%)	Uptake PCA spn. (%)
Nil	100 ± 7	100 ± 8	100 ± 6	100 ± 7
Actinomycin D (200 µg/ml)	56 ± 15 x	93 ± 6	42 ± 10 xx	94 ± 0.1
Actinomycin D (1,000 µg/ml)	42 ± 14 xx	96 ± 6	42 ± 8 xx	97 ± 2
Cycloheximide (200 µg/ml)	98 ± 13	94 ± 3	81 ± 8	101 ± 8
Cycloheximide (1,000 µg/ml)	65 ± 10 x	93 ± 6	55 ± 7 x	95 ± 9

Values are means + SEM (n = 4)

x $p < 0.005$ xx $p < 0.0001$. The remaining data were not significantly different from control release results ($p > 0.05$).

TABLE 5.2

Effect of Protease Inhibitors on LHRH Release from the rat hypothalamus in vitro

Additions	LHRH release (%)	LHRH hypothalamic content. (%)	Incorporation PCA ppt. (%)	Uptake PCA spn. (%)
Nil	100 ± 17	100 ± 6	100 ± 8	100 ± 8
TLCM (200 µg/ml)	95 ± 21	86 ± 8	15 ± 10 xx	92 ± 3
TPCM (200 µg/ml)	91 ± 18	137 ± 5	18 ± 9 xx	95 ± 4

Values are means ± SEM . (n = 4)

xx $p < 0.0001$. The remaining data were not significantly different from control release results ($p > 0.05$).

TABLE 5.3

Effect of Inhibitors of Microtubule and Microfilament Contraction on LHRH Release from the rat hypothalamus *in vitro*

Additions	LHRH release (%)	LHRH hypothalamic content (%)	Incorporation PCA ppt (%)	Uptake PCA spn. (%)
Nil	100 ± 5	100 ± 14	100 ± 12	100 ± 6
Colchicine (100 µg/ml)	109 ± 2	80 ± 6	108 ± 13	108 ± 2
Vinblastine (100 µg/ml)	113 ± 9	114 ± 8	62 ± 10 *	103 ± 2
Vincristine (100 µg/ml)	95 ± 6	105 ± 13	102 ± 7	111 ± 5
Cytochalasin B (100 µg/ml)	106 ± 2	115 ± 9	92 ± 9	99 ± 3

Values are means ± SEM. (n = 4)

* $p<0.005$. The remaining data were not significantly different from control release results ($p>0.05$).

5.3. DISCUSSION

In the presence of inhibitors of protein synthesis there was a decrease in LHRH release from the incubated hypothalamus. The hypothalamic LHRH content was unchanged. Thus, it appears that LHRH destined for secretion is either synthesized de novo or the neurosecretory process itself is directly or indirectly dependent on the synthesis of proteins. When protein synthesis was inhibited indirectly by inhibition of oxidative phosphorylation LHRH release increased markedly. This suggests loss of cellular integrity with the consequent release of cellular hormone content.

The failure of typsin/chymotrypsin inhibitors to effect secretion of LHRH would appear at first sight to argue against the postulate that putative prohormonal LHRH is cleared to LHRH by a tryptic-like enzyme (Millar et al, 1978). Clearly the chemicals entered the cell as protein synthesis was impaired. However, trypsin inhibitors have also been shown to be ineffective in inhibiting conversion of proinsulin (Steiner et al, 1967) and pro PTH Kemper et al, 1972) by endogenous tryptic-like enzymes. This suggests that the specificity of these enzymes are different from that of trypsin. Alternatively, the inhibitors may not enter the secretory granules in which cleavage occurs. It is also possible that large stores of decapeptide LHRH are present in the hypothalamus and that inhibition of cleavage of newly synthesized prohormone will have little effect on the amount released (Fink, 1979). Millar (personal communication) has demonstrated that prohormonal LHRH in sheep and rat hypothalamus constitutes less than 1% of the total LHRH.

Vincristine, vinblastine and cytochalasin B did not inhibit the release of LHRH from the incubated hypothalami. This suggests that release is not dependent on the microfilament/microtubule system which is sensitive to these drugs, or that the rate of complex formation between the tubulin and drugs is slow and is longer than the incubation period (Wilson et al, 1974).

6. THE EFFECT OF NEUROTRANSMITTERS ON LHRH AND IRS RELEASE FROM THE INCUBATED RAT HYPOTHALAMUS.

INTRODUCTION

The abundance of neurotransmitters in the ME and other hypothalamic regions has been well documented and numerous physiological studies have implicated them in the regulation of anterior pituitory function (Müller et al, 1977). In this chapter only the regulation of LH/FSH and GH secretion by neurotransmitters will be discussed. The central noradrenergic systems exerts an important stimulatory effect on LH secretion (Krulich, 1979). The serotoninergic system is generally thought to inhbit LH secretion while the role of the dopaminergic system remains controversial although it does influence LH secretion (Mueller et al, 1976; Drouva and Gallo, 1977; Vale et al, 1977; Krulich, 1979). The central noradrenergic, serotoninergic and dopaminergic systems all appear to have a stimulatory role in GH secretion in primates (Krulich, 1979). However, some controversy exists as to the exact physiological role of dopamine in rat GH secretion (Vijayan et al, 1978). No evidence is available to implicate a direct effect of biogenic amines on pitutary LH/FSH or GH secreting cells. Thus, many of the neurotransmitter effects on pituitary secretion may be mediated via their influences on the secretory activity of the hypothalamic peptidergic neurones.

Noradrenalin, dopamine, acetylcholine and serotonin have been localised to nerve terminals in the stalk median eminence (Müller et al, 1977). Somatostatin and LHRH are also present in nerve endings close to the capillary loops of the hypophyseal portal system (Hökfelt et al, 1974). Axo-axonic contact of LHRH and dopamine containing nerve terminals has been demonstrated in close proximity to the portal vessels (Ajika, 1979). This morphological evidence further supports the concept of synaptic influence of neurotransmitters on the release of hypothalamic neuropeptides which regulate pituitary hormone secretion.

Direct experimental evidence of neurotransmitter mediated release of LHRH and somatostatin from the hypothalamus has been derived from in vitro experiments. A dopamine-induced release of LHRH from hypothalamic nerve terminals (synaptosomes) has been reported by Bennett et al (1975). Subsequently dopamine- and noradrenalin-induced release of LHRH from hypothalamic fragments and the isolated

hypothalamus was demonstrated (Rotsztejn et al, 1976, 1977; Negro-Vilar et al, 1978, 1979; Ojeda et al, 1979). The effect of the catecholamines on LHRH release is apparently steroid dependent since it was only observed on ME fragments from intact and not castrated rats. Thus the effect of a neurotransmitter could be modified by the sex, endocrine status and age of the experimental animal. In addition the dopamine and noradrenalin release of LHRH from the ME could not be demonstrated from hypothalamic fragments which included the arcuate and ventromedial nuclei (Negro-Vilar et al, 1979). This suggests that the release of LHRH from ME terminals is tonically inhibited by neurones with cell bodies located in the MBH. Such recurrent inhibitory or facilitatory neural circuits may play an important role in the modulation of LHRH release into the portal vessels.

Dopamine and noradrenalin have been reported to stimulate somatostatin release from hypothalamic tissue in vitro (Negro-Vilar et al, 1978; Maeda and Frohman, 1980). Somatostatin release from rat hypothalamic synaptosomes was stimulated by dopamine (10^{-6}M) (Wakabayashi et al, 1977). However, Bennett et al (1979) showed that dopamine, noradrenalin and serotonin did not increase somatostatin release from rat hypothalamic synaptosomes. The stimulatory effects of dopamine and noradrenalin on somatostatin release are difficult to align with their effects in vivo. However, it is possible that there is a simultaneous stimulation of GRF release which overrides the inhibitory effect of somatostatin on GH release.

To try to elucidate the effects of neurotransmitters on the release of LHRH and somatostatin from rat hypothalami, the following experiments were conducted in our laboratory concurrently with experiments utilising synaptosomes isolated from ovine stalk ME. This should distinguish effects taking place at the nerve terminal from those which require the intact, integrated neuronal circuits present in the whole hypothalamus. The effect of a wide range of biogenic amines (10^{-7}-10^{-5}M) on the LHRH and IRS release from the incubated hypothalamus was studied.

6.1. METHOD

Hypothalami, dissected as previously described, were preincubated for 45 min in 1 ml of Hank's medium. The incubation was continued for 60 min in 0.5 ml Hank's medium containing the particular neurotransmitter (10^{-5}M or 10^{-7}M). Vitamin C (5×10^{-4}M) was added to the incubation medium as an antioxidant. Preincubation with the neurotransmitter did not enhance the response. At the end of the incubation period the release of LHRH and IRS into the medium was assayed. Incorporation and uptake of the [^{3}H]-amino acid hydrolysate by the hypothalamic tissue was monitored (see Chapter 2).

6.2. RESULTS

LHRH release was increased by melatonin and decreased by GABA (Table 6.1). Serotonin, noradrenalin, adrenalin, L-DOPA, dopamine, acetylcholine and histamine were without effect on LHRH release from incubated rat hypothalami.

IRS release was increased by melatonin, serotonin and adrenalin, and decreased by histamine (Table 6.1). Noradrenalin, L-DOPA, dopamine, acetylcholine and GABA were without effect on IRS release from incubated rat hypothalami.

TABLE 6.1

Effect of neurotransmitters on LHRH and IRS release from the incubated rat hypothalamus

Additions	LHRH release % of control	IRS Release % of control
nil	199 ± 1111	100 ± 3
L-DOPA	97 ± 5	120 ± 12
Dopamine	110 ± 4	110 ± 9
Noradrenalin	100 ± 11	94 ± 10
Adrenalin	87 ± 6	215 ± 4 xx
Histamine	100 ± 5	61 ± 8 x
Serotonin	85 ± 9	190 ± 2 xx
Melatonin	180 ± 12 xx	171 ± 3 xx
GABA	52 ± 11	100 ± 2
Acetylcholine	93 ± 4	103 ± 5

Concentration of all neurotransmitters 10^{-7}M, similar results were obtained at concentrations of 10^{-5}M.
Results are expressed as mean ± SEM, n=5.

x $p<0.005$, xx $p<0.001$. The remaining data were not significantly different from control release results ($p>0.05$).

6.3. DISCUSSION

LHRH release was stimulated by melatonin in accordance with the results of Kao and Weisz (1977) who reported a similar finding. Since melatonin has been found to have anti-gonadotrophic actions when administered to rats (Kamberi, 1973) these findings appear paradoxical. It is clear from the present results that melatonin does not inhibit LHRH release. Melatonin, also increased LHRH release from SME synaptosomes (Kewley, 1981). Taken in conjunction with the present results it appears that this indolamine exerts a stimulatory effect at the LHRH ME nerve terminal.

GABA decreased LHRH release in keeping with the inhibitory role of this neurotransmitter. GABA causes a selective increase in permeability of the plasma membrane to chloride ions, thus tending to stabilise the membrane potential which would prevent a depolarisation induced release of neuropeptide.

IRS release was stimulated by melatonin, serotonin and adrenalin. This has not previously been reported. Melatonin also increased IRS release from SME synaptosomes while serotonin and adrenalin were without effect (Kewley, 1981). The physiological significance of the increase in IRS release by the indolamines cannot be explained at present. The increase in IRS release attributed to adrenalin is in accordance with the low plasma GH levels observed during an intravenous infusion of adrenalin (Hertelendy et al, 1969). The inhibitory response of histamine on IRS release has not previously been reported and is of interest in view of the fact that few substances have been shown to inhibit IRS release. The physiological significance of this finding cannot however, be explained.

Dopamine and noradrenalin have been reported to stimulate LHRH and somatostatin release from the isolated hypothalamic tissue (Rotsztejn et al, 1976, 1977; Ojeda et al, 1979; Negro-Vilar et al, 1978, 1979; Maeda and Frohman, 1980). However, on 6 separate occasions we were unable to show any significant effect of these catecholamines on LHRH and IRS release from the incubated hypothalamus.

Since other neurotransmitters effected the release of LHRH and IRS the lack of effect of the catecholamines cannot be accounted for by an ineffective rate of diffusion and uptake into the hypothalamus. The differences in response may be due to the widely different incubation conditions used. It is also possible that the sex, age and endocrine status of the rats used in the present experiments influenced the LHRH release in response to the catecholamines (Vale et al, 1977).

Interactions between peptidergic and nonpeptidergic neurons almost certainly occurs within the hypothalamus (Müller et al, 1977). In addition, some peptidergic neurons are known to contain a classical neurotransmitter as well e.g. substance P and serotonin are present in the same neuron in rat medulla oblongata (Hökfelt et al, 1980).

Thus, a neurotransmitter released from a peptidergic nerve terminal may feedback on the same neuron to regulate secretion in addition to the conventional synaptic contact between neurons. In the present chapter we have clearly demonstrated that some biogenic amines do influence the release of LHRH and IRS from the incubated hypothalamus. These data are compatible with the concept that LH/FSH and GH release from the pituitary may be modulated by monoamine regulation of hypothalamic LHRH, IRS and GRF secretion.

7. THE EFFECT OF NEUROPEPTIDES ON LHRH AND IRS RELEASE FROM THE INCUBATED RAT HYPOTHALAMUS.

INTRODUCTION

Neuropeptides are known to influence the release of anterior pituitary hormones and it is thought that they may affect the release of other neuropeptides (Vale et al,1977; Moss,1979). Substance P, neurotensin, TRH, LHRH, somatostatin, VIP, CCK, the enkephalins, vasopressin and oxytocin, have been localised within nerve terminals (Hökfelt et al,1980). However, a neurotransmitter role has only been thoroughly substantiated for substance P (Krivoy et al,1979).

The hormones of the gastro-intestinal tract, gastrin, glucagon, insulin, substance P, VIP, CCK, secretin and bombesin are also present in the CNS (Krieger and Liotta,1979; Zimmerman,1979). Likewise, somatostatin, neurotensin and the enkephalins originally found in the brain have since been localised in the gut. Thus, apart from, or together, with their hormonal function of peptide release into the general circulation these hormones may have additional neuromodulator or neurotransmitter roles within the CNS.

Extensive maps for the distribution of substance P, enkephalins, neurotensin, CCK and bradykinin have been published but it is still not clear whether two or more neuropeptides can reside in a single neuron (Hökfelt et al, 1980). There is evidence to suggest that ACTH and β endorphin are present in the same neurons in the hypothalamus (Block et al,1978;Watson et al,1978). Recently, Hökfelt et al(1980)have observed TRH-like immunoreactivity in substance P neurons in the rat medulla oblongata. Thus it is apparent that a peptidergic neuron may contain not only a classical neurotransmitter but also other neuropeptides exemplifying the complexity of the neurosecretory process.

Met-enkephalin stimulates GH and inhibits LH release <u>in vivo</u> (Beaumont and Hughes,1979; Drouva et al,1980). However, it is not clear whether these effects are mediated at the hypothalamic or hypophyseal level. Recently, met-enkephalin inhibition of K^+-stimulated LHRH and somatostatin release from MBH fragments was

demonstrated (Drouva et al, 1980). Thus regulation at the hypophyseal level could account for alteration of the pituitary release of LH and GH as reported *in vivo* (Beaumont and Hughes, 1979).

This section describes the effects of a wide range of neuropeptides (200-500 ng/ml) on the release of LHRH and IRS from incubated rat hypothalami. These experiments were undertaken to see whether other neuropeptides modulated the release of LHRH and IRS and thus, indirectly, the release of the associated trophic hormones from the pituitary.

7.1. METHODS

Hypothalami, dissected as previously described (See Chapter 2 ·) were pre-incubated for 45 min in 1 ml of Hank's medium. The incubation was continued for 60 min in 0,5 ml Hank's medium containing the particular neuropeptide (200-500 ng/ml). Release was not enhanced when the hypothalami were preincubated with neuropeptides.

At the end of the incubation period the amount of LHRH and IRS released into the medium was assessed by radioimmunoassay. Incorporation and uptake of $[^3H]$-amino acid hydrolysate by the hypothalamic tissue was also monitored (See Chaper 2).

7.2 RESULTS

The basal release of LHRH from the incubated hypothalamus was decreased in the presence of vasopressin and oxytocin (Table 7.1). LHRH release appeared to be enhanced by β-endorphin. However, these high values were due to interference of β-endorphin in the LHRH radioimmunoassay. LHRH release was not altered by TRH, somatostatin-14, neurotensin, substance P, met-enkephalin and leu-enkephalin Table 7.1).

The basal release of IRS was increased by TRH, LHRH, neurotensin and substance P (Table 7.1). IRS was not altered by vasopressin oxytocin, met-enkephalin, leu-enkephalin, and β-endorphin.

TABLE 7.1

Effect of neuropeptides on LHRH and IRS release from incubated rat hypothalami.

	LHRH RELEASE	IRS RELEASE
ADDITIONS	% CONTROL	% CONTROL
Control	100 ± 6	100 ± 5
Substance P	92 ± 7	173 ± 5 **
Neurotensin	104 ± 9	125 ± 5 *
TRH	102 ± 4	189 ± 2 **
LHRH	-	250 ± 15 **
SS-14	101 ± 3	-
Met-enkephalin	93 ± 10	100 ± 3
Leu-enkephalin	108 ± 5	93 ± 7
Vasopressin	61 ± 9 *	107 ± 5
Oxytocin	66 ± 9 *	89 ± 9
β-endorphin	-	110 ± 10

Concentration of all neuropeptides 500 ng /ml

Results are expressed as mean ± SEM n = 5

* $p < 0.005$; ** $p < 0.001$. The remaining data were not significantly different from control release results ($p > 0.05$)

7.3 DISCUSSION

Vasopressin and oxytocin both caused a decrease in LHRH release. Pavel et al (1979) reported that the plasma LH level was not affected by intraventricular administration of either vasopressin or oxytocin (10^{-3}- 10^{-4}M). However, AVT (arginine vasotocin) (10^{-4} pg) decreased plasma LH level suggesting that this effect is mediated by inhibition of LHRH release from the hypothalamus by interference with serotonin transmission in the brain. Our results suggest that oxytocin and vasopressin have a direct inhibitory effect on LHRH release which is not consistent with the *in vivo* effects of Pavel.
Higher concentrations of oxytocin and vasopressin (500 ng/ml) were used in the present experiments to facilitate penetration of the neuropeptide into the tissue. It is possible that oxytocin and vasopressin mimic the effects of AVT. However, it seems unlikely that the effects seen are mediated via serotonin, since LHRH release from the incubated hypothalamus was insensitive to serotonin.

Neither oxytocin or vasopressin had any effect on IRS release indicating that the observed result was a specific effect on LHRH release.

Neurotensin and substance P both increased IRS release from the incubated hypothalamus. These results are in agreement with those of Sheppard et al (1979) and Maeda and Frohman (1980) and further support the suggestion that the decrease in serum GH and TSH levels following intraventricular injection of substance P or neurotensin is due, at least in part, to a stimulation of hypothalamic somatostatin release (Rivier et al, 1977; Chihara et al, 1978; Maeda and Frohman 1978). In this laboratory, it has been shown that substance P increased the basal release of IRS from ovine SME synaptosomes whereas neurotensin decreased the basal and K^+-stimulated release of somatostatin (Kewley, 1981 ,).

Taken in conjunction with the present findings, this suggests that there are stimulatory receptors for substance P on the somatostatin nerve terminals of the somatostatin neuron. In contrast, neurotensin binding receptors at the nerve terminal of somatostatin neurons are inhibitory. Thus, the stimulatory effect of neurotensin on the intact hypothalamus may override the inhibitory effect of this neuropeptide on somatostatin release from the nerve terminal. This suggests that at least two receptor sites for neurotensin exist on the intact neuron.

It has been suggested that met-enkephalin can modulate the release of the peptides by interacting with a specific opiate receptor located on LHRH and somatostatin neurons. Met- and leu-enkephalin did not effect the basal LHRH and IRS release from incubated hypothalami in agreement with Rotsztejn et al (1978) and Sheppard et al (1979(a)).

These findings all report the effect of the enkephalins on <u>basal</u> release of neuropeptides from the incubated hypothalamus. It is possible that met-enkephalin failed to penetrate the tissue. However, it seems more likely that the opiates only effect the stimulated release of neuropeptides. Met-enkephalin significantly decreased the K^+ - stimulated release of LHRH and somatostatin from MBH fragments (Drouva et al, 1980). These results suggest that met-enkephalin can modulate release of neuropeptides by intereacting with a specific opiate receptor found on LHRH and somatostatin neurons. Alternatively, met-enkephalin may effect LHRH and somatostatin release indirectly by inhibiting dopamine secretion. The latter is known to stimulate release of LHRH and somatostatin from the incubated hypothalamus (Rotsztejn, 1976, 1977, 1978; Negro-Vilar et al, 1978; Berelowitz et al, 1980). Met-enkephalin also inhibited the K^+-stimulated release of IRS from ovine SME synaptosomes (Kewley, 1981). This suggests that inhibitory receptors for met-enkephalin exist on the nerve terminals of somatostatin neurons.

Central administration of TRH has been reported to inhibit GH secretion (Chihara et al, 1976).

The stimulatory action of TRH on IRS release from the incubated hypothalamus in the present experiments provides an explanation for the effect of the neuropeptide on GH secretion. Somatostatin was reported to inhibit TRH release from organ cultures of rat hypothalami (Hirooka et al,1978; Hollander et al,1980). Together these *in vitro* results suggest a possible short-loop feedback system operating in the hypothalamus which may be an additional factor in modulating the hypothalamic-pituitary-thyroid axis.

An interesting finding, is the increased release of a IRS in the presence of LHRH, but the physiological relevance of this is difficult to assess.

In summary, a number of neuropeptides were shown to effect LHRH and IRS release from incubated hypothalami. These data support the concept of a neuromodulator or neurotransmitter role for neuropeptides in regulating hypothalamic and other CNS neuropeptide hormone secretion. In particular some known systemic effects such as the stimulatory effect of substance P and neurotensin on GH, TSH, FSH and LH release may be mediated via influences on LHRH and IRS release from the hypothalamus.

8. APPLICATION OF THE INCUBATED HYPOTHALAMIC SYSTEM TO STUDY THE HYPOTHALAMIC-PITUITARY-GONADAL-AXIS.

INTRODUCTION

The feedback actions of androgens on LH and FSH secretion are potentially mediated directly via feedback at the pituitary level or indirectly via altering release of hypothalamic releasing factors. Androgens can act directly on the anterior pituitary lobe both *in vitro* (Kao and Weisz, 1975; Drouin and Labrie, 1976) and *in vivo* (Kingsley and Bogdanove, 1973). However, the relative contribution of feedback regulation at the pituitary and at the hypothalamic level is unknown. Investigations of this nature have been hampered by the inability to satisfactorily measure LHRH *in vivo*. Nansel et al (1979) have demonstrated that direct androgen feedback on the pituitary is rapid and sensitive enough to play a part in the physiological regulation of LH release. However, it is unlikely that this effect functions independently of an indirect (brain mediated) feedback mechanism.

Little conclusive evidence exists concerning the effects of gonadal hormones on LHRH release from the hypothalamus. Castration of male rats decreases hypothalamic LHRH content. Treatment of castrated rats with testosterone prevents this decrease in hypothalamic LHRH content (Shin et al, 1974, 1976; Cher et al, 1977). These changes in hypothalamic LHRH content may reflect alterations of either LHRH synthesis or secretion or both.

In addition to gonadal hormone feedback at the pituitary and/or hypothalamic level, short-loop feedback systems may also operate. Gonadotrophic hormones may influence LHRH release and LHRH may even regulate its own secretion by autoregulation. Thus studies were undertaken to investigate the effects of the steroid hormones, testosterone and β-oestradiol, on LHRH release from the hypothalamus isolated from intact rats (with normal endogenous steroid levels) and castrated rats (decreased endogenous steroid levels). Short loop feedback regulation on the hypothalamus was studied by monitoring the effect of *in vitro* addition of the pituitary hormones LH, FSH and PRL (prolactin) and an LHRH analogue, des-Gly^{10}-D-Leu^{6} ethylamide LHRH, on LHRH release from the hypothalamus isolated from intact rats. In all experiments IRS release was also measured.

8.1. METHOD

Male Long Evans rats, 200-250g were used for all experiments. Animals were castrated under ether anaesthesia and were left for two days before treatment was commenced. This was to ensure clearance of all endogenous testicular steroid hormones. The animals were then injected subcutaneously, daily, for 7 days with 100 µl linseed oil either alone or containing 100 µg testosterone proprionate or 1 µg β-oestradial. Twenty four hours after the final injection rats were decapitated and the basal LHRH and IRS release from the isolated hypothalamus was monitored as described in the Chapter 2.

The effect of in vitro addition of testosterone (20-50 ng/ml), β-oestradiol (20-50 pg/ml), ovine LH (1-100 ng/ml), FSH (500 ng/ml) and des-Gly^{10}-D-leu^{6} ethylamide LHRH (10-50 ng/ml) on hypothalamic LHRH and IRS release was examined. Hypothalami were dissected as previously described and then preincubated in 1 ml Hank's medium for 45 min. Thereafter, they were incubated for 60 min in 0,5 ml Hank's medium. All test substances were added at the beginning of the 60 min incubation period. At the end of the incubation period the release of LHRH and IRS into the medium was assayed and uptake and incorporation into protein of [^{3}H]-amino acids by hypothalamic tissue was monitored (See Chapter 2). Des-Gly^{10}-D-leu^{6} ethylamide LHRH did not cross react with LHRH antiserum 1076 (0%) and therefore at the concentration used did not interfere in the radioimmunoassay and allowed specific determination of released LHRH.

8.2 RESULTS

Hypothalami taken from male rats castrated 9 days earlier released significantly less LHRH into the incubation medium than did hypothalami from control intact rats (Fig. 8.1).

Hypothalami of castrated rats given either 100 µg testosterone proprionate or 1 µg β-oestradiol daily for 7 days, released amounts of LHRH into the medium similar to that from hypothalami of castrates and significantly less than hypothalami of intact rats (Fig. 8.1).

Hypothalami of intact rats given 1 µg β-oestradiol daily for 7 days, released significantly more LHRH into the medium ($p < 0.01$) than hypothalami of the intact control rats (Fig. 8.1). IRS release from hypothalami of castrated rats given 1 µg β-oestradiol daily for 7 days, was significantly more than released from rat hypothalami isolated from any of the other experimental groups (Fig. 8.1). Hypothalami taken from castrated rats or castrated rats given 100 mg testosterone proprionate daily for 7 days, released similar amounts of IRS to that released from hypothalami of the intact control rats (Fig 8.1).

LHRH release from the incubated hypothalamus isolated from intact male rats was not altered by the *in vitro* addition of testosterone proprionate, β-oestradiol, LH, FSH, or LHRH analogue (Fig 8.2). IRS release was, however, markedly enhanced by the *in vitro* addition of β-oestradiol while testosterone proprionate, LH, FSH, and LHRH were without effect.

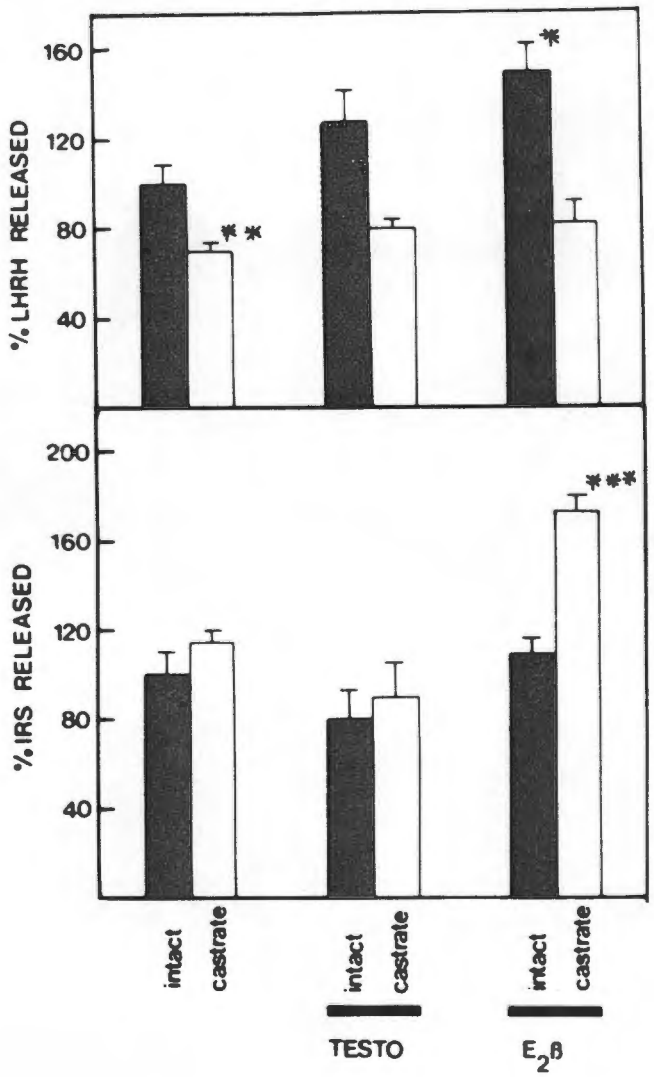


FIG 8.1

Effect of in vivo administration of steroids on LHRH and IRS release from the rat hypothalamus in vitro.
Values are means ± S.E.M. (n=5). Significant differences * $p<0.10$, ** $p<0.025$, *** $p<0.01$

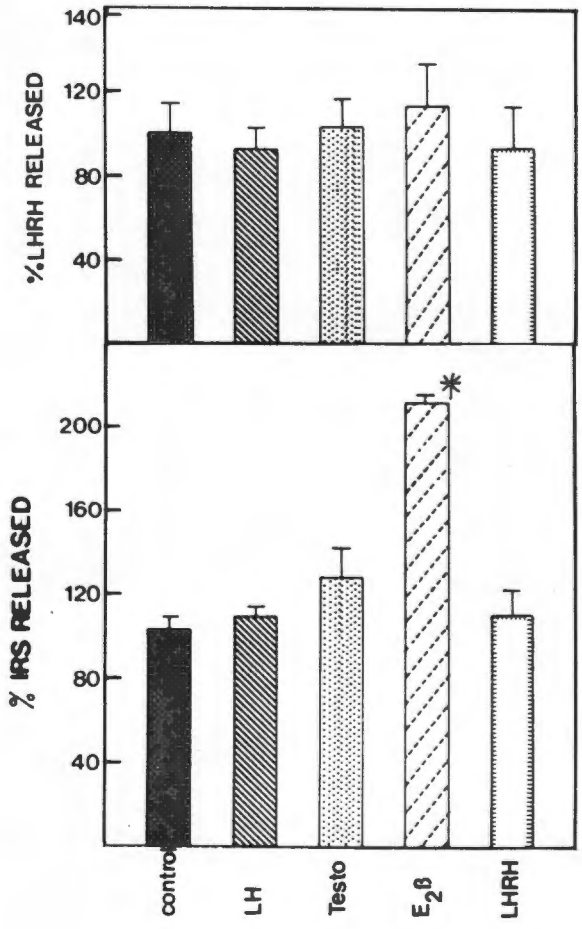


FIG 8.2

Effect of LH, Testo, LHRH analogue and β-oestradiol on LHRH and IRS release from the rat hypothalamus in vitro.

Values are means ± S.E.M. (n=5). Significant differences * $p<0.0005$

8.3. DISCUSSION

It is well established that LHRH stimulates LH and FSH release while testosterone inhibits secretion of the two gonadotrophic hormones (Labrie et al, 1979). The decrease in LHRH release from incubated hypothalami of castrated rats was not overcome by treatment of castrates with testosterone or β-oestradiol. Treatment with β-oestradiol increased IRS release indicating that the steroid penetrated the hypothalamus. The decrease in basal LHRH release from the incubated hypothalamus of castrated rats is in agreement with Rudenstein et al (1979). More recently Dyer et al (1980) have reported that castration reduced the amount of LHRH released from the incubated hypothalamus in response to electrical stimulation. In addition they confirmed the earlier findings that there was also a decrease in hypothalamic LHRH content. (Shin & Howitt, 1979; Chen et al, 1977; Kalra et al, 1977; Rudenstein et al, 1979). These data may appear paradoxical since after castration the negative feedback effect of gonadal hormones on the hypothalamic - hypophyseal system is abolished and peripheral gonadotrophic hormone levels are usually elevated. It is anticipated that hypothalamic LHRH release, particularly the stimulated release, would be facilitated in castrated rats. Clearly, our findings do not concur with the classical view of feedback regulation. It is also possible that the post castration rise in systemic LH and possibly FSH levels, may deplete the amount of LHRH available for release. However, a similar decrease in LHRH content of and release from incubated hypothalami was reported for hypophysectomised rats (Rudenstein et al, 1979). This effect was prevented by administration of testosterone to the hypophysectomised animals. These results suggest that the effects of castration on hypothalamic LHRH release are not mediated via the increase in gonadotrophic hormone levels.

An enhanced pituitary responsiveness to LHRH within 4 hours of castration has recently been reported (Nansel et al, 1979). However, the indirect hypothalamic response to steroid withdrawal does not occur until 6-8 hours post castration. It is concluded that the slower hypothalamic response is the rate limiting component of

the overall brain-pituitary reponse to androgen withdrawal. In the present study 48 hours were allowed for clearance of endogenous gonadal steroid hormone. The decrease in the circulating levels of these hormones following castration and prior to commencement of steroid treatment could have altered the normal hypothalamic LHRH activity. This concept is supported by the observed decrease in the electrically stimulated release of LHRH from the hypothalamus of testosterone treated, castrated rats (Dyer et al, 1980). In these experiments, three weeks were allowed for the clearance of endogenous gonadal hormones prior to treatment of rats with 1.25 mg testosterone proprionate for three days. This treatment is sufficient to inhibit secretion of gonadotrophic hormones from the pituitary. In contract, Rudenstein et al (1979) commenced steroid treatment of castrated rats immediately after castration. Furthermore, in hypophysectomised animals, unless testosterone treatment commenced immediately, there was a decrease in the amount of LHRH released from the incubated hypothalami of these animals. In view of these findings the time allowed for clearance of endogenous gonadal hormones clearly effects the response of the hypothalamus to subsequent treatment with steroids.

In the hypothalamus testosterone can be metabolized to dihydrotestosterone (Lieberburg and Mc Ewan, 1977) and to β-oestradial (Selmanoff et al, 1977) which suggests that the stimulatory effect of testosterone on LHRH release may require the conversion of testosterone to dihydrotestosterone or β-oestradiol. The demonstration that β-oestradiol did not enhance LHRH release from hypothalami isolated from castrated rats suggests that the failure of testosterone to increase LHRH release did not result from a failure of tissue to aromatize testosterone to β-oestradiol. In intact rats β-oestradiol significantly increased LHRH release over basal secretion. It is possible that testosterone alone was insufficient for normalisation of LHRH release, and other gonadal hormones, such as inhibin, maybe involved. Another possibility is that castration reduces the number of brain receptors for the gonadal hormones.

In view of the findings presented earlier in this thesis viz. that the K+-stimulated release of LHRH is Ca^{2+} dependent and the basal release decreased in the absence of extracellular Ca^{2+} (see chapter 4), it may be concluded that there is some measure of physiological control over the release of the neuropeptide in vitro. Thus it is most unlikely that the basal LHRH release observed in the present experiments merely reflects leakage from the hypothalamus. Clearly a more detailed study on the effects of castration on the K^{+}-stimulated release of LHRH from the incubated hypothalamus would be of value.

Certain neurons projecting to portal capillaries in the median eminence are directly or indirectly sensitive to changes in the level of gonadal steroids (Stoeckart et al, 1975). Moreover, gonadal steroid treatment exerts a profound change in central catecholamine activity, most noteably an increased turnover of dopamine (Kizer et al, 1978) and noradrenalin (Simpkins et al, 1979) 1980) in MBH neurons. This suggests that catecholamines play a role in the regulation of MBH LHRH activity. Thus, although MBH may be the focal site of testosterone action (Kalra and Kalra, 1978) the direct effect of testosterone on the LHRH neuron may be mediated by an intermediary neurotransmitter or neuromodulator.

Hypothalami of castrated rats treated with β-oestradiol released significantly more IRS into the incubation medium than hypothalami of castrated rats, or of intact rats treated with β-oestradiol or testosterone. This suggests that the stimulatory effect of β-oestradiol on IRS is prevented by the presence of testosterone and/or other gonodal hormones. Whether this is due to a direct effect such as the decrease in endogenous testosterone or an indirect effect is unknown. Somatostatin and oestrogen-containing neurons are present in close proximity in the paraventricular and arcuate nuclei of the rat hypothalamus (Stumpf and Sar, 1977). In the intact rat the androgens may exert an inhibitory effect on somatostatin neurons while the positive effect of oestrogen is only shown when androgens are removed.

It seems unlikely that the effect of β-oestradiol is due to the somatostatin neurons being more superficially situated in the hypothalamus as they show similar distribution of LHRH-containing neurons (Stumpf and Sar, 1977; Terry and Martin, 1978). Thus potentially there is equal opportunity for interaction with both sets of neurons. In view of a similar effect of β-oestradiol on IRS release in the *in vitro* and *in vivo* experiments it is likely that there is a direct effect of β-oestradiol on IRS release from the hypothalamus which does not require chronic exposure to the oestrogen.

9. CONCLUDING DISCUSSION

Considerable indirect information on the secretion of LHRH and somatostatin has accumulated by measurement of the pituitary hormones which they regulate. However, there is considerably less known about the direct release of these neuropeptides from the hypothalamus. The incubated hypothalamus, hypothalamic frag-ments/ slices, hypothalamic organ culture, hypothalamic synaptosomes and perifused hypothalami are all invitro systems which have been used to study factors influencing neuropeptide release from the hypothalamus (Rotsztejn, et al 1976, 1977, 1978; Negro-Vilar et al, 1978, 1979; Ojeda et al, 1979; Hirooka et al, 1978; Hollander et al, 1980; Bennett et al, 1975, 1979; Wakabayashi et al, 1977; Berelowitz et al, 1978, 1980; Sheppard et al, 1979, 1980; Maeda and Frohman 1980; Richardson et al, 1980; Hartter and Ramirez, 1980). However conditions for maintaining the viability of the tissue have not been clearly established. In the results presented in this thesis it has been shown that monitoring protein synthesis in the incubated hypothalamus is a clear and reliable index of hypothalamic functional viability.

Stimulus-secretion-coupling involves depolarisation of the plasma membrane and leads to an increased membrane ion permeability. This permits influx of Ca^{2+} into the cytoplasm from the extracellular fluid through voltage-dependent channels in the plasma membrane. This Ca^{2+} influx initiates the cascade of events culminating in the release of neuropeptide from the cell (Douglas 1975, Thorn et al, 1978). LHRH and IRS release from isolated hypothalami into the incubation medium was stimulated by membrane-depolarising concentrations of potassium and ouabain. This release was Ca^{2+}-dependent and was inhibited when Ca^{2+} was omitted from the incubation medium. However there are clearly differences in the Ca^{2+} requirements for the K^{+}-stimulated release of LHRH and IRS. Verapamil completely blocked K^{+}-stimulated LHRH release but only partially blocked the release of IRS. Moreover, this suggestion of differences in the mechanism of release of the two neuropeptides was emphasised by the different effects of the ionophores. X537A stimulated both LHRH and IRS release, only the LHRH release was inhibited by verapamil.

Similarly ionophore A23187 stimulated IRS release alone. This release was calcium-dependent but was not blocked by verapamil. Although these results again suggest a difference in release mechanisms for the two neuropeptides the results cannot necessarily be interpreted as indicating differences in Ca^{2+} requirements since the ionophores have diverse effects on membrane properties (Cochrane and Douglas, 1975; Klausner et al, 1979; Brown et al, 1980).

Although release of LHRH and IRS into the incubation medium cannot be equated with secretion *in vivo* it was demonstrated that LHRH and IRS release can be induced by membrane depolarisation (60 mM KCl) and Ca^{2+} influx. This suggests that the release of the neuropeptides in the system described is under the control of at least some of the same physiological factors that influence the secretion of most hormones *in vivo*.

In recent years it has become clear that neurotransmitters and neuropeptides can act in many different brain regions, sometimes even evoking opposite responses at different sites, to regulate hormone release from the pituitary or the hypothalamus (Moss, 1979; Krulich, 1979). The putative role of neuropeptides as central neurotransmitters or neuromodulators implies that they can effect the release of other neuropeptides. Thus, in the present experiments, it is suggested that oxytocin and vasopressin have a modulatory role in regulating the release of LHRH. Similarly, neurotensin and substance P increased IRS release. The regulation of IRS release at the hypothalamic level by these neuropeptides explains, in part, the observed decrease in serum GH and TSH levels following intraventricular injection of neurotensin or substance P (Rivier et al, 1977; Chihara et al, 1978). A similar modulatory influence on LHRH and IRS release was observed with neurotransmitters.

The release of LHRH and IRS from the hypothalamus can be modified by many different factors such as the age, sex, endocrine status of the animal and steroid priming. However, the final effect of a neuropeptide or neurotransmitter on LHRH and IRS release may depend on the differential effect of a second neuromodulator

at a different site of action. In view of the demonstration of axo-axonic and dendro-dendritic contacts of hypothalamic peptidergic neurons with catecholamine containing neurons (Ajika, 1979) we have concurrently investigated peptide release from the incubated hypothalamus and from hypothalamic synaptosomes. Thus we were able to differentiate events involving the entire neuron from those occurring at the nerve terminal.

A direct androgen feedback mechanism on the regulation of LH release has recently been demonstrated (Nansel et al, 1979). It appears that this mechanism cannot operate independently of an indirect (brain-mediated) feedback mechanism. The criterion of an indirect feedback mechanism is evidence that feedback signals produce appropriate alteration in the hypophysiotropin release (Nansel et al, 1979). Castration and/or androgen treatment have only recently been shown to alter LHRH release from the incubated hypothalamus in vitro (Rudenstein et al, 1979) and to decrease hypothalamic LHRH content (Shin and Howitt, 1974, 1976; Chen et al, 1977; Kalra et al, 1977; Rudenstein et al, 1979; Dyer et al, 1980). While β-oestradiol and testosterone did not effect LHRH secretion when added to the incubation medium in vitro, the in vivo modulation of steroid hormones did effect LHRH release from the hypothalamus isolated from treated animals. Castration induced a significant decrease in LHRH release which was not overcome when treatment with testosterone proprionate or β-oestiodiol, commenced 2 days post castration. The increase in serum LH levels following castration has been ascribed to increased sensitivity of the pituitary to less than normal concentrations of LHRH (Nansel et al, 1979). Administration of LHRH to castrates, enhanced this LH secretion while the administration of testosterone to castrates decreased the LH response to LHRH. Our results suggest that the fall in endogenous gonadal hormones in the two days post castration, and prior to treatment, alters the hypophysiotropic activity. This effect cannot be overcome by the subsequent treatment with testosterone or β-oestradiol. These data suggest a direct action of the gonadal hormones on hypothalamic hypophysiotropic releasing activity which is in agreement with Nansel et al (1979). These data also support the hypothesis that gonadal steroids may maintain LHRH secretion in vivo. In addition these findings suggest

that LHRH release is not affected by acute administration of testosterone and β-oestradiol and that feedback regulation is associated with chronic exposure to the hormones.

Thus the present thesis has demonstrated the use of the incubated hypothalamus for studying the *in vitro* release of LHRH and IRS. It has described both similarities and differences in the release of LHRH and IRS and shown that the mechanism of release of the two neuropeptides is clearly different. Used in conjunction with *in vivo* experiments the isolated hypothalamus provides a valuable tool for unfolding the nature of physiological control of endocrine function and in particular feedback inhibition. Thus the hypothalamus provides a useful approach to the elucidation of the mechanisms of hormonal homeostasis.

REFERENCES

ACHER, R. 1979. Angew. Chem. Int. Ed. Engl. 18 : 846

AJIKA, K. 1979. J. Anatomy 128 : 331

AKOPYAN, T.N., Arutunyan, A.A., Lajtha, A., Galayan, A.A. 1978. Neurochem. Res. 3 : 89

AKOPYAN, T.N., Arutunyan, A.A., Oganisyan, A.I., Lajtha, A., Galayan, A.A. 1979. J. Neurochem. 32 : 629

ARAKI, S., Ferin, M., Zimmerman, E.A., Van de Wiele, R.L. 1975. Endocrinol. 96 : 644

ARIMURA, A., Sato, H., Kumasaka, T., Worobec, R.B., Debeljuk, L., Dunn, J., Schally, A.V. 1973. Endocrinol. 93 : 1092

ARIMURA, A., Debeljuk, L., Schally, A.V. 1974. Endocrinol. 95 : 323

ARIMURA, A., Sato, H., Du Pont, A., Nishi, A., Schally, A.V. 1975(a), Science 189 : 1009

ARIMURA, A., Sato, H., Coy, D.H., Schally, A.V. 1975(b). Proc. Soc. Exp. Biol. Med. 148 : 784

BAKER, P.F. 1972. Prog. Biophys. Mol. Biol. 24 : 177

BARNEA, A., Neaves, W.B., Porter, J.C. 1977. Endocrinol. 100 : 1068

BARTKE, A., Steele, R.E., Musto, N., Caldwell, B.V. 1973. Endocrinol. 92 : 1223

BAUER, K., Horsthemke, B., Krusatschek, H., Nowak, P., Kleinkauf, H. 1979. Hoppe-Seyler's Z. Physiol. Chem. 360 : 229

BEAUMONT, A., Hughes, J. 1979. Ann. Rev. Pharmacol. Toxicol. 19 : 245

BENNETT, G.W., Edwardson, J.A., Holland, D., Jeffcoate, S.L., White, N. 1975. Nature 257 : 323

BENNETT, G.W., Edwardson, J.A., Marcano De Cotte, D., Berelowitz, M., Pimstone, B.L., Kronheim, S. 1979. J. Neurochem. 32 : 1127

BERGLAND, R.M., Page, R.B. 1979. Science 204 : 18

BERELOWITZ, M., Kronheim, S., Pimstone, B.L., Sheppard, M.C. 1978. J. Neurochem. 31 : 1537

BICKNELL, R.J., Shofield, J.G. 1976.Feb. Lett. 68 : 23

BIGDELI, H., Snyder, P.J. 1978. Endocrinol. 103 : 281

BLACKWELL, R.E., Guillemin, R. 1973. Ann. Rev. Physiol. 35 : 357

BLOCH, B., Bugnon, C., Fellman, D., Lenys, D. 1978. Neurosci. Lett. 10 : 147

BLOBEL, G., Dobberstein, B. 1975. J. Cell Biol. 67 : 835

BLAUSTEIN, M.P., Kendrick, N.C., Fried, R.C., Ratzlaff, R.C. 1977. In : Approaches to Cell Biology. Society for Neuroscience Symposia Vol. II Ed. Cowan, W.M., Ferrendelli, J.A. (Society for Neuroscience Bethesda) Pg. 172

BORGEAT, P., Labrie, F., Drouin, J., Belanger, A., Immer, H., Sestanj, K., Nelson, V., Gotz, M., Schally, A.V., Coy, D.H., Coy, E.J. 1974. Biochem. Biophys. Res. Commun. 56 : 1052

BRADBURY, M.W.B., Burden, J., Hillhouse, E.W., Jones, M.T. 1974. J. Physiol (London) 239 : 261

BRAZEAU, P., Ling, N., Esch, F., Bohlen, P., Benôit, R., Guillemin, R. 1980. C.R. Acad. Sci. Paris. t290 D1367

BROOKS, C.McC. 1938. Am. J. Physiol. 121 : 157

BROWN, E.M., Gardner, D.G., Aurbach, G.D. 1980. Endocrinol. 106 : 133

BROWNSTEIN, M., Arimura, A., Sato, H., Schally, A.V., Kizer, J.S. 1975. Endocrinol. 96 : 1456

BURGUS, R., Dunn, T.F., Desiderio, D., Guillemin, R. 1969. C.R. Acad. Sci. Paris 269 : 1870

BURGUS, R., Dunn, T.F., Desiderio, D., Ward, D.N., Vale, W., Guillemin, R. 1970. Nature 226 : 321

BURGUS, R., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, R., Fellows, R., Blackwell, R., Vale, W., Guillemin, R. 1972. Proc. Natl. Acad. Sci. U.S.A. 62 : 278

CHEN, H.T., Geneau, P., Meites, J. 1977. Proc. Soc. Exp. Biol. Med. 156 : 127

CHEUNG, C.Y., Weiner, R.L. 1976. Endocrinol. 99 : 914

CHEUNG, W.Y. 1980. Science 207 : 19

CHIHARA, K., Kato, V., Ohgo, S., Iwasaki, Y., Abe, H., Maeda, K., Imura, H. 1976. Endocrinol. 98 : 1047

CHIHARA, K., Arimura, A., Coy, D.H., Schally, A.V. 1978. Endocrinol. 102 : 281

COCHRANE, D.E., Douglas, W.W. 1975. Br. J. Pharmac. 54 : 400

COOKE, J.D., Quastel, D.M.J. 1973. J. Physiol. (London) 228 : 435

COY, D.H., Coy, E.J., Arimura, A., Schally, A.V. 1973. Biochem. Biophys. Res. Commun. 54 : 1267

CRIGHTON, D.B., Schneider, H.P.G., McCann, S.M. 1970. Endocrinol. 87 : 323

CURRY, D.L., Bennett, L.L. 1976. Proc. Natl. Acad. Sci. U.S.A. 73 : 248

DOUGLAS, W.W., Poisner, A.M. 1964. J. Physiol. 172 : 1

DOUGLAS, W.W., Sorimachi, M. 1972. Brit. J. Pharmacol. 45 : 143P

DOUGLAS, W.W. 1975. In : Calcium Transport in Contraction and Secretion. Ed. Carafoli, E. (Elsevier Amsterdam) Pg. 167

DREIFUSS, J.J. 1975. Ann. N.Y. Acad. Sci. 248 : 185

DROUIN, J., Labrie, F. 1976. Endocrinol. 98 : 1528

DROUVA, S.V., Gallo, R.V. 1977. Endocrinol. 100 : 792

DROUVA, S.V., Epelbaum, J., Tapia-Arancibia, L., Laplante, E., Kordon, C. 1980. Europ. J. Pharmacol. 61 : 411

DUPONT, A., Alvardo-Urbina, G., Cate, J., Labrie, F. 1978. Can. J. Physiol. Pharmacol. 56 : 840

DYER, R.G., Mansfield, S., Yates, J.O. 1980. Exp. Brain. Res. 39 : 453

FINK, G. 1979. Ann. Rev. Physiol. 41 : 571

FINNEY, D.J. 1974. Statistical Methods in Biological Assay. Griffin. London.

FRANCHIMONT, P., Chari, S., Hagelstein, M.T., Duraiswami, S. 1975. Nature 257 : 402

FRIDKIN, M., Hazum, E., Baram, T., Lindner, H.R., Koch, V. 1977. In : Peptides. Ed. Goodman, M., Meienhafer, J. (Halstead Press, New York) Pg. 193

FRIEDGOOD, H.B. 1936. In : Symp. on Endocrine Glands. Harvard Tercent. Celebrations 1936. Cited from Textbook of Endocrinology (1950) Ed. Williams, R.H. (W.B. Saunders Philadelphia) Pg. 644

GAGE, P.W., Quastel, D.M.J. 1965. Nature 206 : 625

GAINER, H., Brownstein, M.J. 1978. In : Cell Virology of hypothalamic neurosecretion. Eds. Vincent, J.D., Kordon, C. (Editions du Centre National de la Recherche Scientifique Paris) Pg. 525.

GAINER, H., Russell, J.T., Fink, D.J. 1979. In : Brian Peptides : A New Endocrinology. Ed. Cotto, A.M., Peck, E.J., Boyd III, A.E. (Elsevier Holland) Pg. 139

Goddard, E.A. 1978. Med. Biochem. Hons. Project. (Unpublished).

GREEN, J.D., Harris, G.W. 1947. J. Endocrinol. 5 : 136

GREEN, J.D., Harris, G.W. 1949. J. Physiol. (London) 108 : 359

GREENWOOD, G.C., Hunter, W.M., Glover, J.S. 1963. Biochem. J. 89 : 14

GRIFFITHS, E.C., Hooper, K.C., Hopkinson, C.R.N. 1973. Acta Endocrinol. (Kbh) 74 : 49

GRIFFITHS, E.C., Hooper, K.C., Jeffcoate, S.L., White, N. 1975. Acta Endocrinol. (Kbh) 79 : 209

GRIFFITHS, E.C., Hooper, K.C., Jeffcoate, S.L., Holland, D.T. 1976. Hormone Res. 7 : 218

GRIFFITHS, E.C., Jeffcoate, S.L., Holland, D.T. 1977. Acta Endocrinol. (Kbh) 85 : 1

GRIFFITHS, E.C., Jeffcoate, S.L., Thorne, J., White, N. 1978. J. Physiol. (London) 275 : 28p

GRIFFITHS, E.C., Kelly, J.A. 1979. Mol. Cell. Endocrinol. 14 : 3

GROSS, D. 1976. Endocrinol. 98 : 1408

GUYTON, A.C. 1976. In : Textbook of Medical Physiology (W.B. Saunders Philadelphia)

HARRIS, G.W. 1937. Proc. Roy. Soc. B. 122 : 374

HARRIS, G.W. 1955. In : Neural Control of the Pituitary Gland (Edward Arnold, London)

HARRIS, G.W. 1944. MD Thesis University of Cambridge cited in Harris (1955)

HARTTER, D.E., Ramirez, V.D. 1980. Endocrinol. 107 : 375

HENDRIKS, S., Millar, R.P., Pimstone, B.L. 1975. S.A. Med. J. 49 : 1559

HERBERT, D. 1976. Endocrinol. 98 : 1554

HERTELENDY, F., Machlin, L., Kipnis, D.M. 1969. Endocrinol 84 : 192

HINSEY, J.C., Markee J.E. 1933. Proc Soc. Exp. Biol. (N.Y.) 31 : 270

HIROOKA, V., Hollander, C.S., Suzuki, S., Ferdinand, P., Juan, S.I. 1978. Proc. Natl. Acad. Sci. U.S.A. 75 : 4509

HÖKFELT, T., Efendic, S., Johansson, O., Luft, R., Arimura, A. 1974 Brain Res. 80 : 165

HÖKFELT, T., Efendic, S., Hellerström, C., Johansson, O., Luft, R., Arimura, A. 1975. Acta. Endocrinol. (Kbh) 80 : 200

HÖKFELT, T., Fuxe, F., Goldstein, M., Johansson, O., Fraser, H., Jeffcoate, S.L. 1976. In : Anatomical Neuroendocrinology ed. Stumpf, W.E., Grant, L.D. (Karger Basel)

HÖKFELT, T., Elde, R., Johansson, O., Terenius, L., Stein, L. 1977. Neuro. Sci. Lett. 5 : 25

HÖKFELT, T., Johansson, O., Ljungdahl, A., Lundberg, J.M. Schultzberg, M. 1980. Nature 284 : 515

HOLLANDER, C.S., Greene, L.W., Rosman, L., Yamauchi, K., Richardson, S., Thaw, C., D'Elleto, R. 1980 Clin. Res. 28 : 479 A

HOPKINS, C.R., Walker, A.M. 1978. Mol. Cell. Endocrinol. 12 : 189

HORSTHEMKE, B., Bauer, K. 1980. Biochemistry 19 : 2867

HUDSON, D., Pickering, A., McLaughlin, J.L., Matthews, E., Sharpe, R., Fink, G., McIntyre, I., Szelke, M. 1976. Vth. Int. Congress of Endocrinology, Hamburg. Abstract Book 18, Abstract No. 43

HUGHES, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., Morris, H.R. 1975. Nature 258 : 577

HYONO, A., Hendriks, Th.,Daemen, F.J.M., Bonting, S.L. 1975. Biochem. Biophys. Acta. 389 : 34

ISHIBASHI, F., Sato, T., Onari, K., Kawate, R. 1979. Endocrinol. (Jpn) 26 : 585

ISRAEL, M., Dunant, V., Manaranche, R. 1979. Prog. in Neurobiol. 13 : 237

IVERSEN, L.L. 1979. Sci. Am. 241 : 118

JONAS, H.A., Burger, H.G., Cumming, I.A., Findlay, J.K., Dekretser, D.M. 1975. Endocrinol. 96 : 384

JONES, D.A., McIlwain, H. 1971. J. Neurochem. 18 : 41

KALRA, S.P., Kalra, P.S., Mitchell, E.O. 1977. Endocrinol. 100 : 201

KALRA, P.S., Kalra, S.P. 1978. Life Sci. 23 : 65

KAMBERI, I.A. 1973. Prog. Brain Res. 39 : 261

KAO, L.W., Weisz, J. 1975. Endocrinol. 96 : 253

KAO, L.W., Weisz, J. 1977. Endocrinol. 100 : 1723

KELLY, J.A., Griffiths, E.C., Jeffcoate, S.L., Hopkinson, C.N.R. 1979. Biochem. Soc. Trans. 7 : 76

KEMPER, B., Habener, J.F., Potts, J.T. Jr., Rich, A. 1972. Proc. Natl. Acad. Sci. U.S.A. 69 : 643

KERDELHUE, B., Valens, M., Langlois, Y., Elobel, A., Reinberg, A. 1978. Hormone Res. 9 : 70

KEWLEY, C.F. 1981. PhD. Thesis. U.C.T.

KINGSLEY, J.R., Bogdanove, E.M. 1973. Endocrinol. 93 : 1398

KIZER, J.S., Palkovits, M., Brownstein, M.J. 1976. Endocrinol. 98 : 311

KIZER, J.S., Humm, J., Nicholson, G., Greeley, G., Youngblood, W. 1978 Brain Res. 120 : 113

KLAUSNER, R.D., Fishman, M.C., Karnovsky, M.J. 1979. Nature. 281 : 81

KNISATSCHEK, H., Bauer, K. 1979. J. Biol. Chem. 254 : 10936

KOCH, Y., Boram, T., Chobsieng, P. 1974. Biochem. Biophys. Res. Commun. 61 : 95

KRIEGER, D.T., Liotta, A.S. 1979. Science 205 : 366

KRIVOY, W.A., Couch, J.R., Henry, J.L., Stewart, J.M. 1979. Fed. Proc. 38 : 2344

KRULICH, W. 1979. Ann. Rev. Physiol. 41 : 603

KRULICH, L., Illner, P., Fawcell, C.P., Quijada, M., McCann, S.M. 1971. In : Growth and Growth Hormone ed. Pecile, A., Muller, E.E. (Excerpta Medica Amsterdam). Pg. 306

LABRIE, F., Bouther, M., Pelletier, G., Borgeat, P., Lemay, A., Gauge, J.J. 1973. Endocrinol. 93 : 903

LABRIE, F., Borgeat, P., Drouin, J., Beaulieu, M., Lagace, L., Ferland, L., Raymond, V. 1979. Ann. Rev. Physiol. 41 : 555

LARSSON, L., Golterman, N., de Magistris, L., Rehfeld, J.F., Schwartz, T.W. 1979. Science 205 : 1393

LECLERC, R., Pelletier, G., Puviani, R., Arimura, A., Schally, A.V. 1976. Mol. Cell. Endocrinol. 4 : 257

LI, C.H., Chung, D. 1976. Proc. Natl. Acad. Sci. U.S.A. 73 : 1145

LIEBERBURG, I., McEwan, B.S. 1977. Endocrinol. 100 : 588

LLINAS, R.I.R. 1977. In : Approaches to the Cell Biology of Neurons. Society for Neuroscience Symposia Vol. II ed. Cowan, W.M., Ferrendelli, J.A. (Society for Neuroscience Bethesda) Pg. 139

LOUDES, C., Joseph-Bravo, P., Le Blanc, P., Kordon, C. 1978. Biochem. Biophys. Res. Commun. 83 : 921

McCANN, S.M. 1962. Am. J. Physiol. 202 : 395

McILWAIN, H. 1952. Biochem. J. 52 : 289

McKELVY, J.F., Le Blanc, P., Loudes, C., Perrie, S., Grimm-Jorgensen, Y., Kordon, C. 1976. Biochem. Biophys. Res. Commun. 73 : 507

MAEDA, K., Frohman, L.A. 1978. Endocrinol. 103 : 1903

MAEDA, K., Frohman, L.A. 1980. Endocrinol. 106 : 1837

MALAISSE, W.J., Devis, G., Somers, G. 1977. Experienta 33 : 1035

MARCANO DE COTTE, D., de Menezes, C.E.L., Bennett, G.W., Edwardson, J.A. 1980. Nature 283 : 487

MARKS, N. 1976. In : Subcellular Mechanisms in Reproductive Endocrinology Ed. Naftdin, F., Ryan, K.J., Davies, J.

MARKS, N. 1977. In : Peptides in Neurobiology. Ed. Gainer, H. (Plenum Press New York) Pg. 221

MARSHALL, J.C., Odell, W.D. 1975. Proc. Soc. Exp. Biol. Med. 149 : 351

MILLAR, R.P., Aehnelt, C., Rossier, G. 1977. Biochem. Biophys. Res. Commun. 74 : 720

MILLAR, R.P., Denniss, P., Tobler, C., King, J.C., Schally, A.V., Arimura, A. 1978. In : Cell Biology of hypothalamic neurosecretion. Eds. Vincent, J.D. Kordon, C. (Editions du Centre National de la Recherche Scientifique Paris) Pg. 487

MILLAR, R.P. 1978. J. Endocrinol. 77 : 429

MOSS, R.L. 1979. Ann. Rev. Physiol. 41 : 617

MUELLER, G.P., Simpkins, J., Meites, J., Moore, K.E. 1976. Neuroendocrinol. 20 : 121

MULLER, E.E., Nisticó, G., Scapagnini, U. 1977. Neurotransmitters and Anterior Pituitary Function (Academic Press, New York)

NAIR, R.M.G., Barrett, J.F., Bowers, C.Y., Schally, A.V. 1970. Biochemistry 9 : 1103

NAKAZATO, Y., Douglas, W.W. 1974. Nature 249 : 479

NANSEL, D.D., Aujer, M.S., Meinzer, W.H., Bogdanove, E.M. 1979. Endocrinol. 104 : 524

NEGRO-VILAR, A., Ojeda, S.R., Arimura, A., McCann, S.M. 1978. Life Sci. 25 : 1493

NEGRO-VILAR, A., Ojeda, S.R., McCann, S.M. 1979. Endocrinol. 104 : 1749

NETTER, F.H. 1965. In : Endocrine and selected metabolic diseases. Ed. Farsham, P.H. (Ciba, New York) Vol. 4:4

OHTA, M., Narahashi, T., Keeler, R.F. 1973. J. Pharmacol. and Exp. Therap. 184 : 143

OJEDA, S.R., Negro-Vilar, A., McCann, S.M. 1979. Endocrinol. 104 : 617

OLIVER, C., Mical, R.S., Porter, J.C. 1977. Endocrinol. 101 : 598

OUCHINNIKOV, Y.A. 1979. Europ. J. Biochem. 94 : 321

PALKOVITS, M., Arimura, A., Brownstein, M.J., Schally, A.V., Saavedra, J.M. 1974. Endocrinol. 95 : 554

PARSONS, J., Erlandsen, S., Hegre, O., McEvoy, R., Elde, R.P. 1976. J. Histochem. Cytochem. 24 : 872

PAVEL, S., Juca, N., Calb, M., Goldstein, R. 1979. Endocrinol. 104 : 517

PELLETIER, G., Labrie, F., Puviani, R., Arimura, A., Schally, A.V. 1974. Endocrinol. 95 : 314

PELLETIER, G. 1976. In : Hypothalamus and Endocrine Functions. Ed. Labrie, F., Meites, J., Pelletier, G. (Plenum Press, New York) Pg. 433

PETERSON, R.E., Guillemin, R. 1974. Am. J. of Med. 57 : 501

POPA, G.T., Fielding, V. 1930. J. Anat. 65 : 88

PRADAYROL, L., Jörnvall, H., Mult, V., Ribet, A. 1980. Febs. Lett. 109 : 55

QUIJADA, M., Krulich, L., Fawcett, C.P., Sundberg, P.K., McCann, S.M. 1971. Fed. Proc. 30 : 197

RAHAMIMOFF, H., Abramovitz, E. 1978. Febs. Lett. 89 : 223

REED, P.W., Lardy, H.A. 1972. J. Biol. Chem. 247 : 6970

RICHARDSON, S.B., Hollander, C.S., D'Eletto, R., Greenleaf, P.W., Thaw, C. 1980. Endocrinol. 107 : 122

RIVIER, C., Brown, M., Vale, W. 1977. Endocrinol. 100 : 751

RORSTAD, O.P., Epelbaum, J., Brazeau, P., Martin, J.B. 1979. Endocrinol. 105 : 1083

ROTSZTEJN, W.H., Charli, J.L., Pattou, E., Epelbaum, J.E., Kordon, C. 1976 Endocrinol. 99 : 1663

ROTSZTEJN, W.H., Charli, J.L., Pattou, E., Kordon, C. 1977. Endocrinol. 101 : 1475

ROTSZTEJN, W.H., Drouva, S.V., Pattou, E., Kordon, C. 1978. Nature 274 : 281

RUDENSTEIN, R.S., Bigdeli, H., MacDonald, M.H., Snyder, P.J. 1979. J. Clin. Invest. 63 : 262

RUFENER, C., Orci, L., Nordmann, J.J., Rouiller, C. 1972. Gen. Comp. Endocrinol. 18 : 621

RUSSELL, J.T., Thorn, N.A. 1973. Acta. Physiol. Scand. 87 : 20A

SACHS, Haller, E.W. 1968. Endocrinol. 83 : 251

SANDOW, J., Heptner, W., Niemann, E. 1973. Arch. Pharmac. Exp. Path. 277 : R64

SCHALLY, A.V., Arimura, A., Kastin, A.J., Matsuo, H., Baba, Y., Redding, T.W., Nair, R.M.G., Debeljuk, L., White, W.F. 1971. Science 173 : 1036

SCHALLY, A.V., Arimura, A., Kastin, A.J. 1973. Science 179 : 341

SCHALLY, A.V., Dupont, A., Arimura, A., Redding, T.W., Linthicum, G.L. 1975. Fed. Proc. Fed. Am. Soc. Exp. Biol. 35 : 584

SCHALLY, A.V., Huang, W.Y., Chang, R.C.C., Arimura, A., Redding, T.W., Millar, R.P., Hunkapillar, M.W., Hood, L.E. 1980. Proc. Natl. Acad. Sci. U.S.A. in the press

SCHLAEPFER, W.W. 1977. Brain Res. 136 : 1

SETCHELL, B.P., Jacks, F. 1974. Endocrinol. 62 : 675

SELMANOFF, M.K., Bradkin, L.D., Weiner, R.I., Siiteri, P.K. 1977. Endocrinol. 101 : 841

SHEPPARD, M.C., Kronheim, S., Pimstone, B.L. 1979(a). J. Neurochem. 32 : 647

SHEPPARD, M.C. 1979(b). PhD. Thesis, U.C.T.

SHEPPARD, M.C. 1980. In : Neuropeptides : Biochemical and Physiological Studies. Ed. Millar, R.P. (Churchill Livingstone, Edinburgh)

SHIN, S.H., Howitt, C.J., Milligan, J.V. 1974. Life Sci. 14 : 2491

SHIN, S.H., Howitt, C.J. 1976. Neuroendocrinol. 21 : 165

SIMPKINS, J.W., Huang, H.H., Advis, J.P., Meites, J. 1979. Biol. Reprod. 60 : 625

SIMPKINS, J.W., Kalra, P.S., Kalra, S.P. 1980. Neuroendocrinol. 30 : 94

SKOU, J.C. 1965. Physiol. Rev. 45 : 486

SOMJEN, G.G. 1979. Ann. Rev. Physiol. 41 : 159

SPIESS, J., Vale, W. 1978. Metabolism. 27 : 1175

SPIESS, J., Vale, W. 1980. Biochemistry. 19 : 2861

STEINER, D.F., Cunningham, D.D., Spigelman, Z., Aten, B. 1967. Science. 157 : 697

STEVENS, C.F. 1979. Sci. Am. 241 : 48

STOECKART, R., Jansen, H.G., Kreike, A.J. 1975. Cell. Tiss. Res. 155 : 11

STUMPF, W.E., Sar, M. 1977. Fed. Proc. 36 : 1973

SUNDBERG, D.K., Knigge, K.M. 1978. Brain Res. 139 : 89

TAN, A.T., Tsang, D., Renaud, L.P., Martin, J.B. 1977. Brain Res. 123 : 193

TAUBENHAUS, M., Saskin, S. 1941. Endocrinol. 29 : 958

TERRY, L.C., Martin, J.B. 1978. Ann. Rev. Pharmacol. and Toxicol. 18 : 111

THORN, N.A., Russell, J.T., Torp-Pedersen, C., Treiman, M. 1978. Ann. N.Y. Acad. Sci. U.S.A. 307 : 618

VALE, W., Grant, G., Amoss, M., Blackwell, R., Guillemin, R. 1972. Endocrinol. 91 : 562

VALE, W., Ling, N., Rivier, J., Villarreal, J., Rivier, C., Douglas, C., Brain, M. 1976. Metabolism 25 : 1401

VALE, W., Rivier, C., Brown, M. 1977. Ann. Rev. Physiol. 39 : 473

VERITY, M.A., Brown, W.J., Ganong, M.K. 1979. J. Neurochem. 32 : 1295

VIJAYAN, E., Krulich, L., McCann, S.M. 1978. Neuroendocrinol. 26 : 174

WAKABAYASHI, I., Miyazawa, Y., Kanda, M., Miki, N., Demura, R.H., Shizume, K. 1977. Endocrinol. (Jpn) 24 : 601

WATSON, S.J., Akil, H., Richard, C.W., Barchas, J.D. 1978. Nature 275 : 226

WHEATON, J.E., Krulich, L., McCann, S.M. 1975. Endocrinol. 97 : 30

WHITE, W.F., Hedlund, M.T., Wilber, G.G., Rippel, R.H., Johnson, E.S., Wilber, J.F. 1974. Endocrinol. 94 : 1422

WILLIAMS, J.A., Wolff, J. 1971. Proc. Natl. Acad. Sci. U.S.A. 67 : 1901

WILSON, L., Bornburg, J., Mizel, S., Grisham, L., Creswell, K. 1974. Fed. Proc. 33 : 158

WISLOCKI, G.B., King, L.S. 1936. Am. J. Anat. 58 : 421

ZIMMERMAN, E.A., Hsu, K.C., Ferin, M., Kozlowski, G.P. 1974. Endocrinol. 95 : 1

ZIMMERMAN, E.G. 1979. Fed. Proc. 38 : 2286

ZINGG, H.H., Patel, Y.C. 1979. Biochem. Biophys. Res. Commun. 90 : 466

ZINGG, H.H., Patel, Y.C. 1980. Biochem. Biophys. Res. Commun. 93 : 1274