THYROTROPHIN RELEASING HORMONE, SOMATOSTATIN AND LUTEINIZING HORMONE RELEASING HORMONE: ASPECTS OF THEIR SYNTHESIS, RELEASE AND ACTIONS

by

William John Sheward

A thesis
submitted for the degree of
Doctor of Philosophy
University of Edinburgh
Summer, 1986

ABSTRACT

The aim of this thesis was to investigate some aspects of the hypothalamic control of hormone release from the anterior pituitary, and the immunoreactive forms (IR) of neuropeptides released *in vivo* into rat hypophysial portal blood with special emphasis on somatostatin (SS), luteinizing hormone releasing hormone (LHRH) and thyrotrophin releasing hormone (TRH).

The tetradecapeptide, somatostatin-14 (SS-14), is formed by the cleavage of a larger precursor molecule to produce a number of IR forms with intermediate molecular weight. SS-14, the N-terminally extended form, SS-28, and SS-28(1-12) are released into hypophysial portal vessel blood and the release of each form was increased (≈ 5 fold) by electrical stimulation of the median eminence (ME). These observations together with data on their biological activity, suggest that SS-28 as well as SS-14, is an hormonally active peptide.

High performance liquid chromatography (HPLC) combined with radioimmunoassay showed that LHRH-IR in rat hypophysial portal blood co-eluted as a single peak with synthetic LHRH and with LHRH-IR in rat and mouse hypothalami. No LHRH could be detected in hypothalamic extracts from hypogonadal (*hpg*) mice, in spite of which the pituitary gland of the *hpg* mouse contains a small amount of bioactive as well as immunoreactive LH which can be released by exogenous LHRH. The priming effect of LHRH can also be elicited in the *hpg* mouse, and these data show that previous exposure of gonadotrophes to LHRH is not required for the synthesis of a small amount of LH, or the releasing and priming actions of LHRH. Studies on the secretion of LHRH and LH during suckling in lactating rats, and after the administration of naloxone or tetrahydrocannabinol (THC) to female rats at different times during the oestrous cycle showed that THC reduced LHRH output and blocked the pro-oestrous surge of LH but naloxone had no effect on plasma LH concertrations or LHRH release. Peripheral plasma concentrations of LH and portal plasma concentrations of LHRH were low in lactating animals and were not reduced by suckling.

Immunoreactive TRH in portal blood was resolved by HPLC into 3 peaks of about equal magnitude, one of which corresponded to synthetic TRH. The two other immunoreactive peaks in portal blood did not correspond in retention time to any known metabolite of TRH and, since they were not present in the hypothalamus, could not be attributed to a stored TRH precursor. The injection of anti-TRH serum blocked the thyrotrophin but not the prolactin responses to electrical stimulation of the ME or paraventricular nuclei in male or pro-oestrous female rats, or in response to suckling in the lactating rat. Thus, TRH is not crucial for prolactin release in either male or female rats.

The secretion of anterior pituitary hormones was also investigated in male rats in which the catecholamine (CA)-containing nerve terminals of the median eminence and neurointermediate lobe were lesioned by of 6-hydroxydopamine. The results showed that these CA neurons inhibit the secretion of thyrotrophin and adrenocorticotrophin, but may not play an important role in the control of gonadotrophin secretion in the male.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor George Fink for giving me the opportunity to conduct these studies and for his unfailing encouragement and advice throughout the course of the project. Many thanks are due to my friends and colleagues in the MRC Brain Metabolism Unit and the University Department of Pharmacology; to Dr. A.G. Watts and Dr. A.J. Harmar for introducing me to the technique of high performance liquid chromatography and for all their help and advice, and to Graciela Sanchez Watts and John Bennie for their assistance with radioimmunoassays. My thanks also to Professor Graeme Smith for his collaboration with some of the experiments and for much helpful discussion, and to Drs. R.P. Millar, R. Benoit, T.M. Plant and I.C.A.F. Robinson for their collaboration.

Grateful thanks to Mrs. Jean Hunter and her staff for their expert care of the animals and to Mr. J. Lissimore and Mr. J. Hatton in the workshops for their help, particularly in the manufacture of electrodes. Drs. G.D. Niswender, L.E. Reichert and A.F. Parlow (of the NIADDK), and ICI Pharmaceuticals provided the materials for the majority of the radioimmunoassays. The antiserum to TRH was generously supplied by Dr. H.M. Fraser, MRC Reproductive Biology Unit to whom I am also grateful for useful discussion. Many thanks to Jo Donnelly for deciphering my writing and typing this manuscript.

Finally I should like to thank Mum and Dad for all their support over many years, this thesis is for them and:

"To all friends around the Wrekin".

LIST OF PUBLICATIONS

Some of the results presented in this thesis have been published as follows:

Fink, G., Sheward, W.J. and Charlton, H.M. (1982). Priming effect of luteinizing hormone releasing hormone in the hypogonadal mouse. J. Endocr. 94, 283-287.

Smith, G.C., Sheward, W.J. and Fink, G. (1982). Effect of 6-hydroxydopamine lesions of the median eminence and neurointermediate lobe on the secretion of pituitary hormones in the male rat. Brain Res. 246, 330-333.

Fink, G., Fraser, H.M., Harmar, A.J. and Sheward, W.J. (1982). Thyrotropin-releasing hormone like immunoreactivity in rat hypophysial portal blood not confined to pGlu-His-Pro NH_2. J. Physiol. 334 124P.

Millar, R.P., Sheward, W.J., Wegener, I. and Fink, G. (1983). Somatostatin-28 is an hormonally active peptide secreted into hypophysial portal vessel blood. Brain Res. 260, 334-337.

Sheward, W.J., Harmar, A.J., Fraser, H.M. and Fink, G. (1983). Thyrotropin-releasing hormone in rat pituitary stalk blood and hypothalamus: studies with high performance liquid chromatography. Endocrinology 113, 1865-1869.

Sheward, W.J., Benoit, R. and Fink, G. (1984). Somatostatin-28 (1-12)-like immunoreactive substance is secreted into hypophysial portal vessel blood in the rat. Neuroendocrinology 38, 88-90.

Fink, G., Fraser, H.M. and Sheward, W.J. (1984). Effect of passive immunization with anti-TRH serum on electrically stimulated release of prolactin and TSH in the male rat. J. Physiol. 346, 123P.

Fink, G., Sheward, W.J. and Plant, T.M. (1984). The hypogonadal mouse pituitary contains bioactive LH. J. Reprod. Fert. 70, 277-280.

Fink, G., Fraser, H.M. and Sheward, W.J. (1984) Release of TSH and prolactin in response to suckling in anaesthetized lactating rats passively immunized with anti-TRH serum. J. Physiol. 357, 98P.

Sheward, W.J., Watts, A.G., Fink, G. and Smith, G.C. (1985) Effects of intravenously administered 6-hydroxydopamine on the content of monoamines in the median eminence and neurointermediate lobe of the rat. Neurosci. Lett. 55, 141-144

Sheward, W.J., Fraser, H.M. and Fink, G. (1985) Effect of immunoneutralization of thyrotrophin-releasing hormone on the release of thyrotrophin and prolactin during suckling or in response to electrical stimulation of the hypothalamus in the anaesthetized rat. J. Endocr. 106, 113-119.

Sheward, W.J., Harmar, A.J. and Fink, G. (1985) LHRH in the rat and mouse hypothalamus and rat hypophysial portal blood: confirmation of identity by high performance liquid chromatography. Brain Res. 345, 362-365.

I declare that the studies presented in this thesis are the result of my own independent investigation with the exception of:

i) Chromatographic separation and radioimmunoassay of somatostatin-14 and -28, and radioimmunoassay for SS-28(1-12) were carried out by Dr. R.P. Millar (University of Capetown) and Dr. R. Benoit (San Diego) respectively (Chapter 3) and radioimmunoassay of oxytocin (Chapter 8) by Dr. I.C.A.F. Robinson (Mill Hill).

ii) Bioassay of LH was carried out by Dr. T.M. Plant (Pittsburgh) (Chapter 7).

iii) Experiments described in Chapter 9 were done in collaboration with Professor G.C. Smith (Melbourne).

This work has not been, and is not being concurrently submitted for candidature for any other degree.

CONTENTS

	page
Abstract	iii
Acknowledgements	iv
List of publications	v
CHAPTER 1: Introduction	1
1.1 Neurohumoral control of anterior pituitary hormone secretion	2
1.2 Thyrotrophin releasing hormone (TRH)	5
1.3 Somatostatin	10
1.4 Luteinizing hormone releasing hormone (LHRH)	14
1.5 Neural control of prolactin secretion	20
1.6 Catechol- and indoleaminergic innervation of the hypothalamus	23
1.7 Biosynthesis of neuropeptides	27
1.8 Aims of this thesis	29
CHAPTER 2: Materials and Methods	
2.1 Animals	31
2.2 Surgical and experimental procedures	32
2.3 Fluorescence histochemistry	40
2.4 High performance liquid chromatography (HPLC)	42
2.5 Radioimmunoassays and protein binding assays	47
CHAPTER 3: The Immunoreactive Forms of Somatostatin Released into Hypophysial Portal Vessel Blood	
3.1 Introduction	56
3.2 Materials and methods	58
3.3 Results	59
3.4 Discussion	61

page

CHAPTER 4: Thyrotrophin Releasing Hormone in Hypophysial Portal Blood, Hypothalamus and Pituitary Gland: Studies with High Performance Liquid Chromatography

4.1 Introduction 66
4.2 Materials and methods 68
4.3 Results 71
4.4 Discussion 74

CHAPTER 5: Effect of Anti-TRH Serum on the Release of Thyrotrophin and Prolactin During Suckling or in Response to Electrical Stimulation of the Hypothalamus

5.1 Introduction 82
5.2 Materials and methods 84
5.3 Results 87
5.4 Discussion 88

CHAPTER 6: Chromatographic Studies of Immunoreactive LHRH in the Rat and Mouse Hypothalamus and in Rat Hypophysial Portal Blood

6.1 Introduction 99
6.2 Materials and methods 100
6.3 Results 102
6.4 Discussion 102

CHAPTER 7: The Priming Effect of LHRH, and Determination of the Biological Activity of LH in the Hypogonadal (hpg) Mouse

7.1 Introduction 106
7.2 Materials and methods 107
7.3 Results 110
7.4 Discussion 112

page

CHAPTER 8: Effect of Δ^1-Tetrahydrocannabinol, Naloxone or the Suckling Stimulus on the Release of LHRH into Hypophysial Portal Vessel Blood

8.1 Introduction 118
8.2 Materials and methods 122
8.3 Results 125
8.4 Discussion 128

CHAPTER 9: The Effects of Intravenously Administered 6-Hydroxydopamine on the Content of Monoamines in the Median Eminence and Neurointermediate Lobe, and on Pituitary Hormone Secretion in the Male Rat

9.1 Introduction 140
9.2 Materials and methods 142
9.3 Results 143
9.4 Discussion 146

CHAPTER 11: Summary and Conclusions 155

References 166

Appendices

CHAPTER 1

Introduction

The role of the hypothalamus in the control of hormone secretion from the anterior pituitary gland was suggested by early observations of the effects of electrical stimulation of the hypothalamus and of pituitary stalk section, and the transplantation of the pituitary gland to sites distant from the median eminence. Such studies led to the hypothesis that the release of anterior pituitary hormones was controlled by inhibitory and facilitatory substances which are released from nerve terminals in the median eminence and transported by way of the hypophysial portal vessels to the anterior pituitary gland (Green and Harris, 1947; Harris, 1955,1972). A crucial step in the development of this hypothesis was the isolation and characterization of the peptide hypothalamic releasing (or release inhibiting) factors (or hormones). The availability of pure preparations of the synthetic peptides enabled the production of specific antisera and development of radioimmunoassays (RIA). By combination of these assays with techniques which enabled the dissection of discrete brain nuclei (Palkovits, 1973), the presence of the releasing factors was demonstrated in regions of hypothalamic and extrahypothalamic brain indicating that these peptides might also serve a neurotransmitter role in the central nervous system. The cell bodies of the neurons which project to the median eminence have been demonstrated by immunohistochemistry and, from studies of the effect of various lesions and transections, the possible routes of some of these pathways have been mapped. The development of techniques for the exposure of the hypophysial portal vessels and the collection of portal blood from the cut pituitary stalk of anaesthetized rats, permitted direct studies of the release of

hypothalamic peptides *in vivo*. The physiological importance of known or putative hypothalamic-pituitary regulatory neuropeptides has also been determined by the administration of specific antisera to neutralize the endogenous peptides (passive immunization/immunoneutralization). Pharmacological and histochemical studies have broadly identified the role of monoamine and amino acid transmitters in the regulation of hypothalamic peptide secretion. Recent studies have focussed attention on the synthesis of the hypothalamic peptides and shown that the peptides are synthesized as components of larger precursors which are processed by enzymatic degradation. These precursor molecules may contain several copies of a single peptide or a number of structurally unrelated peptides which may share common functions. Cleavage at different sites within the precursor molecule may generate different products or, as is the case with somatostatin, extended forms of the peptide which are themselves biologically active.

1.1 NEUROHUMORAL CONTROL OF ANTERIOR PITUITARY HORMONE SECRETION

The history of the development of the neurohumoral hypothesis has been extensively reviewed (Harris, 1955, 1972; Fink, 1976). Briefly, the first descriptions of the prominent blood vessels which run along the pituitary stalk and terminate in fine capillary networks in the anterior pituitary gland and median eminence (ME) were recorded by Popa and Fielding (1930) and their observations of these 'hypophysial portal vessels' were subsequently confirmed by Wislocki and King (1936). For many years the direction of blood flow through the vessels was a subject of debate (Popa and Fielding,

1930, 1933; Wislocki, 1937, 1938), but this was finally resolved by direct observations in anaesthetized amphibians (Houssay, Biasotti and Sammartino, 1935; Green, 1947) and rats (Green and Harris, 1949) which showed that blood flowed from the hypothalamus to the pituitary gland. Several workers have since suggested the presence of a vascular link between the anterior and posterior (neural lobe) pituitary gland and also suggest that some blood may pass from the anterior pituitary gland to the ME, possibly by way of the neural lobe (Torak, 1964; Ambach, Palkovits and Szentagothai, 1976; Oliver, Mical and Porter, 1977; Bergland and Page, 1978, 1979). Direct observations of pituitary blood flow in the aneasthetized pig have demonstrated a limited flow of blood from the neural lobe to the anterior pitutary but failed to demonstrate the passage of blood from the anterior pituitary gland through the neural lobe to the ME (Page, 1983).

The physiological importance of the vascular link connecting the hypothalamus and the pituitary gland was demonstrated in a series of elegant studies which investigated the effects of pituitary stalk section and of the grafting of pituitary tissue to sites distant from the sella turcica. Section of the pituitary stalk was followed by rapid vascular regeneration across the cut and restoration of pituitary function (e.g. the majority of animals showed oestrous cyclicity and became pseudopregnant in response to sterile matings (Harris, 1950). When the regeneration of the vascular connection was prevented by insertion of a small plate in the cut, animals remained in anoestrous and the gonads became atrophied (Harris, 1950). Transplantation studies, in which pituitary gland tissue was

grafted into hypophysectomized rats, demonstrated the restoration of normal pituitary function when the graft was placed under the ME and revascularized by the hypophysial portal vessels but not when the grafts were placed under the temporal lobe of the brain where they became vascularized by the cerebral and dural vessels (Harris and Jacobsohn, 1952). These results were confirmed and extended in further experiments by Nikitovitch-Winer and Everett (1958, 1959) who, by a two step operation, showed that there was a loss of reproductive function when pituitary tissue was grafted under the kidney capsule whereas normal function was restored by the later retransplantation of the pituitary beneath the median eminence.

The criteria established by Geoffrey Harris (1972) for the demonstration of a hypothalamic releasing factor are "a) that the substance is extractable from hypothalamic or stalk: median eminence tissue; b) that it is present in hypophysial portal blood in greater amounts that in systemic blood; and c) that varying concentrations of the substance in portal blood are related to varying secretion rates of one of the anterior pituitary hormones under a number of different experimental and environmental conditions". These criteria have been fully satisfied for LHRH. Although somatostatin and TRH are largely undetectable in peripheral blood, both have been shown to be present in physiologically active concentrations in hypophysial portal blood. Whilst there is much indirect evidence to suggest that the secretion rates of somatostatin and TRH vary under experimental conditions, in contrast to the extensive studies of LHRH release, there have been few direct studies of the release of these peptides into portal blood under varying physiological

conditions.

1.2 THYROTROPHIN RELEASING HORMONE (TRH)

1.2.1 Isolation and characterization of TRH

In the 1960's intense efforts were directed to the isolation of TRH from large quantities of porcine or bovine hypothalami. Finally in 1966 a small quantity of TRH was isolated and shown to contain only three amino acids (histidine, proline and glutamic acid) which were present in equimolar quantities (Schally, Bowers, Redding and Barrett, 1966). Further work in the laboratories of Schally and Guillemin resulted in the determination of the amino acid sequence and structure of porcine and bovine TRH respectively, and the subsequent chemical synthesis of this molecule (Boler, Enzmann, Folkers *et al*, 1969; Burgus, Dunn, Desiderio and Guillemin, 1969; Burgus, Dunn, Ward *et al*, 1969; Folkers, Enzmann, Boler *et al*, 1969; Burgus, Dunn, Desiderio *et al*, 1970). From these, and other studies, TRH was shown to be the tripeptide amide -

(pyro)Glu-His-Pro NH_2.

1.2.2 Hypothalamic distribution of TRH

Radioimmunoassay of the TRH content of microdissected brain nuclei showed high concentrations of immunoreactive TRH in many hypothalamic and extrahypothalamic brain areas. Within the hypothalamus the largest concentration was in the ME but high concentrations were also found in regions adjacent to the third ventricle including the periventricular, paraventricular (PVN) and arcuate nuclei (Brownstein, Palkovits, Saavedra, Bassiri and Utiger, 1974). Losses during tissue processing due to the high solubility

of TRH prevented detailed immunohistochemistry until it was found that these technical difficulties were overcome by the application of a rapid tissue fixation technique using acreolin (Lechan and Jackson, 1982). High concentrations of TRH were demonstrated along the length of the ME in the external layer adjacent to the portal vessels and in the vascular organ of the lamina terminalis (OVLT) and posterior pituitary gland. In animals pre-treated with colchicine in order to block axonal transport, the perikarya of the TRH-containing neurons were demonstrated in several hypothalamic areas including the suprachiasmatic preoptic nucleus and dorsomedial nucleus. The highest numbers of TRH-perikarya were contained in the parvocellular division of the PVN where many cells were seen in the anterior and medial subdivisions, which had neuronal processes directed ventromedially towards the third ventricle. Using retrograde tracers (horseradish peroxidase and true blue) injected into the external layer of the ME, it has been shown that cell bodies in the parvocellular division of the PVN project to the ME (Wiegland and Price, 1980; Lechan, Nestler, Jacobson and Reichlin, 1980) and it is likely that the TRH-containing cell bodies in the PVN are the origin of the TRH fibres terminating in the ME.

1.2.3 Regulation of thyrotrophin secretion

The role of the hypothalamus in the control of thyrotrophin secretion was demonstrated by early experiments which relied upon measuring changes in thyroid function as an index of thyrotrophin secretion: e.g. the ability of grafted pituitary tissue to maintain normal thyroidal weight and histology when the graft was placed under the ME, but not when transplanted under the temporal lobe

(Harris and Jacobsohn, 1952), and the increased release of ^{131}I from the thyroid which followed electrical stimulation of the anterior hypothalamus (Harris and Woods, 1958). The development of RIAs for thyrotrophin facilitated more detailed studies and the hypothesis was formed that thyrotrophin output depends upon a balance between the stimulatory effects of TRH and the negative feedback exerted by the thyroid hormones which modulate the pituitary responsiveness to TRH (Brown-Grant, 1969; Reichlin, Martin, Mitnick et al, 1972; Morley, 1981).

The basal concentration of thyrotrophin in the plasma was decreased after passive immunization with anti-TRH sera (Koch, Goldhaber, Fireman et al, 1971; Szabo, Kovathana, Gordon and Frohman, 1978; Harris, Christianson, Smith et al, 1978; Fraser and McNeilly, 1983), but passive immunization only partially reduced the hypersecretion of thyrotrophin which follows thyroidectomy (Harris et al, 1978; Szabo et al, 1978). The plasma concentration of thyrotrophin was reduced by deafferentation of the MBH (Hefco, Krulich, Aschenbrenner, 1975; Fukuda and Greer, 1977) and lesions of the PVN (Martin, Boshans, Reichlin, 1970; Aizawa and Greer, 1981). Conversely, plasma thyrotrophin concentration was elevated by electrical stimulation of either the ME or PVN (Martin and Reichlin, 1972).

Since early experiments which showed that sectioning the pituitary stalk of the rat prevented the hypertrophy of the thyroid in response to cold exposure (Uotila, 1939; Brolin, 1945), a large number of studies have shown that increased thyrotrophin secretion followed short or long term cold exposure. The dependence of this

effect upon TRH release was shown by blockade of the response by passive immunization with anti-TRH serum (Szabo and Frohman, 1977; Harris et al, 1978). In conscious rats in which a push-pull cannula had been implanted into the ME, there was a significant increase in TRH release into the perfusate when the rats were exposed to cold (Arancibia, Tapia-Arancibia, Assenmacher and Astier, 1983). Bilateral electrolytic lesions of the paraventricular nuclei abolished the thyrotrophin response to cold (Ishikawa, Kakegawa and Suzuki, 1984).

Many studies have shown that the thyroid hormones decrease basal and TRH stimulated secretion of thyrotrophin predominantly by a direct action on the pituitary gland (e.g. Averill, 1969; Bowers, Schally, Enzmann, Boler and Folkers, 1970; Reichlin, Martin, Boshans et al, 1970; Bowers, Schally, Reynolds and Hawley, 1967). The major hormone produced by the thyroid is thyroxine (T_4), though some triiodothyronine (T_3) is also produced. Conversion of T_4 to the more potent T_3 occurs by monodeiodination in the anterior pituitary and in other peripheral sites (Larsen, Dick, Markovitz, Kaplan and Gard, 1979; Connors and Hedge, 1981). In addition, T_4 may also be monodeiodinated to produce the less-important reverse T_3 (Baxter, Eberhardt, Apriletti et al, 1979). Approximately only 0.5% T_3 and 0.05% T_4 are present in plasma as free hormone, the majority is bound to plasma binding proteins (Brown-Grant, 1969). T_3 is approximately ten times more potent than T_4 in the inhibition of the synthesis and release of thyrotrophin (Larsen and Frumess, 1977). Both the feedback and the metabolic effects of the thyroid hormones require the entry of the hormones into the target

cell (in the case of the pituitary gland, the thyrotrophe) and binding to a nuclear binding site which has greater affinity for T_3 than T_4 (Oppenheimer, 1979). Evidence obtained from _in vitro_ studies suggests that T_3 may induce the synthesis of a protein which blocks thyrotrophin release (Gard, Bernstein and Larsen, 1981). It has also been shown that T_3 may decrease the number of the binding sites for TRH on the thyrotroph (Connors and Hedge, 1981; Hinkle, Perrone and Schonbrunn, 1981).

There have been few studies of direct effects of the thyroid hormones on TRH output, but an action of T_3 and T_4 on the hypothalamus to regulate TRH secretion cannot be discounted. In the majority of studies there were no consistent changes in hypothalamic TRH content after the administration of thyroid hormones, or after thyroidectomy or hypophysectomy (e.g. Kordon, Marcus, Winokur and Utiger, 1977). The increased ability of rat hypophysial portal blood to stimulate the _in vitro_ release of thyrotrophin in response to electrical stimulation of the anterior hypothalamus was not prevented by pre-treatment with T_3 (Wilbur and Porter, 1970). Similarly, no consistent differences were reported in the concentration of immunoreactive TRH in portal blood from normal, hypothyroid or thyroid hormone-treated rats (Ching and Utiger, 1983). In support of a hypothalamic feedback mechanism, Belchetz, Gredley, Bird and Himsworth (1978) showed that bilateral injection of T_3 into the dorsomedial nuclei or lateral hypothalamic area of hypothyroid monkeys resulted in a rapid decrease in serum thyrotrophin concentration; the low dose of T_3 used produced only a small change in thyrotrophin secretion when injected directly into

the pituitary gland.

Somatostatin may also participate in the regulation of thyrotrophin secretion. Somatostatin inhibits both basal and TRH-stimulated release of thyrotrophin (Vale, Rivier, Brazeau and Guillemin, 1974; Belanger, Labrie, Borgeat *et al*, 1974). Conversely, passive immunization with anti-somatostatin serum resulted in elevated plasma thyrotrophin concentrations (Arimura and Schally, 1976; Chihara, Arimura, Chihara and Schally, 1978) and enhanced the thyrotrophin response to cold exposure (Ferland, Labrie, Jobin, Arimura and Schally, 1976). Hypothalamic somatostatin content is decreased in hypothyroid rats and restored to normal by treatment with T_3 (Berelowitz, Maeda, Harris and Frohman, 1980). Berelowitz *et al* (1980) also demonstrated that T_3 stimulated the *in vitro* release of somatostatin from hypothalami from normal rats and showed that somatostatin secretion from hypothalami from hypothyroid rats was reduced.

1.3 SOMATOSTATIN

1.3.1 Isolation and characterization of somatostatin

Extracts of ovine hypothalami were shown to contain a substance which inhibited the release of growth hormone (GH) from the rat anterior pituitary gland (Krulich, Dhariwal and McCann, 1968). Subsequently a tetradecapeptide (H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH), which was later termed, somatostatin, was isolated from sheep and pig hypothalami and shown to inhibit the release of immunoreactive GH *in vivo* and *in vitro* (Brazeau, Vale, Burgus *et al*, 1973; Schally, Dupont, Arimura *et al*, 1976).

Somatostatin was also shown to inhibit the secretion of insulin (Alberti, Christensen, Iversen et al, 1973; Mortimer, Carr, Lind et al, 1974), glucagon (Mortimer et al, 1974), gastrin (Bloom, Mortimer, Thorner et al, 1974) and somatostatin (Vale et al, 1974; Belanger et al, 1974).

1.3.2 Distribution

Somatostatin has been shown by immunohistochemistry to be widely distributed throughout the CNS (Johansson, Hokfelt and Elde, 1984) and, in addition, its presence has been demonstrated in peripheral nerves and secretory cells of the gastro-intestinal tract and in the D cells of the pancreatic islets (Arimura, Sato, Dupont, Nishi and Schally, 1975; Hokfelt, Johansson, Efendic, Luft and Arimura, 1975; Hokfelt, Efendic, Hellerstrom, Johansson, Luft and Arimura, 1975). The highest concentrations of immunoreactive somatostatin in the CNS are found in the ME, where a high concentration of somatostatin-containing fibres form a dense plexus surrounding the hypophysial portal vessels in the external layer (Johansson et al, 1984); smaller concentrations of somatostatin are present in the pre-optic area, medial basal hypothalamus (MBH) and amygdala. Within the hypothalamus, somatostatin-containing cell bodies are found in high numbers in the anterior hypothalamic periventricular nucleus (extending caudally into the parvocellular portion of the PVN); fewer numbers of immunoreactive cells are also present in the arcuate, suprachiasmatic nucleus and ventromedial nucleus (Johansson et al, 1984; Kawano, Daikoku and Saito, 1982). The ME content of somatostatin is depleted by complete or anterolateral deafferentation of the MBH suggesting that the innervation of the

median eminence arises from cell bodies outside of the MBH (Brownstein, Arimura, Fernandez-Durango et al, 1977; Epelbaum, Willoughby, Brazeau and Martin, 1977; Kawano et al, 1982). Krisch (1978) suggested that fibres from the cell bodies in the hypothalamic periventricular nucleus follow a short direct route, along the wall of the third ventricle, to the median eminence. A similar direct pathway was proposed for the projections of the lesser number of somatostatin-containing cell bodies in the preoptic periventricular nucleus (Jew, Leranth, Arimura and Palkovits, 1984). However, from studies of the ME content of somatostatin after various transections, it has been suggested that the somatostatin fibres may leave the hypothalamic periventricular nucleus and project to the ME by way of a loop, through the lateral hypothalamus, which joins the medial forebrain bundle, and enters the MBH mainly in the lateral retrochiasmatic area near the ventral surface (Epelbaum, Tapia-Arancibia, Herman, Kordon and Palkovits, 1981; Makara, Palkovits, Antoni and Kiss, 1983). Some innervation of the ME from fibres originating in the amygdala has also been reported (Crowley and Terry, 1980; Terry and Crowley, 1986).

1.3.3 Regulation of growth hormone secretion

The secretion of GH from the anterior pituitary is regulated by an interaction between the inhibitory and facilitatory influences of somatostatin and a growth hormone releasing factor (GHRH) respectively. The precise details of the interactions between these two peptides is not firmly established. Although many earlier studies had indicated the presence of a hypothalamic factor responsible for the stimulation of GH release (e.g. Deuben and

Meites, 1964; Krulich et al, 1968), it is only recently that peptides (hp GRF-44 and hp GRF-40) with high GH-releasing activity have been isolated and characterized from human pancreatic tumour cells (Guillemin, Brazeau, Bohlen et al, 1982; Rivier, Spiess, Thorner and Vale, 1982). In the rat, the GHRH isolated and characterized from the hypothalamus was found to be a 43 amino acid peptide which showed 67%-70% homology with the sequence of the human GHRH peptides. (Spiess, Rivier and Vale, 1983).

Growth hormone is secreted from the anterior pituitary gland in a pulsatile manner characterized by high amplitude pulses of short duration which occur at approximately 3 to 4 h intervals (Tannenbaum and Martin, 1976). Passive immunization with anti-somatostatin serum resulted in increased basal GH secretion, but did not alter the pulsatile secretion of GH (Ferland et al, 1976; Terry and Martin, 1981) whereas administration of a monoclonal antibody raised against a purified preparation of GHRH resulted in abolition of GH pulses both in normal and in anti-somatostatin treated rats (Wehrenberg, Brazeau, Lubin, Bohlen and Guillemin, 1982). Tannenbaum and Ling (1984) proposed that superimposed upon a tonic release of somatostatin and GHRH were additional rhythmic surges of each peptide, about 180° out of phase with each other, and with a periodicity of 3-4 h. Direct evidence for episodic secretion of these peptides was provided by studies of their secretion into hypophysial portal blood in rats anaesthetized with an anaesthetic combination (Ketamine and Xylazine) which does not block pulsatile GH release (Plotsky and Vale, 1985). It was also suggested that somatostatin may directly, or indirectly, inhibit GHRH secretion,

such that surges of GHRH only occur in the presence of diminished somatostatin secretion (Plotsky and Vale, 1985).

The hypothalamic content of somatostatin is reduced following hypophysectomy - an effect which was reversed or prevented by GH administration (Baker and Yen, 1976; Hoffman and Baker, 1977; Fernandez-Durango, Arimura, Fishback and Schally, 1978; Terry and Crowley, 1980). Daily administration of GH to normal rats increased both the hypothalamic content and the release of somatostatin, and conversely, somatostatin content and release were reduced in animals in which plasma concentrations of GH were reduced by either hypophysectomy or by administration of anti-GH serum (Berelowitz, Firestone and Frohman, 1981). Intracerebroventricular injection of GH resulted (after a latent period of 30-45 minutes) in increased secretion of somatostatin into hypophysial portal blood (Chihara, Minamitani, Kaji, Arimura and Fujita, 1981). The negative feedback regulation of GH secretion implied by these studies, may be mediated either by the direct effect of changes in plasma growth hormone concentration or indirectly by way of the altered synthesis of the hepatic peptide, somatomedin C. GH regulates the production of the somatomedin C (the mediator of the effects of GH on muscular and skeletal growth) and somatomedin C both stimulates the release of somatostatin from the hypothalamus _in vitro_ and directly inhibits the pituitary release of GH (Berelowitz, Szabo, Frohman _et al_, 1981).

1.4 LUTEINIZING HORMONE RELEASING HORMONE (LHRH)

1.4.1 Isolation and characterization of LHRH

Data from several laboratories indicated the presence in the

hypothalamus of a substance capable of releasing LH and FSH from the anterior pituitary gland (e.g. McCann, Taleisnik and Friedman, 1960; Campbell, Feuer and Harris, 1964; Schally and Bowers, 1964). Finally, Schally and his co-workers succeeded in isolating LHRH from porcine hypothalami (Matsuo, Baba, Nair, Arimura and Schally, 1971) and showed it to be a decapeptide with the sequence -

(pyro) Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly NH_2;

a finding confirmed by Guillemin for ovine LHRH (Burgus, Butcher, Amos _et al_, 1972). Since it stimulates the release and synthesis of both LH and FSH, LHRH has long been regarded as the sole hypothalamic releasing factor for both hormones, however, recent data indicates that the possibility of a separate FSH-RH still cannot be discounted. For example, the pro-oestrous surge of FSH was incompletely blocked by the administration of a LHRH-antagonist (Condon, Heber, Stewart, Sawyer and Whitmoyer, 1984). Condon _et al_ (1984) also suggested that the surge in the plasma concentration of FSH which occurs early on the morning of oestrus (at about the time at which ovulation occurs) is completely independent of the hypothalamic release of LHRH, though in this context it should be noted that an increase in the concentration of LHRH in hypophysial portal plasma does occur at this time (Sarkar, Chiappa, Fink and Sherwood, 1976). Lesion of the paraventricular nuclei-dorsal anterior hypothalamus resulted in diminished secretion of FSH, but did not reduce either the plasma concentration of LH or the median eminence content of LHRH (Lumpkin and McCann, 1984). Passive immunization with anti-LHRH serum, or the administration of LHRH-antagonists, blocks the pulsatile secretion of LH seen in

gonadectomized animals, but whilst these treatments reduced the plasma concentration of FSH, neither the frequency nor amplitude of the FSH pulses was affected (de Paolo, 1985; Culler and Negro-Vilar, 1986) suggesting that although LHRH regulates the baseline secretion of FSH, generation of pulsatile FSH secretion is controlled by an LHRH independent mechanism. There is also biochemical evidence which indicates the presence of a bio-active FSH-RH which was partially separated from the LHRH decapeptide by gel filtration of rat hypothalamic extracts (Mizunuma, Samson, Lumpkin et al, 1983).

1.4.2 LHRH pathways

Immunohistochemical studies have shown that within the hypothalamus the highest density of LHRH-containing nerve terminals are located in the lateral palisade zone of the ME, though in addition a dense plexus of LHRH terminals are also found in the OVLT (King and Gerall, 1976; Fuxe Hokfelt, Andersson et al, 1978). Studies in which the afferent connections to the MBH were severed, indicate that most of the LHRH in the ME is derived from cell bodies outside of this area (Weiner, Pattou, Kerdelhue and Kordon, 1975; Brownstein, Arimura, Schally, Palkovits and Kizer, 1976). LHRH-containing cell bodies have been demonstrated in both the anterior and mediobasal hypothalamus of many species, but in the mouse and rat the presence of LHRH-immunoreactive cell bodies in the MBH is controversial (Krey and Silverman, 1983). However, the presence of LHRH cell bodies in the preoptic-suprachiasmatic area of the rat is well established (e.g. Barry, Dubois and Poulain, 1973; Setalo, Vigh, Schally, Arimura and Flerko, 1976; Kawano and Daikoku, 1981; Rethelyi, Vigh, Setalo et al, 1981; Witkin, Paden and

Silverman, 1982). The cell bodies are not arranged in compact nuclei but are diffusely aggregated across several nuclear groups which extend from the septal nuclei and diagnonal band of Broca, through the preoptic region and into the anterior hypothalamic area.

There was a marked reduction in the content of LHRH in the ME after bilateral knife cut lesions were placed in the lateral hypothalamus between the pre-optic area and the ME, whereas midline cuts were ineffective (Jew et al, 1984) suggesting that most of the fibres to the ME follow a lateral hypothalamic route through the medial forebrain bundle. Immunohistochemical studies have shown that the fibres from the cell bodies in the preoptic area to the ME form two major divisions (preoptico infundibular tracts) which converge in the basal hypothalamus close to the ME: a mediobasal path which descends through the hypothalamus along the wall of the third ventricle, and a lateral path which projects to the ME by way of the medial forebrain bundle (e.g. Kawano and Kaikoku, 1981; Rethelyi et al, 1981). More detailed studies indicate that within these major divisions are a number of diffuse fibre pathways which may differentially innervate different portions of the ME (Rethelyi et al, 1981; Hoffman and Gibbs, 1982). Some of the fibres may course through the OVLT (where some fibres may terminate) before continuing to the ME by way of either the ventral surface of the optic chiasm or the floor of the third ventricle (Hoffman and Gibbs, 1982); this observation may explain the decrease in ME content of LHRH seen following lesions of the OVLT (Samson and McCann, 1979). The failure to lesion the LHRH tract which passes beneath the optic chiasm (Rethelyi et al, 1971; Hoffman and Gibbs, 1982) may also

explain the finding of some LHRH remaining in the MBH after 'total' hypothalamic deafferentation (e.g. Weiner, Pattou, Kerdelhue and Kordon, 1975).

1.4.3 Regulation of gonadotrophin secretion

The capacity of hypophysial portal blood to release LH was shown initially by bioassay (Fink, Nallar and Worthington, 1967; Fink and Harris, 1970). After the development of a specific RIA for LHRH (Nett, Akbar, Niswender, Hedlund and White, 1973), Fink and Jamieson (1976) were able to demonstrate greater concentrations of immunoreactive LHRH in hypophysial portal than in peripheral blood; results which were confirmed by Eskay, Mical and Porter (1977). The release of LHRH was increased significantly by the application of an electrical stumulus applied to the medial preoptic area (Fink and Jamieson, 1976) using stimulation parameters which were optimal for the release of LH (Jamieson and Fink, 1976). The effectiveness of the stimulation was greater when the stimulus was applied to the ME (Chiappa, Fink and Sherwood, 1977). The responsiveness of the preoptic area to stimulation varied during the oestrous cycle such that responsiveness increased between di-oestrus and pro-oestrus and reached a peak at around 13.00 h of pro-oestrus (Sherwood, Chiappa and Fink, 1976) and could be enhanced by administration of oestradiol benzoate or testosterone (Sherwood *et al*, 1976).

In male rats and for the greater part of the oestrous cycle in the female, the plasma concentrations of LH and FSH are kept low by the negative feedback effects of the gonadal steroids (McCann, 1962; Goodman, 1978; Fink, 1979a,b). In the majority of female rats maintained under controlled lighting conditions (14 h light and 10 h

dark), ovulation occurs once every four days in response to a spontaneous LH surge. This LH surge is, in turn, triggered by a daily neural signal (Everett and Sawyer, 1950) which is only expressed in response to the prolonged elevation in the plasma concentration of oestradiol-17β (Knobil, 1974; Legan and Karsch, 1975). The plasma concentration of oestradiol-17β, secreted from the ovary, begins to increase slowly on the afternoon of dioestrus, and, after a more rapid increase during the morning of pro-oestrus, reaches a peak early in the afternoon of pro-oestrus (Ferin, Tempone, Zimmering and Van der Wiele, 1969; Aiyer and Fink, 1974; Fink, 1977). This increase in plasma oestradiol concentration results in a massive increase in the pituitary responsiveness to LHRH (Aiyer, Fink and Greig, 1974; Aiyer and Fink, 1974) and triggers the surge of LHRH (Sarkar, Chiappa, Fink and Sherwood, 1976; Sherwood, Chiappa, Sarkar and Fink, 1980; Ching, 1982). The responsiveness of the anterior pituitary gland to LHRH is further enhanced by the increased secretion of ovarian progesterone during the LH surge (Aiyer and Fink, 1974; Feder, Brown-Grant and Corker, 1971; Fink and Henderson, 1977a; Turgeon and Waring, 1981) and the "priming effect" of LHRH (Aiyer, Chiappa and Fink, 1974). The priming effect may serve to co-ordinate maximum output of LHRH with peak responsiveness to LHRH, thereby ensuring the massive ovulatory surge (Fink, Stanley and Watts, 1983) which occurs between 17.00 and 19.00 h. A second, and smaller in magnitude, surge of LHRH occurs at the time of maximum sexual activity and ovulation at around 02.00 h on the morning of oestrus (Sarkar et al, 1976) and may be related to the prolonged surge of FSH. The plasma concentration of

FSH begins to rise at the same time as LH, but maximum FSH secretion is not achieved until early in the morning of oestrus (Daane and Parlow, 1971; Fink and Aiyer, 1974). The pro-oestrous gonadotrophin surge appears to be terminated mainly by the fall in LHRH concentration in hypophysial portal blood (Blake, 1976) which may be due to an inhibitory action of progesterone (Freeman, Dupke and Croteau, 1976; Drouva, Laplante and Kordon, 1983).

Studies of the effects of LHRH on the synthesis and release of gonadotrophins have been facilitated by the discovery of a mutant (hpg) mouse which is deficient in hypothalamic LHRH and as a consequence has a marked reduction in pituitary gonadotrophins and hypogonadism (Cattanach, Iddon, Charlton, Chiappa and Fink, 1977). Administration of LHRH to hpg mice (most effectively by multiple daily injection) resulted in an increased pituitary content of gonadotrophins and gonadal development, and demonstrated that in these mice LHRH is a more potent stimulator of FSH than of LH synthesis (Charlton, Halpin, Iddon et al, 1983). Charlton et al (1983) also showed that in the hpg female mouse the major site of oestrogen feedback was the anterior pituitary gland, whereas in the male, testosterone exerted its inhibitory effects at the hypothalamus or some other brain site.

1.5 NEURAL CONTROL OF PROLACTIN SECRETION

The secretion of prolactin is regulated by complex interactions between a number of inhibitory and facilitatory factors which are released by the hypothalamus (Leong, Frawley and Neill, 1983). In mammals the predominant influence of the hypothalamus on prolactin

secretion is inhibitory as was indicated initially by the presence of functional lactotrophes in pituitary glands transplanted under the kidney capsule (Nikitovitch-Winer and Everett, 1958, 1959). Increased plasma concentrations of prolactin were measured after deafferentation of the MBH (Blake, Scaramuzzi, Norman, Hilliard and Sawyer, 1973) or after pituitary stalk section (Kanematsu and Sawyer, 1973; Diefenbach, Carmel, Frantz, Ferin, 1976) and the presence of a prolactin inhibitory factor (PIF) was demonstrated in hypothalamic extracts (e.g. Talwalker, Ratner and Meites, 1963; Kragt and Meites, 1967). In general, indoleamines appear to stimulate, and catecholamines inhibit, prolactin secretion (Meites, Lu, Wuttke et al, 1972; Weiner and Ganong, 1978) and a large number of studies have suggested that DA is the major PIF (Ben-Jonathan, 1980; Leong et al, 1983). Dopamine and DA-agonists are effective inhibitors of prolactin release both in vivo and in vitro (Vale, Rivier and Brown, 1977) and DA receptors have been identified in the anterior pituitary gland (e.g. Calabro and MacLeod, 1978; Cronin and Weiner, 1979; Heiman and Ben-Jonathan, 1982). DA is present in hypophysial portal blood (Ben-Jonathan, Oliver, Weiner, Mical and Porter, 1977; Plotsky, Gibbs and Neill, 1978; Ben-Jonathan, Neill, Arbogast, Peters and Hoefer, 1980) in concentrations which are sufficient to inhibit prolactin secretion (Gibbs and Neill, 1978). However, DA does not appear to be the sole PIF (Leong et al, 1983) and in low concentrations DA may even facilitate prolactin release (Denef, Manet and Dewals, 1980). Several other factors also have been shown to have PIF activity, including γ-aminobutyric acid (Schally, Redding, Arimura, Dupont and Linthicum, 1977; Enjalbert,

Ruberg, Arancibia et al, 1979a). The cyclic dipeptide metabolite of TRH, diketopiperazine, has also been claimed to inhibit prolactin release in vitro (Enjalbert, Ruberg, Arancibia et al, 1979b; Prasad, Wilbur, Akerstrom and Banerji, 1980) but had no effect when injected in vivo (Emerson, Alex, Braverman and Saffan, 1981). Recently the 56 amino acid peptide found at the C-terminus of the LHRH precursor and called GnRH associated peptide (GAP), was also reported to inhibit prolactin release (Nikolics, Mason, Szonyi, Ramachandran and Seeburg, 1985).

The plasma concentration of prolactin is increased by a number of different stimuli including suckling (Grosvenor, Mena and Whitworth, 1979a), stress (Valverde, Chieffo and Reichlin, 1973; Shin, 1979), and mating (Gunnet and Freeman, 1983). A surge of PRL also occurs on the afternoon of pro-oestrus (Amenomori, Chen and Meites, 1970) which, like LH, is dependent upon an increase in the plasma concentration of oestrogen (Neill, Freeman and Tillson, 1971; Freeman, Reichert and Neill, 1972). In a number of these situations the abrupt withdrawal of PIF inhibition is insufficient to account for the rapid increase in the rate of secretion of prolactin (e.g. Valverde et al, 1973; Plotsky and Neill, 1982b). The presence of prolactin releasing factors (PRF) has been demonstrated in hypothalamic extracts (Dular, La Bella, Vivian and Eddie, 1974; Rivier and Vale, 1974). Many peptides have been shown to release prolactin and suggested as physiologically active PRF's, including TRH (Tashjian, Barowsky, Jensen, 1971), vasoactive intestinal peptide (Kato, Iwasaki, Iwasaki et al, 1978; Shaar, Clemens and Dininger, 1979; Enjalbert, Arancibia, Ruberg et al, 1980), peptide

histidine isoleucine (Kaji, Chihara, Kita et al, 1985a), and oxytocin (Lumpkin, Sampson and McCann, 1983).

1.6 CATECHOL- AND INDOLEAMINERGIC INNERVATION OF THE HYPOTHALAMUS

There is considerable evidence for the involvement of central aminergic neurons in the regulation of hormone secretion from the anterior and posterior pituitary gland (e.g. Fink and Geffen, 1978; Weiner and Ganong, 1978, Moos and Richard, 1982; Mason, 1983). A detailed description of the aminergic innervation of all the hypothalamic nuclei is beyond the scope of this chapter, but a brief account follows of the major aminergic pathways to the hypothalamus and the distribution of catechol- and indoleamine-containing nerve terminals in the ME and pituitary gland. Changes in amine turnover in the ME and pituitary gland have been shown to accompany alterations in the concentration of hormones in peripheral blood. Aminergic nerve fibres terminating in the ME may regulate anterior pituitary hormone secretion either by way of direct axo-axonic contact with the terminals of peptide secreting axons or by direct action on the cells of the anterior pituitary gland following the release of amines into hypophysial portal blood. Aminergic neurons also participate in the control of hormone secretion from the intermediate lobe of the pituitary gland by way of direct synaptic contacts with the secretory cells, for example the inhibition of melanocyte-stimulatory hormone secretion by dopamine (Tilders, Van der Woude, Swaab and Mulder, 1979; Munemura, Eskay, Kebanian and Long, 1980).

1.6.1 Dopamine pathways

The DA pathways in the brain have been the subject of numerous reviews (e.g. Moore and Bloom, 1978; Palkovits, 1981; Bjorklund and Lindvall, 1984). Most of the DA terminals in the hypothalamus originate from intrahypothalamic cell bodies; however, one extrahypothalamic pathway (the mesocortical system) may have projections to the ME from its cell bodies in the ventral tegmentum and substantia nigra (Kizer, Palkovitz and Brownstein, 1976). There are two intrahypothalamic DA pathways - the incerto-hypothalamic system and the tuberoinfundibular system. The incerto-hypothalamic fibre system (Lindvall, Bjorklund, Moore and Stenevi, 1974; Bjorklund, Lindvall and Nobin, 1975) can be topographically subdivided into two - in the caudal part, cell bodies are found in the caudal thalamus, posterior hypothalamic area and medial zona incerta and in the rostral part, cell bodies in the periventricular nucleus (Bjorklund _et al_, 1975). The fibres from this system are short and locally projecting (Bjorklund _et al_, 1975). The cell bodies of the tuberoinfundibular system are located in the arcuate nucleus and the anterior portion of the periventricular nucleus (Dahlstrom and Fuxe, 1964; Hokfelt and Fuxe, 1972; Bjorklund, Falck, Nobin and Stenevi, 1973a). The axons from these cell bodies project to the neural and intermediate lobes of the pituitary gland (Smith and Fink, 1972) and to the median eminence (Bjorklund, Falck, Hromek, Owman and West, 1970; Smith and Fink, 1972; Jonsson, Fuxe and Hokfelt, 1972; Bjorklund _et al_, 1973a,b; Ajika and Hokfelt, 1975).

Dopamine accounts for about 70% of the total catecholamine

content of the ME (Cuello, Horn, Mackay and Iversen, 1973). Within the ME the greatest concentration of DA terminals is present in the lateral portions of the external layer (Bjorklund et al, 1970; Lofstrom, Jonsson and Fuxe, 1976a). In the pituitary gland, the largest content of DA is in the neural lobe, with smaller concentrations in the anterior lobe (Saavedra, Palkovits, Kizer, Brownstein and Zivin, 1975). Pituitary stalk section shows that the DA content of both the neural and intermediate lobes originates from the tuberohypophysial DA system (Saavedra, 1985).

1.6.2 Noradrenaline pathways

In contrast to DA, no NA containing cell bodies have been identified within the hypothalamus; deafferentation of the medial hypothalamus results in a major reduction of NA content (Brownstein, Palkovitz, Tappaz, Saavedra and Kizer, 1976). There are two principal NA neuron systems in the brain, which are comprised of those neurons which have their cell bodies in the locus coeruleus and those with cell bodies in the lateral tegmentum (Swanson and Hartman, 1975; Moore and Card, 1984). Although the majority of the NA input to the nuclei of the hypothalamus (including the ME) originates from the cell bodies in the lateral tegmentum (Moore and Card, 1984), several nuclei (the supra-optic, dorsomedial, paraventricular and periventricular nuclei) also receive fibres from the locus coeruleus (Segal, Pickel and Bloom, 1974; Jones and Moore, 1977; Moore and Card, 1984).

The NA containing terminals in the ME are mainly located in the internal layer (Bjorklund et al, 1970; Lofstrom et al, 1976). In addition, NA has been detected biochemically in both the neural and

intermediate lobe of the pituitary (Saavedra et al, 1975). Whilst all of the NA present in the intermediate lobe is probably of central origin, approximately 40% of * may be derived from fibres of the sympathetic nervous system which originate in the superior cervical ganglia (Saavedra, 1985).

1.6.3 5-Hydroxytryptamine pathways

It was difficult to demonstrate 5-HT-containing terminals in the hypothalamus using fluorescence histochemistry due to the relative insensitivity of the technique for this amine. However, with biochemical (Saavedra, Palkovits, Brownstein and Axelrod, 1974), autoradiographic (Chan-Palay, 1977; Parent, Descarries and Beaudet, 1981) and immunohistochemical (Steinbusch, 1981; Steinbusch and Nieuwenhuys, 1981) techniques it was possible to show the widespread distribution of 5-HT and 5-HT-containing nerve terminals throughout the hypothalamus with high concentrations present in the suprachiasmatic and ventromedial nuclei. The 5-HT neurons which terminate in the hypothalamus, and pituitary, originate from the dorsal and medial raphe nuclei which are located in the midbrain (Dahlstrom and Fuxe, 1964; Fuxe, 1965). The hypothalamic content of 5-HT is reduced to approximately one third by lesion of the dorsal raphe nuclei (Palkovits, Saavedra, Jacobovitz et al, 1977) or by deafferentation of the MBH (Brownstein, Palkovits, Tappaz, Saavedra and Kizer, 1976). The presence of intrahypothalamic 5-HT-containing cell bodies has been reported (e.g. Chan-Palay, 1977; Beaudet and Descarries, 1979; Parent et al, 1981). However, there are doubts as to the specificity of the techniques used in these studies (Fuxe et al, 1978; Steinbusch and Nieuwenhuys, 1981) and no 5-HT cell bodies

*the NA in the neural lobe

were found in the hypothalamus in the immunohistochemical studies of Steinbusch and Nieuwenhuys (1981).

Autoradiography using ^{3}H-5-HT (Calas, Alonso, Arnauld and Vincent, 1974) and immunohistochemistry (Steinbusch and Nieuwenhuys, 1981) reveal an accumulation of 5-HT in the vicinity of the hypophysial portal vessels in the external layer of the ME. Biochemical studies also have shown a high content of 5-HT in the neural lobe and smaller quantities in the anterior pituitary gland (Saavedra, Palkovits, Kizer, Brownstein and Zivin, 1975) and immunohistochemistry (Steinbusch and Nieuwenhuys, 1981) showed 5-HT-containing fibres in the neural lobe, but not in the intermediate lobe or anterior pituitary. Since in the latter study a high concentration of the 5-HT staining fibres were in the region of the neural lobe adjacent to the intermediate lobe, it was suggested that these fibres may be involved in the regulation of peptide release from the intermediate lobe. Although the immunoreactive 5-HT fibres of the neural lobe disappear after pituitary stalk section, as measured biochemically, approximately half the 5-HT remains (Friedman, Kreiger, Mezey et al, 1983) and this is probably accounted for by the 5-HT content of non-neuronal structures such as mast cells and platelets (Palkovits, Mezey, Chiueh et al, 1986).

1.7 BIOSYNTHESIS OF NEUROPEPTIDES

Most biologically active peptides are synthesized as a component of a larger molecular weight precursor which is formed by ribosomal synthesis using a messenger RNA (mRNA) template. For some

neuropeptides both the amino acid sequence of the precursor molecule and the nucleotide sequence of the encoding mRNA are now known. The primary product of the translation of mRNA is a polypeptide (pre-pro-peptide), the N-terminal of which consists of a hydrophobic signal peptide (about 15-30 amino acids in length) which is believed to promote the passage of the pro-peptide, through the membrane of the rough endoplasmic reticulum (RER) into the cisternae. The signal peptide is cleaved from the remainder of the molecule by the action of a metallo-endopeptidase which is associated with the inner membrane of the RER (e.g. Jackson and Blobel, 1977; Zwizinski and Wickner, 1980). The pro-peptide is translocated from the cisternae of the RER into the Golgi apparatus where it is packaged into secretory granules. Further processing occurs in the secretory granules, though for some peptides some proteolytic cleavage may occur first in the Golgi apparatus.

Within the pro-peptide molecule, subsequent proteolytic cleavage usually occurs at sites which are denoted by pairs of basic amino acids - generally Lys-Arg, but also Arg-Arg and Lys-Lys. Exceptions to this occur with the cleavage at a single Arg residue in pro-vasopressin to generate neurophysin II and a C-terminal glycopeptide (Land, Schutz, Schmale and Richter, 1982) and the cleavage of somatostatin-28 from pro-somatostatin. Peptides may be modified in one or more ways either before or after cleavage by glycosylation, phosphorylation, acetylation of the N-terminus, amidation of the C-terminus, formation of disulphide bonds between cysteine residues, and by the cyclization of glutamic acid to form pyroglutamate. Sites for the amidation of a C-terminal are

signalled by a glycine residue at the N-terminal side of a basic amino acid pair - the enzyme necessary for this conversion has been demonstrated (Bradbury, Finnie and Smythe, 1982).

Precursors may contain more than one active peptide within their sequence and may be differentially processed in different tissues. An example of this is pro-opiomelanocorticotrophin which is processed in the anterior pituitary gland and intermediate lobe to yield β-lipotrophin (β-LPH), adrenocorticotrophin (ACTH) and an N-terminal fragment; in the intermediate lobe further processing of ACTH generates α-melanotrophin (α-MSH) and corticotrophin-like intermediate lobe peptide (CLIP) whereas β-LPH is cleaved to produce γ-LPH and β-endorphin (Roberts, Phillips, Rosa and Herbert, 1978). Precursors may also contain more than one copy of an active peptide, e.g. six copies of methionine-enkephalin and one copy of the leucine-enkephalin are contained within the sequence of pro-enkephalin A (Gubler, Seeburg, Hoffmann, Gage and Udenfriend, 1982).

1.8 AIMS OF THIS THESIS

The objectives of this thesis were to examine the immunoreactive forms of the hypothalamic peptides, LHRH, TRH and somatostatin, which are released into hypophysial portal vessel blood and to study some aspects of the hypothalamic control of anterior pituitary hormone secretion. The technique of passive immunization, with an anti-TRH serum, was used to examine the role of TRH in mediating the prolactin response to suckling in lactating rats and the increased secretion of thyrotrophin and prolactin in response to electrical stimulation of the median eminence or paraventricular nuclei in male

rats. The priming effect of LHRH was investigated in normal and in hypogonadal mice to determine whether previous exposure of the anterior pituitary gland to normal levels of LHRH, and a normal pituitary content of LH, were necessary for the priming and the releasing actions of LHRH. The bioreactivity of the small amount of immunoreactive LH present in the anterior pituitary gland of hypogonadal mice was also studied using an *in vitro* bioassay. Direct measurements of LHRH release into rat hypophysial portal blood were made to examine (i) the mechanism underlying the suppression of LH release during lactation, (ii) the mechanism by which Δ^1-tetrahydrocannabinol blocks the pro-oestrous surge of LH, and (iii) the role of endogenous opioid peptides in the regulation of LHRH release in pro-oestrous or oestrous rats. In a final series of experiments, 6-hydroxydopamine was used to lesion the terminals of the catecholaminergic neurons in the median eminence and neuro-intermedial lobe of the pituitary gland to examine the role of these neurons in the regulation of basal hormone secretion in the male rat.

CHAPTER 2

Materials and Methods

2.1 ANIMALS

The animals used for the majority of the experiments were adult male or female Wistar rats (180-300 g body weight) supplied by the Edinburgh University Centre for Laboratory Animals. For some of the experiments described in Chapters 6 and 9, female (180-300 g) or lactating female (300-500 g) Wistar Cobs (caesarian originated barrier sustained) rats were purchased from Charles River U.K. Ltd. (Margate, Kent). At least 10 days were allowed for acclimatization to the animal house before animals were used in experiments. Normal and hypogonadal (hpg) male and female mice were bred in the Department of Pharmacology from stocks obtained from the Department of Human Anatomy, University of Oxford.

All animals were maintained under controlled lighting (lights on 05.00 h - lights off 19.00 h) and temperature (22°C) and allowed free access to food (for rats, Diet 41B, Oxoid, Basingstoke; for mice, Oxoid breeding diet) and tap water. Chlortetracycline hydrochloride (40 mg/l) was added to the drinking water of rats with electrode implants, and the diet of these animals was supplemented with bread and milk.

The oestrous cycles of female rats were determined by daily inspection of vaginal smears and only animals which showed regular 4-day oestrous cycles were used. Each day of the cycle was termed either metoestrus, dioestrus, pro-oestrus or oestrus depending on the predominant cell type present in the smear. The characteristic smears for each day were:

metoestrus - predominantly leucocytes together with some epithelial and cornified cells

dioestrus - leucocytes

pro-oestrus - clumps of nucleated epithelial cells

oestrus - cornified epithelial cells.

2.2 SURGICAL AND EXPERIMENTAL PROCEDURES

2.2.1 Anaesthetics

The following anaesthetics were used:

(A) 2,2,2-tribromoethanol (Aldrich Chemical Company, Gillingham, Kent) in amylene hydrate (tert. amyl alcohol; BDH, Poole, Dorset) 1 g/ml (formerly available as 'Avertin'); 2 ml diluted with 8 ml of absolute alcohol and 90 ml 0.9% saline solution. The dose used was 1.0 ml/100 g body weight administered by intraperitoneal (i.p.) injection.

(B) Urethane (ethyl carbamate; Sigma, Poole, Dorset), administered i.p. as a 10% solution (w/v) in 0.9% saline solution at a dose of 1.0 to 1.2 g/kg body weight.

(C) Saffan (Glaxovet Ltd., Uxbridge; formerly supplied as 'Althesin') - 9 mg/ml alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione) and 3 mg/ml alphadolone acetate (21-acetoxy-3α-hydroxy-5α pregnane-11,20-dione) solubilized in saline by 20% w/v polyoxyethylated castor oil.

(D) Anaesthetic ether - purchased from Macfarlane Smith Ltd., Edinburgh.

(E) Sodium pentobarbitone - purchased as Sagatal (May & Baker Ltd., Dagenham) and consisted of 60 mg/ml sodium pentobarbitone in alcohol:propylene glycol vehicle (10:20 v/v). The dose used was 40-50 mg/kg body weight injected i.p.

2.2.2 Collection of Peripheral Blood

Anaesthetized animals were placed and secured in a supine position and a small incision (approximately 1.0-2.0 cm long) was made longitudinally over one or both external jugular veins (EJV). The tissue overlying the vein was gently retracted to expose the vein. A 25 gauge hypodermic needle was introduced into the vein through the pectoralis major muscle and the appropriate volume of blood (generally 0.5 ml) was withdrawn into a syringe which had been heparinized by rinsing with a small volume of heparinized saline (1000 IU/ml). Immediately after collection, blood samples were expelled into plastic tubes (LP2, Luckhams Ltd., Burgess Hill, Sussex) which had been cooled on ice. The samples were centrifuged at 4°C and the plasma aspirated with a glass pipette and stored in plastic tubes (PT0944, Luckhams Ltd.) at -40°C.

2.2.3 The Collection of Hypophysial Stalk Blood

Hypophysial portal vessel blood was collected using the method described by Worthington (1966) and modified by Fink and Jamieson (1976). The method was based on the transpharyngeal approach used by Green and Harris (1949) to expose and visualize the hypophysial portal vessels.

The animal was anaesthetized with either urethane or Saffan and placed in a supine position on a rat operating board. A midline incision was made from the lower lip, extending apporoximately 5 cm caudally. The subcutaneous tissues and salivary glands were separated in the midline to expose the muscles overlying the trachea. A 1 cm longitudinal incision was made in these muscles and the trachea exposed and freed from the surrounding tissue to enable

the passage of a small silk suture (3/0) underneath. A small transverse incision was made in the trachea and one end of a short (1.5 cm) length of polythene tubing (2.08 mm o.d., 1.57 mm i.d.) inserted through the incision and tied in position with the suture. The floor of the mouth was then injected with a 2% w/v solution of lignocaine (Xylocaine; Astra Pharmaceuticals, Watford, Herts). A long suture was placed through the tip of the tongue and gentle traction applied to it. A tight ligature was placed around each mandibular ramus in order to prevent bleeding from branches of the lingual arteries. Long sutures were placed through each of the flaps of the divided lower lip and were used for lateral retraction of the two sides of the jaw. The lower jaw was then divided in the midline, starting between the incisor teeth and continuing caudually, dividing the musculature of the floor of the mouth and oral mucosa along the left and right side of the mandible thus resulting in mobilization of the tongue. The extent of these incisions was limited so as to avoid damage to the lingual arteries. A suture was placed around the base of the tongue to prevent bleeding, and with the aid of a suture through its tip, the tongue was retracted and secured in position over the chest, thereby resulting in visualization of the soft palate and the epiglottis. To ensure haemostasis small pieces of oxidised cellulose gauze (Surgicel; Johnson & Johnson, Slough) were placed on the cut edges of muscle and bone where the mandible and floor of the mouth had been cut. From this stage onwards, the preparation was continued with the aid of a Zeiss binocular operating microscope. A small volume (0.1-0.2 ml) of lignocaine was injected into the soft palate

which was then incised with an iris electrocautery (Weiss and Son, London). The incision extended longitudinally along the midline from about 1 mm anterior to the epiglottis, to the posterior edge of the hard palate, and laterally to the pterygoid processes of the sphenoid. The mucosa overlying the basosphenoid was removed with a small pledget of cotton wool. A midline hole was drilled in the basosphenoid bone from the sphenoid-basosphenoid suture extending rostrally to the basophenoid-presphenoid suture. During drilling the hole was packed with bone wax (Ethicon Ltd., Edinburgh) in order to prevent bleeding from the spongy bone layer. The hole was drilled deep enough to expose the inner table of bone which, when fully exposed along the full length of the trough, was carefully 'polished' around its edge with a fine drill. When sufficiently thin to yield to touch, the inner table of bone was gently removed with watchmaker's forceps thus exposing the dura mater overlying the portal vessels. Bleeding was controlled by applying gentle pressure with a piece of cotton wool for a few minutes. A fragment of a stainless steel razor blade held in a pin vice was used to make a "V"-shaped cut in the dura and arachnoid mater. The cuts were started over the pituitary gland and were extended rostrally lateral to the portal vessels; the apex formed by the cuts lay just rostral to the median eminence. Cerebrospinal fluid which entered the operating field after the dura and arachnoid mater had been cut, was absorbed with small pieces of cotton wool. The portal vessels were fully exposed by reflecting the flap of dura mater and arachnoid mater formed by the cuts. After ensuring haemostasis, the animal was injected intravenously with 2500 IU of heparin (heparin

sodium [mucous] 5000 IU/ml; Weddel Pharmaceuticals, London) by way of the external jugular vein. Using fine iridectomy scissors (Weiss and Son, London) the pituitary stalk was cut as close as possible to the pituitary gland so as to include as many of the portal vessels as possible. The blood issuing from the cut stalk collected in the trough in the basosphenoid bone and was carefully removed using a Pasteur pipette. To prevent the inclusion of any fragments of pituitary stalk tissue the blood which collected during the first 5 min after stalk section was discarded. Thereafter the collection was continued for 15 min periods into either small ice-cold plastic tubes (LP2; Luckhams Ltd.) or into ice-cold ethanol. At the end of the collection period the volume of blood was recorded as was, when appropriate, the volume of plasma obtained after centrifugation. Unextracted plasma, and alcohol extracts of plasma or blood were stored at -40°C until required for assay or high performance liquid chromatography.

2.2.4 Electrode Preparation, Implantation and Stimulation Parameters

Unipolar and bipolar electrodes were constructed (Jamieson and Fink, 1976; Chiappa, Fink and Sherwood, 1977) from 10-13 mm lengths of platinum wire (0.125 mm in diameter) which were insulated to within 0.3 mm of the tip with close-fitting glass capillary tubing. A short length of insulated copper wire was soldered onto the upper end of the electrode. The electrode assembly was mounted in a Teflon jig and fixed in place with self-curing dental acrylic cement (Simplex rapid; Howmedica, London). For bipolar electrodes, which were used for stimulation of the paraventricular nuclei (PVN) the electrode tips were separated by 1.0 mm.

For implantation of PVN stimulating electrodes, male rats (200–220 g body weight) were anaesthetized with tribromoethanol-amylene hydrate and positioned with the head immobilized in a stereotaxic frame in which the incisor bar was 5 mm higher than the ear bars. A midline incision was made in the scalp to expose the frontal and parietal bones, a hole (4 x 4 mm) drilled in the frontal bones immediately anterior to the bregma, and the dura mata revealed; care was taken to avoid damage to the superior sagittal sinus. Bleeding during any point of the operative procedure was controlled with gentle pressure with cotton wool soaked in 0.9% saline solution. Three small stainless steel screws (approximately 1.5 mm in diameter) were driven into small drill holes made around the outside margin of the exposed area. The dura mata was carefully ruptured and the electrode implanted (co-ordinates; 6.4 mm anterior, -1.6 mm vertical and 0.5 mm on either side of the midline; de Groot, 1959) with the aid of a triplanar micromanipulator (Prior U.K.). The electrode assembly was secured to the skull using dental cement which was carefully applied round the electrode and screws. Once the cement had set firmly, the animal was removed from the stereotaxic frame and the scalp sutured. Animals were used 3–10 days after implantation.

For median eminence (ME) stimulation the median eminence was exposed using the transpharyngeal approach (2.2.3) at the time of the experiment and a unipolar electrode placed onto the ME under visual control and with the aid of a triplanar micromanipulator. Care was taken to avoid damage to the hypophysial portal vessels. A metal clip attached to the muscles of the jaw served as an

indifferent electrode.

The electrical stimulus (Fink and Jamieson, 1976) for both ME and PVN preparations was produced by a Neurolog constant current stimulator (Digitimer, Welwyn Garden City, Herts), and consisted of accurately balanced trains (30 s on, 30 s off) of biphasic rectangular pulses with a frequency of 50 Hz, pulse amplitude of 500 μA and pulse duration of 1 ms. The stimulus was monitored with a calibrated oscilloscope (Model 14D-15; Scopex, Letchworth, Herts) and for most experiments was applied for one or two 15 min periods.

At the end of the experiment, the heads were removed from animals with PVN electrode implants, trimmed and fixed in 4% phosphate buffered formalin. Subsequently the electrodes were carefully removed after disolving the dental cement with chloroform, and the brains dehydrated in alcohol and embedded in paraffin wax. Serial 10 μm sections were cut, stained with haematoxylin and eosin and examined for correct placement of the electrode tips.

2.2.5 Preparation and Implantation of Oestradiol-Containing Capsules

Silastic capsules containing oestradiol-17β were prepared according to the method of Karsch, Dierschke, Weick, Yamaji, Hotchkiss and Knobil (1973) and as used by Henderson, Baker and Fink (1977). 'Silastic' medical grade tubing (2.6 mm internal diameter, 4.9 mm outer diameter; catalogue number 601-321, Dow Corning Corporation, Michigan, U.S.A.) was cut into 3 mm lengths. One end of the capsule was sealed with 'Silastic' elastomer which was made by mixing catalyst and elastomer (approximately 1:1000 v/v; catalogue number 382, Dow Corning Corporation). The 4-5 mm length of the inside of the capsule was then packed with crystalline

oestradiol-17β (Sigma, Poole) and the open end then sealed with elastomer. Capsules were implanted subcutaneously into the middle of the back of animals through a small skin incision made on the back of the neck. The capsules were incubated in donor animals (long-term ovariectomized rats) for at least 24 hours before their removal and immediate re-implantation into experimental animals. By pre-incubation of the capsules in donor rats, the initial high spikes of plasma oestradiol-17β, observed in animals during the first 24 h after implantation of non-incubated capsules, were avoided (Karsch et al, 1973).

2.2.6 Preparation of Hypothalamic and Pituitary Homogenates

Animals were decapitated and the brains quickly dissected onto an ice-cold glass microscope slide. A block of hypothalamic tissue, which extended from the anterior edge of the optic chiasma to the caudal edge of the mammillary body and laterally to the hypothalamic sulci and 2 mm deep, was removed using a fine pair of scissors. For assay of hypothalamic peptides the block of tissue was weighed using a torsion balance, transferred to 1 ml of ice-cold 0.1 N HCl and homogenized in an all glass, hand held, homogenizer (Jencons, U.K.). For determination of catecholamines, only the SME (median eminence together with the remains of the pituitary stalk) was dissected. The SME was removed by gently holding the stalk in watchmaker's forceps and, using iridectomy scissors, undercutting an area of the ME about 2 mm wide, from the stalk-ME junction and as deep as the floor of the third ventricle. The pituitary was removed from its dural capsule and the neurointermediate lobe (NIL) dissected from the anterior pituitary. SME and NIL samples were

stored in 2 ml screw top ampules (Sterilin, Teddington, catalogue number 506) in liquid nitrogen. The anterior pituitary was weighed and homogenized in 1 ml of 0.9% (w/v) saline solution. Hypothalamic and anterior pituitary homogenates were stored at -40°C until required for assay.

2.3 FLUORESCENCE HISTOCHEMISTRY

Several methods have been developed to enable the visualization of catechol- and indoleamine-containing nerve terminals and cell bodies in the central and peripheral nervous system by the production of fluorescent derivatives which are formed as a result of condensation reactions between the amines and formaldehyde or glyoxylic acid (e.g. Falck, Hillarp, Thieme and Torp, 1962; Lindvall and Bjorklund, 1974; Corrodi and Jonsson, 1976). By perfusion of the brain with a mixture of formaldehyde and gluteraldehyde (the 'FAGLU' technique) the brain is rapidly fixed and the fluophores, which are formed at room temperature remain stable even when the tissue is maintained in aqueous solution (Furness, Heath and Costa, 1978). The FAGLU method provides a relatively simple and reliable technique for the examination of catecholamine distribution in serial sections of rat brain, and was used in the experiments described in Chapter 9 to investigate the extent of the 6-hydroxydopamine-induced lesions of the mediobasal hypothalamus.

2.3.1 Apparatus and Solutions for Perfusion

The perfusion apparatus, which was only slightly modified from that described by Furness *et al.* (1978), consisted of two 1 litre aspirator bottles which were connected to an 18 gauge hypodermic

needle by way of a three way tap, and also connected to a sphygmomanometer bulb and mercury manometer to maintain and monitor the perfusion pressure respectively. All connections were made using short lengths of wide bore, thick-walled tubing. The one bottle contained a 1% (w/v) solution of sodium nitrite and the other contained the fixative solution (4% formaldehyde ['Analar' grade, BDH Chemicals Ltd., Poole] - 0.5% gluteraldehyde ['Electron Microscope' grade, TAAB Laboratories, Reading] in 0.1 M sodium phosphate buffer, pH 7.0).

2.3.2 Perfusion of Animals and Preparation of Brain Sections

Animals were anaesthetized with sodium pentobarbitone and injected with heparin (4000 IU/kg body weight i.p.), and immobilized on an operating board. The thorax was opened to expose the heart and 4 mg gallamine triethiodide (Flaxedil, May and Baker, Dagenham) injected into the heart. The tip of the 18 gauge needle was inserted into the left ventricle and held in position with a clamp across the ventricle. A second clamp was placed upon the abdominal aorta. The vena cava was cut and the blood flushed out by perfusing the animal with the sodium nitrite solution for 1-2 min. The perfusion was then continued with fixative for a further 10 min at a perfusion pressure of 120 mm Hg. When the perfusion was completed (after about 200 ml of fixative had been used) the fixed brain was quickly removed and transferred to approximately 50 ml of ice-cold fixative and stored in the dark at 4°C for 3-5 days until sectioned. The brain was trimmed to a 5 mm thick block which contained the hypothalamus and was mounted onto a glass slide with Loctite quick setting adhesive (B10-904, Loctite Corporation,

Newington). The block was held in position until set by applying gentle pressure with a glass plate for about 5 min, after which 30 μm thick sections were cut at room temperature using a 'Vibrotome' (Model G, Oxford Laboratories, California) (Furness, Costa and Blessing, 1977). Throughout the sectioning procedure the blocks were kept moist with ice-cold fixative. Sections were removed from the blade of the vibrotome using a fine brush, transferred to a dish of fixative and floated onto glass microscope slides. The sections were dried for 30 min over phosphorous pentoxide in a dessicator in the dark and mounted in 'Fluoromount' (Gurr, Essex). Fluorescence was examined using a Leitz Orthoflux fluorescence microscope* with epi-illumination provided by a 200 W high pressure mercury lamp (Wotan, Germany) and photographed on Tri-X, 400 ASA film (Kodak).

2.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

2.4.1 HPLC separation of peptides

Reversed-phase HPLC, used in combination with sensitive radioimmunoassays, provides a powerful technique for qualitative and quantitative studies of biologically important peptides (e.g. O'Hare and Nice, 1979; Cody, Wilkes and Hruby, 1984; Herman, Lang, Unger, Bayer and Ganten, 1984), and was used in the following studies to examine the immunoreactive forms of TRH (Chapter 4) and LHRH (Chapter 6) which are present in the hypothalamus and released into hypophysial portal vessel blood.

2.4.1.1 Sample Preparation

Freeze- or vacuum-dried tissue or blood extracts were redissolved in 1.4 ml 0.1% trifluoroacetic acid (TFA; Rathburn

*using BG 12/3 mm primary filter and Leitz K510 or K530 secondary filters.

Chemicals Ltd., Walkerburn, Scotland) and centrifuged briefly (two minutes at full speed* in a MSE Micro Centaur centrifuge) to remove particulate material. One millilitre of the supernatant was applied to the HPLC column and the remaining solution evaporated to dryness, re-constituted in assay buffer, and assayed for total immunoreactive LHRH or TRH content. In a few samples, a small amount of fine particulate material remained in suspension and this was removed before sample injection by filtration through 0.2 μm regenerated cellulose filters (BAS Inc., West Lafayette, Ind.).

2.4.1.2 Chromatography

The chromatographic separation of peptides in tissue and blood extracts was achieved using an Altex HPLC system as described below. The mobile phase was pumped through the system at a constant flow rate of 1 ml/min by two Altex 110A pumps. Samples were applied onto the column either manually by way of an Altex 210 injection valve fitted with a 1 ml injection loop or automatically using an Altex model 500 autosampler. The analytical column consisted of a 4.6 x 250 mm column packed with 5 μm Ultrasphere ODS (Altex, Berkely, CA); this main column was protected by a 2.1 x 50 mm pre-column packed with Co Pell ODS (Whatmans). Separation was achieved with the aid of a linear gradient produced with the aid of an Altex 421 controller. The gradient changed from 100% solvent A to 100% solvent B over 50 minutes, where

Solvent A was 0.1% (v/v) aqueous TFA (HPLC grade water; Fisons, Loughborough)

Solvent B was 0.1% (v/v) TFA in acetonitrile (HPLC grade S; Rathburn Chemicals Ltd.)

*13,400 x g.

The eluant from the column was passed through an Altex spectrophotometer flow cell in an Hitachi model 100-10 spectrophotometer and the absorbance at 210 nm recorded on a Gilson N2 chart recorder. As a result of the large number of absorbing compounds present in portal blood, the spectrophotometer trace provided little information about the peptides in portal blood extracts, but was useful for monitoring column performance and examining the retention times of peptide analogues during screening runs before radioimmunoassay. Finally, the outflow from the column was passed to an LKB 2111 multirac fraction collector and fractions were collected every 0.3 min (maximum of 180 fractions). All the connections between the various components of the HPLC system were made with the shortest possible lengths of narrow bore stainless steel tubing. The collected fractions were stored at -40°C until vacuum dried over phosphorous pentoxide, reconstituted in assay buffer and assayed for LHRH or TRH-like immunoreactivity.

2.4.2 <u>HPLC with Electrochemical Detection (LCED)</u>

HPLC with electrochemical detection was used for the simultaneous determination of noradrenaline (NA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) in extracts of the SME and NIL of 6-OHDA lesioned rats (Chapter 9). Full details of the principles of continuous flow voltametry and theory of the electrochemical reactions involved in electrochemical detection have been described by Adams (1969), Weber and Purdy (1978) and Mefford (1981). Briefly, the method depends upon the ability of the amines under study to undergo oxidation when subjected to a fixed potential.

Application of the oxidative potential results in the loss of two electrons from each catechol- or indoleamine molecule to the detector electrode. The concentration of the amine in solution can be determined since the molar concentration is proportional to the current generated by this oxidative mechanism.

2.4.2.1 Sample Preparation

Samples for LCED (SME and NIL dissected as described in 2.2.6) were homogenized, using glass hand-held homogenizers, in 80 µl of 0.2 M perchloric acid in 10^{-4} M cysteine solution to which had been added 400 pg/20 µl of n-acetyl dopamine (n-AcDA; Sigma, U.K.) as internal standard (IS). The homogenates were centrifuged at 2000 g for 5 minutes, the supernatant was removed immediately before injection and filtered through 0.2 µm regenerated cellulose filters (BAS Inc., West Lafayette, Ind.) using BAS MF 1 centrifugal microfilters.

2.4.2.2 Chromatography and Detection

Separation of the catechol- and indoleamines in the samples was achieved on a 250 x 4.6 mm analytical column of Ultrasphere ODS (5 µm) (Altex, Berkley, CA) protected with a 50 x 4.6 mm Ultrasphere ODS precolumn. 20 µl of either standards or filtered tissue homogenate were applied to the column by way of a Rheodyne 7125 injection valve fitted with a 20 µl sample loop. An Altex 110A pump was used to deliver the mobile phase to the system at a constant rate of 1 ml/min. The mobile phase (Mayer and Shoup, 1983, as modified by Watts, 1982) consisted of 0.1 M sodium acetate - 0.1 M sodium citrate buffer, methanol (HPLC grade; Rathburn Chemicals Ltd., Walkerburn, Scotland) and tetrahydrofuran (Spectrophotometric

grade and inhibitor free; Aldrich Chemical Company, Gillingham) (95:5:2, v/v) adjusted to pH 5.0 by the addition of 6.5 g sodium hydroxide, and with the addition of 100 mg/l of sodium octanesulphonic acid (Fisons, Loughborough) as ion-pair reagent. Before the inclusion of tetrahydofuran, the buffer was filtered by passage through 0.22 μm Millipore$^{(R)}$ filters and degassed under vacuum. Eluant from the analytical column was passed through a LC-17 glassy carbon transducer cell connected to a LC-4A amperometric detector. The working potential of the electrode was maintained at + 0.70 V with respect to an Ag/AgCl reference electrode and the generated current amplifed and displayed on a Tarkan 600 chart recorder set at a range of 1 V.

Fresh standard solutions of NA, DA, DOPAC, 5-HT, 5-HIAA and n-AcDA (Sigma, U.K.) were prepared daily in 0.2 M perchloric acid in 10^{-4} M cysteine solution from 100 μg/ml stock solutions (stock solutions were stored in the dark at 4°C for a maximum of 2 months). Standards were routinely injected at concentrations of 50, 100, 200 and 2000 pg/20 μl injection and the standard curves were linear over a range of 20-2000 pg/injection. The lower limit of sensitivity of the assay was taken as three times the amplitude of baseline noise and was between 15 and 25 pg/injection. Typical LCED traces produced by two of the standard mixtures, and a portion of a typical standard curve are show in Figures 2-1 and 2-2, respectively.

2.4.2.3 Identification of Compounds and Calculation of Results

Routinely, peaks in traces from sample extracts were identified on the basis of similar retention time to the known compounds in the standard mixtures. However, in a series of initial experiments,

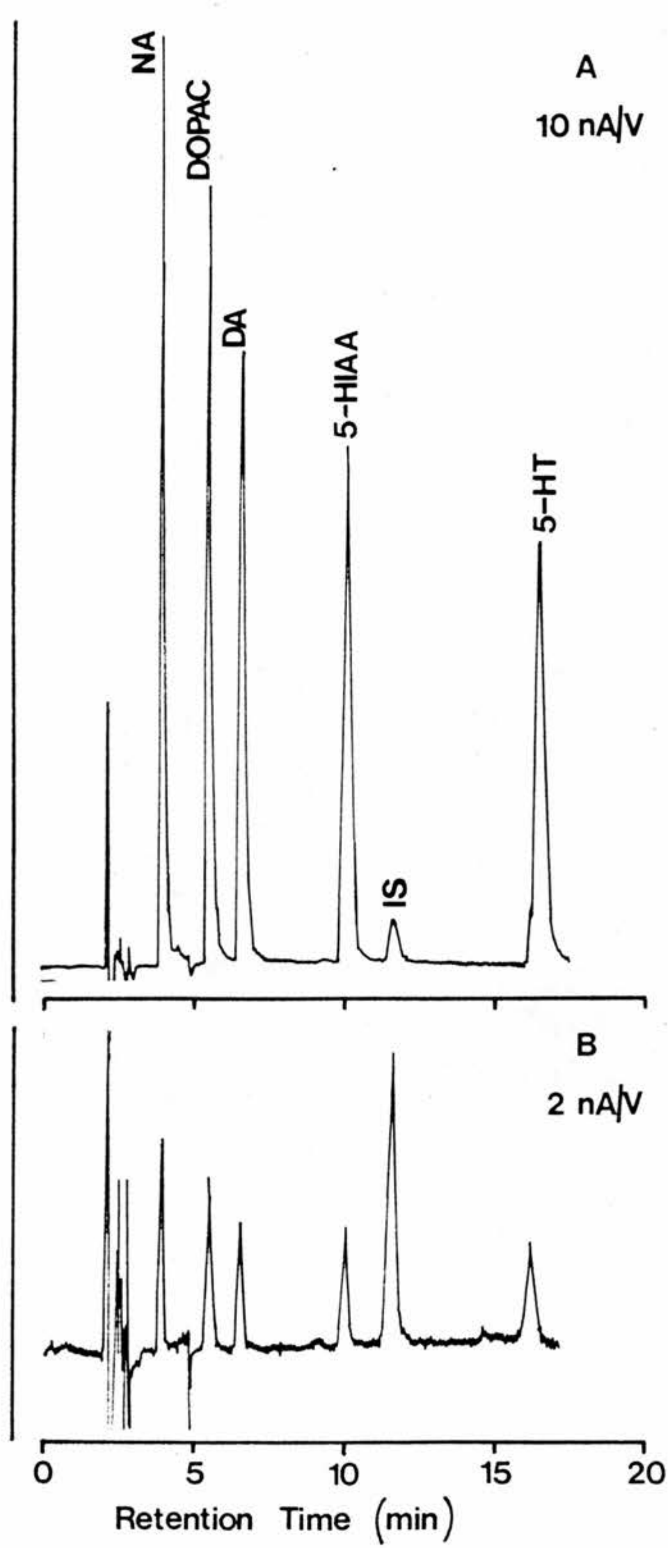


FIGURE 2-1: Standard chromatograms for (A) 2 ng/20 μl and (B) 100 pg/20 μl of noradrenaline (NA), dopamine (DA), 3,4 dihydroxyphenyl-acetic acid (DOPAC), 5-hydroxyindole acetic acid (5-HIAA) and 5-hydroxytryptamine (5-HT) with 1 ng/20 μl of n-acetyl dopamine (IS - internal standard).

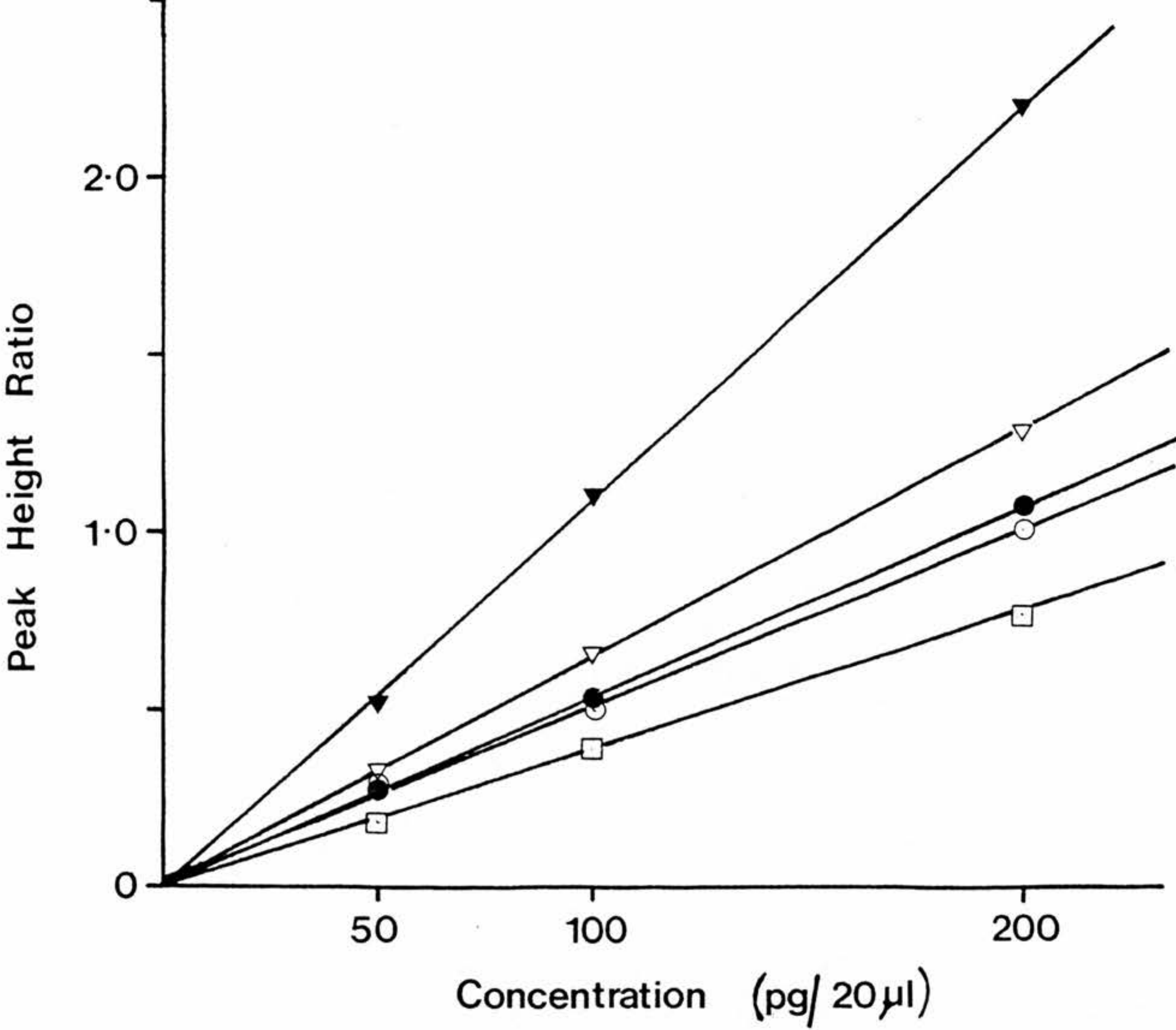


FIGURE 2-2: Standard curves for noradrenaline (▼), 3,4 dihydroxyphenylacetic acid (▽), dopamine (●), 5-hydroxytryptamine (□) and 5-hydroxyindole acetic acid (○), calculated by peak height ratio relative to the internal standard. All standard curves were linear up to 2 ng/20 µl injection.

extracts of NIL and SME samples were chromatographed on the LCED system and analysed using two different oxidation potentials (+0.60 and + 0.45 V). Since the compounds being measured differ in their abilities to oxidise at different applied potentials, by comparing the response ratios of the unknown compounds and standards at two potentials, the identity of the compounds in the sample can be confirmed (Blank, 1976; Mefford, 1981; Mayer and Shoup, 1983). Table 2-A shows that the response ratios in the tissue extracts were similar to those produced by the standard compounds and indicate that each of the peaks analysed in the tissue extracts is due to the presence of a single compound with similar electrochemical properties to the standard compounds.

The heights of all the identified peaks above the baseline of the chart record were measured and standard curves were constructed for each of the amines by linear regression of peak height ratio (PHR) against concentration where

$$\text{PHR} = \frac{\text{Peak height of standards}}{\text{Peak height of the internal standard}}$$

As the same quantity of IS was added to both samples and standards, once the PHR was measured for the unknown sample, it was possible to calculate the amine concentration in the sample directly from the standard curve whilst simultaneously correcting for variations in injection volume and the recovery through the extraction procedure.

2.5 RADIOIMMUNOASSAYS AND PROTEIN BINDING ASSAYS

The techniques and general principles of protein binding assays and radioimmunoassays (RIA) have been the subject of numerous reviews (Midgley, Niswender and Rebar, 1969; Kirkham and Hunter,

Table 2-A Response ratios (peak height at + 0.60 V/peak height at + 0.45 V) of compounds detected by ECD in the median eminence and in the neurointermediate lobe of the pituitary (Mean ± S.E. of the mean of 4-5 determinations).

Compound	Standard	Median Eminence	Neurointermediate lobe
Noradrenaline	2.08 ± 0.06	2.08 ± 0.01	2.01 ± 0.08
Dopamine	1.48 ± 0.02	1.46 ± 0.01	1.45 ± 0.04
DOPAC	3.59 ± 0.11	3.82 ± 0.06	3.99 ± 0.14
5-HT	1.85 ± 0.03	1.79 ± 0.06	1.87 ± 0.05
5-HIAA	3.33 ± 0.08	3.52 ± 0.21	2.93 ± 0.11

1971; Odell and Daughaday, 1971; Yalow and Berson, 1971; Ekins, 1974; Jaffe and Behrman, 1979; Yalow, 1980) and only a brief description of the general methods for RIA will be outlined here. Individual details of the methods of preparation and purification of radiolabelled hormones and the composition of the buffers used in the assays are given in the Appendices. The principle of RIA is the competition between radio-labelled and unlabelled antigen for binding sites on a finite number of specific antibody molecules. The binding of the added labelled antigen is inversely proportional to the concentration of the unlabelled antigen in the sample, which can hence be determined.

Hormones were labelled with ^{125}I (Na ^{125}I, 100 mCi/ml; Amersham International U.K.) using a modification of the chloramine-T method (Greenwood, Hunter and Glover, 1963). With the exception of the TRH and corticosterone assays, for which bound and free hormone were separated by alcohol precipitation of the antibody-hormone complex or dextran-coated charcoal separation respectively, double antibody radioimmunoassay techniques (Utiger, Parker and Daughaday, 1962) were used throughout. In all assays the bound hormone was retained for counting, either by liquid scintillation counting or using a Berthold Mag 310 Gamma counter (Scotlab Instrument Sales, Bellshill, Scotland).

Assay results were calculated either by the inbuilt machine programme of the Berthold counter, or manually by linear regression from standard curves constructed with logit B/Bo as ordinate and log concentration as abscissa, where B/Bo represents the ratio of bound to total bound and,

$$\text{logit } B/Bo = \log_e \left[\frac{B/Bo}{(1 - B/Bo)} \right]$$

Upper and lower limits of detection of the assay were taken as the concentrations at which 10 and 90%, respectively, of the labelled antigen was bound to the primary antibody. The reproducibility and reliability of the assays were determined by the inclusion of two known quality control pools in each assay, thereby enabling calculation of inter- and intra-assay coefficients of variation (CV) (Rodbard, 1971). The pools consisted of plasma from ovariectomized rats (LH and FSH, low and high pools), male rat plasma to which had been added either acid extracts of rat hypothalamic tissue (LHRH high pool) or synthetic LHRH (LHRH low pool), or plasma from unstressed or ether-stressed male rats (prolactin and TSH, low and high pools); for the TRH radioimmunoassay, the pools consisted of solutions of the synthetic tripeptide in assay buffer. Additional quality control data were obtained by monitoring total counts, blanks and total bound (both as percentages of total counts) and the calculated equivalent concentration of hormone corresponding to 20%, 50% and 80% of total bound.

Samples for all assays were aliquoted into disposable plastic test-tubes (LP3, Luckhams Ltd.) using automatic pipettes (Pipetman, Gilson) and reagents dispensed using a Micromedic automatic pipetting system.

2.5.1 Radioimmunoassay of Thyrotrophin Releasing Hormone

The TRH content of hypophysial portal blood extracts or HPLC fractions was determined using an anti-TRH serum (number 420) raised and characterized by Dr. Hamish Fraser (MRC Reproductive Biology

Unit, Edinburgh) (Fraser and McNeilly, 1982). The antiserum was raised in sheep immunized using a TRH-bovine serum albumin (BSA) conjugate. The cross-reaction of this antiserum with TRH, analogues of TRH and with some other hypothalamic peptides are summarized in Table 2-B.

Synthetic TRH (Peninsula Laboratories, San Carlos, California) was used for standards (range 2-800 pg/assay tube) and for iodination. [^{125}I]-TRH was prepared and purified (Joseph-Bravo, Charli, Palacios and Kordon, 1979). Samples and standards, diluted with phosphate-buffered saline (PBS) pH 7.4 with 0.25% BSA, were pre-incubated with antiserum for 24 h at 4°C, after which radiolabelled TRH was added (5000 cpm/tube). The final antiserum concentration was 1:120,000 in an assay volume of 400 µl. After a further 36 h incubation at 4°C, antibody-bound and free ^{125}I-TRH were separated by the addition of 1.4 ml ice-cold ethanol. Typical standard curves, which also demonstrate the parallel displacement of radiolabelled TRH by hypothalamic, or hypophysial portal blood extracts, are shown in Figures 2-3 and 2-4. The lower limit of detection of the assay ranged from 2-4 pg/tube, and the inter- and intra-assay coefficients of variation were 3.8% and 2.6% (for quality control of 9 pg/tube), and 5.2% and 10.8% (for quality control of 174 pg/tube), respectively. Other quality control data is shown in Figure 2-5.

2.5.2 Radioimmunoassay of Luteinizing Hormone Releasing Hormone (LHRH)

LHRH was measured in hypophysial portal blood and fractions recovered from the HPLC of hypothalamic or portal blood, using the

Table 2-B Cross-reaction of TRH, analogues of TRH and other hypothalamic peptides with ovine anti-TRH serum number 420

Peptide	Relative cross-reactivity (%)
pGlu - His - $ProNH_2$	100
pGlu - Phe - $ProNH_2$	100
pGlu - Lys - $ProNH_2$	12.5
pGlu - Met - $ProNH_2$	1.4
pGlu - His - ProOH	< 0.005
pGlu - Leu - ProOH	< 0.005
pGlu - Ala - ProOH	< 0.005
pGlu - His - Pro-propylamide	0.03
H.His - $ProNH_2$	0.004
pGlu - His - $TrpNH_2$	0.05
Glu(BOC) - His - $ProNH_2$	0.96
pGlu - Lys(BOC) - $ProNH_2$	0.26
LHRH	< 0.005
Somatostatin-14	< 0.005
Oxytocin	< 0.005
Corticotrophin releasing hormone	< 0.005
Substance P	< 0.005
β-Endorphin	< 0.005

From data in Fraser and McNeilly (1982) and H.M. Fraser (personal communication).

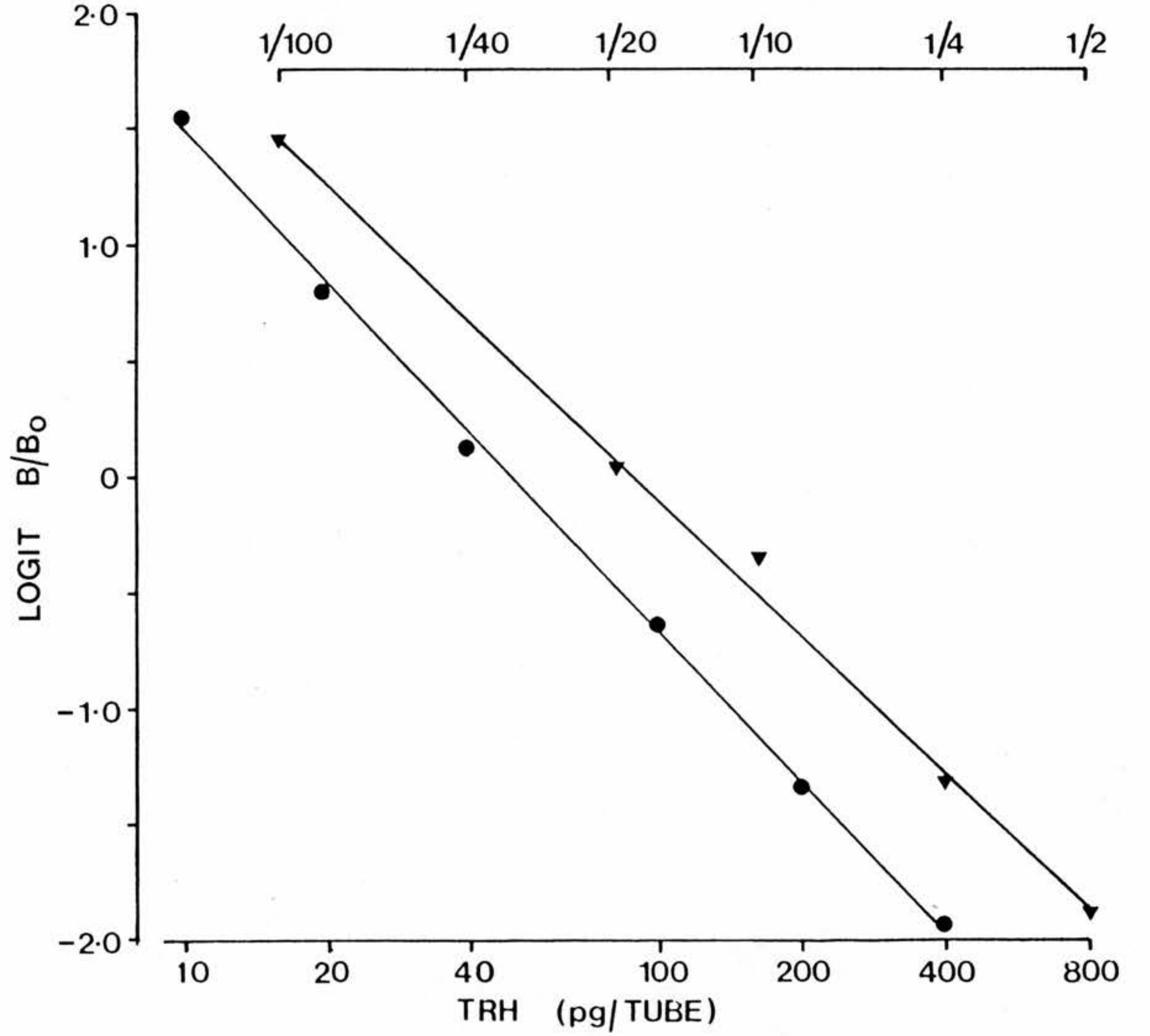


FIGURE 2-3: Standard curve from a representative assay for thyrotrophin releasing hormone (TRH ●) and demonstration of the parallel displacement of radiolabelled TRH by varying dilutions of hypothalamic extracts (▼). Each point represents the mean of 3 replicates.

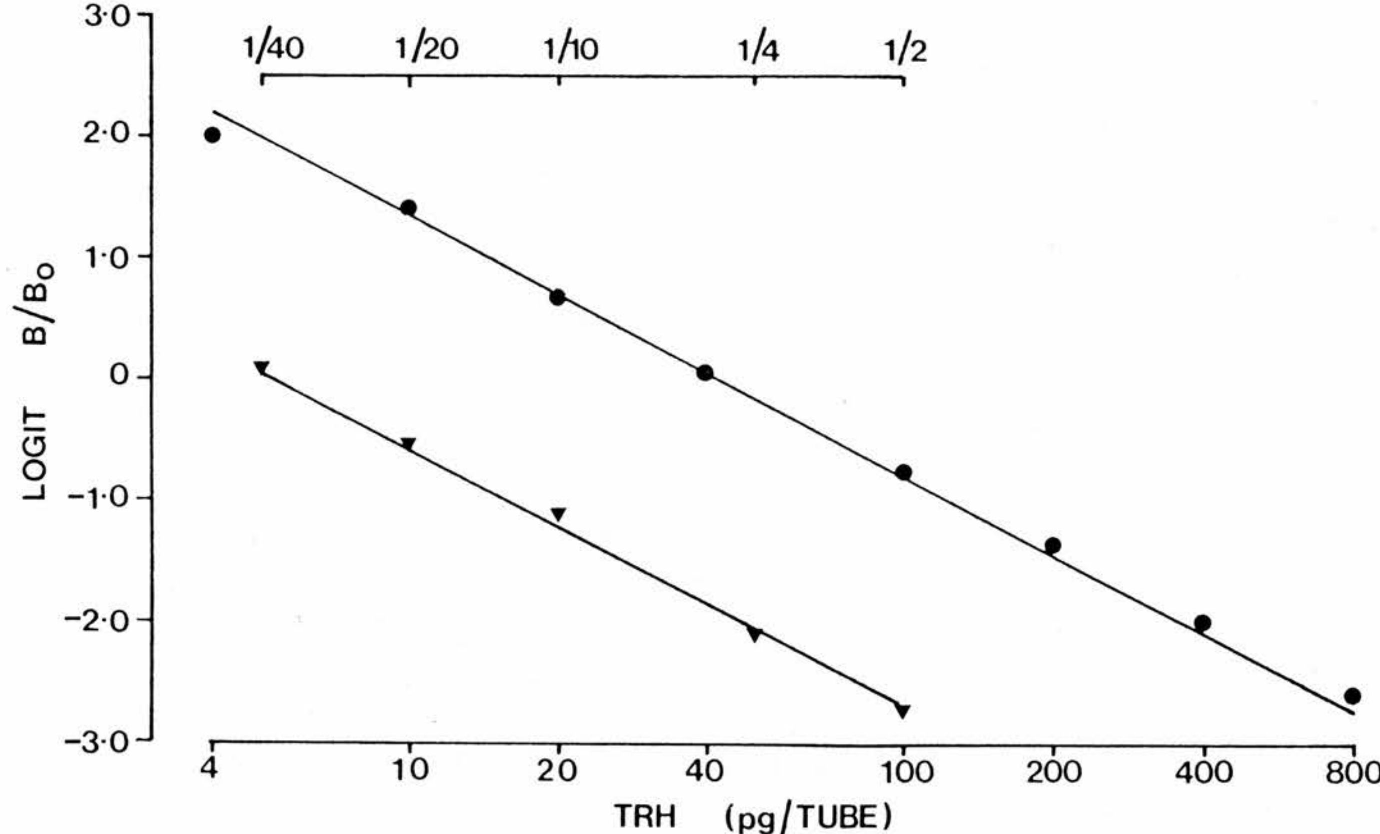


FIGURE 2-4: Standard curve from a representative assay for thyrotrophin releasing hormone (TRH ●) and demonstration of the parallel displacement of radiolabelled TRH by varying dilutions of extracts of hypophysial portal blood (▼). Each point represents the mean of 3 replicates.

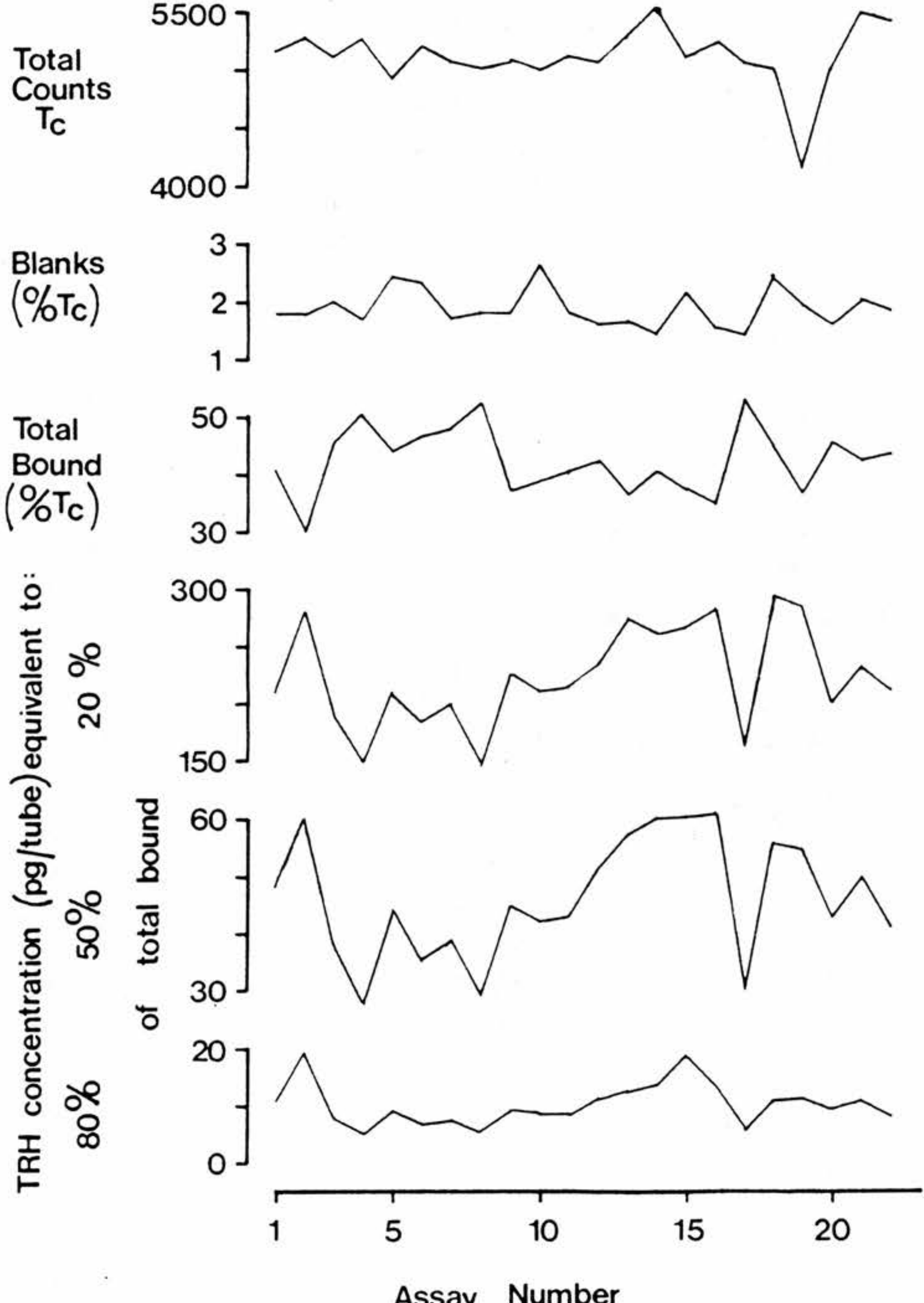


FIGURE 2-5: Quality control data for thyrotrophin releasing hormone (TRH) radioimmunoassays.

double antibody radioimmunoassay developed by Nett, Akbar, Niswender, Hedlund and White (1973) as used and described by Fink and Jamieson (1976) and Chiappa and Fink (1977). The primary antibody was either the R-42 anti-LHRH antiserum (provided by Dr. G.D. Niswender, NIADDK) which was raised in a rabbit against a conjugate of the free acid form of LHRH conjugated to BSA, or antiserum HC-6 which was raised in rabbits by G. Fink against a LHRH-haemocyanin conjugate. The R-42 antiserum recognizes the whole LHRH molecule and does not cross-react with a series of fragments of LHRH although it does recognise the free-acid (Nett <u>et al</u>., 1973; Fink and Jamieson, 1976). The cross-reactions of the HC-6 antiserum are summarized in Table 2-C. Synthetic LHRH (ICI Pharmaceuticals, Macclesfield, U.K.) was used for standards and for iodinations. Samples for assay were diluted with PBS/0.1% Gelatine buffer and incubated for 24 h at 4°C with antiserum (added at a working dilution of 1/90,000 [HC-6] or 1/80,000 [R-42]; all antisera were diluted with PBS/0.5 M disodium EDTA/0.5% normal rabbit serum, pH = 7.0) and ^{125}I labelled LHRH (5000 cpm/tube) before the separation of bound and free hormone by incubation for a further 24 h with the second antibody (ARGG, anti-rabbit gamma globulin raised in donkeys, Scottish Antibody Production Unit, Carluke) added at a working dilution of 1:20 in PBS. A typical standard curve for an assay in which the HC-6 antiserum was used is illustrated in Figure 2-6. Only a small number of LHRH assays were performed and, therefore, full quality control data cannot be given, but the assays conformed to the quality control parameters previously established.

UNIV. EDINBURGH. EX BIBL.

Table 2-C Cross-reaction of LHRH, analogues of LHRH, and other hypothalamic peptides with the HC-6 anti-LHRH serum

Peptide		Relative cross-reactivity (%)
LHRH		100%
LHRH fragments:	1-9	< 0.05
	1-8	< 0.05
	1-6	< 0.05
	1-2 & 9-10	< 0.05
	3-10	2.8
	3-9	< 0.05
	7-10	3.3
[Gly-OH^{10}]-LHRH		< 0.002
[Trp^{7},Leu^{8},Gly-OH^{10}]-LHRH		0.07
[D-Phe^{2},Pro^{3},D-Phe^{6}]-LHRH		23
[D-pGly^{1},D-Phe^{2},D-Trp^{3,6}]-LHRH		3.5
[D-Lys^{6}]-LHRH		63
[D-Ala^{6}]-LHRH		100
Des-Gly^{10}[Pro^{9}]-LHRH ethylamide		0.15
Des-Gly^{10}[D-Trp^{6},Pro^{9}]-LHRH ethylamide		0.4
Des-Gly^{10}[D-Leu^{6},Pro^{9}]-LHRH ethylamide		0.11
TRH		< 0.001
CRF		< 0.001
GHRH		< 0.001

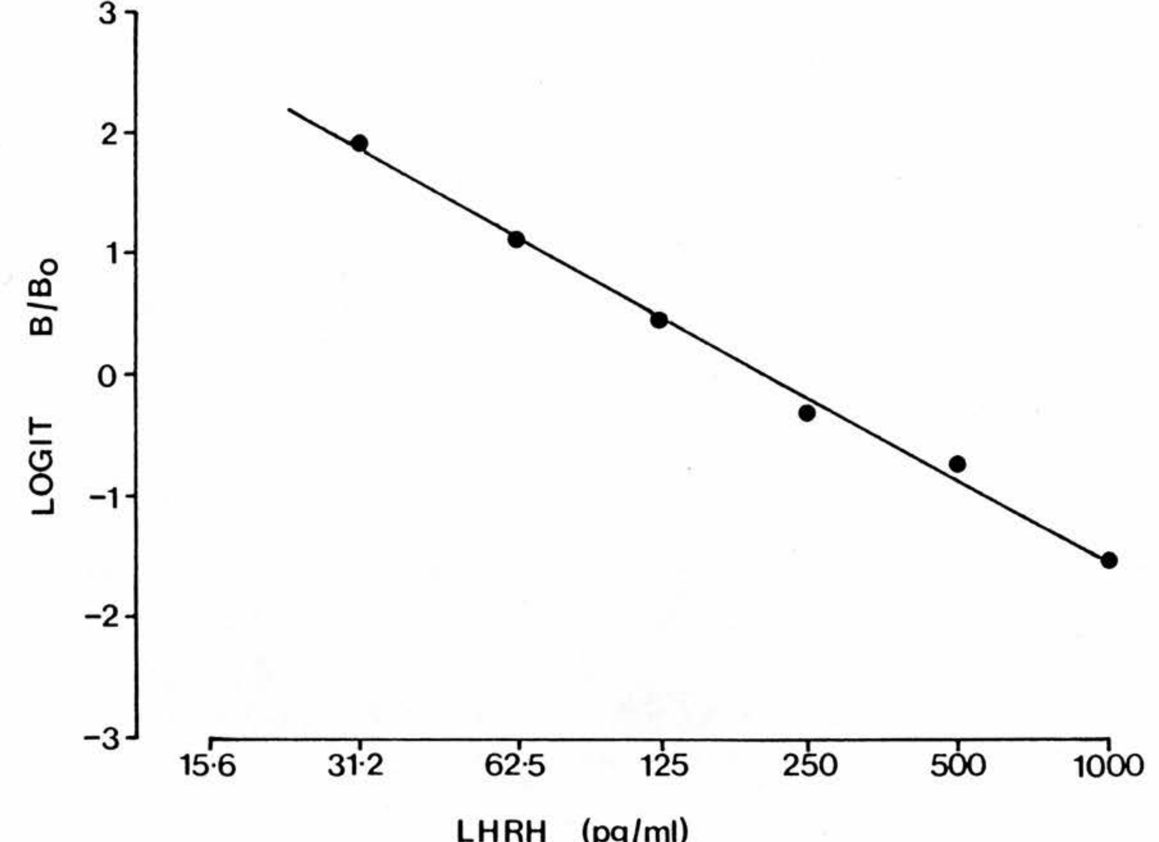


FIGURE 2-6: Standard curve from a representative assay for luteinizing hormone releasing hormone (LHRH) obtained using the HC-6 anti-LHRH serum as the primary antibody. Each point represents the mean of 3 replicates.

2.5.3 Radioimmunoassay of Thyrotrophin (TSH)

Plasma or pituitary concentrations of thyrotrophin were determined by double antibody RIA using materials supplied by NIADDK. The samples obtained in the experiments described in Chapter 9 were assayed as used and described by Pickering (1978) and Pickering and Fink (1979b) using NIADDK-rat TSH RP-1 as reference material. Samples and standards were incubated with antibody for 24 h at 4°C before addition of ^{125}I labelled thyrotrophin (10,000 cpm/tube). After a further 48 h incubation, ARGG was added to separate antiserum-bound and free hormone. The majority of assays were performed using a modification of this assay as described below.

Samples and standards were diluted with PBS/1% BSA and incubated at 4°C for 6 days with rabbit anti-rat TSH-S-2 added at a working dilution of 1/2500 and I^{125} labelled thyrotrophin (rTSH-I-6). Bound and free hormone were separated by incubation for 24 h with ARGG at 4°C. The standard for these assays was NIADDK-rTSH-RP-2 which is 176 times more potent than the RP-1 reference preparation. Inter- and intra-assay CV were 4.8 and 6.5%, respectively. Figures 2-7 and 2-8 show a typical standard curve and summarize the quality control data for the assays included in this thesis.

2.5.4 Radioimmunoassay of Prolactin

Plasma and pituitary concentrations of prolactin (PRL) were determined by double antibody RIA using materials supplied by the NIADDK as used by Pickering and Fink (1979b) and Pickering (1978). The standard curve (range 0.5-64 ng/ml) was prepared from rat-PRL-RP-1 and rat-PRL-I-5 was used for iodination. Standards and

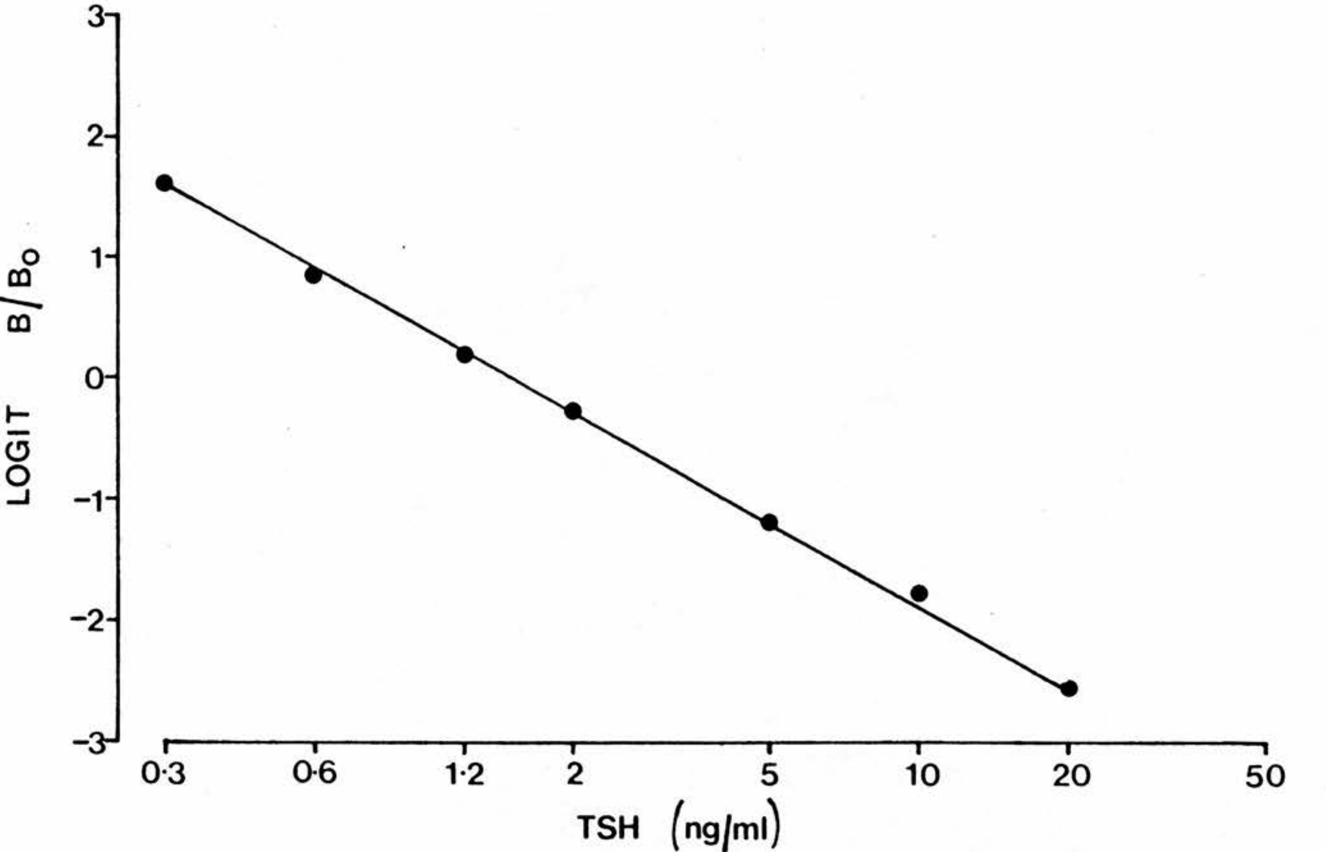


FIGURE 2-7: Standard curve from a representative assay for thyrotrophin (TSH) obtained using the NIADDK-rTSH-RP-2 reference preparation as standard. Each point represents the mean of 3 replicates.

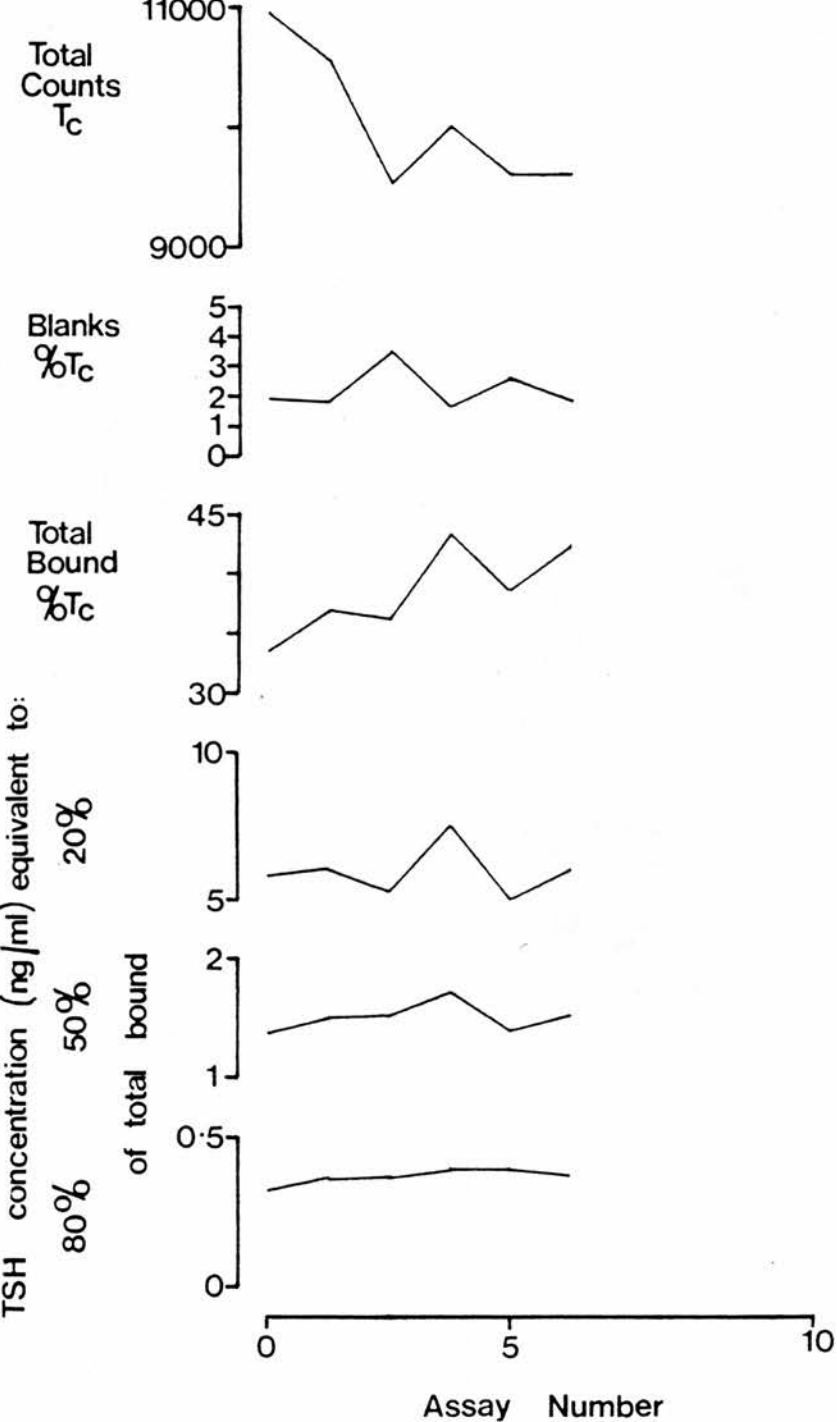


FIGURE 2-8: Quality control data for the thyrotrophin (TSH) radioimmunoassays.

samples for the assay were suitably diluted in assay buffer (PBS/1% BSA) and incubated with the antiserum (anti-rat-PRL-S5, added at a working dilution of 1:5000) for 24 h at 4°C before the addition of ^{125}I-PRL (approximately 10000 cpm/tube). After further incubation for 48 h at 4°C, the antiserum-bound hormone was separated from the free hormone using ARGG (SAPU) at a working dilution of 1:20. A typical standard curve is shown in Figure 2-9 and quality control data in Figure 2-10. Inter- and intra-assay C.V. were 5.3% and 1.4% respectively. For plasma samples assayed in duplicate in 2-20 µl aliquots, the lower limits of detection were 15-50 ng/ml.

2.5.5 Radioimmunoassay for Luteinizing Hormone

LH was measured in rat and mouse plasma and pituitary samples using the ovine-ovine RIA developed by Niswender, Midgley, Monroe and Reichert (1968). The protocol used was that described by Aiyer (1974) and Aiyer and Fink (1974). Ovine-LH (LER-1056-C2) provided by Dr. L.E. Reichert, Jr. (NIADDK) was used for iodination and ovine LH (NIH-LH-S18) used to prepare the standards (range 0.25-16 ng/ml). The anti-ovine LH antiserum (GDN-15) was provided by Dr. G.D. Niswender (NIADDK).

Samples and standards were diluted in PBS/1% BSA and pre-incubated with antiserum (added at a working dilution of 1:6000) for 24 h at 4°C before the addition of ^{125}I-LH (10,000 cpm/tube). The second antibody (ARGG, SAPU 1:20) was added after a further 48 h incubation period at 4°C. A typical standard curve is reproduced in Figure 2-11, and quality control data in Figure 2-12. The inter- and intra-assay coefficients of variation were 7 and 9% respectively. For a 50 µl plasma sample the lower limit of

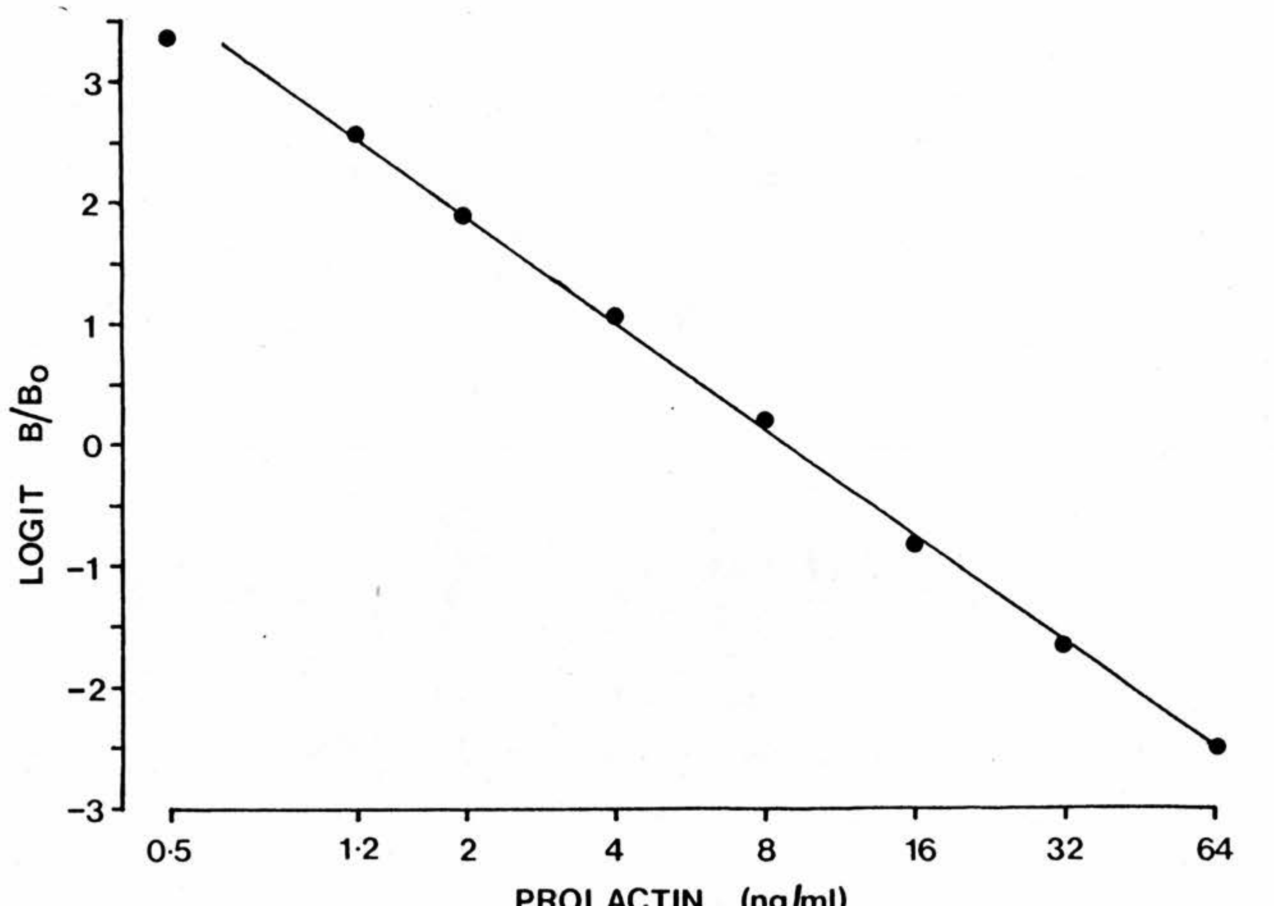


FIGURE 2-9: Standard curve from a representative assay for prolactin. Each point represents the mean of 3 replicates.

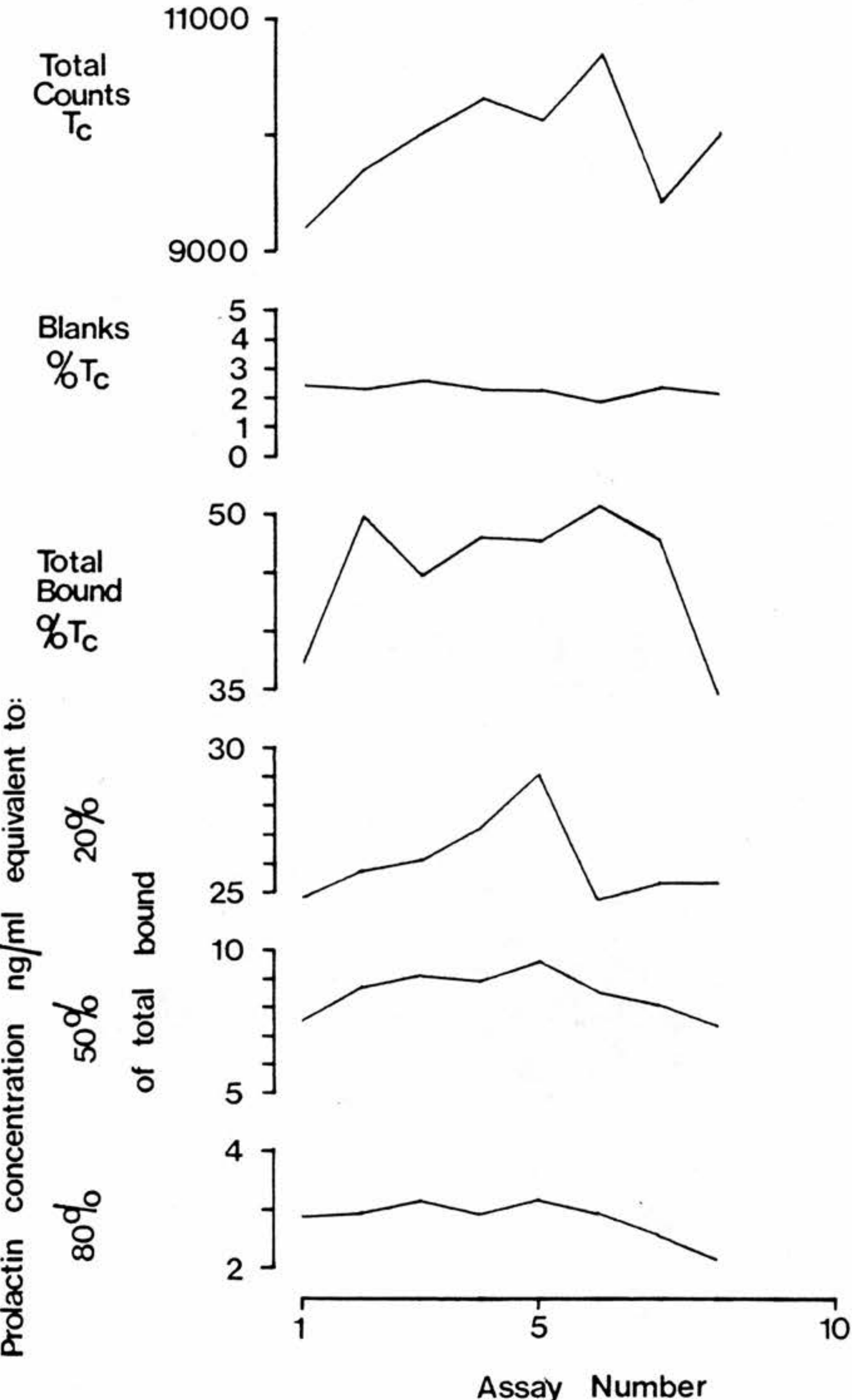


FIGURE 2-10: Quality control data for the prolactin radioimmunoassays.

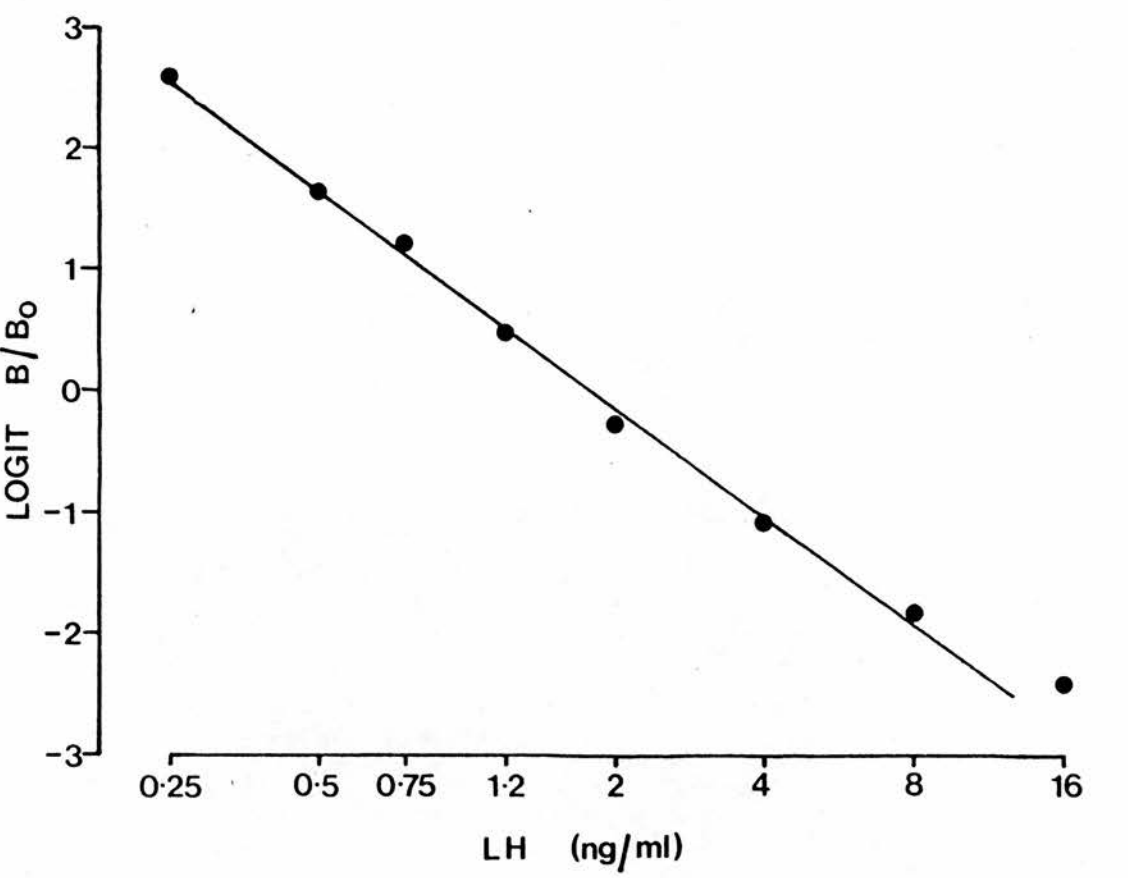


FIGURE 2-11: Standard curve from a representative assay for luteinizing hormone (LH). Each point represents the mean of 3 replicates.

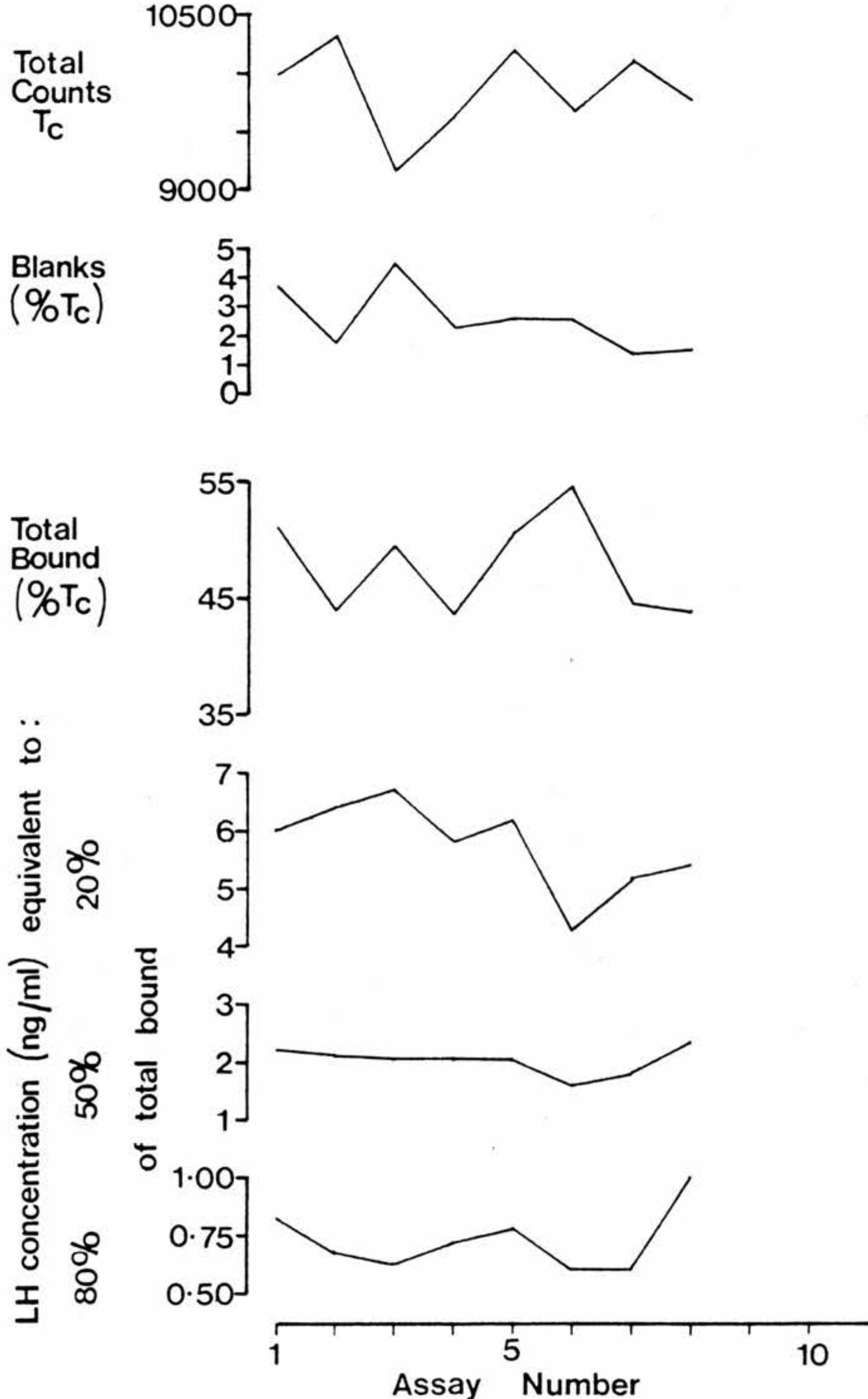


FIGURE 2-12: Quality control data for the luteinizing hormone (LH) radioimmunoassays.

sensitivity of the assay was generally 0.8 ng LH/ml.

2.5.6 Radioimmunoassay of FSH

The concentration of FSH in rat plasma and pituitary samples was determined by RIA using materials provided by the NIADDK according to the method of Danne and Parlow (1971) as used by Chiappa (1976) and Sarkar (1979). NIADDK-rat-FSH-I 1 was used for iodination and rat-FSH-RP-1 for the preparation of the standard curves. Standards and samples were diluted with PBS/1% BSA and incubated at 4°C with antiserum (NIADDK-anti-rat-FSH-S6, used at a working dilution of 1:2500) and ^{125}I FSH (12000 cpm/tube). After 6 days incubation, antibody bound and free hormone were separated using ARGG. A representative standard curve is shown in Figure 2-13. All samples were incubated in a single assay for which the intra-assay coefficient of variation was 7.1%.

2.5.7 Measurement of Plasma Corticosterone

Plasma corticosterone was estimated using the competitive protein binding assay (Chiappa and Fink, 1977; Chiappa, 1976) which was based on the method described by Corker, Naftolin and Richards (1971).

Progesterone, which would otherwise have interfered in the assay, was removed from the plasma samples by extraction with petroleum ether. The aqueous phase from this extraction contained corticosterone which was then extracted with diethyl ether. The aqueous phase from the second extraction was discarded and the ether removed by evaporation under a stream of nitrogen. The samples were redissolved in TRIS buffer (pH 8.0) for assay. The efficiency of the extraction was monitored by the recovery of 1000 cpm of

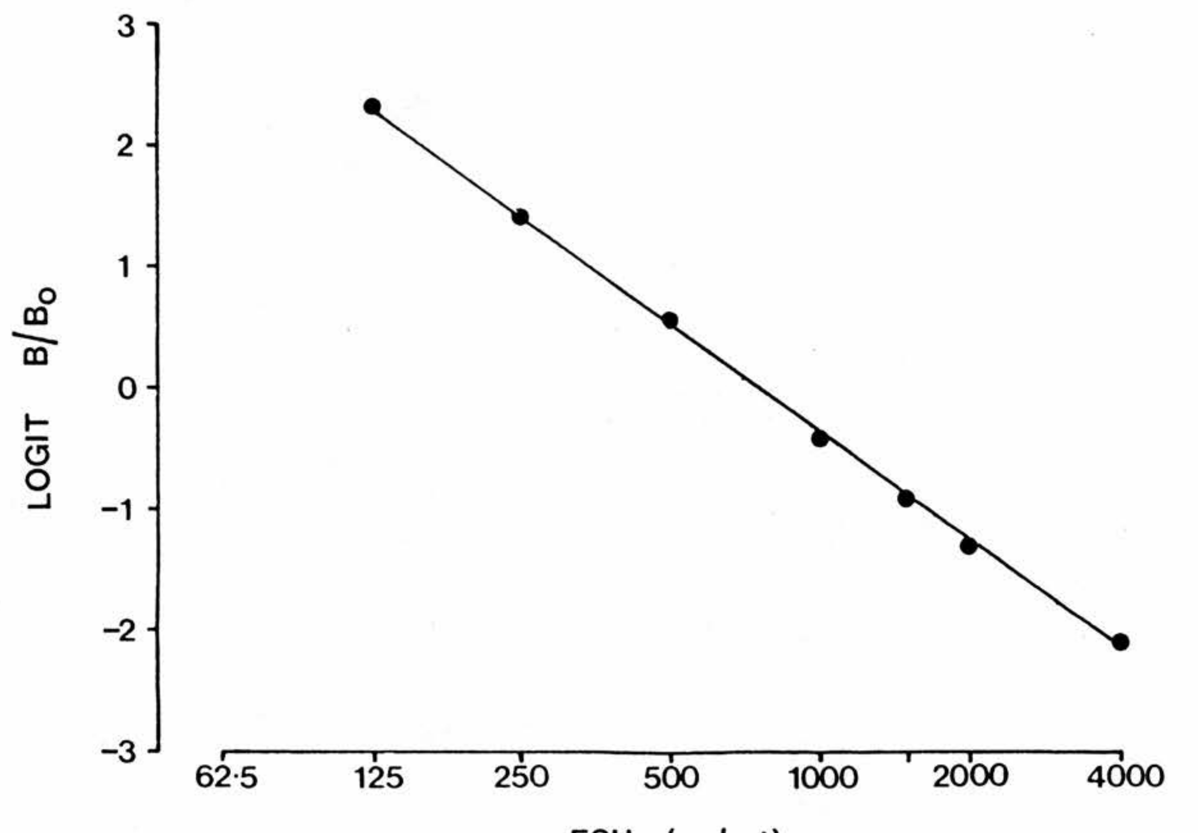


FIGURE 2-13: Standard curve of the follicle stimulating hormone (FSH) radioimmunoassay. Each point represents the mean of 3 replicates.

^{3}H-corticosterone added to each plasma sample at the start of the extraction procedure. The final concentration of corticosterone measured in the assay was corrected for losses during extraction.

The assay was performed in clean glass test tubes (75 x 10 mm; Gallenkamp). Extracted samples and standards (Corticosterone, Sigma, U.K.) together with corticosterone binding globulin (human female plasma, diluted 1:500) and ^{3}H-corticosterone (1α, 2α [n]-^{3}H corticosterone, Amersham International, UK; approximately 10,000 cpm/tube) were incubated for 45 min at 45°C followed by 10 min at 4°C. Bound and free hormone were separated by addition of dextran-coated charcoal (Norit-A activated charcoal and dextran T-70; Sigma, UK). A typical standard curve is shown in Figure 2-14.

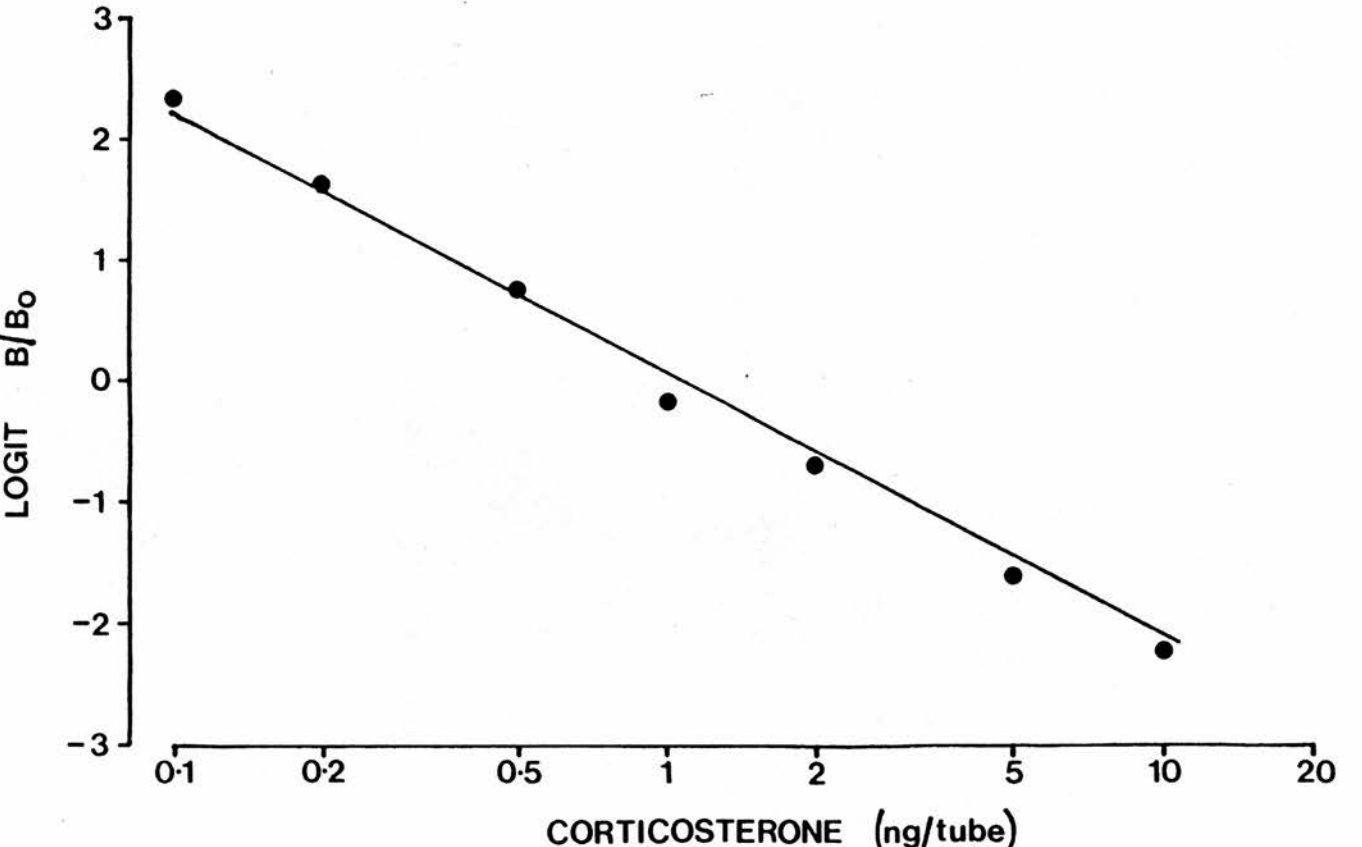


FIGURE 2-14: Standard curve of the corticosterone competitive binding assay. Each point represents the mean of duplicate determinations.

CHAPTER 3

The Immunoreactive Forms of Somatostatin Released Into Hypophysial Portal Vessel Blood

3.1 INTRODUCTION

The tetradecapeptide somatostatin-14 (SS-14) is produced by cleavage of a larger precursor molecule from which a number of immunoreactive (IR) forms of intermediate molecular weight are also formed. The sequence of Pre-Pro-SS has been determined by the cell-free translation of messenger RNA (mRNA) from rat medullary thyroid carcinoma (Goodman, Aron and Roos, 1983) and shown to be a peptide containing 116 amino acids (molecular weight, 12,737 daltons) with the SS-14 sequence at the C-terminus (amino acid residues 102-116). Removal of the signal peptide (residues 1-14) yields Pro SS (92 amino acid residues, 10,388 daltons; Goodman *et al*., 1983) and further cleavage of this may generate at least 7 other peptides (Benoit, Ling, Alford and Guillemin, 1982b; Benoit, Bohlen, Esch and Ling, 1984) as summarized in Figure 3-1. The N-terminally extended form of SS-14, comprised of 28 amino acids (SS-28; Fig. 3-2), had first been identified in the intestine (Pradayrol, Jornvall, Mutt and Ribet, 1980) and was later characterized in the hypothalamus (Esch, Bohlen, Ling *et al*, 1980; Schally, Huang, Chang, Arimura *et al*, 1980; Spiess, Villarreal and Vale, 1981). Since a pair of basic amino acid residues is interposed between the SS-14 sequence and the extension, SS-28 was thought to be a precursor of SS-14. However, SS-28 proved to be more potent than SS-14 in inhibiting the secretion of growth hormone (Brazeau, Ling, Esch *et al*, 1981), prolactin and insulin (Meyers, Murphy, Redding, Coy and Schally, 1980; Tannenbaum, Ling and Brazeau, 1982) and this suggests that SS-28 may be a regulatory hormone in its own right as well as a precursor of SS-14.

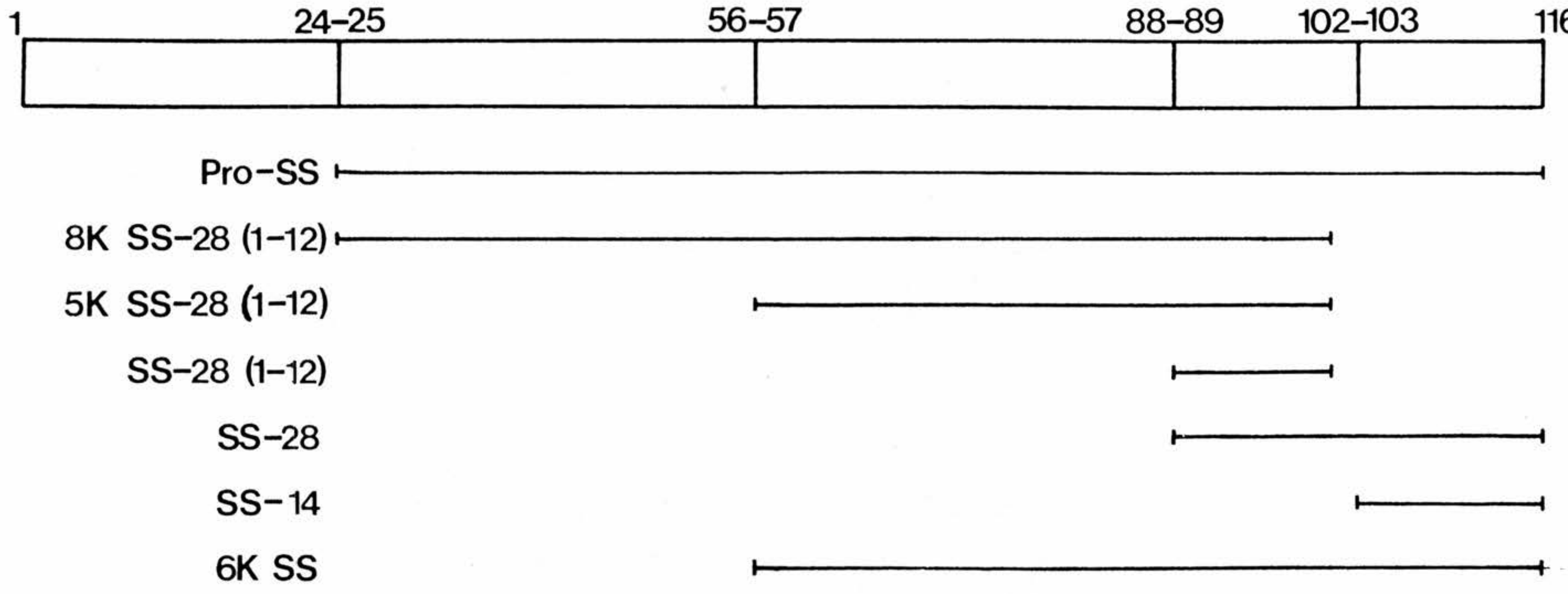


FIGURE 3-1: Diagram illustrating the relationship between Pre-Pro somatostatin (Goodman *et al*, 1983) and 7 peptides derived from it (Benoit *et al*, 1982b, 1984).

FIGURE 3-2 Amino acid sequences of somatostatin-28, somatostatin-14 and somatostatin-28(1-12).

Somatostatin-28

Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

Somatostatin-28(1-12)

Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu

Somatostatin-14

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

SS-28(1-12) (Fig. 3-2), the N-terminal fragment of SS-28, has also been characterized in the hypothalamus and pancreas of the rat (Benoit, Bohlen, Ling et al, 1982a) and shown by radioimmunoassay to be present in extrahypothalamic areas of the nervous system and in the gastrointestinal tract (Benoit et al. 1982b; Benoit, Ling, Bakhit et al, 1982c).

Earlier studies (e.g. Chihara, Arimura and Schally, 1979) showed that SS-14 was released into hypophysial portal blood, confirming a likely role for SS-14 in the neural control of the secretion of growth hormone and other anterior pituitary hormones such as thyrotrophin and prolactin. Similarly, before SS-28 and SS-28(1-12) can be accepted as hypothalamic-pituitary releasing hormones it must be shown that they are released from the hypothalamus in physiologically active quantities. SS-28 can be released in vitro from synaptosomal preparations of the median eminence by depolarizing stimuli or by Ca^{2+} ionophores (Kewley, Millar, Berman and Schally, 1981), and calcium dependent release of SS-28(1-12) from rat hypothalamic slices has also been demonstrated in vitro (Bakhit, Benoit and Bloom, 1983a). Hypophysial portal plasma has been shown by gel filtration to contain several peaks of immunoreactive SS (Gillioz, Giraud, Conte-Devolx et al, 1979; Chihara et al, 1979). One of the peaks corresponded to SS-14, but the additional immunoreactive peaks were not identified in these studies. The following experiments were carried out to determine whether SS-28 and SS-28(1-12) like immunoreactivity (IR) were present in hypophysial portal vessel blood in order to assess whether these peptides may serve as physiological neurohormones.

3.2 MATERIALS AND METHODS

Male Wistar rats (200-350 g body weight) were supplied and maintained under controlled conditions as described in Chapter 2 (2.1). Animals were anaesthetized with urethane, and hypophysial portal vessel blood was collected for four consecutive periods of 15 min each (2.2.3). During the second and fourth period of collection an electrical stimulus was applied to the median eminence by way of a unipolar glass insulated electrode (2.2.4). The portal blood was collected into ice cold tubes containing a solution (10 µl for each 100 µl of blood) of 10^{-2} M Bacitracin (Sigma, U.K.), 1000 KIU Trasylol (Bayer, U.K.) and 0.1 M EDTA (B.D.H., Poole). At the end of collection the blood samples were centrifuged at 4°C and the plasma was extracted twice with 2 ml ethanol-1.0 N-HCl (95/5: v/v). The samples were stored at -40°C, dried under vacuum, and subsequently reconstituted in the appropriate buffer for chromatography or RIA. The significance of differences between mean SS concentrations was determined by the Mann Whitney U test (Siegel, 1956).

3.2.1 HPLC of Somatostatin-14 and 28

Dried extracts of hypophysial portal blood were sent to Dr. R.P. Millar at the University of Capetown where they were reconstituted in 500 µl of 0.05 M ammonium acetate buffer (pH 5.5) of which 50 µl were used for the assay of total somatostatin and the remaining 450 µl chromatographed using a Walters HPLC system. The sample was applied on to a cation exchange column (40 x 250 mm Partisil PXC 10/25 SCX; Whatman) and eluted with a stepwise gradient of increasing concentrations of ammonium acetate buffer in 10%

methanol, as shown below:

1-15 minutes 0.05 M ammonium acetate in 10% ethanol, pH 5.5

16-30 minutes 0.2 M ammonium acetate in 10% ethanol, pH 4.6

31-60 minutes 0.5 M ammonium acetate in 10% ethanol, pH 4.1;

the flow rate was 1.6 ml/min throughout. The fractions eluting from the column were freeze dried and the somatostatin content determined by RIA using an antiserum (number 774) which recognizes the mid-region of the molecule and hence cross-reacts with both SS-28 and SS-14 (Kewley, Millar, Berman and Schally, 1981).

3.2.2 Determination of SS-28(1-12) by radioimmunoassay

The extracts of corresponding samples of either hypophysial portal or peripheral plasma (derived from samples of peripheral blood withdrawn from the external jugular vein either before, or immediately after, the onset of the first period of median eminence stimulation) from 5 animals were pooled, dried under vacuum and sent to Dr. R. Benoit in San Diego for assay. The dried samples were reconstituted in 0.05 M ammonium acetate pH 5.5; this solution was centrifuged at 12000 x g for 10 min and the pH of the supernatant adjusted to pH 7.0 with NaOH solution. After a further centrifugation, SS-28(1-12) was determined by radioimmunoassay (Benoit et al. 1982c) using antiserum S 320 which recognizes the C-terminus of SS-28(1-12) but shows no cross-recativity with SS-14 and negligible cross-reactivity with SS-28 (< 0.01%).

3.3 RESULTS

Figure 3-3 shows that electrical stimulation of the median eminence produced a large increase in both the total amount of SS

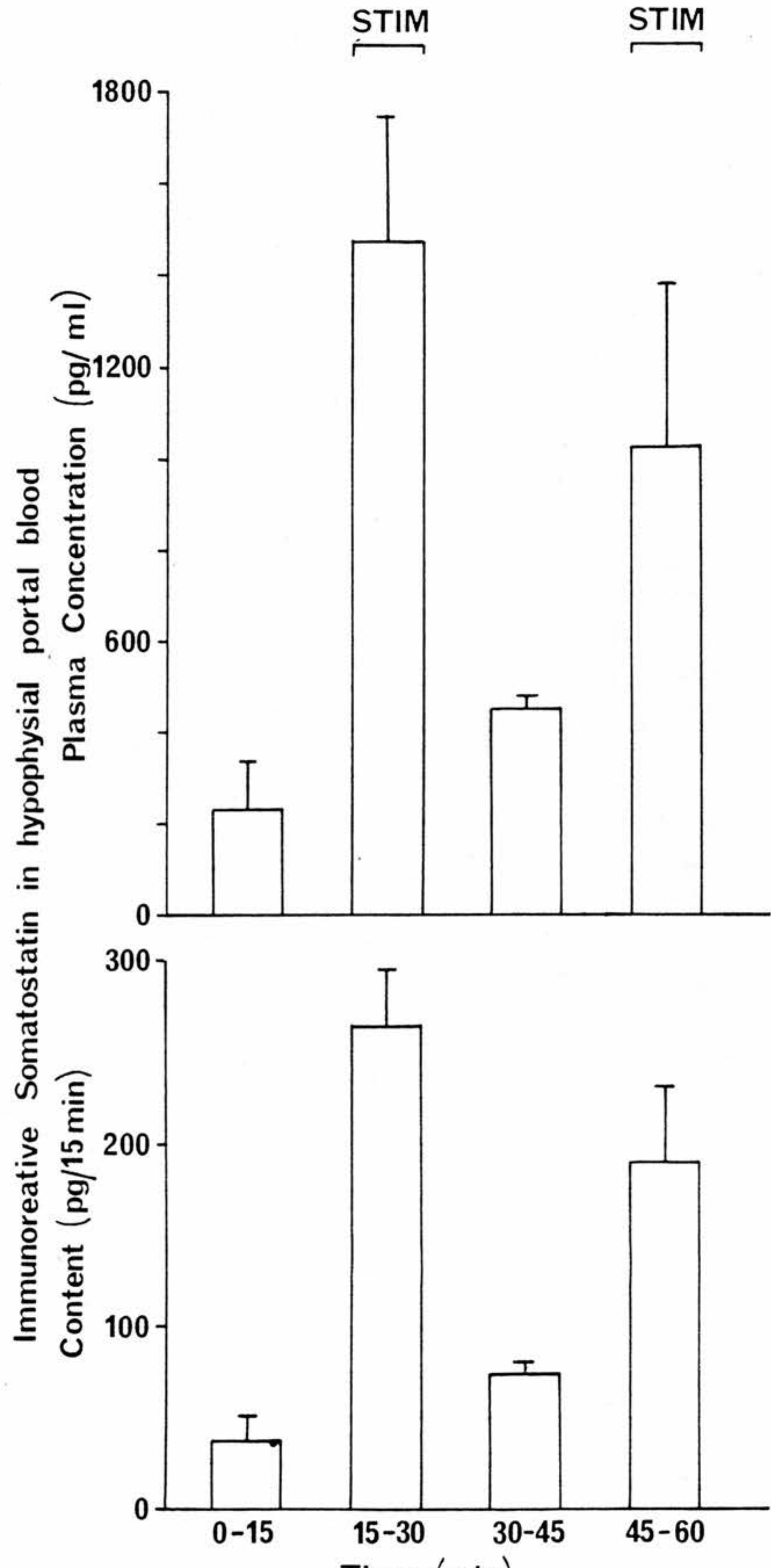


FIGURE 3-3: The mean (± SEM) concentrations of immunoreactive somatostatin per millilitre of plasma and the total amount released into hypophysial portal plasma collected from 6 animals during 4 consecutive periods of 15 min. An electrical stimulus was applied to the median eminence during the second and fourth periods of collection (STIM).

released per unit time and the concentration of SS per millilitre of hypophysial portal plasma ($P < 0.001$ for the first and $P < 0.05$ for the second stimuli. Cessation of the hypothalamic stimulation resulted in a prompt drop in SS output to a level similar to that before the onset of stimulation; application of the second stimulus resulted in a further increase in SS output which was lower than in response to the first, but not significantly so. With the antiserum 774 and the HPLC conditions used in these experiments, the SS-IR in hypophysial portal blood was resolved into two components which eluted in precisely the same positions as SS-14 and SS-28 respectively (Figs. 3-4 and 3-5). There was no significant difference in the relative secretion of the two peptides into portal blood collected during electrical stimulation of the median eminence compared with that secreted after the stimulus had been switched off (Fig. 3-4). Using the same extraction and assay procedures, the concentration of SS detected in extracts of peripheral blood, sampled from either the inferior vena cava or aorta, was 35 pg/ml.

Figure 3-6 shows that SS-28(1-12)-IR was present in extracts of hypophysial portal blood and that ME stimulation produced a 4-5 fold increase ($P < 0.005$) both in the concentration and the total amount of SS-28(1-12) released per unit time. The response to the second stimulus was significantly ($P < 0.005$) less than that to the first stimulus. In peripheral plasma the concentrations of SS-28(1-12) were below the limit of detection (< 150 pg/ml) in all but two of the samples in which the concentrations of SS-28(1-12) were 192 and 242 pg/ml for pre- and post-stimulation samples, respectively.

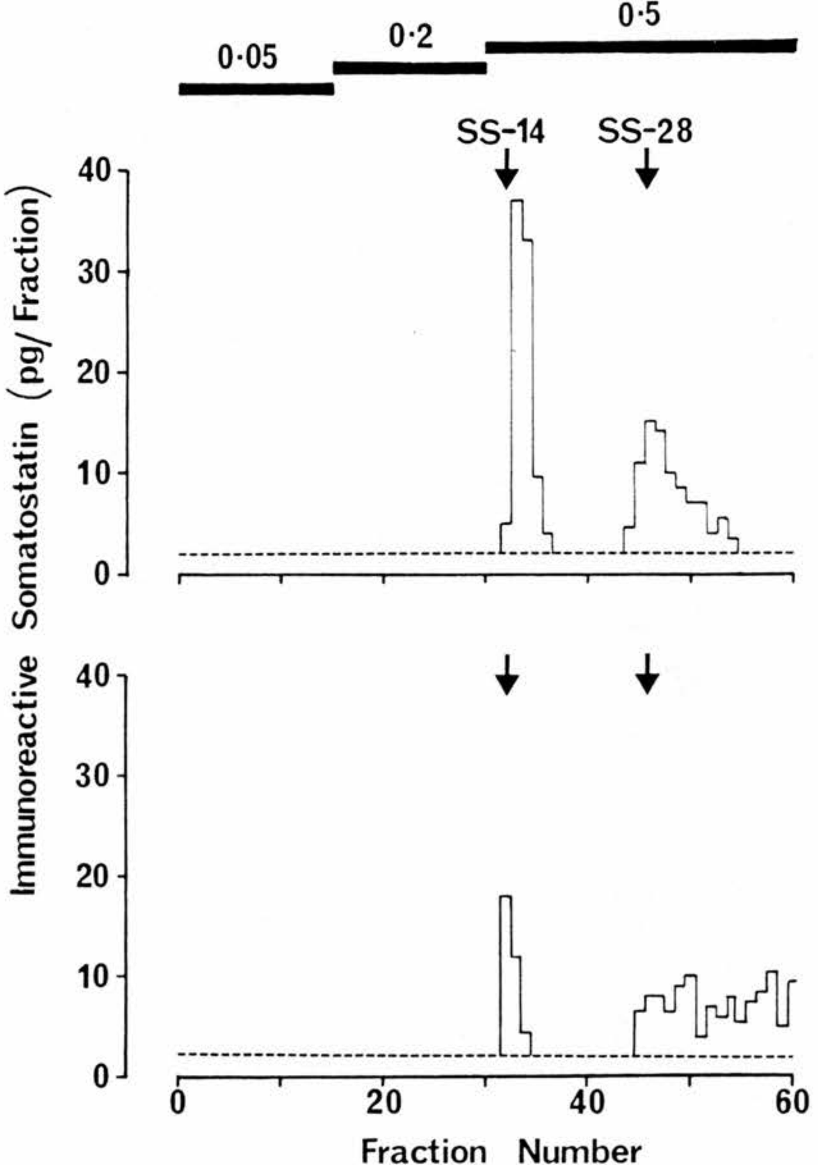


FIGURE 3-4: Representative profiles of somatostatin immunoreactivity in HPLC fractions of extracts of portal plasma collected during (upper trace) and after (lower trace) the application of the electrical stimulus to the median eminence. Heavy bars denote the increasing concentrations of ammonium acetate buffer (molarity shown) used to elute the immunoreactive somatostatin from the HPLC column. The total amounts of immunoreactive SS-14 and SS-28 detected were respectively 90 and 92 pg during the stimulus and 35 and 52 pg after the stimulus.

Arrows indicate the elution positions of synthetic SS-14 and SS-28.

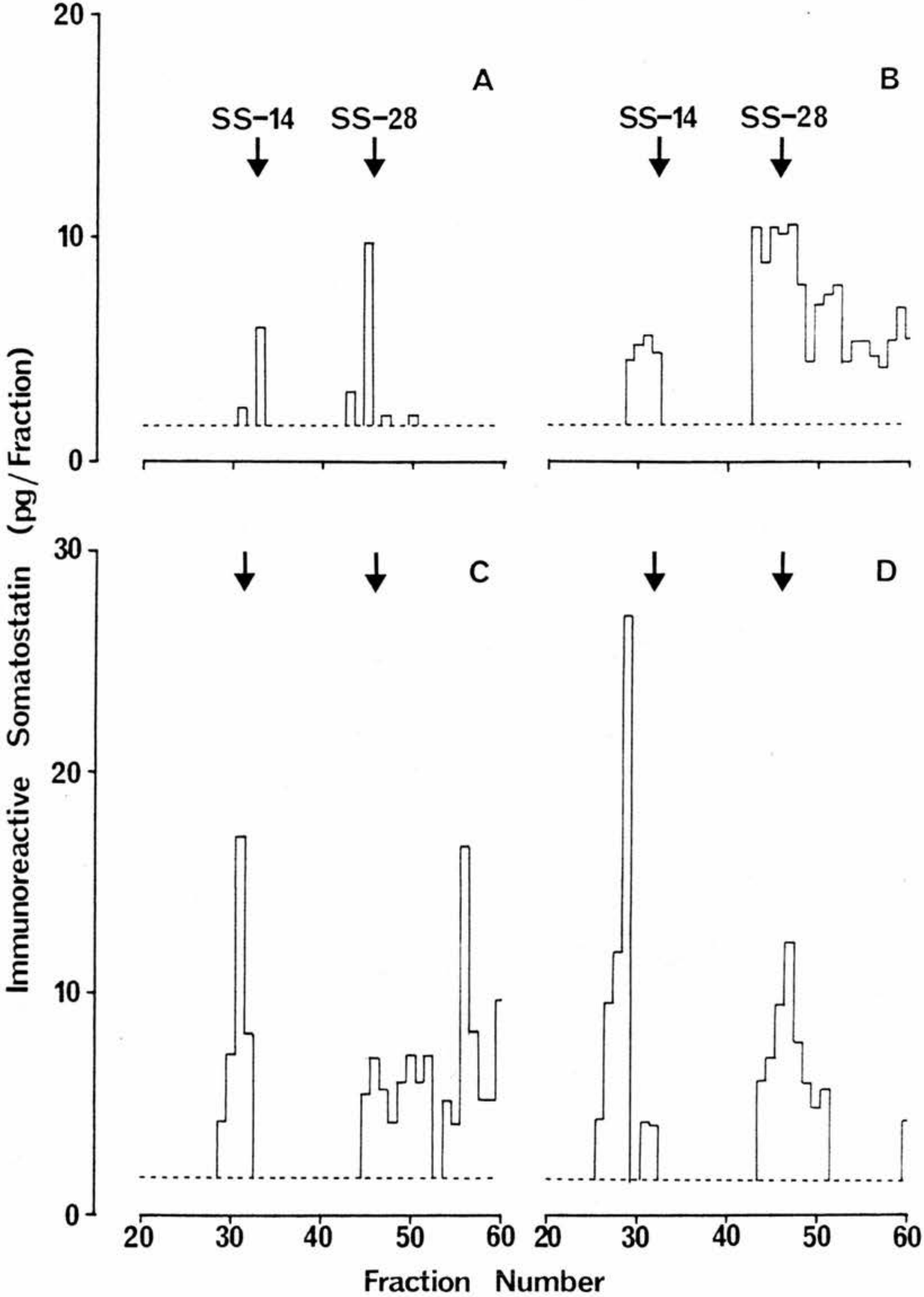


FIGURE 3-5: Profiles of somatostatin immunoreactivity in HPLC fractions of extracts of portal plasma collected before (A and B) and during (C and D) the application of the electrical stimulus to the median eminence. Elution conditions as described in Figure 3-4.

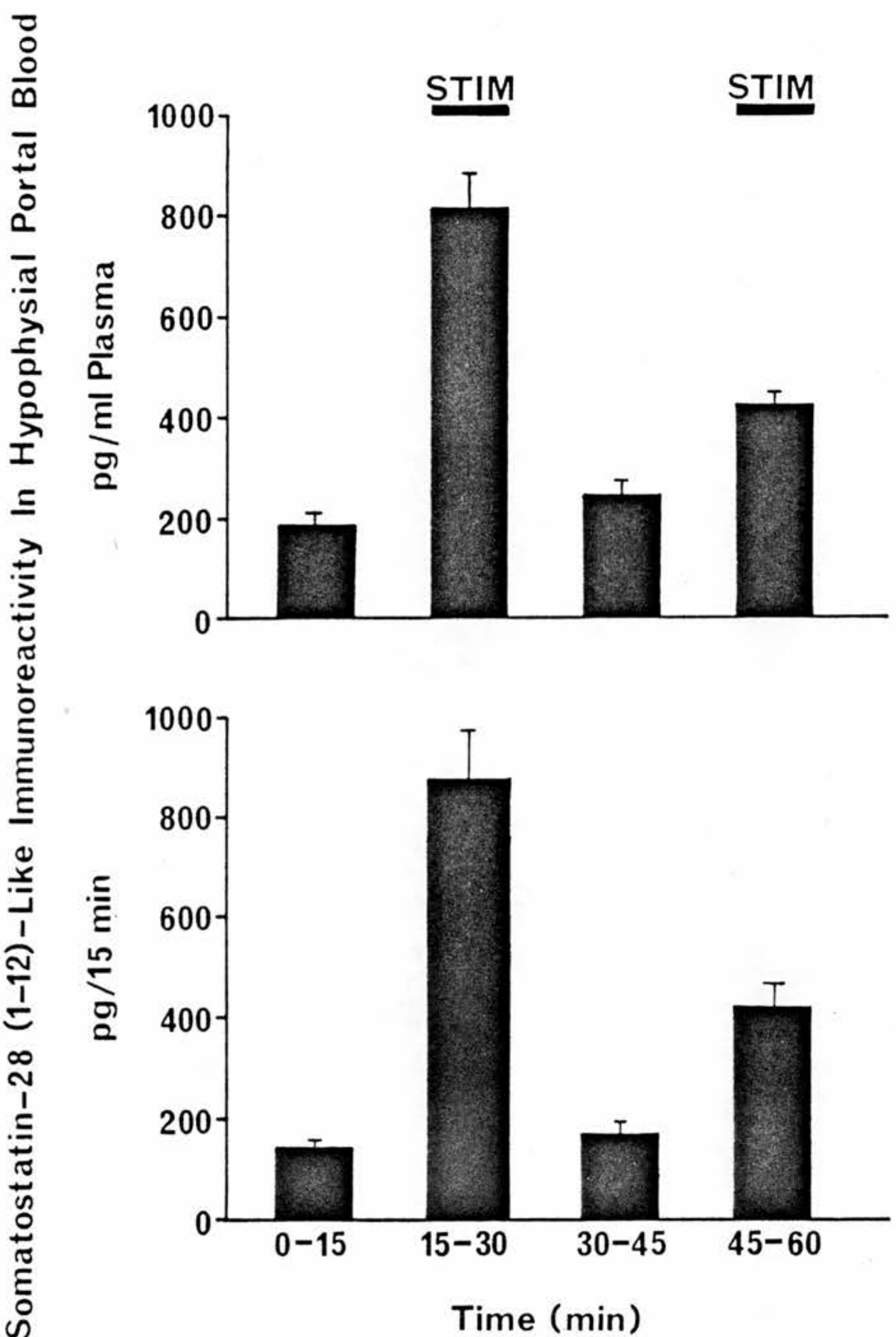


FIGURE 3-6: Mean (±SEM) concentration of SS-28(1-12)-like IR in hypophysial portal vessel plasma and the total amount released during successive 15 min periods of blood collection. Each mean value is based on estimations of five separate pooled extracts: each pool was comprised of samples collected from 5 animals. An electrical stimulus was applied to the median eminence during the second and fourth collection periods, denoted by STIM.

3.3 DISCUSSION

The concentration of SS in portal blood and the amount of SS secreted per unit time in the period of collection before stimulation of the median eminence (Fig. 3-3) were of the same order as those found by Chihara *et al*. (1979b) and Gillioz *et al*. (1979). The 6 to 7-fold increase in both the plasma concentration and the secretion rate of SS produced by stimulation of the median eminence was similar to that reported for preoptic stimulation (Chihara, Arimura, Kubli-Garfias and Schally, 1979a). Median eminence stimulation produced a similar increase also in the concentration and secretion rate of luteinizing hormone releasing hormone (LHRH) (Sherwood, Chiappa and Fink, 1976). Cessation of hypothalamic stimulation resulted in a prompt drop in total SS output to levels recorded before application of the stimulus, and the application of second stimulus resulted in another increase in SS output that was not significantly different from the response to the first stimulus.

The amount of somatostatin in portal blood was only sufficient to analyse by a single HPLC chromatographic system which provides the best resolution of somatostatin molecular forms. However, the two peaks of SS-IR in hypophysial portal blood extracts eluted in an identical manner to that released from median eminence synaptosomes. The immunoreactive material released from synaptosomes has been characterized in a series of different chromatographic systems (Kewley *et al*., 1981); on Sephadex G-25 the SS contained in, and secreted from, median eminence synaptosomes eluted as two major immunoreactive peaks, a 3K species co-eluting

with SS-28 and a 1.6K species co-eluting with SS-14. The 3K species on HPLC was shown to elute identically with SS-28 in both a reverse phase and in the same cation exchange system used for separating the extract of hypophysial portal plasma (Fig. 3-4). Similarly, the SS-14 (1.6K) species behaved in an identical manner to synthetic SS-14 in this HPLC system.

In the rat hypothalamus and amygdala, SS-14 and SS-28 represent approximately 60% and 10% respectively of the total SS content (Pierotti and Harmar, 1985). Similarly, SS-14 is reported as the major immunoreactive form of SS released from the hypothalamus _in vitro_ in response to either a reduction in glucose concentration (Lengyel, Kruseman, Grossman, Rees and Besser, 1984) or depolarizing stimuli (Pierotti, Harmar, Tannahill and Arbuthnott, 1985). By contrast, SS-14 and SS-28 are present in the median eminence in approximately equimolar proportions (Pierotti and Harmar, 1985) and the proportion of SS-28 released from the ME _in vitro_ is also higher (Kewley _et al_, 1981; Pierotti _et al_, 1985). Figure 3-4 shows that the increase in total SS output was due to increased secretion of both SS-28 and SS-14; the cross-reaction of antiserum 774 with SS-28 on a molar basis is only 30 per cent (Kewley _et al_., 1981) and, therefore, these results (Fig. 3-5), which underestimate the amount of SS-28 secreted, suggest that, _in vivo_, SS-28 is probably secreted in greater amounts than SS-14.

Srikant and Patel (1981) suggested that the greater potency of SS-28 compared with SS-14 in inhibiting hormone release (Meyers _et al_., 1980; Brazeau _et al_, 1981; Rodrigues-Arno, Gomez-Pan, Rainbow _et al_, 1981) was due, at least in part, to the fact that the

pituitary binds SS-28 with an affinity that is 3.2 times greater than that for SS-14. The longer half-life of SS-28 in plasma may also contribute to its apparently greater potency *in vivo* (Patel and Wheatley, 1983). The present data taken together with those of Srikant and Patel raise the possibility that SS-28 is a major hypothalamic factor that inhibits growth hormone release and that SS-14 may be an active fragment. Some hormones such as ACTH, although themselves highly active and functionally important, are capable of being processed further to biologically active molecules such as α-MSH. By analogy, SS-28 should therefore be regarded as a potential precursor for SS-14 as well as an important hormone in its own right. In view of the different potencies (Meyers *et al*., 1980; Schally *et al*., 1980; Rodrigues-Arno *et al*., 1981), actions (e.g. SS-28 but not SS-14 inhibits the FSH response to LHRH; Millar, Klaft, Barron, Levitt and Ling, 1982) and possibility of different receptor populations (Srikant and Patel, 1981) for SS-14 and SS-28, alterations in the relative production and secretion of the two hormones may play a significant role in the physiological regulation of pituitary hormone secretion.

Radioimmunoassay with the S320 antiserum, showed that like SS-28 and SS-14, SS-28(1-12)-IR is present in hypophysial portal blood in greater concentrations than in peripheral blood and that its release into portal blood can be increased significantly by ME stimulation. Although the S320 antiserum used in the radioimmunoassay does not cross-react significantly with either SS-28 or SS-14, it does recognize two other SS-28(1-12)-IR like peptides in the hypothalamus, with higher molecular weights, 5K and 8K SS-28(1-12),

(Benoit _et al_., 1982c; Benoit _et al_., 1984), and conceivably these larger forms may also be released into portal vessel blood and may account for up to 20 to 25 per cent of the immunoreactivity measured in this study.

The reduced release of SS-28(1-12) into portal blood in response to the second compared with the first ME stimulus is similar to the result obtained in studies on LHRH (Fink and Jamieson, 1976). The amount of SS-28(1-12) released during the first stimulus (~ 170 pg/animal; Fig. 3-5) was small relative to the hypothalamic content of the peptide (~ 45 ng; Benoit _et al_., 1982b). Therefore, as in the case of LHRH (Sherwood, Chiappa, Sarkar and Fink, 1980), the reduced response to the second stimulus is likely to have been due to a decrease in the readily releasable pool of SS-28(1-12) and/or a change in Ca^{2+} channel permeability (e.g. Nordmann, 1976) rather than a reduction in the total hypothalamic content of SS-28(1-12).

The biological activity of SS-28(1-12) is still to be determined. However, there is some evidence to suggest that the processing and release of SS-28 (1-12) may be dissociated from that of SS-14. The ratios of SS-28, SS-28 (1-12) and SS-14 vary in different regions of the brain; results of immunohistochemical studies taken together with other data reveal that, whilst all three species are present in hypothalamic nerve terminals, in the neocortex and hippocampus SS-28 is confined mostly to cell bodies, and SS-28 (1-12) is preferentially located in the nerve terminals (Morrison, Benoit, Magistretti, Ling and Bloom, 1982; Morrison, Benoit, Magistretti and Bloom, 1983). Cysteamine (2-mercapto-

ethylamine) produces a dose-dependent reduction in total SS throughout the brain (Sagar, Landry, Millard et al, 1982) and increases basal secretion of growth hormone (Millard, Sagar, Badger and Martin, 1983). Bakhit, Koda, Benoit, Morrison and Bloom (1984) have shown that administration of this compound to rats selectively reduced both the hypothalamic content, and K^+ evoked release *in vitro*, of SS-14, but did not affect the content and release of SS28 (1-12), suggesting that selective release of SS-14 and SS-28(1-12) may occur under certain conditions. However the specificity and mechanism of action of cysteamine are controversial. Bakhit, Benoit and Bloom (1983b) showed a selective depletion by cysteamine of the brain content of SS-14, but not SS-28 and suggested that this effect was produced by the release of SS-14 rather than by interference of cysteamine with the SS radioimmunoassay. Sagar *et al* (1982) reported a depletion of both SS-14 and SS-28 following cysteamine administration and other studies have indicated that cysteamine may render SS non-immunoreactive by an interaction with the disulphide bond thereby apparently depleting both SS-14 and SS-28, but not linear molecules such as SS-28(1-12) (Patel and Pierzchala, 1985).

Taken together the present results obtained *in vivo* confirm the results obtained from release studies *in vitro* (Kewley *et al*., 1981; Bakhit *et al*, 1983) in showing that, in addition to SS-14, SS-28 and SS-28(1-12) are secreted into hypophysial portal vessel blood. There is much evidence to support a physiological role of SS-28 independent of its possible role as a precursor of SS-14, however it remains to be established whether SS-28(1-12) has any functional significance or is purely a non-functional fragment formed with, and co-secreted with, SS-14.

CHAPTER 4

Thyrotrophin Releasing Hormone in Hypophysial Portal Blood, Hypothalamus and Pituitary Gland: Studies with High Performance Liquid Chromatography

4.1 INTRODUCTION

The detailed mechanism for the synthesis of thyrotrophin releasing hormone (TRH) in the mammalian brain remains to be elucidated. An early report suggested that TRH might be synthesized by the action of soluble enzymes in the cytoplasm rather than by ribosomal synthesis (Mitnick and Reichlin, 1972) but these findings were not substantiated by work in other laboratories (Bauer and Lipmann, 1976; Rupnow, Hinkle and Dixon, 1979) and it is now generally accepted that, like the larger peptide molecules, TRH is derived by the cleavage and post-translational modification of a ribosomally-synthesized precursor (McKelvy, 1983; Griffiths and Millar, 1983). The *in vitro* incorporation of ^{3}H proline into TRH has been demonstrated in explants of guinea-pig hypothalamus (McKelvy, 1974; McKelvy, Sheridan, Joseph, Phelps and Perrie, 1975) and in newt hypothalamus and forebrain (Grimm-Jorgensen and McKelvy, 1974). Using frog brain, which contains a high concentration of TRH (Jackson and Reichlin, 1977b,1979), Rupnow, Hinkle and Dixon (1979) demonstrated the presence of a macromolecule which yielded TRH after chemical or enzymatic treatment.

Studies using complementary DNA-cloning techniques have now resulted in the determination of the partial sequence of the amino terminal of a pre pro-TRH isolated from the skin of *Xenopus laevis* (Richter, Kawashima, Egger and Kreill, 1984) and, more recently, of a pre pro-TRH isolated from rat hypothalamus (Lechan, Wu, Jackson *et al*, 1986). The deduced rat TRH precursor, of 255 amino acids, contained five copies of the sequence

Lys-Arg-Gln-His-Pro-Gly-Lys (or Arg)-Arg.

The 123 amino acid polypeptide deduced from the partial sequence of the frog precursor, contained three copies of the above sequence, together with a fourth TRH-containing sequence at the carboxyl terminal -

Lys-Arg-Gln-His-Pro

(Richter et al, 1984). Other than the repeating sequences encoding TRH, there was no homology between the rat and frog precursors (Lechan et al, 1986). The tetrapeptide, Gln-His-Pro-Gly, is generated by cleavage at the pairs of Lys-Arg (or Arg-Arg) residues, and from this intermediate, TRH can be formed by the spontaneous or enzymatic modification of the glutamine residue to pGlu (Ortowski, Richman and Meister, 1969; Abraham and Podell, 1981). The glycine may be required for the enzymatic amidation of the proline residue (Bradbury, Finnie and Smythe, 1982) and the enzymes required for this conversion have been demonstrated in rat brain (Kizer, Busby, Cottle and Youngblood, 1984); alternatively the amide group might be transferred to pGlu-His-Pro by a transamidase (Griffiths and Millar, 1983).

There are no features of the TRH precursor isolated from frog skin or rat brain which suggest the possibility of other known biologically active peptides being generated during processing (Richter et al, 1984; Lechan et al, 1986). In addition to TRH, several non-TRH containing peptides ranging in size from 10 to 49 amino acids may also be generated from the rat precursor, but whether these are released and are bioactive remains to be determined (Lechan et al, 1986). Some of the TRH-like immunoreactivity (IR) as measured by radioimmunoassay in

extrahypothalamic sites and body fluids does not correspond to the authentic tripeptide (e.g. Youngblood, Humm and Kizer, 1979; Busby, Youngblood, Humm and Kizer, 1981b). The aims of the following experiments were to use HPLC in conjunction with RIA to determine whether the TRH-IR measured in portal vessel blood, the hypothalamus and pituitary gland, was due to one or more peptides, and to examine the release of TRH in response to electrical stimulation of the hypothalamus. The electrical stimulus used in these studies was applied to either the median eminence (ME), in order to stimulate directly the terminals of the TRH neurones or the paraventricular nuclei (PVN) in which are located the cell bodies of the TRH neurones (Jackson and Reichlin, 1974; Lechan and Jackson, 1982).

4.2 MATERIALS AND METHODS

4.2.1 TRH-like immunoreactivity in hypophysial portal vessel blood

Hypophysial portal vessel blood was collected for 15 - 60 min periods from adult male Wistar rats anaesthetized with urethane, as described in Chapter 2. Since TRH is rapidly degraded in blood samples at 37°C (Jackson, Papapetrou and Reichlin, 1979) blood from the cut pituitary stalk was removed every 30 to 60 seconds (depending upon the rate of flow of blood) and transferred to a glass centrifuge tube containing 4 ml of ice-cold absolute alcohol (Fink, Koch and Ben Aroya, 1982). The contents of the tube were thoroughly mixed at regular intervals (generally with each addition of blood to the tube) throughout the collection period. In some experiments the pituitary gland was removed after the stalk had been cut; after haemostasis had been achieved, heparin was injected, the

stalk re-cut and pituitary stalk blood was collected into ethanol on ice for either two consecutive periods of 30 min each or for a single collection period of 60 min. Peripheral blood samples (0.5 ml) withdrawn from the external jugular vein (EJV) during collection were extracted in the same manner as stalk blood. The blood samples were centrifuged (3000 rpm at 4°C for 15 min) and the supernatants removed. The precipitate was resuspended in a further 4 ml of ice-cold ethanol, centrifuged again and the supernatant combined with that from the first centrifugation. The ethanol extracts of stalk or peripheral blood were stored at -40°C and subsequently evaporated to dryness at 35°C under vacuum or under a stream of nitrogen gas.

4.2.2 Immunoreactive TRH in the hypothalamus and pituitary gland

For studies on hypothalamic TRH-IR, blocks of hypothalamic tissue were dissected (2.2.6) from the brains of animals which had been killed by decapitation. The blocks of tissue were homogenised and extracted in pairs in either 1 ml 0.1 N HCl or in 4 ml ice-cold ethanol. One to three whole pituitary glands were homogenized in 1 ml ethanol. After freeze or vacuum drying (acid and ethanol extracts, respectively), extracts were prepared for HPLC as described below. Some hypothalamic extracts were re-dissolved in 200 µl of distilled water, mixed for 30-60 sec with 1 ml peripheral blood (taken from the EJV of a rat anesthetized with urethane and injected with heparin) and then extracted for TRH as described for stalk blood. In order to determine the recovery of the extraction procedure a similar experiment was carried out in which synthetic TRH (Penninsula Laboratories, California) was added to, and

extracted from, peripheral blood. In a second series of experiments hypothalami were homogenised in 200 μl of either 0.9% saline or distilled water and either freeze-dried, or incubated for 5 min at 37°C with 5 ml peripheral blood or 5 ml distilled water. At the end of the incubation period the blood was extracted with ice cold ethanol and both the ethanol and aqueous extracts dried as described above.

4.2.3 Release of TRH in response to electrical stimulation of the hypothalamus

Hypophysial portal vessel blood, from male Wistar rats anaesthetized with urethane (2.2.3), was collected for 3 or 4 consecutive 15 min periods and extracted in ice-cold ethanol as described above. During the second and also (when collected) the fourth collection period, an electrical stimulus was applied to either the ME by way of a unipolar electrode positioned in the ME at the time of the experiment or to the PVN using a bipolar electrode which had been stereotaxically implanted 3-10 days previously (2.2.4). In preliminary experiments with ME stimulation, the collection of portal blood immediately followed the surgical preparation of the animals, but in subsequent experiments with either ME or PVN stimulation there was a 4 h delay between the exposure of the dura and the later exposure of the portal vessels (for rationale, see Chapter 5). The electrical stimulus was applied for 15 min; in control animals the electrodes were implanted, but no stimulating current was passed. Peripheral blood samples (0.5 ml) were withdrawn from the EJV immediately before, and at the end of, the first period of stimulation and were extracted in ice-cold

ethanol. In order to relate the amount of TRH released into portal blood to the hypothalamic TRH content, at the end of the experiment the rats in which the ME had been stimulated, were decapitated, the hypothalami dissected (2.2.6) and homogenized in 0.1 N hydrochloric acid. For comparison, control hypothalami were removed from unanaesthetized rats killed by decapitation. The heads of animals bearing PVN electrodes were fixed in 4% phosphate buffered formalin for confirmation of correct electrode placement (2.2.4). Extracts of peripheral or portal blood were stored at -40°C until dried under vacuum and reconstituted in either PBS/0.25% BSA for RIA (2.5.1) or in 0.1% trifluoracetic acid (aqueous) for application to an HPLC column.

4.2.4 High performance liquid chromatography and radioimmunoassay

The dried tissue or blood extracts were dissolved in 1.4 ml 0.1% TFA in water and centrifuged briefly. The supernatants were applied to the HPLC column and eluted with a linear gradient (2.4.1); the fractions eluting from the column were vacuum dried and the TRH-IR content determined by RIA (2.5.1). In addition, TRH and several synthetic analogues and related compounds, particularly those (Lys^2 TRH, Phe^2 TRH and [$3Me\text{-}His^2$] TRH) which showed high cross-reactivity with the anti-TRH antiserum, were run on the HPLC under identical conditions and their retention times determined.

4.3 RESULTS

HPLC resolved the TRH-IR of pituitary stalk blood into 3 components (Fig. 4-1) the first of which eluted in the same position as synthetic TRH. Table 4-A shows that the stalk blood content of

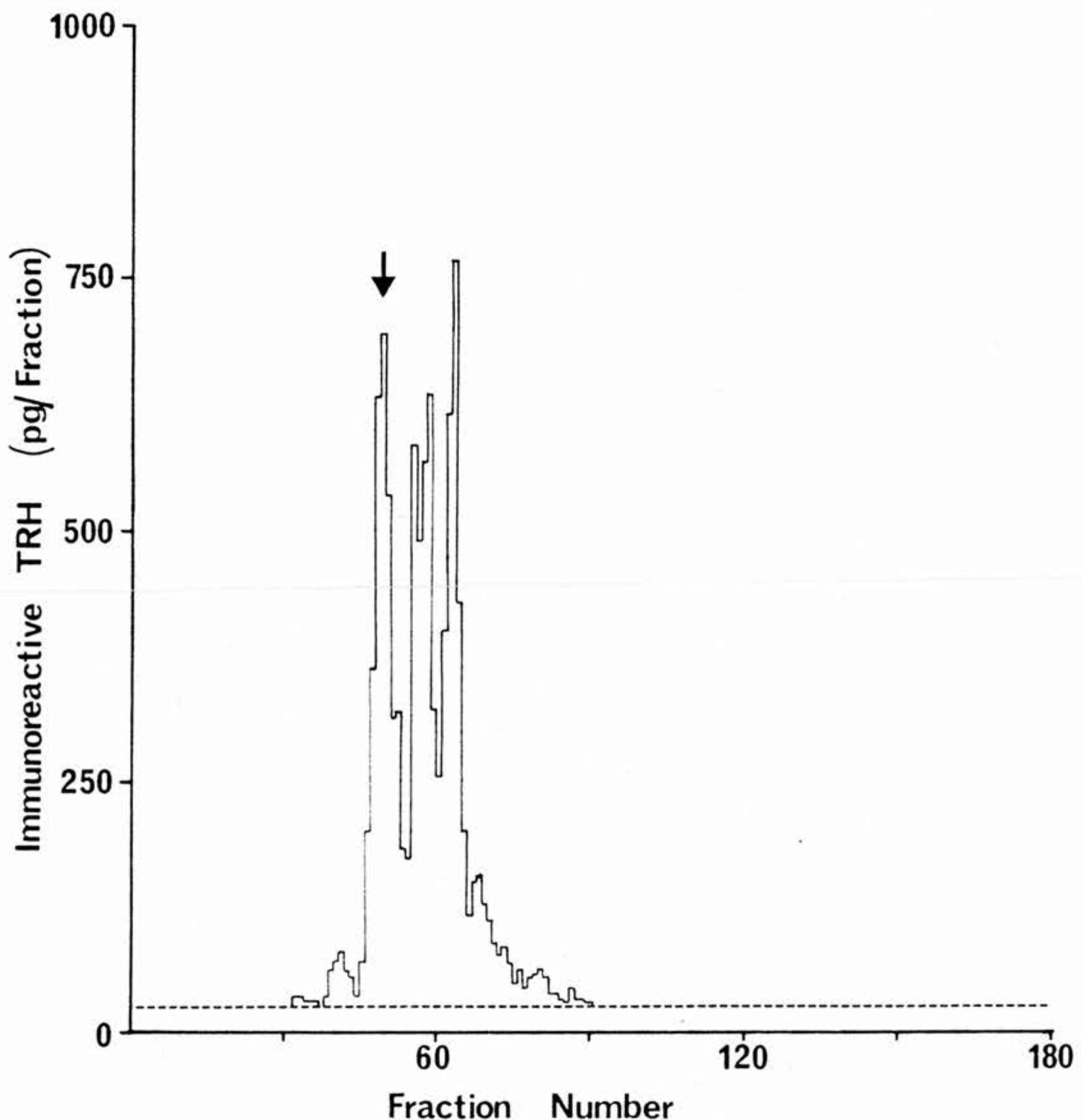


FIGURE 4-1: Representative HPLC profile of TRH-like immunoreactivity in an ethanol extract of hypophysial portal blood. The blood was collected for a single, 1 hour collection period. The arrow indicates the position at which synthetic TRH eluted from the HPLC column. The dotted line indicates the lower limit of detection of the assay. Similar results were obtained in a total of 39 separate HPLC runs.

TABLE 4-A Mean ± SEM content of TRH-IR in each of the three peaks of material recovered after HPLC of 4 successive 15 min periods of stalk blood collection. The first peak corresponded in retention time to authentic TRH (n = 5).

Collection Period	TRH-IR (pg/15 min collection period)		
	Peak 1	Peak 2	Peak 3
0 - 15 min	758 ± 247	596 ± 160	568 ± 117
15 - 30 min	447 ± 69	396 ± 70	417 ± 71
30 - 45 min	246 ± 61	212 ± 41	272 ± 41
45 - 60 min	216 ± 57	184 ± 49	244 ± 57

each of the three components decreased in successive 15 min collection periods. The mean total amount of TRH-IR released in 1 hour (Table 4-A) was 4556 pg of which 1667 pg (37%) corresponded to authentic TRH. Efficiency of the extraction procedure, as assayed by recovery of synthetic TRH from peripheral blood, was 70%. All three immunoreactive peaks were present in extracts of stalk blood collected from animals in which the pituitary gland had been removed (Fig. 4-2A). In peripheral blood, levels of TRH were generally below the limit of detection of the assay, but, when detectable, the TRH-IR was also resolved into 3 components which eluted in a position similar to those of stalk blood (not shown). By contrast, the TRH-IR of extracts of pituitary tissue (Figure 4-2B) and of hypothalamic extracts (Figure 4-3A) eluted as a single peak in an identical position to that of authentic TRH. Only a single IR peak was recovered when hypothalamic extracts or homogenates were added to (Fig. 4-3B), or incubated with (Fig. 4-4), peripheral blood. In addition to TRH the anti-TRH serum cross-reacted significantly with Lys^2 TRH, Phe^{2}TRH (Chapter 2, Table 2-B) and at about 25% with [3Me-His^2]TRH (Fig. 4-5) there was no cross-reaction with pGlu-His-Gly (Anorexigenic peptide; Trygstad, Foss, Edminson, Johansen and Reichelt, 1978). Figures 4-3 and 4-6 show that Lys^2 TRH eluted in a similar position to TRH while Phe^2 was retained well beyond TRH; [3Me-His^2]TRH eluted just before the second peak.

Figure 4-7 illustrates typical results from a preliminary study of TRH release into hypophysial portal blood, before, during and after electrical stimulation of the ME. In this experiment there was no 4 h delay period between the completion of the surgery and

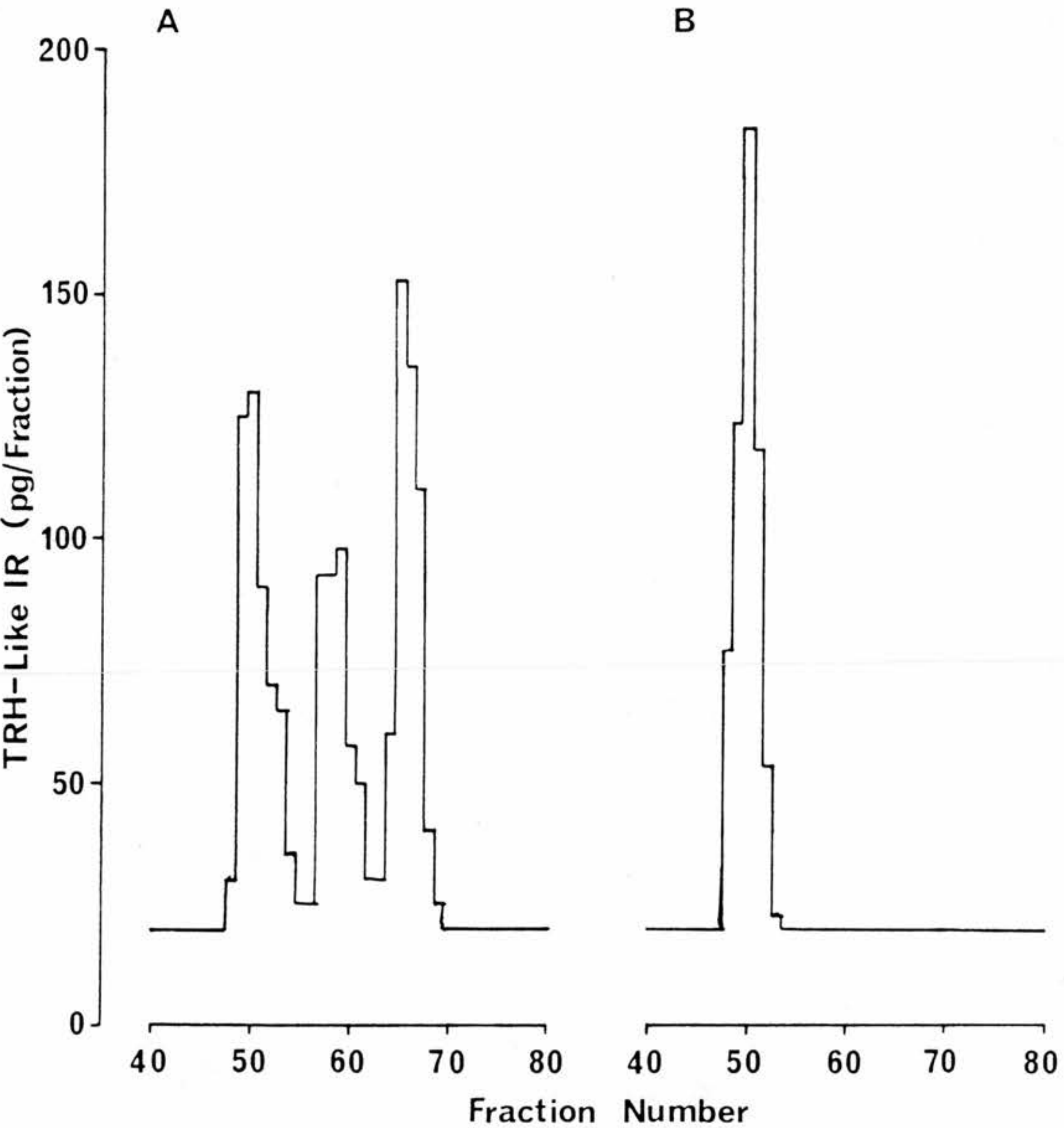
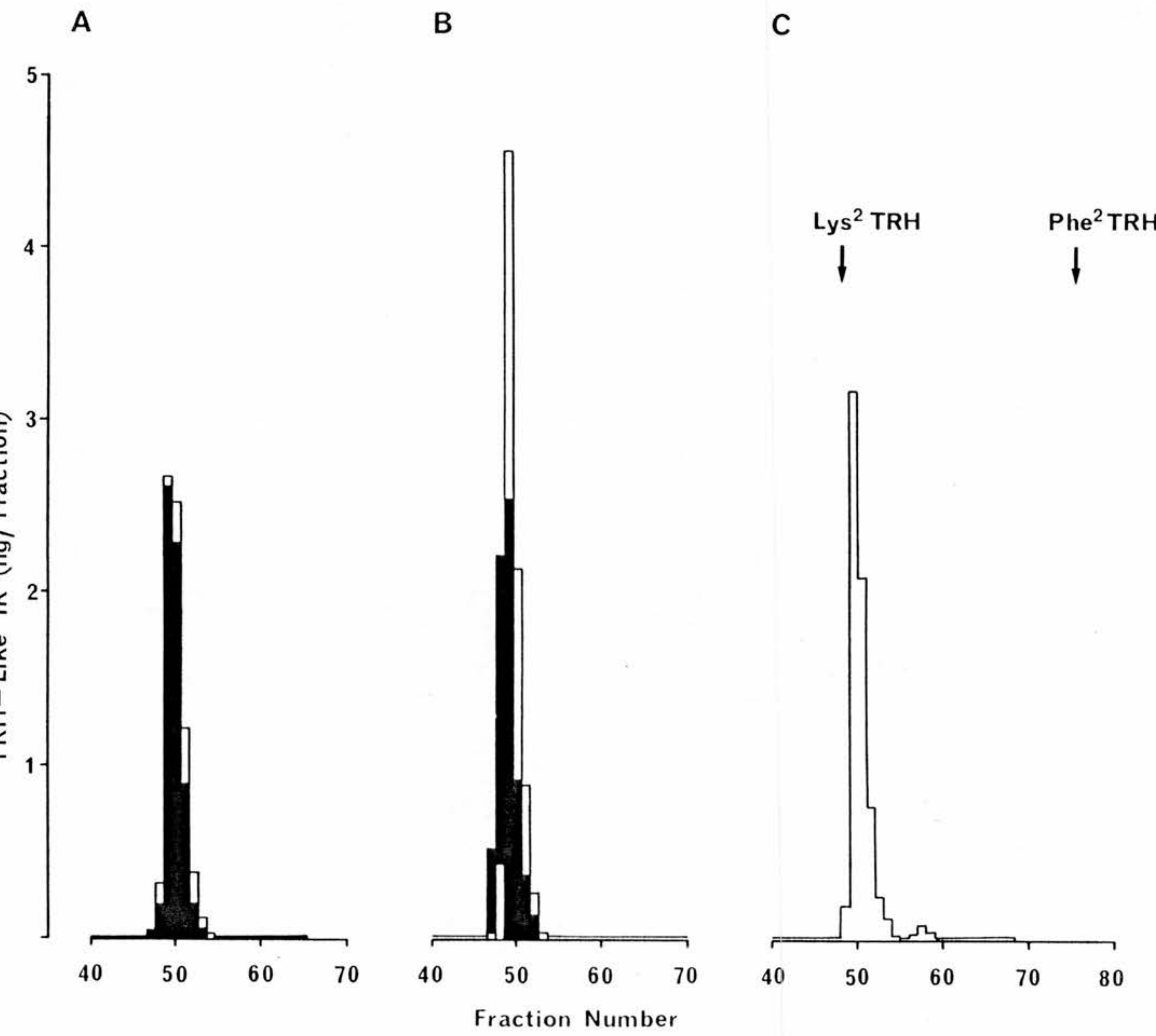


FIGURE 4-2: A: HPLC profile of TRH-IR in an extract of stalk blood obtained from an animal in which the pituitary had been removed before stalk blood collection. Similar profiles were obtained in three experiments.
B: HPLC profile of pituitary TRH-IR (similar results were obtained in two separate experiments).


A
B
C
TRH-Like IR (ng/ Fraction)
5
4
3
2
1
40
50
60
70
80
Fraction Number
Lys² TRH
Phe² TRH

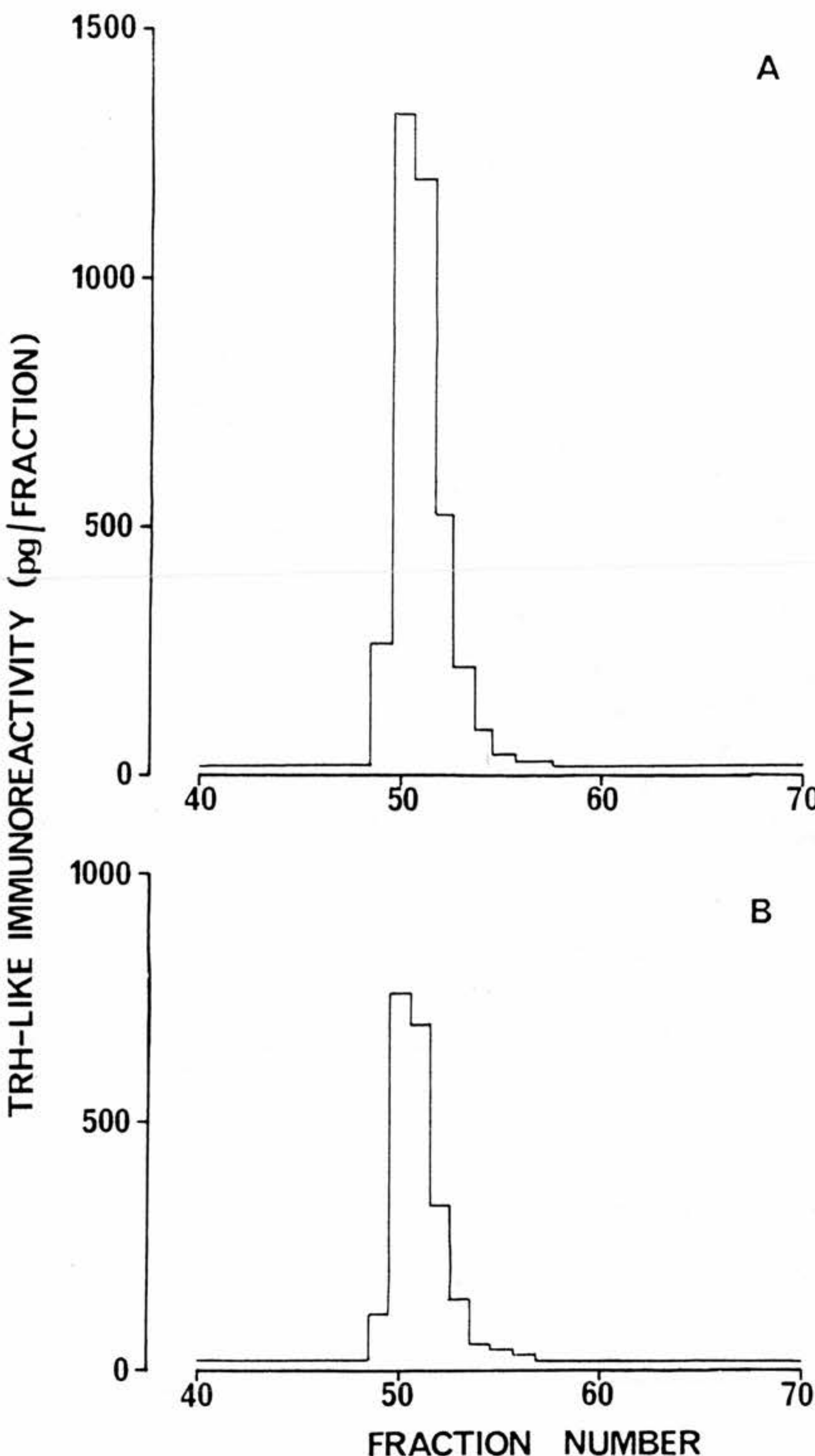


FIGURE 4-4: HPLC profiles of TRH-IR in (A) rat hypothalamus homogenized in 200 µl of water, (B) aqueous extract of hypothalamus incubated in water at 37°C for 5 minutes.

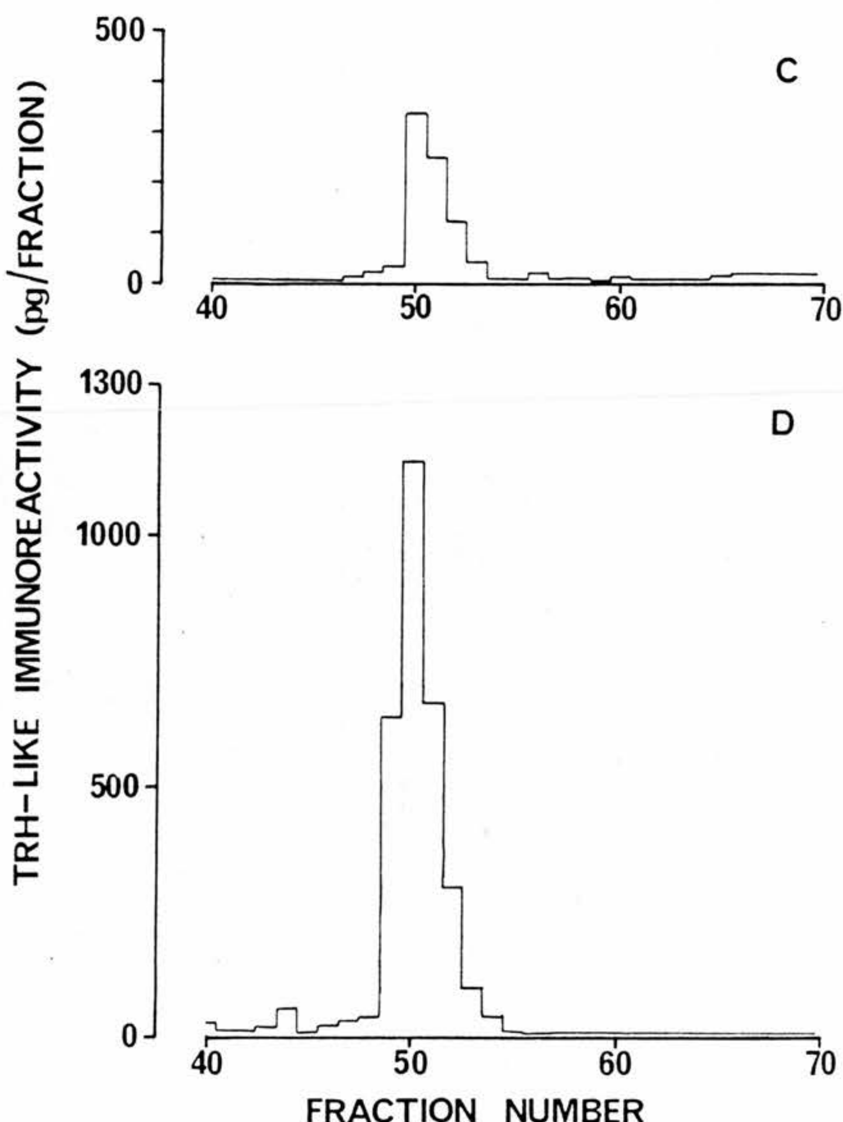


FIGURE 4-4 (continued): HPLC profiles of TRH-IR in (C) aqueous extracts of hypothalamus incubated with 5 ml of peripheral blood at 37°C for 5 minutes before extraction in ice-cold ethanol, or (B) saline extracts of hypothalamus incubated with peripheral blood and extracted as described above.

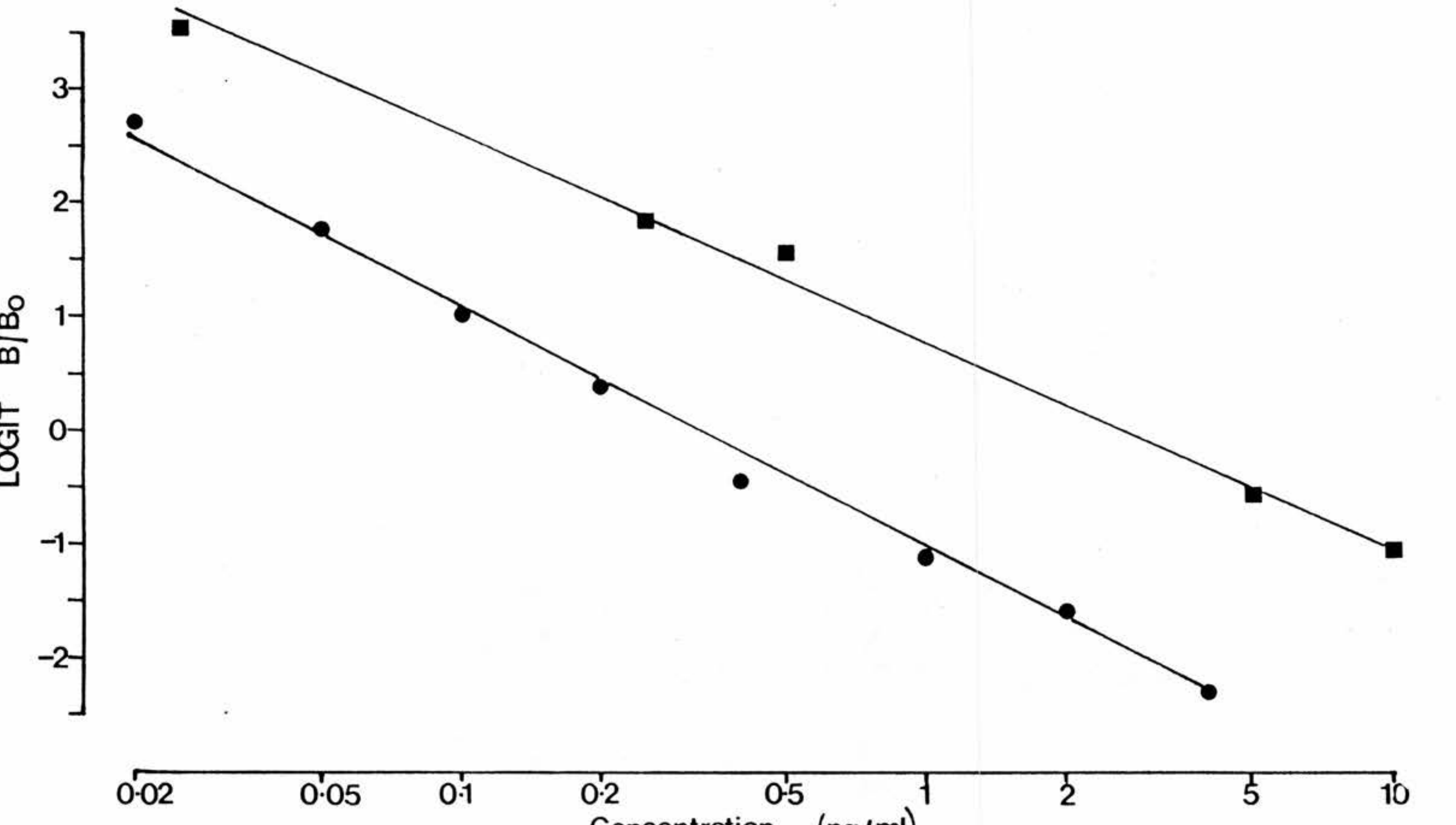


FIGURE 4-5: Parallel displacement of ^{125}I-TRH binding by synthetic TRH (●) and [3Me-His]^2 TRH (■) showing the 25% cross-reactivity of [3Me-His]^{2}TRH with the anti-TRH serum.

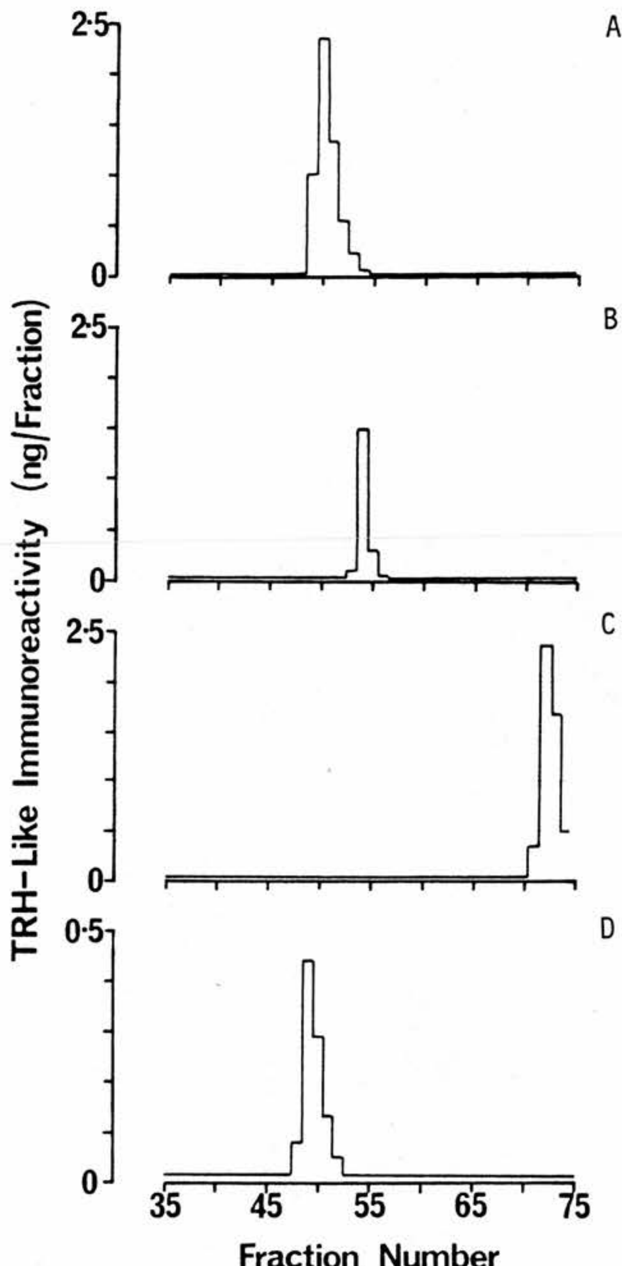


FIGURE 4-6: HPLC elution profiles of (A) TRH, (B) [3Me-His]²TRH, (C) Phe²TRH and (D) Lys²TRH.

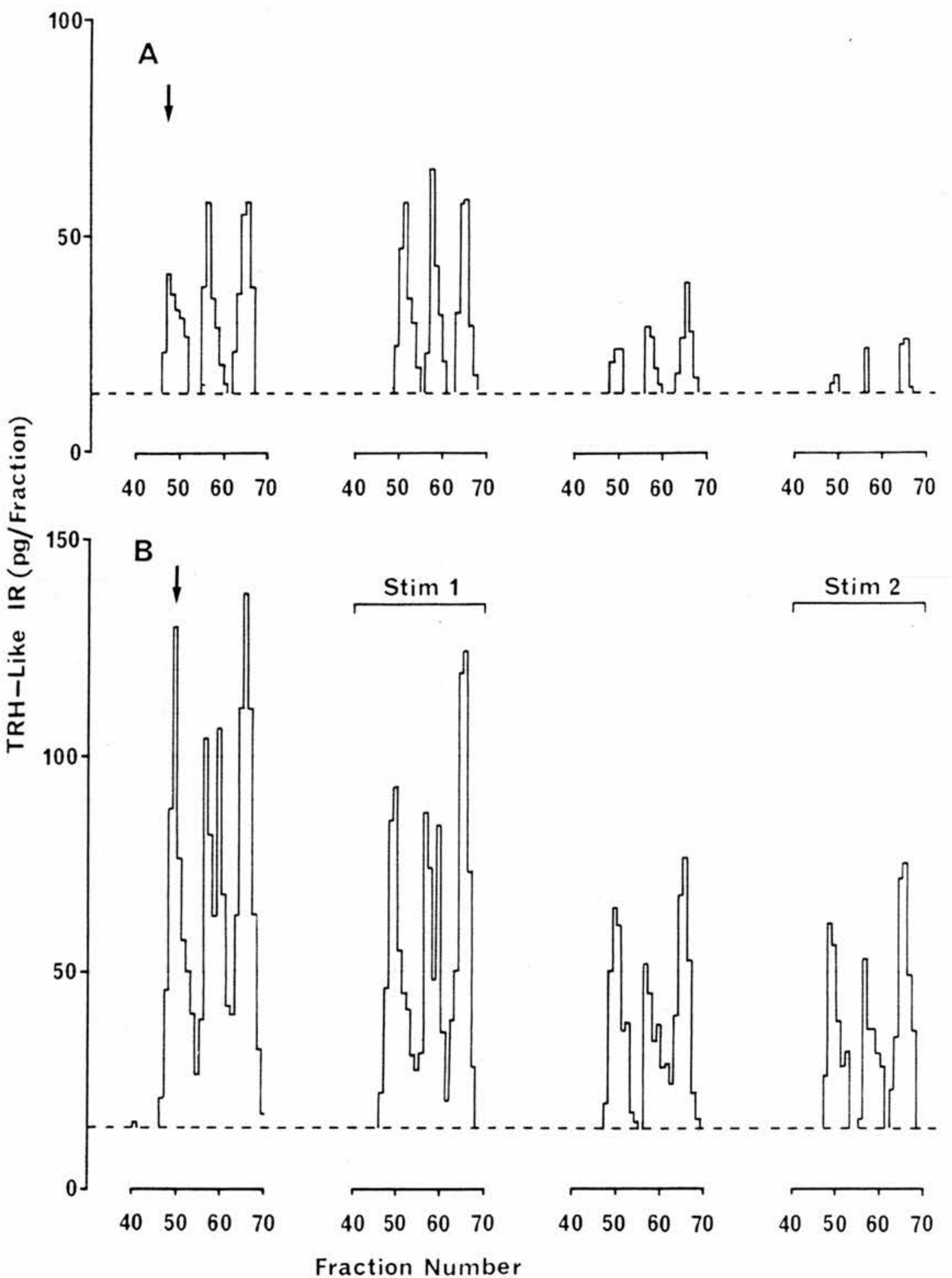


FIGURE 4-7: HPLC profiles of TRH-IR in ethanol extracts of hypophysial portal vessel blood. The blood was collected for four consecutive 15 min collection periods. In (A) an electrode was positioned in the median eminence, but no current was passed; in (B) an electrical stimulus was applied to the median eminence during the second and fourth collection period. Arrows indicate the position at which synthetic TRH eluted from the HPLC column; the dotted line shows the lower limit of detection of the assay. Similar results were obtained from a total of three stimulated and two unstimulated rats.

the application of the stimulus. As shown above, HPLC resolved the TRH-IR into three separate components, each of which decreased in magnitude in each of the four successive collection periods. Electrical stimulation did not alter the rate of release of any of the three peaks of IR material. Similarly, there were no major changes in total TRH-IR release into portal blood after stimulation of the the PVN or the ME in rats in which there had been a 4 h delay period between the initial surgical preparation of the animal and cutting the pituitary stalk (Table 4-B). HPLC of extracts of portal blood collected from PVN stimulated rats revealed a small increase in the size of the first IR Peak (i.e. that which corresponded in retention time to authentic TRH) in response to stimulation (Table 4-C); however, this was mainly related to the increase in the rate of blood flow although the portal vessels during stimulation rather than an increase in the concentration of TRH in hypophysial portal vessel blood. The secretion of TRH-IR was lower in extracts of portal blood collected after a 4 h delay period than in extracts of blood collected from rats in which the pituitary stalk was sectioned immediately after its exposure (Table 4-D). With the exception of the PVN stimulated group of animals there was a significant decrease in the TRH-IR content of portal blood extracts over the four 15 min collection periods ($P < 0.02$-$P < 0.05$). Despite the relatively high rate of TRH-IR release into portal blood, there were no major changes in hypothalamic TRH content (Table 4-D). TRH-IR was not detectable (< 30 pg/ml) in all extracts of peripheral blood except for samples obtained from a total of eight rats from which portal blood was collected immediately after the completion of surgery. In

Table 4-B Mean ± SEM total content of TRH-IR in extracts of hypophysial portal vessel blood collected for four successive 15 min periods from urethane-anaesthetized male rats. Animals were maintained under anaesthesia for 4 h after the exposure of the dura before the pituitary stalk was exposed and the vessels cut for blood collection. Animals had either been previously implanted with bipolar electrodes in the paraventricular nuclei (PVN) or had a unipolar electrode positioned in the median eminence (ME) at the time of the experiment. The electrical stimulus was applied during the second and fourth collection periods; in control animals the electrodes were positioned, but no current was passed. Numbers in brackets refer to the number of animals in each group.

		TRH-IR (pg/15 min collection period)			
		0–15 min	15–30 min	30–45 min	45–60 min
ME stimulated	(n=11)	539 ± 114	409 ± 55	278 ± 55	229 ± 26
	(n=8)†	323 ± 30	330 ± 21	212 ± 22	224 ± 32
ME Non-stimulated	(n=11)	250 ± 41	240 ± 42	198 ± 38	156 ± 29
PVN stimulated	(n=7)	404 ± 76	439 ± 73	433 ± 123	384 ± 105
PVN Non-stimulated	(n=7)	416 ± 107	306 ± 80	240 ± 58	224 ± 68
	(n=6)†	317 ± 51	235 ± 44	203 ± 53	165 ± 41

†Values recalculated after exclusion of animals in which TRH-IR content in the first 15 min period was > 1000 pg; in contrast to the remaining animals, TRH-IR content fell rapidly during subsequent collection periods.

Table 4-C Content of TRH-IR in each of the three peaks of material recovered after HPLC of three successive 15 min periods of hypophysial stalk blood collection. The first peak coincides in retention time with authentic TRH. The data shown were obtained from six individual rats all of which were previously implanted with bipolar electrodes in the paraventricular nuclei (PVN). After exposing the dura, animals were maintained under urethane anaesthesia for 4 h before the exposure of the pituitary stalk and blood collection. An electrical stimulus was applied to the PVN of four of the animals (A-D) during the second collection period (15-30 min).

Animal period)	Collection Period (min)	Blood volume (μl)	TRH-IR (pg/15 min collection		
			Peak 1	Peak 2	Peak 3
A	0-15	550	57	48	43
	15-30*	650	76	51	59
	30-45	300	N/D	23	N/D
B	0-15	250	49	40	65
	15-30*	400	144	39	99
	30-45	210	41	44	67
C	0-15	100	23	N/D	N/D
	15-30*	200	46	N/D	17
	30-45	100	N/D	N/D	N/D
D	0-15	100	49	46	76
	15-30*	150	73	52	58
	30-45	100	25	N/D	33
E	0-15	400	92	71	119
	15-30	300	66	52	67
	30-45	300	32	62	80
F	0-15	150	114	101	115
	15-30	100	63	57	86
	30-45	150	32	23	45

N/D Not detectable

* Electrical stimulus applied (for stimulus parameters see 2.2.4)

Table 4-D Mean ± SEM total TRH-IR release into hypophysial portal vessel blood during an hour of blood collection from electrically stimulated and non-stimulated rats with electrodes positioned in the median eminence (ME). Collection of blood commenced either immediately after, or following a delay of 4 h after, the surgical preparation of the animals. The content (pg) and concentration (pg/mg wet weight) of TRH in the hypothalami taken from these animals at the end of the collection period and from control, unanaesthetised rats killed by decapitation are also shown. Numbers in brackets refer to the number of animals in each treatment group.

	Total TRH-IR released into portal vessel blood (pg/60 min)	Hypothalamic TRH Content (pg)	Hypothalamic TRH Concentration (pg/mg wet weight)
ME stimulated (no delay)	> 5000 (5)	6754 ± 382 (5)	388 ± 21 (5)
ME unstimulated (no delay)	> 5000 (7)	7481 ± 435 (7)	443 ± 18 (7)
ME stimulated (4 h delay)	1456 ± 214† (11)	6466 ± 258 (11)	407 ± 15 (9)
ME unstimulated (4 h delay)	844 ± 146 (11)	7782 ± 333 (10)	426 ± 17 (10)
Unanaesthetised rats killed by decapitation	N/A	7292 ± 266 (16)	375 ± 14 (16)

† 1089 ± 87 if exclude the results from 3 animals in which TRH release in the initial 15 min collection period remained above 1000 pg/15 min. N/A Not applicable.

these eight animals peripheral blood TRH-IR concentrations were 198 ± 19 pg/ml and 215 ± 29 pg/ml for the samples of blood withdrawn from the EJV 15 and 30 minutes into the portal blood collection period respectively; since when measurable there was no significant difference in the TRH content of samples of peripheral blood from stimulated and unstimulated rats, the data from both groups of animals have been combined.

4.4 DISCUSSION

These results show that HPLC can resolve the TRH-IR in extracts of rat pituitary stalk blood into three components, the first of which has an identical retention time to the synthetic tripeptide. Although only about 37% of total TRH-IR corresponded to authentic TRH, the absolute amount of TRH released into stalk blood, 1667 pg/h (Table 4-A), is in the same range as the values reported previously (Eskay, Oliver, Ben-Jonathan and Porter 1975; Fink et al. 1982) and is still very large relative to the amounts of LHRH (Sherwood, Chiappa, Sarkar and Fink, 1980) and somatostatin (Chapter 3) released. The amount of authentic TRH-IR in hypothalamus (about 5 ng; Fig. 4-3A) was also similar to that published before for crude extracts (Reichlin, Saperstein, Jackson, Boyd and Patel, 1976; Vale, Rivier and Brown, 1980; Fink et al. 1982). Thus the percentage of neuropeptide released relative to that stored (Fig. 4-3A) was about 34% for authentic TRH compared with about 0.6% for LHRH and 0.3% for somatostatin (Fink et al. 1982). The additional peaks of immunoreactivity which were detected in the portal blood extracts are unlikely to correspond to known metabolites of TRH since the

antiserum used for the radioimmunoassay does not cross-react significantly with either the free acid derivative of TRH or the cyclized dipeptide, diketopiperazine (Table 2.B). The antiserum shows little or no cross-reaction with analogues of TRH in which either of the terminal amino acids are modified or replaced, but does cross-react with molecules in which the histidyl residue is altered; the cross-reaction between Phe^{2}TRH and the TRH antiserum was 100%, but Figs. 4-3 and 4-6 show that Phe^{2}TRH is unlikely to contribute significantly to the TRH-IR in stalk blood. Lys^2 TRH, which eluted in the same position as TRH, has not been reported to be synthesised in the brain. [3Me-His^2]TRH, which was tested because of its relatively high cross-reaction with the anti-TRH serum, could not account for the TRH-IR of stalk blood, and is in any case not detectable in hypothalamus (Pekary, Morley and Hershman, 1978).

In addition to its widespread distribution throughout the central nervous system and spinal cord (Brownstein, Palkovits, Saavedra, Bassiri, Utiger, 1974; Jackson and Reichlin, 1974; Winokur and Utiger, 1974), TRH-IR has been detected by RIA in a variety of peripheral organs and body fluids from a number of mammalian and non-mammalian species. Thus the presence of TRH-IR has been reported in the pancreas and gastrointestinal tract (Morley, Garvin, Pekary and Hershman, 1977; Leppaluoto, Koivulsalo and Kraama, 1978), the retina (Schaeffer, Brownstein and Axelrod, 1977), the pineal gland (Kellokumpu, Vuolteenaho and Leppaluoto, 1980), reproductive system (Pekary, Meyer, Vaillant and Hershman, 1980; Pekary, Richkind and Hershman, 1983), breast milk (Baram, Koch and Hazum, 1977),

peripheral blood and urine (Jackson and Reichlin, 1974; Oliver, Charvet, Codaccioni and Vague, 1974), and frog skin and blood (Jackson and Reichlin 1977b, 1979). Although most of the TRH-IR in the mammalian CNS, as assessed by HPLC or other chromatographic techniques, appears identical to the authentic tripeptide (Kreider, Winokur and Utiger, 1979; Jackson, 1980; Kellokumpu, et al. 1980; Parker, 1981; Emson, Bennett and Rossor, 1981), the identity of the immunoreactive material found in some peripheral organs and body fluids has been questioned (Emerson, Frohman, Szabo and Thakkar, 1977; Youngblood, Humm and Kizer, 1979; Youngblood, Humm, Lipton and Kizer, 1980). In contrast, Engler, Scanlon and Jackson (1981) have shown that TRH-IR in the peripheral blood of neonatal rats, which is higher than in the adult, corresponds to authentic TRH, though it is probably derived from the pancreas rather than the hypothalamus. The TRH-IR in human peripheral blood has also been reported to be identical to synthetic TRH (Mallik, Wilber and Pegues, 1982). The differences in the resolving power of the various chromatographic techniques used to investigate TRH-IR in different tissues may account for the discrepancies in the results obtained in different laboratories (Busby, Youngblood, Humm and Kizer, 1981a) and to overcome these difficulties the immunoreactive TRH in a number of peripheral organs was re-examined using a multi-step purification procedure followed by HPLC and thin layer chromatography [TLC] (Busby, Youngblood, Humm and Kizer, 1981b). Using this procedure Busby et al. (1981b) showed that (i) only about 10% of the TRH-IR in partially purified peripheral blood extracts was similar to authentic TRH, (ii) none of the TRH-IR present in human urine,

placenta and in the sheep pineal corresponded to authentic TRH, (iii) in the rat eye, only 57% of the total extracted TRH-IR had a similar retention time to TRH in the HPLC system and of this only 90% was authentic when rechromatographed on TLC, (iv) 83% of the TRH-IR extracted from the pancreas was chemically similar to TRH on HPLC.

Using reverse phase HPLC run under isocratic conditions, Pekary et al (1980) demonstrated the presence of TRH and at least one other homologous peptide distributed throughout the reproductive system of the male rat; the additional IR material co-eluted with TRH during Sephadex G-10 chromatography. In a subsequent study (Pekary et al, 1983) most of the TRH-IR found in semen and extracts of canine prostate, epididymis and testis was due to at least three peptides with an amino acid sequence similar to TRH (possibly in which the histidyl residue was replaced by a neutral amino acid) rather than the authentic peptide. A compound which cross-reacts with anti-TRH serum, but which inhibits the release of prolactin from pituitary explant cultures, (and was not diketopiperazine) has been demonstrated in rat testicular and pituitary tumour tissue (Morley, Meyer, Pekary et al, 1980). Whilst this compound co-eluted with TRH on sephadex G-10 it showed non-identity with TRH on cation exchange chromatography. HPLC has also revealed multiple peaks of TRH-IR in human CSF samples (Biggins, Das, Dodd et al, 1983).

In previous studies, alcohol extracts of hypophysial portal vessel blood were shown to contain a single species of TRH-IR using gel filtration on Sephadex G-25 columns (Fink, Koch and Ben Aroya, 1983) or thin layer electrophoresis (Ching and Utiger, 1983);

techniques which may not give optimal resolution of small TRH-like peptides (Busby et al, 1981a). HPLC has also been used to examine the TRH-IR released from rat hypothalamic and cerebral cortical neurons in response to K^+ depolarization (Scanlon, Robbins, Bolaffi, Jackson and Reichlin, 1983) and, under isocratic conditions, 95% of the IR material eluted in a broad peak in a similar position to authentic TRH.

The present findings of multiple TRH-IR peaks in hypophysial portal vessel blood extracts were not confirmed by a recent study (de Greef, Klootwijk, Karels and Visser, 1985) in which, using HPLC and elution under isocratic conditions, it was shown that all the IR material appeared homogeneous with authentic TRH. One possible explanation for this difference is that as suggested by de Greef et al (1985), the resolution of their HPLC system was less than that employed in the present study. Busby et al. (1981b) suggested that substances were present in crude tissue extracts which caused the appearance of multiple IR peaks and an alteration in the retention time of authentic TRH. However, this cannot explain the multiple TRH-IR peaks shown in stalk blood in the present study, since only one peak of TRH-IR corresponding to authentic TRH was found in hypothalamic extracts whether or not they were mixed or incubated with peripheral blood.

TRH is present in high concentrations in the posterior pituitary gland and to a lesser degree in the anterior pituitary gland (Jackson and Reichlin, 1977a). Figure 4-2 shows that the pituitary TRH-IR is homogeneous, and that the profile of IR in stalk blood was unchanged in animals in which, because the pituitary had

been removed before stalk blood collection, there was no contamination with back-flow blood from the pituitary sinusoids. Thus, the pituitary gland is not a likely source of the two additional peaks.

Early experiments showed that electrical stimulation of the anterior hypothalamus resulted in the increased ability of hypophysial portal blood to stimulate the release of thyrotrophin in *in vivo* or *in vitro* bioassay systems (Averill, Salamin and Worthington, 1966; Wilbur and Porter, 1970). The electrical stimulus used in the present experiments has been shown to result in a 6-7 fold increase in the concentrations in hypophysial portal vessel blood of LHRH (Fink and Jamieson, 1976; Chiappa *et al*, 1977; Sherwood *et al*, 1980) and SS-14, SS-28(1-12) and SS28 (Chapter 3). Despite the effects of the stimulus on thyrotrophin and prolactin release (see Chapter 5), in the present experiments there was no apparent increase in total TRH-IR release into portal blood after ME or PVN stimulation (Table 4-B). The relatively small increase in TRH in response to stimulation (approximately 2 fold) apparent after HPLC of portal blood extracts collected during stimulation of the PVN of rats in which there was a 4 h delay between surgery and cutting the pituitary stalk (Table 4-C) was largely due to the increased flow of hypophysial portal blood during stimulation rather than a change in TRH concentration.

Using the same stimulus as was applied in the present experiments, stimulation of either the ME or PVN did not result in increased release into portal blood of vasoactive intestinal peptide (Brar, Fink, Maletti and Rostene, 1985a) or oxytocin and vasopressin

(Horn, Robinson and Fink, 1985c), although the latter study did show that the stimulus was effective in increasing oxytocin and vasopressin concentrations in systemic plasma. Since the stimulus was effective in releasing thyrotrophin from the anterior pituitary gland it is clear that the apparent lack of a major effect of the stimulus on TRH secretion is not due to either inappropriate stimulation parameters or to 'fatigue' caused by the frequency of stimulation or the length of the trains of pulses (Boer, Cransberg and Dogterom, 1980; Bicknell and Leng, 1981). As noted earlier, the TRH content of portal blood is high relative to hypothalamic content, and decreases rapidly in successive periods of blood collection. Therefore, it is most likely that, as discussed for VIP (Brar et al, 1985a) and oxytocin and vasopressin (Horn et al, 1985c), the lack of any major effect of hypothalamic stimulation on the release of TRH into portal blood is the result of maximal secretion of TRH following sectioning of the pituitary stalk thus rendering it impossible to drive the system further by electrical stimulation.

In conclusion, the source of the two peaks in addition to authentic TRH in hypophysial portal blood remains unclear. Doubt has been expressed as to the ability of an anti-TRH, such as was used in the present study, to recognise the Glu (or Gln)-His-Pro sequence in a larger precursor due to the specificity of these antisera for the modified amino and carboxy termini (Spindel and Wurtman, 1980; McKelvy, 1983). Chemical analysis, and the determination of the biological potency, of the additional IR peaks was not possible because of the relatively small amount of material

present in portal blood. Conceivably, there may be a precursor which does not react with the antiserum, but on release into stalk blood gives rise to intermediates in TRH synthesis which do cross-react with the antiserum. Alternatively, when TRH enters blood some of it might be changed in a minor way by reactions which may alter the retention time on HPLC. However, at least under these experimental conditions, the studies carried out with peripheral blood to which had been added synthetic TRH or hypothalamic extracts (Fig. 4-4) make the possibility of the generation of the additional IR material by the action of blood borne enzymes appear unlikely.

CHAPTER 5

Effect of Anti-TRH Serum on the Release of Thyrotrophin and Prolactin during Suckling or in Response to Electrical Stimulation of the Hypothalamus

5.1 INTRODUCTION

The stimulation of the nipples during suckling generates a neural signal which is conveyed by the mammary nerves and an ascending pathway to the magnocellular neurons of the paraventricular and supraoptic nuclei resulting in the reflex release of oxytocin and subsequent milk ejection (e.g. Wakerley and Lincoln, 1973; Cross, Dyball, Dyer et al, 1975). In addition, the suckling stimulus also induces a reflex release of prolactin from the anterior pituitary gland (Amenomori, Chen and Meites, 1970). The release of both oxytocin and prolactin may also be evoked in lactating rats by the direct electrical stimulation of the mammary nerve (Mena, Pacheco, Aguayo, Clapp and Grosvenor, 1978; Mena, Pacheco and Grosvenor, 1980). Anaesthesia with urethane does not abolish either the milk ejection reflex (Lincoln, Hill and Wakerley, 1973), nor the prolactin response to suckling (Burnet and Wakerley, 1976; Wakerley, O'Neill and ter Haar, 1978) though in anaesthetized rats the rise in plasma prolactin concentrations occurs much later after the onset of suckling than occurs in conscious animals (Burnet and Wakerley, 1976). Although during suckling, oxytocin release was seen in the absence of prolactin release, the reverse was not observed, suggesting that some component of the milk ejection reflex is necessary to trigger suckling induced prolactin release (Wakerley et al, 1978). The precise details of the neural pathways which mediate these reflexes are not established (Urban, Moss and Cross, 1971; Tindal, Knaggs and Turvey, 1969; Tindal and Knaggs, 1972, 1977; Sutherland and Fink, 1983).

Thyrotrophin releasing hormone (TRH) in addition to its role in regulating the release of thyrotrophin from the anterior pituitary gland, has been shown to release prolactin in a number of species (Bowers, Friesen, Hwang, Guyda and Folkers, 1971; Jacobs, Snyder, Wilber, Utiger and Daughaday, 1971; Convey, Tucker, Smith and Zolman, 1973; Fell, Findlay, Cumming and Goding, 1973; Kelly, Bedirian, Baker and Friesen, 1973; Rivier and Vale, 1974; Deis and Alonso, 1975). Although low concentrations of TRH are effective for the release of prolactin *in vitro* from rat pituitary tumour cells (Tashjian, Barowsky and Jensen, 1971) or pituitary cells obtained from hypothyroid rats (Vale, Blackwell, Grant and Guillemin, 1973) the doses of TRH required for the *in vivo* release of prolactin from the rat anterior gland are high (Blake, 1974; Burnet and Wakerly, 1976) and it has not been established whether TRH is physiologically involved in the regulation of prolactin release in this species (Leong, Frawley and Neill, 1983).

The technique of immunoneutralization of a peptide by the administration of a specific antiserum (passive immunization) has been used previously in many studies to examine the physiological function of hypothalamic peptides including TRH (e.g. Szabo & Frohman, 1977; Fraser & McNeilly, 1982; Wehrenberg, Brazeau, Luben, Bohlen and Guillemin, 1982; Tannenbaum and Ling, 1984). The aims of the following experiments were to determine the effect of electrical stimulation of the hypothalamus on the release of thyrotrophin and prolactin from the anterior pituitary gland and using passive immunization with anti-TRH serum to investigate the role of TRH in mediating these responses and also the prolactin response to the

suckling stimulus in anaesthetized lactating rats.

5.2 MATERIALS AND METHODS

The animals used for the electrical stimulation studies were male or female Wistar rats (Edinburgh University Centre for Laboratory Animals). The female rats had exhibited at least two successive regular 4-day oestrous cycles immediately before the experiment (2.1). Lactating female Wistar Cob rats were supplied by Charles River U.K. Ltd. At the time of their arrival in the animal house, these animals were 4-5 days post partum and were then allowed a further 5 days to acclimatize to the conditions of the animals house. All animals were maintained as described in Chapter 2.

5.2.1 Electrical stimulation and the release of prolactin and thyrotrophin

Adult male Wistar rats (180-300 g body weight) were anaesthetized with urethane* and the ME and pituitary stalk exposed (2.2.3). After exposure of the dura, some animals were maintained under anaesthesia for a 4 h delay period during which the body temperature was regularly monitored by way of a rectal thermometer and maintained at 36-37°C with the aid of a warming lamp. The 4 h delay enabled the plasma concentrations of thyrotrophin and prolactin to return to a low, steady baseline after the stress of surgery, and also allowed comparison with the model used for the suckling studies described below (5.2.3). At the end of the 4 h delay the dura was cut and the ME and pituitary stalk exposed. An electrical stimulus (2.2.4) was applied for 15 min either to the ME or the PVN. In order to maintain the PVN stimulated animals under

*(1-1.2 g/kg body weight i.p.)

the same conditions as animals in which the ME was stimulated, and also to facilitate comparison with the results of the study of TRH release into portal vessel blood (4.2.3), the ME and pituitary stalk were also exposed for 4 h in the PVN stimulated rats before applying the stimulus. In control animals the stimulating electrodes were positioned but no stimulating current was passed. Blood samples (0.5 ml) for subsequent hormone assay were withdrawn from the EJV at 15 min intervals during the 30 min necessary for the surgical preparation of the animal and before and after the 15 min period of stimulation. At the end of the experiment the heads were removed from animals with PVN electrode implants and fixed in phosphate buffered formaldehyde in order subsequently to determine whether the electrode had been correctly placed (2.2.4).

5.2.2 Effect of anti-TRH serum on the responses to electrical stimulation

Male, or pro-oestrous female rats were anaesthetized with urethane and the median eminence (ME) and pituitary stalk exposed using the transpharyngeal approach (2.2.3). In female rats, the anaesthetic was administered and the surgical preparation completed between 06.00 and 08.00 h of pro-oestrus. After the exposure of the dura, all animals were maintained under anaesthesia for a 4 h delay period (as described above) during which animals were slowly injected by way of the external jugular vein with 1 ml of ovine anti-TRH serum (antiserum no. 420; described in section 2.5.1). Control animals were treated similarly with either ovine non-immune serum or ovine anti-BSA-serum. At the end of the 4 h delay period the dura was cut, the ME and pituitary stalk exposed and the

electrical stimulus was applied to the ME or PVN. Blood samples (0.5 ml) for subsequent hormone assay were withdrawn from the external jugular vein 15 min before, and at 15 min intervals after a 15 min period of stimulation. The correct placement of the electrodes in the PVN was confirmed at the end of the experiment and the results obtained from one animal were excluded from the results because of incorrect electrode placement.

5.2.3 Suckling induced prolactin release

The experimental protocol of this study was based on that of Burnet & Wakerley (1976) who showed that in lactating rats anaesthetized with urethane*, a good prolactin surge occurred 2.5-3 h after the beginning of suckling in animals anaesthetized for a total of about 6 h. Lactating female rats 9 to 11 days post-partum were separated from all but one of their young overnight. On the following morning, the mother was anaesthetized with urethane and an hour later was injected intravenously with anti-TRH or anti-BSA serum. Three hours after induction of anaesthesia, a blood sample was withdrawn from the external jugular vein and 7 to 10 pups were then applied to the mother's teats. Further blood samples were then removed at 30 min intervals throughout the period of suckling and the plasma stored at -40°C until assayed for prolactin and thyrotrophin.

5.2.4 Radioimmunoassays and analysis of results

Plasma samples were stored at -40°C until assayed for thyrotrophin (2.5.3) and prolactin (2.5.4). In cases where the values for thyrotrophin or prolactin were below the lower limit of detection of the assay the lower limit, 15-50 ng/ml for prolactin

*(1-1.2 g/kg body weight i.p.)

and 0.3 ng/ml for thyrotrophin, was ascribed to the sample and used in subsequent calculations. The significance of differences between groups was determined by the Mann-Whitney U test (Siegel, 1956).

5.3 RESULTS

5.3.1 Electrically stimulated thyrotrophin and prolactin release

The plasma concentration of thyrotrophin did not alter significantly in rats in which the electrical stimulus was applied to the ME within an hour after the administration of the anaesthetic (Fig. 5-1). However, in the same animals, there was an increase in the plasma prolactin concentration 15 min after the end of the stimulation period ($P < 0.005$ compared with pre-stimulation value; $P < 0.05$ compared with unstimulated control). In rats in which the final exposure of the ME was delayed for 4 h, electrical stimulation of either the ME (Fig. 5-2) or PVN (Fig. 5-3) resulted in significant increases in the plasma concentrations of both thyrotrophin and prolactin. Whilst the thyrotrophin response to stimulation occurred immediately, the peak prolactin response to stimulation of the ME occurred 15 min after the termination of the stimulus. In all treatment groups plasma prolactin concentrations were high after induction of anaesthesia, but declined towards the end of the 30 min period of surgery; the decrease was significant ($P < 0.02$–$P < 0.005$) in all except the ME stimulated (no delay) group. Plasma thyrotrophin concentrations did not alter appreciably during the surgery, but decreased significantly during the 4 h delay period ($P < 0.001$–$P < 0.005$).

Passive immunization with anti-TRH serum blocked the

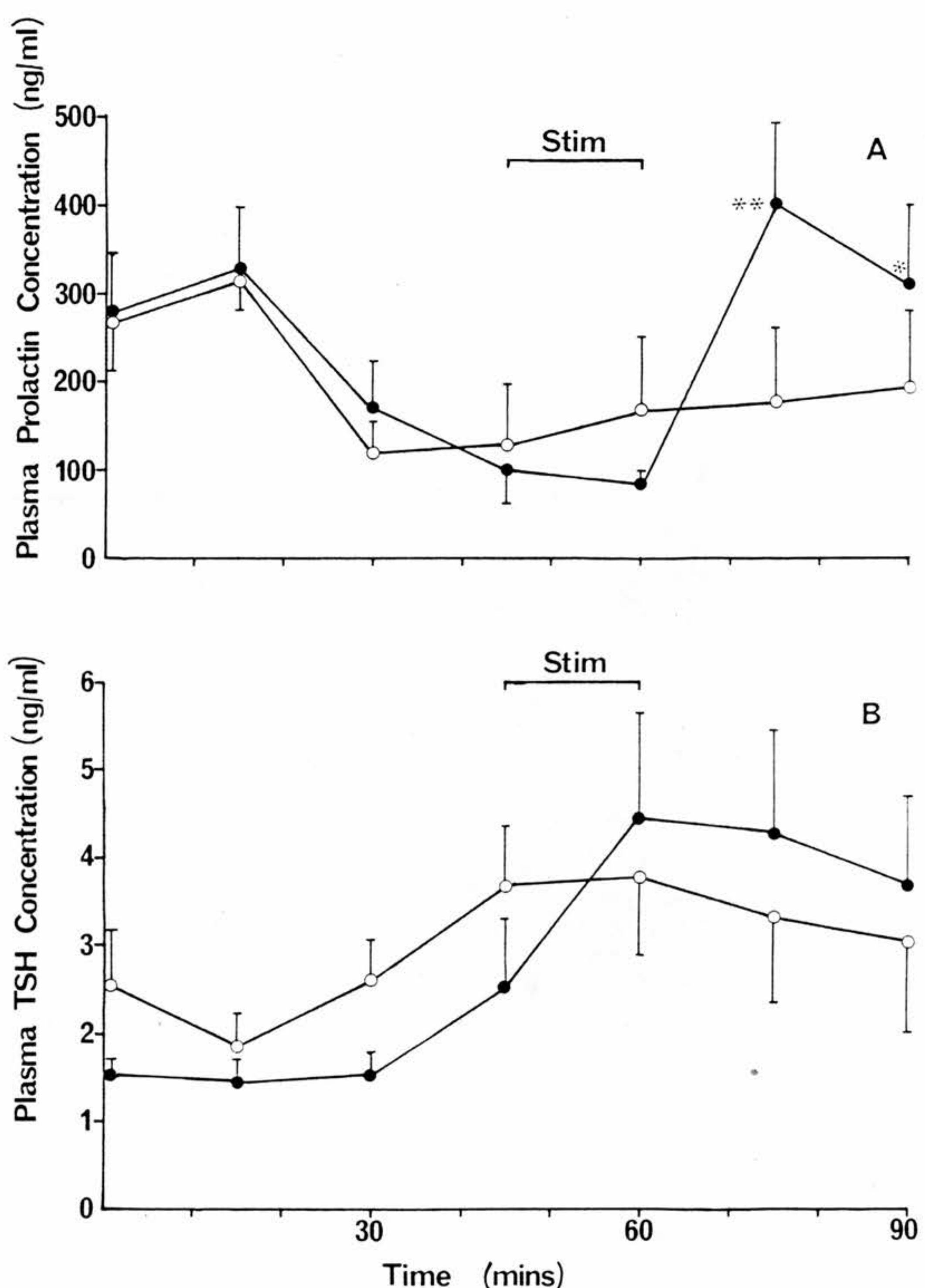
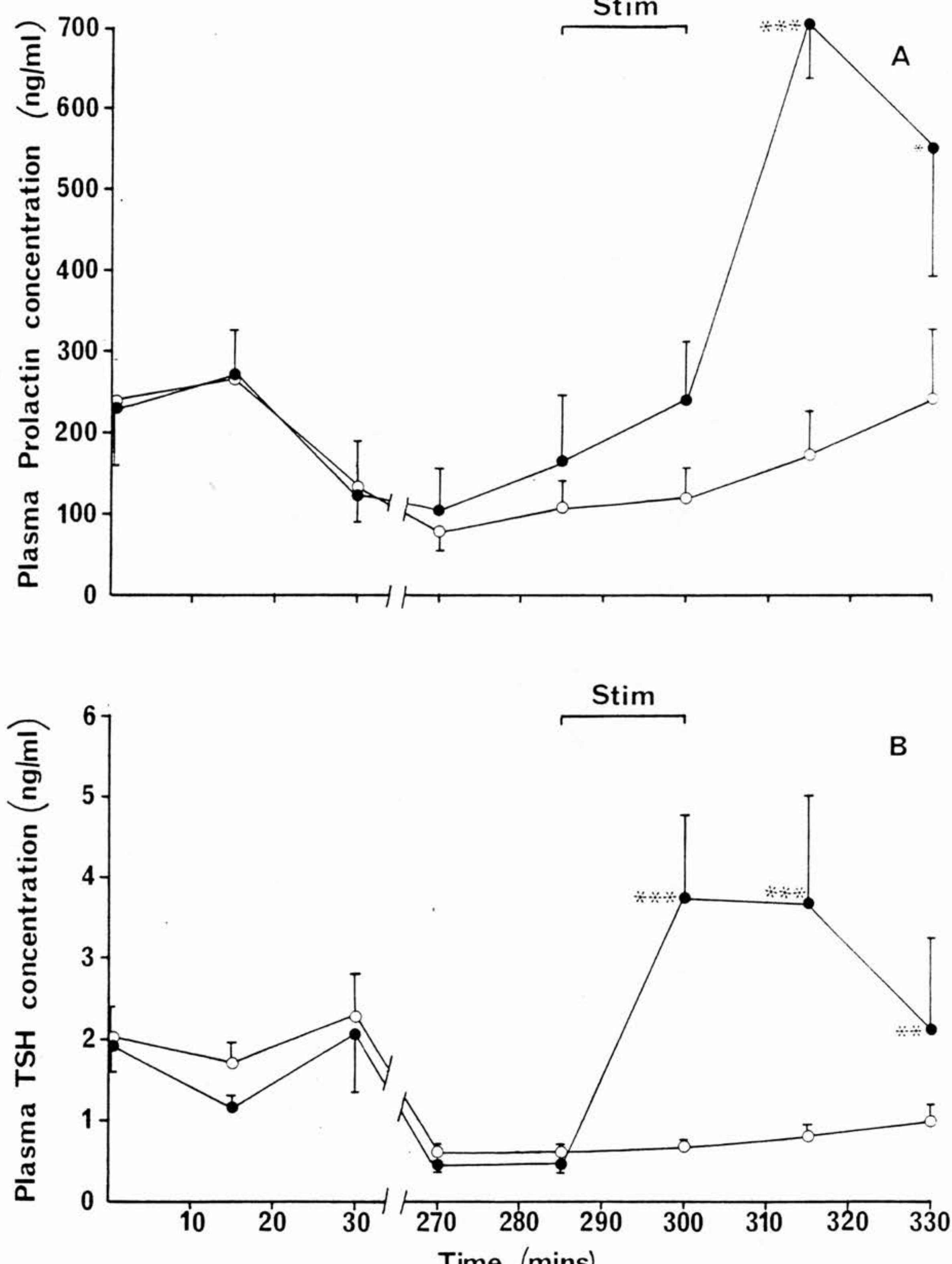


FIGURE 5-1: Mean (± SEM) plasma concentrations of (A) prolactin and (B) thyrotrophin (TSH) in rats in which an electrical stimulus was applied to the median eminence (●) or in control rats (O) in which the electrode was positioned, but no current was passed. The initial blood samples (0, 15 and 30 min) were taken during the exposure of the median eminence and placement of the electrode. The electrical stimulus was applied for 15 min as indicated by the horizontal bars. Seven animals in each treatment group. $^{*}P < 0.05$, $^{**}P < 0.005$ compared with the pre-stimulation concentration.

FIGURE 5-2: Effect of electrical stimulation of the median eminence on the plasma concentrations of (A) prolactin and (B) thyrotrophin (TSH). Stimulated animals are denoted by closed circles; open circles indicate animals in which the electrode was positioned, but no stimulating current was passed. The initial blood samples (0, 15 and 30 min) were taken during the exposure of the dura, animals were maintained under anaesthesia for 4 h before exposure of the median eminence and the placement of the electrode. The electrical stimulus was applied for 15 min as indicated by the horizontal bar. Five-seven animals in each treatment group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with pre-stimulation concentration.


Stim
A
Plasma Prolactin concentration (ng/ml)
700
600
500
400
300
200
100
0
***
*
Stim
B
Plasma TSH concentration (ng/ml)
6
5
4
3
2
1
0
***
***
**
10
20
30
270
280
290
300
310
320
330
Time (mins)

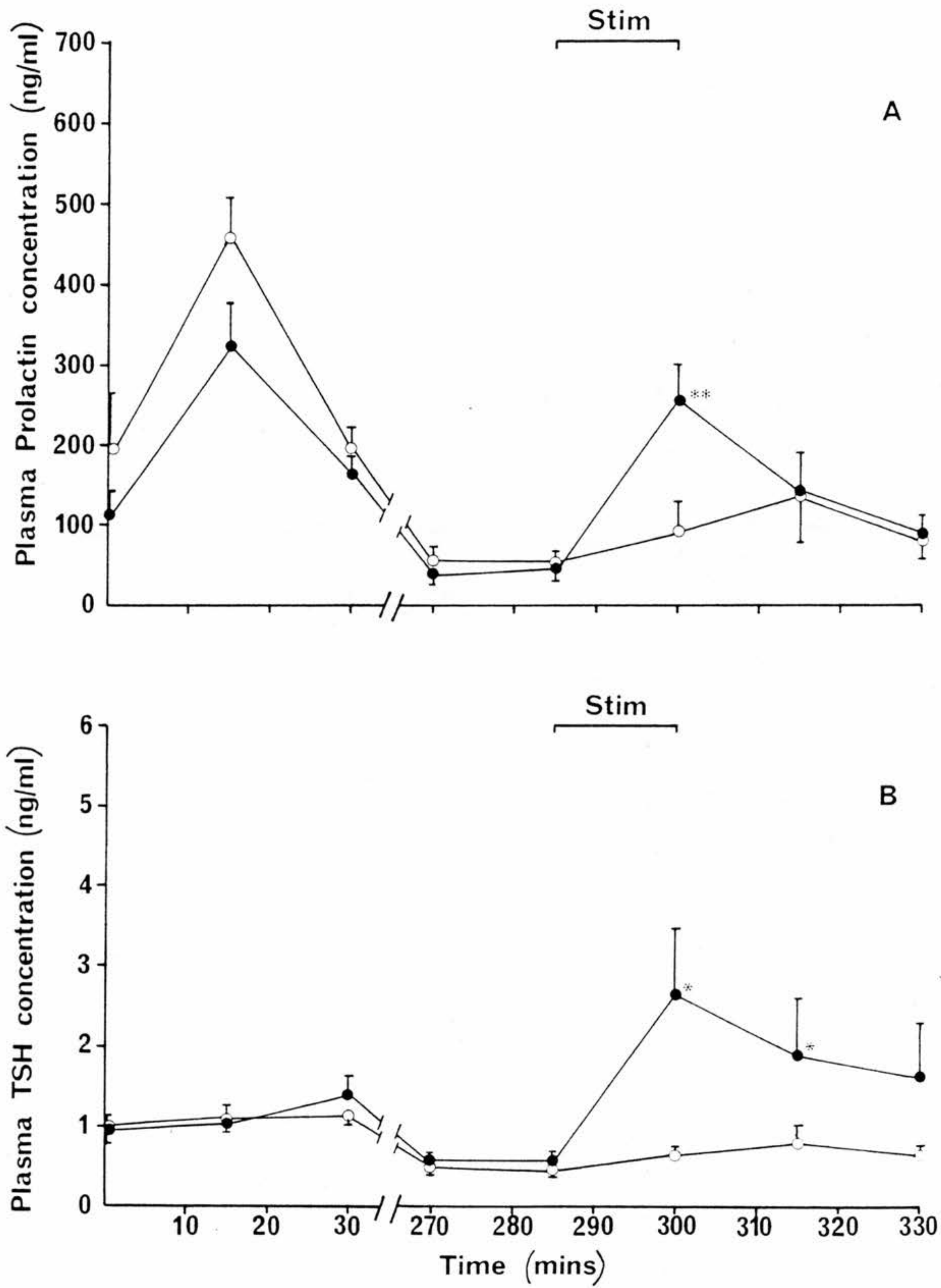
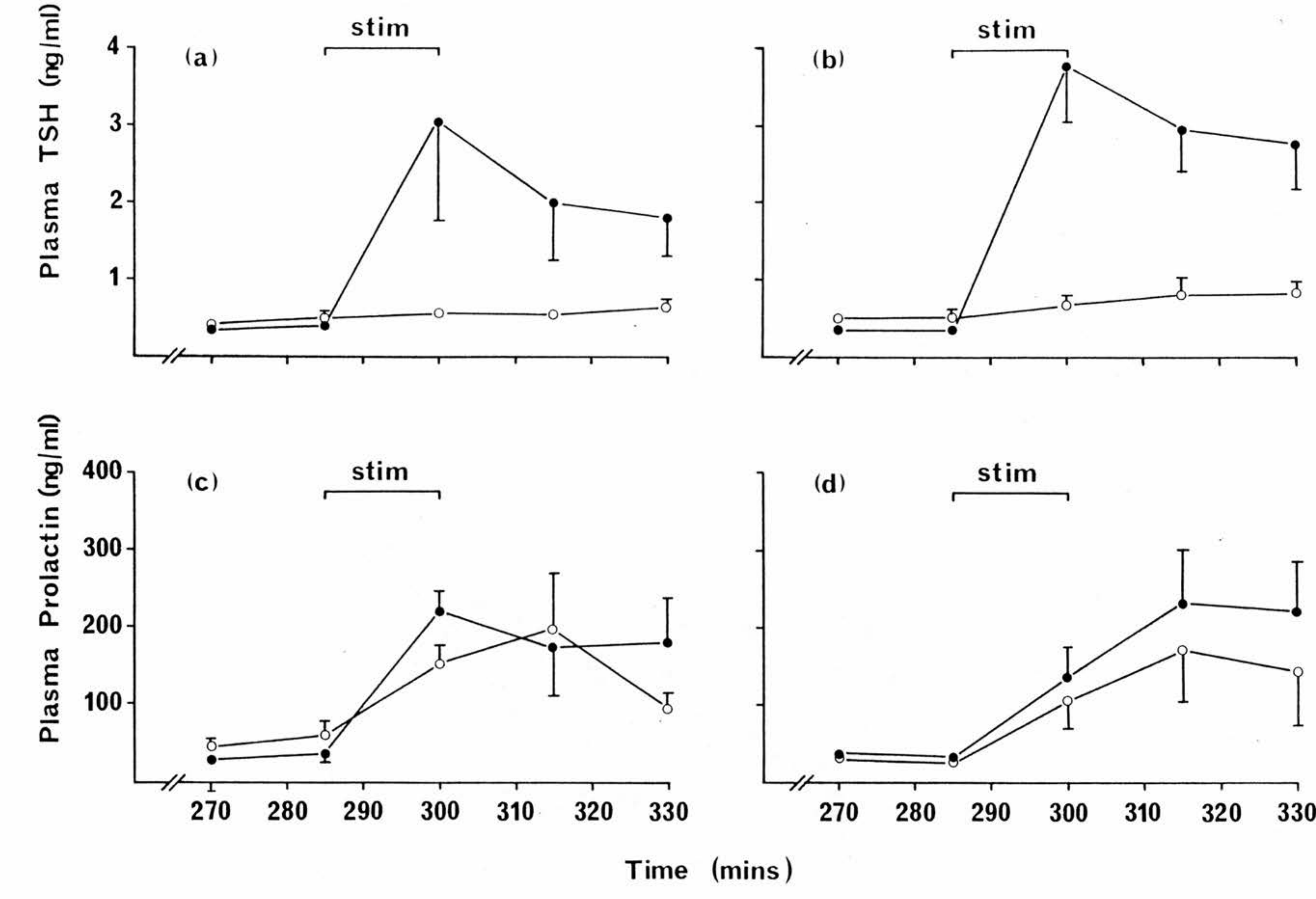


FIGURE 5-3: Effect of electrical stimulation of the paraventricular nuclei on the plasma concentrations of (A) prolactin and (B) thyrotrophin (TSH). Stimulated animals are denoted by (●); (O) indicates animals in which electrodes were implanted, but no stimulating current was passed. The electrical stimulus was applied for 15 min as indicated by the horizontal bars. Seven animals in each treatment group. $*P < 0.02$, $**P < 0.001$ compared with pre-stimulation concentration.

FIGURE 5-4: Mean (± SEM) plasma concentrations of thyrotrophin (TSH) (a and b) and prolactin (c and d) in response to electrical stimulation of the paraventricular nuclei (a and c) or median eminence (b and d) of male rats injected 2 h previously with either anti-TRH (○) or control (●) serum (non-immune). The electrical stimulus was applied for 15 min as described in the text. (n = 5-7 animals/group).


Plasma TSH (ng/ml)
4
3
2
1
(a)
stim
(b)
stim
Plasma Prolactin (ng/ml)
400
300
200
100
(c)
stim
(d)
stim
270
280
290
300
310
320
330
Time (mins)

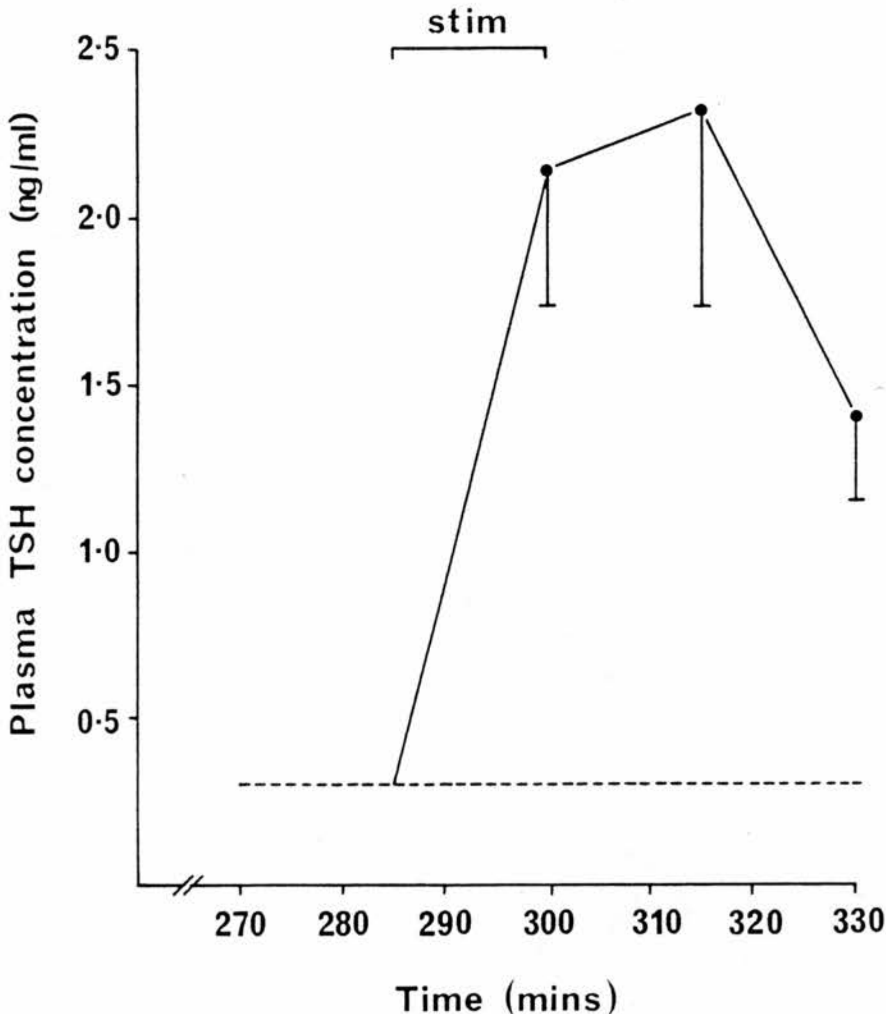


FIGURE 5-5: Mean (± SEM) plasma concentrations of TSH in response to electrical stimulation (horizontal line) of the median eminence in pro-oestrous female rats (n = 5-7/group) injected with control (anti-bovine serum albumin) serum 2 h previously. The blood sample at 285 min was taken immediately before the application of the stimulus. The broken line indicates the lower limit of detection of the assay; in all samples from anti-thyrotrophin-releasing hormone-treated animals the plasma TSH concentration was below the limit throughout the experiment.

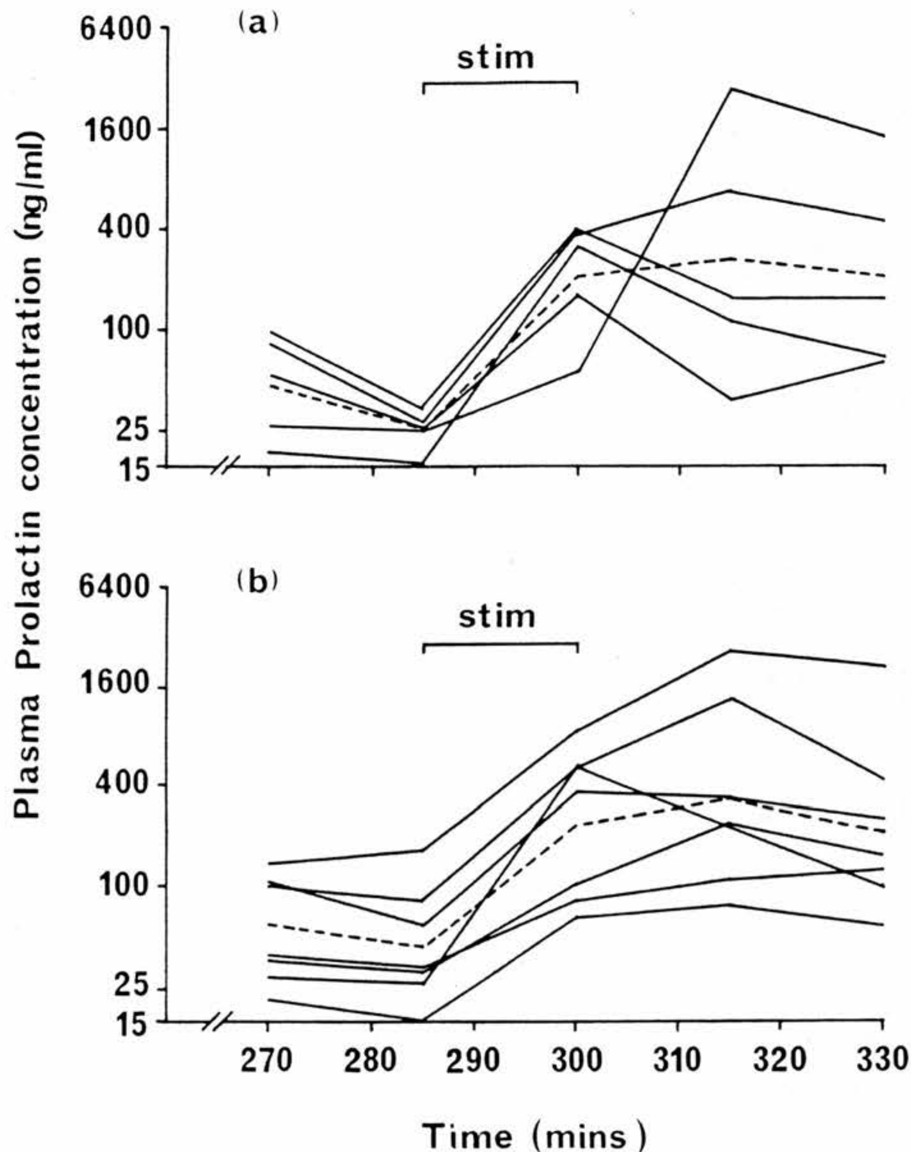


FIGURE 5-6: Plasma prolactin concentrations before and after electrical stimulation (horizontal lines) of the median eminence on the morning of pro-oestrus of individual female rats previously injected with either (A) anti-TRH serum or (B) control (anti-bovine serum albumin) serum. The plasma prolactin concentrations are expressed on a $\log_{10}$ scale; the dotted line indicates the geometric mean prolactin concentration.

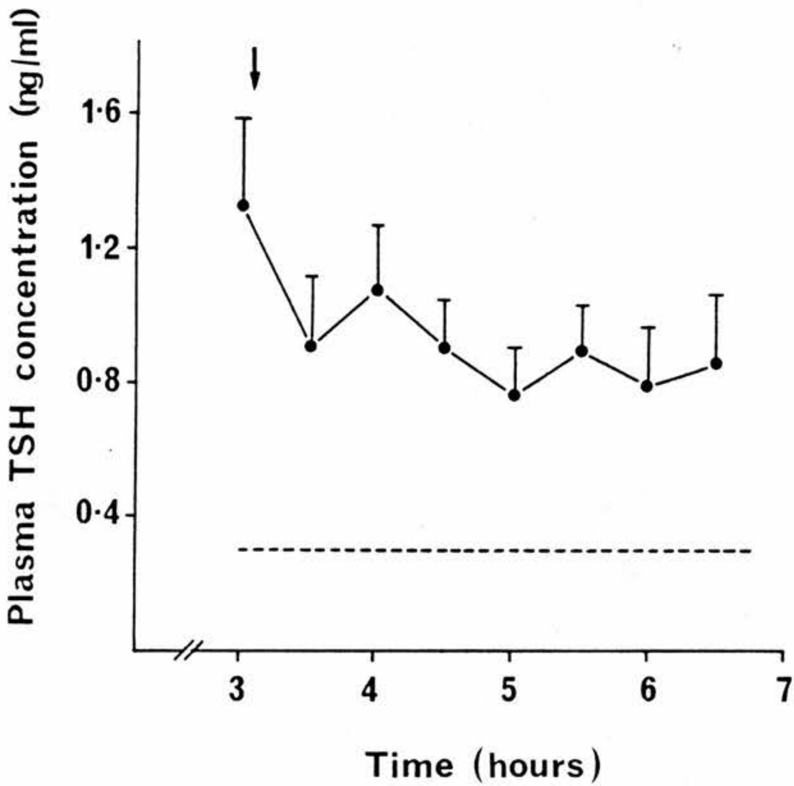


FIGURE 5-7: Mean (± SEM) plasma concentrations (n = 12) of TSH before and during suckling in lactating rats anaesthetized with urethane which had been injected 2 h previously with control (anti-bovine serum albumin) serum. The broken line indicates the lower limit of detection of the assay; in all samples from anti-TRH serum treated animals the plasma TSH concentration was below this limit.

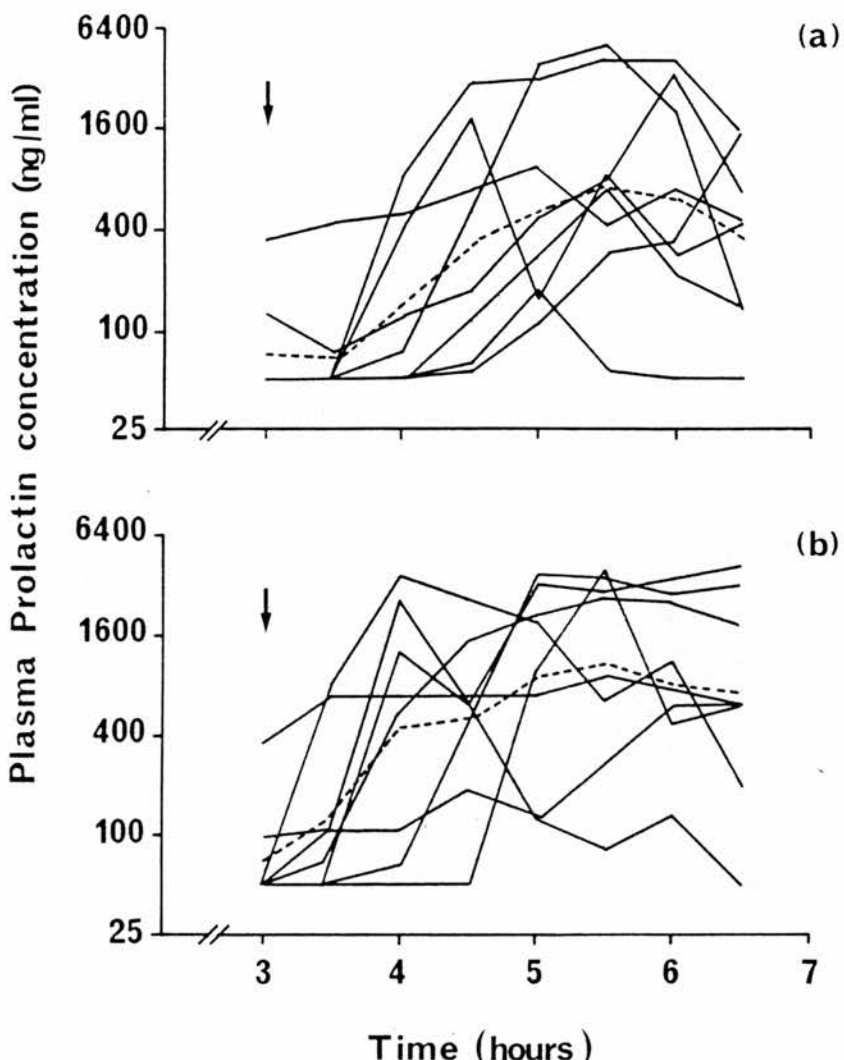


FIGURE 5-8: Plasma prolactin concentrations before and during suckling in individual lactating rats (n = 8) injected with either (a) anti-TRH serum or (b) with control (anti-BSA) serum. The four animals in each group which did not show a significant prolactin reponse to suckling are not included. A $\log_{10}$ scale has been used for the ordinate; the dotted line on each graph indicates the geometric mean of the prolactin concentrations.

Table 5-A. Mean (± S.E.M.) maximal increments (maximal concentrations minus pre-stimulation or pre-suckling concentrations) of the plasma thyrotrophin and prolactin concentrations following electrical stimulation of either the median eminence (ME) or paraventricular nuclei (PVN) or in response to suckling. Five to eight animals per treatment group.

	Mean maximal increments (ng/ml)			
	Plasma thyrotrophin		Plasma prolactin	
	Control	Antiserum treated	Control	Antiserum treated
Male Rats - ME stimulation	3.46 ± 0.70	0.56 ± 0.13**	223.0 ± 61.6	170.4 ± 64.4
PVN stimulation	2.68 ± 1.20	0.15 ± 0.08*	240.5 ± 52.8	127.4 ± 52.0
Female Rats - ME stimulation	2.10 ± 0.57	NC**	341.7[a](2.53 ± 0.22)	487.6[a](2.69 ± 0.22)
Suckling	NC	NC	2004.5[a](3.30 ± 0.13)	1235.9[a](3.10 ± 0.19)

NC = no change detected; (a) Values are geometric means with mean ± S.E.M. $\log_{10}$ increment given in parentheses;
* $p < 0.005$; ** $p < 0.001$; compared with control values (Mann-Whitney U test)

electrically stimulated release of thyrotrophin, but not prolactin (Fig. 5-4; Table 5-A). Similar results were obtained in response to ME stimulation in pro-oestrous rats (Figs. 5-5 and 5-6; Table 5-A), although the prolactin response to stimulation in the pro-oestrous rats was more variable than in the male animals. As in male rats, in female animals the thyrotrophin response to stimulation of the ME was immediate, but the prolactin response was delayed relative to the onset of the stimulus.

5.3.2 Prolactin release in response to suckling

In lactating female rats injected with anti-BSA serum the plasma thyrotrophin concentration did not alter significantly during a period of suckling; in animals treated with anti-TRH serum, plasma thyrotrophin concentrations remained below the detectable limits of the assay throughout (Fig. 5-7). In contrast, plasma prolactin concentrations were increased well above a value of 100 ng/ml or more by suckling in eight out of twelve animals in each of the groups of animals treated with anti-BSA or anti-TRH serum (Fig. 5-8). The peak prolactin response occurred approximately 2.5 h after pups were applied to teats. The mean prolactin response to suckling was lower in the animals treated with anti-TRH serum, but the difference between the responses in these animals was not significantly different from those in animals treated with anti-BSA serum (Table 5-A).

5.4 DISCUSSION

Martin and Reichlin (1972) showed that electrical stimulation of either the ME or PVN resulted in elevated plasma concentrations

of thyrotrophin. This was confirmed by the present experiments in which there was a 4 h delay period between the completion of surgery and the application of the stimulus (Figs. 5-2 and 5-3). However, a thyrotrophin response to stimulation could not be demonstrated in rats in which the stimulus was applied 15 min after the exposure of ME and pituitary gland, although these animals still showed a good prolactin response to stimulation (Fig. 5-1). Stressful stimuli have long been known to inhibit thyrotrophin secretion (e.g. Brown-Grant, Harris and Reichlin, 1954; Ducommun, Vale, Sakiz and Guillemin, 1967) and in the group of animals in which the stimulus was applied (15 min after the completion of surgery), the surgical trauma and stress of anaesthesia may have inhibited the initial response to stimulation.

The results of several studies which used lesion or electrical stimulation of the hypothalamus suggested several hypothalamic areas which may mediate the prolactin responses to stimuli such as mating or suckling. The dorsomedial-ventromedial areas of the hypothalamus facilitate prolactin release, whilst the medial pre-optic area (mPOA) may contain both inhibitory and facilitatory neurones governing prolactin release (e.g. Tindal and Knaggs, 1977; Smith and Gala, 1978; Freeman and Banks, 1980; Gunnet and Freeman, 1985). The present study shows that the ME and PVN are also both effective sites for the electrical stimulation of prolactin release. Tindal and Knaggs (1977) used partially decorticate rats with brain stem transsections (to reduce the requirement for anaesthesia) to show prolactin release in response to stimulation of the mPOA, but failed to show prolactin release after stimulation of sites in or near the

ME or PVN. However, the stimulus used by Tindal and Knaggs (1977) was of lower intensity than that used in the present experiments and was applied as a single train of pulses, 30 seconds in duration. Malvern (1975) showed release of prolactin in response to stimulation of the anterior ME in 3 conscious sheep, whilst stimulation of the posterior ME in a fourth animal appeared ineffective.

In Chapter 4 it was shown that it was not possible to demonstrate an increase in the concentration of TRH in hypophysial portal blood in response to electrical stimulation of the hypothalamus. However, the fact that the i.v. injection of anti-TRH serum blocked the thyrotrophin response to electrical stimulation of either the ME or PVN and suppressed the plasma concentrations of thyrotrophin in lactating female rats, does provides direct evidence in support of the role of TRH as the principal mediator of the neural control of thyrotrophin release from the pituitary gland. In contrast, injections of anti-TRH serum did not block the prolactin response to either electrical stimulation or suckling, although plasma prolactin concentrations were lower (but not significantly so) in some circumstances. These results are similar to those obtained in sheep actively immunized against TRH in which plasma tri-iodothyronine and thyroxine concentrations were suppressed, but in which the seasonal changes in prolactin and the prolactin response to suckling were not significantly reduced. The prolactin response to heat stress, however, was significantly lower in the actively immunized compared with the control ewes (Fraser & McNeilly, 1982). In rats, passive immunization with the ovine

anti-TRH serum used here did not affect significantly the prolactin response to heat stress (Fraser & McNeilly, 1983).

The suckling stimulus resulted in significant increases in the plasma prolactin concentration in the majority of the lactating rats in a manner similar to that described by Burnet & Wakerley (1976). In previous studies using conscious rats (Blake, 1974) or rats anaesthetized with urethane (Burnet and Wakerley, 1976) the prolactin response to suckling was associated with an increase in the plasma concentration of thyrotrophin, although in both studies the magnitude of the thyrotrophin response was much lower than the prolactin response. The present results are in agreement with the results of a recent study (Riskind, Millard and Martin, 1984) in that plasma thyrotrophin concentrations in animals injected with non-immune or anti-BSA serum did not increase during the period of suckling. Several studies have indicated that in man also, the prolactin response to suckling is not accompanied by any change in circulating thyrotrophin concentrations (Gautvik, Weintraub, Graeber, Maloof, Zuckerman and Tashjian, 1973; Jeppsson, Nilsson, Rannevik & Wide, 1976) and Riskind et al. (1984) suggested that thyrotrophin release may be an occasional and unpredictable component of the suckling response. Oxytocin reduced the effectiveness of TRH in releasing thyrotrophin from cultured rat pituitary cells and this led to the suggestion that oxytocin may modulate thyrotrophin release during suckling (Frawley, Leong and Neill, 1985).

Doses of TRH which significantly increased thyrotrophin release in lactating rats did not increase plasma prolactin concentrations

to the values achieved during suckling (Burnet & Wakerley 1976; de Greef & Visser 1981). Grosvenor, Mena and Whitworth (1979a) proposed that suckling induced release of prolactin proceeds in two distinct phases. Initially prolactin is not released directly into the circulation, but the pituitary prolactin stores are rapidly depleted and transformed into a releasable form which is slowly released into the circulation whilst the pituitary stores are repleted (Mena, Pacheco and Grosvenor, 1980). The depletion-transformation stage, which is accompanied by morphological changes in the appearance of the lactotrophs (Shiino, Williams and Rennels, 1972; Chang and Nikitovitch-Winer, 1976; Farquhar, Reid and Daniell, 1976), can be initiated by suckling but not by exposure to ether (Grosvenor, Mena and Whitworth, 1979b) or by TRH (Grosvenor and Mena, 1980). However, once the transformation-depletion stage has been produced by a brief period of suckling both agents could evoke prolactin release. Administration of extracts of stalk-median eminence (SME), dopamine or the dopamine agonist bromocriptine, before the period of suckling prevented both the depletion of prolactin in the pituitary and the rise in plasma prolactin concentration in response to suckling, but neither the SME extract or bromocriptine prevented suckling-induced prolactin release when administered after an initial 10 minute period of suckling had already occurred (Grosvenor, Mena and Whitworth, 1980). Electrical stimulation of the mammary nerve of lactating rats, or suckling, resulted in a brief decrease in the concentrations of dopamine in hypophysial portal blood (de Greef, Plotsky and Neill, 1981; Plotsky and Neill, 1982a) although the

dopamine concentration returned to pre-stimulation values before the maximum prolactin response and the magnitude of the reduction of dopamine release alone was insufficient to fully account for the prolactin release (de Greef et al. 1981; Plotsky and Neill, 1982b). As a result of the foregoing studies it has been proposed that a transient decrease in the secretion of dopamine (DA) into the hypophysial portal vessel blood at the onset of suckling may increase the sensitivity of the pituitary to the prolactin releasing action of TRH (Grosvenor & Mena 1980; de Greef & Visser 1981; Plotsky & Neill 1982). However, Riskind et al. (1984) found no increase in pituitary responsiveness to exogenous TRH after a period of suckling.

Previous studies which have used the technique of passive immunization with anti-TRH serum to investigate the role of TRH in the release of prolactin have produced conflicting results. Koch, Goldhaber, Fireman, Zor, Shani and Tal (1977) showed that passive immunization significantly reduced the prolactin surge on the afternoon of pro-oestrus, but Harris, Christianson, Smith, Fang, Braverman and Vagenakis (1978) reported no change in prolactin despite a suppression of basal thyrotrophin release. However, there are several differences in method which may have contributed to the difference between the results of these two studies. First, different volumes of antisera (which possibly differed in affinity and titre) were injected in the two studies. Harris et al. (1978) used only 0.1 ml whereas Koch et al (1977) used 1.0 ml of antiserum. Secondly, Koch et al (1977) exposed their animals to ether before blood sampling (Koch et al. 1977), and thirdly, Harris

et al (1978) withdrew blood samples at 1300 h, 1500 h and 1700 h whilst Koch et al (1977) sampled blood at 1500 h only. Recent studies, in which hourly blood samples were withdrawn from conscious rats throughout the afternoon of pro-oestrus, have shown that the administration of the same anti-TRH serum as was used in the present study did not affect the magnitude of the spontaneous surge of prolactin on pro-oestrus. However, the onset of the prolactin surge was delayed by approximately 1 h (Horn, Fraser & Fink 1985b) suggesting that TRH might be involved in the initiation, but not the maintenance of the pro-oestrous prolactin surge.

A possible criticism of the passive immunization technique, which may be invoked to explain some of the discrepancies between the data reviewed above, is that under some circumstances the anti-TRH-serum may not act quickly enough to quench all the TRH released into hypophysial portal blood. However, the results presented in this Chapter show that in animals injected with the anti-TRH-serum, plasma thyrotrophin concentrations remained undetectable during electrical stimulation of the ME in pro-oestrous rats (Fig. 5-2) and suckling in lactating rats (Fig. 5-4).

In sum these results provide direct evidence that TRH mediates the neural control of thyrotrophin release. Several data suggest that under certain conditions TRH may play a role in either initiating and/or maintaining the release of prolactin (e.g. Koch et al. 1977; de Greef & Visser, 1981; Fraser & McNeilly, 1982, 1983; Fink, Koch & Ben Aroya, 1982; Leong et al. 1983, Horn et al. 1985b). While the present data do not exclude such a role for TRH, they do show that TRH is not crucial for the neural control of

prolactin release in the rat. The increase in plasma prolactin concentrations in response to electrical stimulation or the suckling stimulus could be mediated either by the inhibition of inhibitory influences or by the release of a specific prolactin releasing factor (1.5).

The decreased secretion of dopamine into portal blood may provide a powerful stimulus for prolactin release (Leong et al, 1983). There is no direct evidence on the effects of electrical stimulation of the hypothalamus on dopamine release into hypophysial portal blood, but on the basis of studies of electrical stimulation of ME slices in vitro (Sarkar, Gottshall, Meites et al, 1983) it would seem likely that, if anything, the release of dopamine into portal blood would be increased by ME stimulation. In an examination of the prolactin inhibiting activity of porcine ME extracts, a significant portion of the activity found could be accounted for by γ-aminobutyric acid (GABA) (Schally, Redding, Arimura, Dupont and Linthicum, 1977). Anatomical studies have shown that GABA and the enzyme, glutamic acid decarboxylase (GAD) responsible for its synthesis are present throughout different regions of the hypothalamus (Tappaz, Brownstein and Kopin, 1977); immunohistochemical studies with anti-GAD antibodies indicate a plexus of presumed GABA terminals in the ME (Vincent, Hokfelt and Wu, 1982). Receptors for GABA are present in the anterior pituitary and evidence is accumulating in support of a regulatory role of GABA in hormone secretion (e.g. Anderson and Mitchell, 1986). GABA is present in hypophysial portal blood and its release was found to increase 7-8 fold after ME stimulation (Mitchell, Grieve, Dow and

Fink, 1983). The 56 amino acid, GnRH-associated peptide (GAP) identified at the C-terminus of the human placental LHRH precursor, has been reported to inhibit prolactin release in vitro (Nikolics, Mason, Szonyi, Ramachandran and Seeburg, 1985). Conceivably the release of GAP as a result of stimulation of the cell bodies of LHRH neurons in the preoptic area could account for the observations [e.g. Gunnet and Freeman (1985)] of neurons in this area which inhibit prolactin release. However, in the absence of direct measurements of the GAP content of hypophysial portal blood, it seems likely that as for LHRH (Sherwood, Chiappa and Fink, 1976) the secretion of GAP would increase rather than decrease during electrical stimulation of the ME. Nikolics et al (1985) suggested that GAP could account for the inverse relationship between gonadotrophin and prolactin secretion which occurs, for example, during lactation (see Chapter 9). However, there are several lines of evidence which suggest that GAP is unlikely to function as a physiological regulatory of prolactin secretion under all circumstances (Fink, 1985). For example, a surge of prolactin occurs simultaneously with some steroid-induced LH surges and during the spontaneously occurring pre-ovulatory LH surge during the afternoon of pro-oestrus (e.g. Smith, Freeman and Neill, 1975; Horn and Fink, 1985a; de Greef et al, 1985). In addition, it would be predicted that if GAP was the major prolactin inhibiting factor then hpg mice should have elevated pituitary and plasma concentrations of prolactin, but in fact pituitary and plasma prolactin concentrations in hpg mice are similar to those measured in normal male mice and lower than those measured in normal female mice (Charlton, Speight,

Halpin et al, 1983). Thus it seems likely that the increase in plasma prolactin in response to ME stimulation is due to the increased release into hypophysial portal blood of a prolactin releasing factor rather than a decrease in the release into portal blood of transmitters (dopamine and GABA) known to inhibit prolactin release.

Several studies have indicated the presence in the hypothalamus of factors, other than TRH, which may stimulate prolactin release (Valverde, Chieffo & Reichlin, 1972; Dular, LaBella, Vivian & Eddie, 1974; Boyd, Spencer, Jackson & Reichlin, 1976; Szabo & Frohman 1976; Yasuda, Yasuda & Greer, 1984) but the nature of the prolactin releasing factor remains to be determined. However, several studies have suggested that, in some circumstances, vasoactive intestinal peptide (VIP) and peptide histidine isoleucine amide (PHI) may be involved in the physiological regulation of prolactin secretion. VIP and PHI are peptides (containing 28 and 27 amino acid residues respectively) which were originally isolated from porcine intestine, but were subsequently identified in the brain (e.g. Rostene, Leranth, Maletti et al, 1982; Tatemoto, Carlquist, McDonald and Mutt, 1983) and shown to stimulate prolactin release (e.g. Kato, Iwasaki, Iwasaki et al, 1978; Frawley and Neill, 1981; Hokfelt, Fahrenkrug, Tatemoto et al, 1983; Kaji, Chihara, Abe et al, 1984). Cell bodies and fibres containing immunoreactive PHI have been demonstrated in the PVN and external layer of the ME respectively (Hokfelt, Fahrenkrug, Tatemoto, Mutt, Werner, 1982). VIP-containing fibres in the external layer of the ME are normally reported to be scarce but many such fibres were visible in adrenalectomized rats or

lactating rats (Mezey and Kiss, 1985). VIP is present in hypophysial portal blood in concentrations which are sufficient to release prolactin _in vitro_ (Said and Porter, 1978; Brar, Fink, Maletti and Rostene, 1985a). When injected into lactating rats at the start of a period of suckling, anti-VIP serum delayed the onset of the prolactin response without diminishing the magnitude of the response; when injected during the period of suckling the antiserum produced a partial and transient decrease in the plasma concentration of prolactin (Abe, Engler, Molitch, Bollinger-Gruber and Reichlin, 1985). The prolactin response to ether exposure was completely blocked by administration of anti-VIP serum to lactating rats (Abe _et al_, 1985) and was partly blocked in male rats by anti-VIP or anti-PHI sera either when administered alone or in combination (Kaji, Chihara, Kita _et al_, 1985a). Passive immunization of male rats with a combination of anti-PHI and anti-VIP sera also resulted in a reduced prolactin response to the injection of 5-hydroxy-tryptophan and completely blocked this response when dopaminergic control was eliminated by sulpiride administration (Kaji, Chihara, Abe _et al_, 1985b). Thus, both VIP and PHI may participate in the control of prolactin release, but present data do not discount the possibility of additional prolactin releasing factors.

CHAPTER 6

Chromatographic Studies of Immunoreactive LHRH in the Rat and Mouse Hypothalamus and in Rat Hypophysial Portal Blood

6.1 INTRODUCTION

Several chromatographic studies have indicated the presence in hypothalamic extracts of higher molecular weight material with LHRH-like immunoreactivity which may represent either the LHRH precursor or intermediate derivatives of the LHRH precursor (Kerdelhue, Jutisz, Gillessen and Studer, 1973; Fawcett, Beezley and Wheaton, 1975; Barnea and Porter, 1975; Millar, Achnett and Rossier, 1977; Gautron, Pattou and Kordon, 1981). The *in vitro* translation of mRNA from rat, human or mouse hypothalamus resulted in the production of a single polypeptide which was immunologically similar to LHRH and had a molecular weight (in all three species) of 28,000 (Curtis and Fink, 1983; Curtis, Lyons and Fink, 1983). The structure of a smaller sized precursor (molecular weight 10,000 and 92 amino acids in length) was deduced from the cloned complementary DNA sequence derived from human placental messenger RNA (Seeburg and Adelman, 1984). As yet there is no explanation for the discrepancy between the size of precursor described by Seeburg and Adelman (1984) and that reported by Gautron *et al* (1981) and Curtis *et al*, (1983).

Many previous studies have demonstrated the presence of LHRH-IR in hypophysial portal vessel blood and the variation in its release under different physiological conditions, consistent with its role in mediating the neural control of gonadotrophin release (Fink, 1979a; Fink, Stanley and Watts, 1983). There have, however, been no chromatographic studies in which it has been demonstrated that the LHRH-IR of portal blood is similar to the authentic decapeptide, although this was suggested by the fact that serial dilutions of

portal plasma inhibited binding of ^{125}I-LHRH in parallel with serial dilutions of the synthetic decapeptide (Fink and Jamieson, 1976; Eskay et al, 1977). The following series of experiments was undertaken to determine (i) whether as assessed by HPLC the LHRH-IR of hypophysial portal blood is due entirely to the decapeptide, and (ii) the characteristics of LHRH-IR in extracts of hypothalami from rats, from normal mice and also from mutant, hypogonadal (hpg) mice. The hypogonadism of the hpg mouse is inherited as an autosomal recessive trait. The hpg mice have low plasma and pituitary concentrations of gonadotrophins and underdeveloped gonads and accessory sexual organs (Cattanach, Iddon, Charlton, Chiappa and Fink, 1977). In the male the penis remains small, the scrotum underdeveloped, and the minute testes remain undescended and the anogenital distance is reduced. In the female, the vagina does not usually open, the uterus appears thread- like and the ovaries are barely discernible without the aid of a microscope. Although data obtained by RIA of crude hypothalamic extracts suggested that the mutant is totally deficient in LHRH, as for the immunoreactive LHRH in portal blood, this point has not been proven with the aid of HPLC.

6.2 MATERIALS AND METHODS

The animals used were male and female hpg and normal mice and adult male and female Wistar rats. The female rats had shown at least two consecutive 4-day oestrous cycles immediately before experimentation (2.1).

6.2.1. LHRH-like immunoreactivity in hypophysial portal vessel blood

Hypophysial portal blood was collected, from a total of 13 male

rats anaesthetized with urethane (2.2.3), for periods of 45 to 60 minutes. In order to increase the amount of LHRH in hypophysial portal blood, the median eminence was electrically stimulated by way of a unipolar electrode (2.2.4) for 10 minutes during each 15 minute period of blood collection. The blood which issued from the cut pituitary stalk was transferred at 30-60 s intervals to ice-cold tubes and subsequently centrifuged at 4°C. The plasma was then immediately mixed with 4 volumes ethanol/1N HCl (95/5; v/v) and stored at -40°C. Immediately before HPLC the plasma extracts were centrifuged and the supernatants pooled and then dried by rotary evaporation.

6.2.2. Hypothalamic LHRH-like immunoreactivity

For studies of hypothalamic LHRH, blocks of hypothalamic tissue (2.2.6) were removed from animals killed by decapitation. Blocks of tissue were taken in two separate batches; the first from 4 pro-oestrous female (animals killed at 13.00 h) and the second from 5 male rats. The tissues were homogenized in a total of 3 ml of ice-cold 0.1 N HCl. Similarly dissected blocks of hypothalamic tissue were removed from 10 normal or 60 hpg mice and homogenized in 2.5 or 12 ml of ice-cold 0.1 N HCl, respectively. The testes or ovaries of each mouse were carefully checked before the hypothalamus was added to the tissue pool in the homogenizer tube to ensure that the hypothalamus had been placed in the correct category. All hypothalamic extracts were stored at -40°C until dried while frozen.

6.2.3. High performance liquid chromatography

The dried blood or hypothalamic extracts were reconstituted in 1.4 ml of 0.1% trifluoroacetic acid in water and centrifuged

briefly. The supernatants were applied to the HPLC column and eluted with a linear gradient (2.4.1). The fractions eluting from the HPLC column were vacuum dried over phosphorous pentoxide, redissolved in assay buffer and assayed for LHRH using double antibody radioimmunoassays (2.5.2) in which the primary antiserum was either the R-42 or the HC-6 anti-LHRH serum.

6.3 RESULTS

Radioimmunoassay using the R-42 antiserum showed that the LHRH-like immunoreactivity in extracts of rat hypophysial portal vessel plasma (Fig. 6-1A) or of acid extracts of hypothalami from male rats (Fig. 6-2A) or normal mice (Fig. 6-2B) eluted from the HPLC column as a single peak which corresponded in retention time to the synthetic decapeptide (Fig. 6-1B). Similar HPLC profiles of LHRH-like immunoreactivity in extracts of either hypophysial portal plasma (Fig. 6-3) or extracts of hypothalami from male or pro-oestrous female rats (Fig. 6-4) were obtained when assayed with the HC-6 anti-LHRH serum. No immunoreactive LHRH was detectable in any of the HPLC fractions from the hypothalami of *hpg* mice. The recovery of the HPLC system for synthetic LHRH was greater than 90%, and for hypothalamic LHRH was 75-90%. The recovery of the whole system (that is, the extraction of plasma with acid ethanol and subsequent HPLC) for synthetic LHRH added to synthetic plasma was 50%.

6.4 DISCUSSION

The development of sensitive radioimmunoassays for LHRH has enabled many detailed studies of its hypothalamic content and

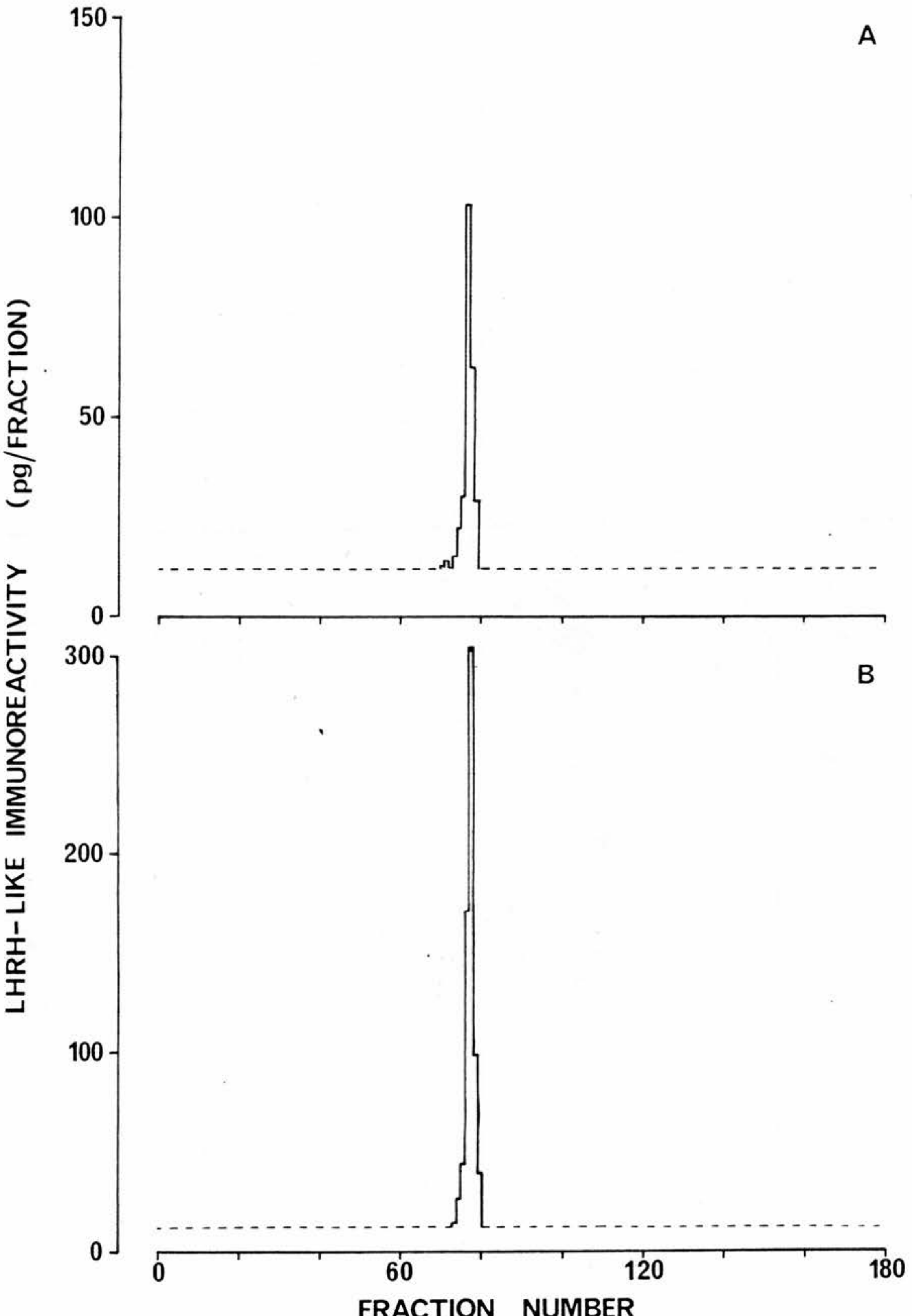


FIGURE 6-1: A: HPLC profile of LHRH-like immunoreactivity, assayed using the R42 antiserum, in an extract of rat hypophysial portal plasma.
B: Elution profile of synthetic LHRH under the same conditions. The broken line in each trace indicates the lower limit of detection of the assay.

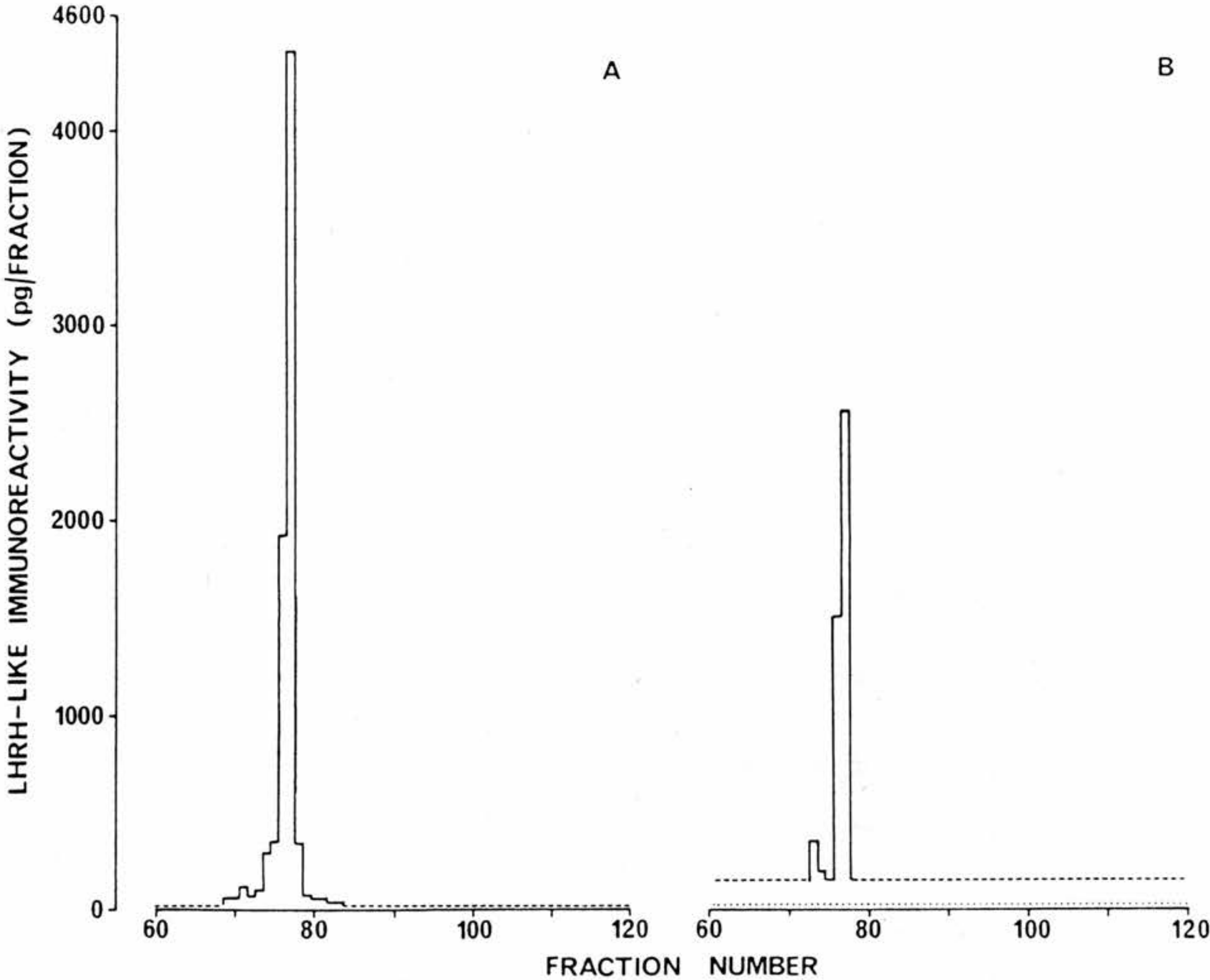


FIGURE 6-2: HPLC profiles of immunoreactive LHRH, assayed using the R42-anti-LHRH serum, in hypothalamic extracts from (A) pro-oestrous female rats and (B) normal mice. The broken line indicates the lower limit of detection of the assay for these samples. The dotted line at the bottom of B is the limit of detection for LHRH in extracts of <u>hpg</u> mouse hypothalamus in which no LHRH was detectable.

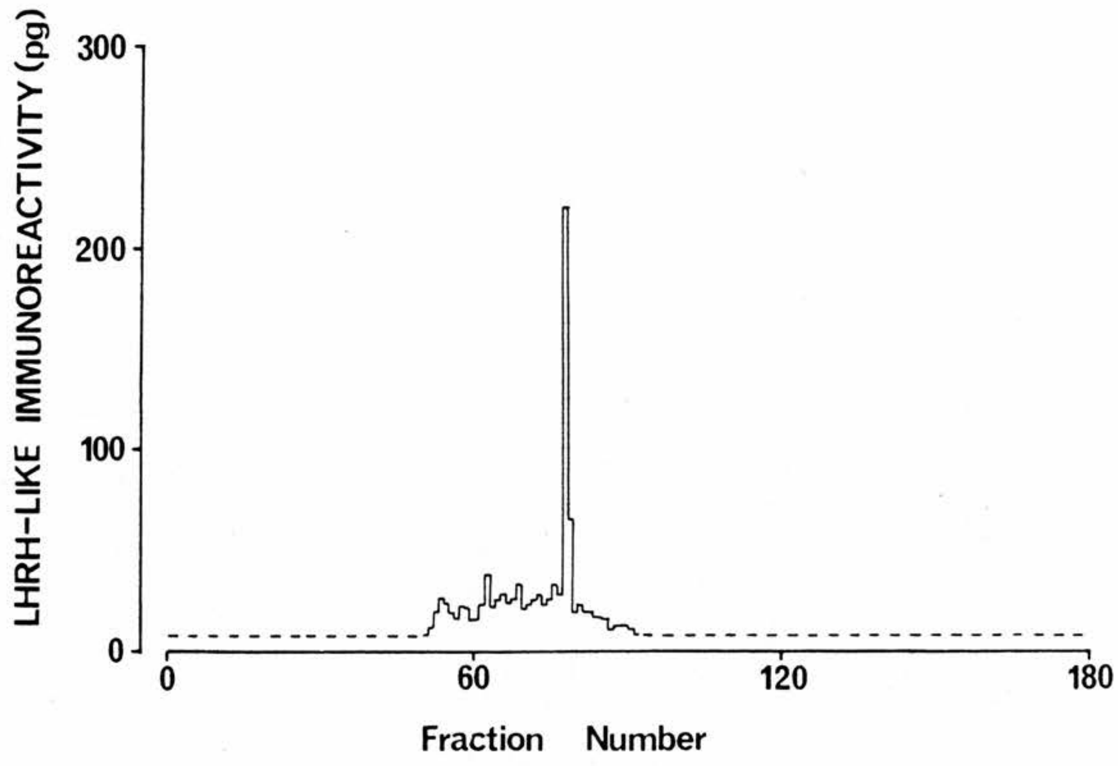


FIGURE 6-3: HPLC profile of LHRH-like immunoreactivity, assayed using the HC-6 antiserum, in an extract of rat hypophysial portal plasma. The broken line indicates the lower limit of detection of the assay for these samples.

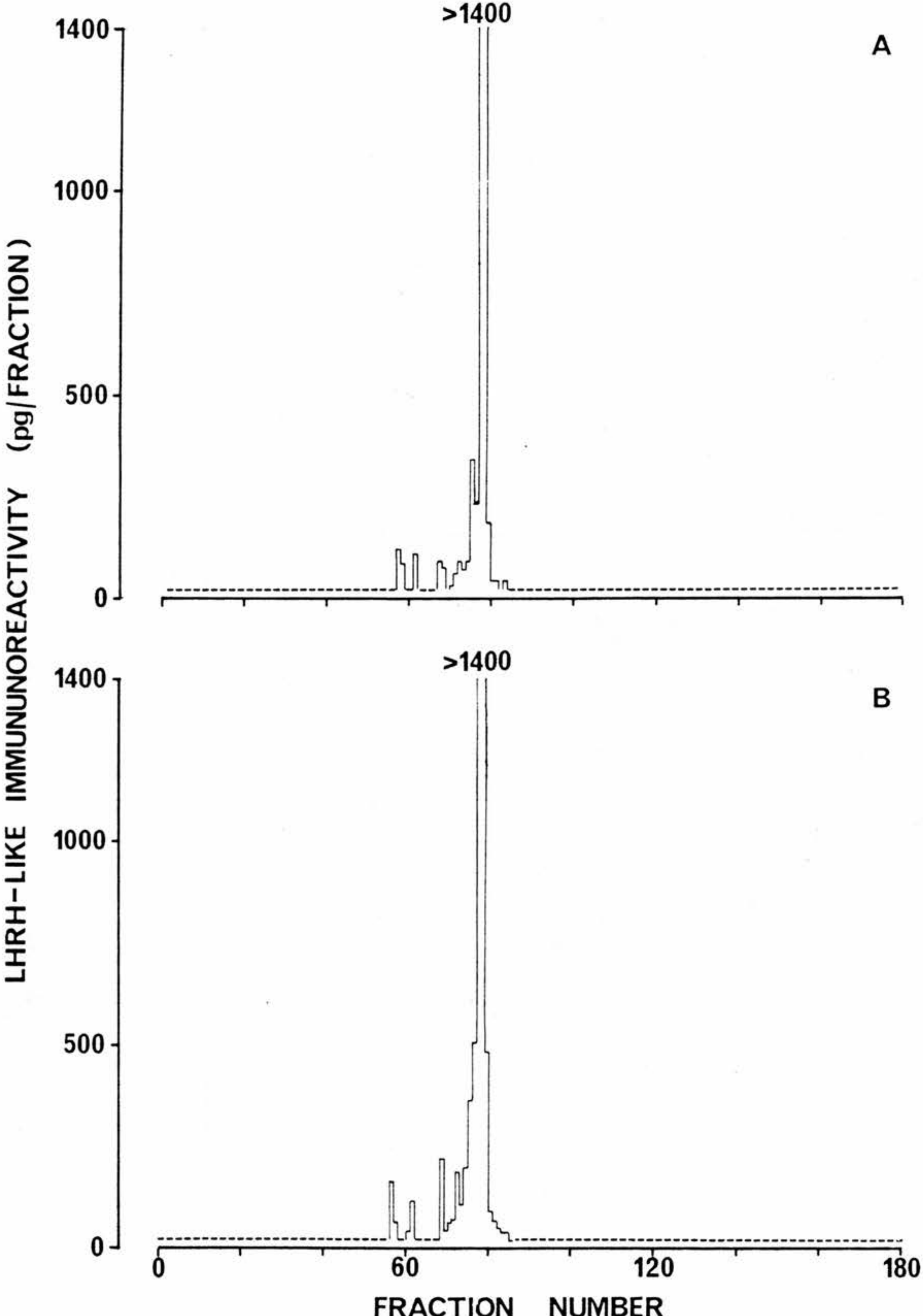


FIGURE 6-4: HPLC profiles of immunoreactive LHRH, assayed using the HC-6 anti-LHRH serum, in hypothalamic extracts from (A) male rats (B) pro-oestrous female rats. The broken line indicates the lower limit of detection of the assay for these samples.

release into hypophysial portal vessel blood (Fink, 1979a; Fink, Stanley and Watts, 1983). Such studies included, for example, investigations of hypothalamic LHRH content in male and female rats (Chiappa and Fink, 1977), the release of LHRH in response to electrical stimulation of the hypothalamus (Fink and Jamieson, 1976; Chiappa, Fink and Sherwood, 1977; Eskay, Mical and Porter, 1977), the preovulatory surge of LHRH during the afternoon of pro-oestrus (Sarkar, Chiappa, Fink and Sherwood, 1976; Sherwood, Chiappa, Sarkar and Fink, 1980) and the steroid modulation of LHRH output (Sarkar and Fink, 1979a, 1980). The present results show that in contrast to somatostatin (Chapter 3) and TRH (Chapter 4) only one peak of LHRH-IR was detected in extracts of hypophysial portal blood or hypothalamus and indicate that the LHRH-IR measured in the previous studies described above was in fact due to the authentic decapeptide.

Previous gel filtration studies of extracts of sheep hypothalami revealed the presence of three peaks of LHRH-IR, which corresponded to the authentic decapeptide and two higher molecular weight (H.M.W.) forms - the largest with a molecular weight greater than 5,000 daltons (Millar, Achnett and Rossier, 1977). By examining the interactions between LHRH-HMW and various antisera raised against differing portions of the LHRH molecule, and also by studying the LHRH-IR formed after digestion of the HMW material with either carboxy- or aminopeptidase, it was concluded that these HMW forms consisted of the LHRH molecule extended at both the N and C-termini (Millar, Wegener and Schally, 1981). Similar gel filtration studies using rat hypothalamic extracts, showed the presence of LHRH and two C-terminally extended forms with molecular

weights of 1,800 and 26,000 daltons (Gautron et al, 1981). Allowing for the removal of a signal peptide the latter value is in agreement with the 28,000 dalton precursor demonstrated by the in vitro translation of poly A^+ mRNA from rat, human or normal mouse brain (Curtis et al, 1983). Subcellular fractionation of crude hypothalamic extracts revealed that only authentic LHRH was present in the synaptosomal fraction, whereas the HMW material was present in the microsomal fraction (Millar et al, 1981; Gautron et al, 1981). Similarly, the LHRH decapeptide was released from synaptosomes in response to a K^+ depolarizing stimulus (Millar et al, 1981). The present results, taken together with the above evidence obtained in synaptosomal preparations, might be taken to suggest that LHRH is processed to completion before entering the portal vessels. However, the possibility remains that under the conditions used in the present study for the extraction and HPLC of LHRH, small quantities of either the LHRH precursor or of intermediate forms of LHRH derived from the precursor may not have been detected.

Radioimmunoassay of crude extracts of hypothalami from hpg mice indicated the absence of detectable LHRH-IR (Cattanach et al, 1977), a finding also shown, indirectly, by the inability of electrical stimulation of the median eminence of hpg mice to raise the plasma concentration of LH (Iddon, Charlton and Fink, 1980), even though administration of exogenous LHRH is an effective stimulus for the release of some of the small amount of LH present in the hpg pituitary (Chapter 7). The present findings confirm the results obtained previously that this mutant does not synthesize any

LHRH-like material. In contrast to normal mice, no polypeptides which were immunoprecipitable with the HC-6 antiserum were produced by the cell free translation of poly A^+ mRNA extracted from the hypothalami from hpg mice (Curtis et al, 1983) suggesting that the lack of LHRH in the mutant was due to a defect in the LHRH gene, transcription or mRNA translation. Recently it has been possible to correct the hpg deficit by the grafting of tissue taken from the pre-optic area of foetal, normal mice into the third ventricle of adult hpg animals, thus resulting in the innervation of the host median eminence with immunohistochemically identified processes from the LHRH-containing cell bodies (Krieger, Perlow, Gibson et al, 1982; Gibson, Charlton, Perlow et al, 1984a). Although these transplants did not fully restitute a normal hypothalamic content of LHRH, the grafts were sufficient to result in increases in plasma testosterone concentrations and weight of the seminal vesicles, full spermatogenesis and some development of the interstitial cells of the testes (Krieger et al, 1982). Similarly, transplantation of pre-optic area tissue taken from normal foetuses (of either sex) into female hpg mice, resulted in vaginal opening and a persistent cornified vaginal smear, enlargement and development of the uteri and ovaries and an increase in pituitary FSH content and plasma LH concentrations (Gibson et al, 1984a). Such animals have been mated and pregnancy maintained for its full duration. Ovulation in hpg mice bearing a graft of normal hypothalamic tissue would appear to be reflex in response to copulation (Gibson, Krieger, Charlton et al, 1984b).

CHAPTER 7

The Priming Effect of LHRH, and Determination of the Biological Activity of LH in the Hypogonadal (hpg) Mouse

7.1 INTRODUCTION

The mechanism of the priming effect of LHRH, the capacity of the decapeptide to increase pituitary responsiveness to itself (Aiyer, Chiappa and Fink, 1974; Fink, Chiappa and Aiyer, 1976) is not known. The priming, but not the releasing, action of LHRH is dependent upon protein synthesis (Edwardson and Gilbert, 1976; de Koning, van Dieten and van Rees, 1976; Pickering and Fink, 1976a, 1977; Curtis, Lyons and Fink, 1985), but the new protein is unlikely to be gonadotrophin since measurements of the amount of LH released compared with that remaining in the pituitary gland suggest that little or no new gonadotrophin is synthesized after the initial exposure to LHRH (de Koning *et al*. 1976; Pickering and Fink, 1979; Speight and Fink, 1981). Curtis *et al* (1985) demonstrated the association of the priming effect with the synthesis of a protein (with molecular weight approximately 69,000) and changes in the isoelectric point of two other proteins. *In vitro*, the priming effect is blocked by treatment with cytochalasin B suggesting that priming may involve a change in contractile proteins (Pickering and Fink, 1979a; Lewis, Morris and Fink, 1985). Conceivably, the priming effect might depend upon an increase in specific LHRH receptors. However, this seems unlikely because the density of the LHRH receptors on anterior pituitary cells decreases at the time of the spontaneous surge of luteinizing hormone (Clayton, Solano, Garcia-Vila, Dufau and Catt, 1980) and once the anterior pituitary gland has been exposed to LHRH a massive amount of LH, equivalent to that released by a second exposure to LHRH, can be released by exposure to either high extracellular K^+ (Pickering and Fink,

1976b) or Ca^{++} ionophores (Pickering and Fink, 1979a). Although steroids do not mediate the priming effect of LHRH, the magnitude of the effect is increased by oestradiol-17β (Aiyer et al. 1974; Meidan, Fink and Koch, 1981).

The absence of hypothalamic LHRH from the hypogonadal mouse (Chapter 6) makes this mutant an ideal model for investigations of the effects of exogenous LHRH in animals which have had no previous exposure to LHRH. The following experiments were designed in order to determine whether previous exposure of the pituitary gland to normal levels of LHRH, and a normal pituitary content of LH, are necessary for the LH releasing action, and the priming effect, of LHRH and for the facilitation of pituitary responsiveness by oestradiol-17β. A second series of experiments was carried out to determine whether the small amount of LH present in the pituitary gland of the hpg mouse was biologically active as assessed by the in vitro production of testosterone by mouse Leydig cells.

7.2 MATERIALS AND METHODS

The animals used were adult hpg or normal mice maintained as described in Chapter 2.

7.2.1 Demonstration of the priming effect of LHRH

At the start of the experiment 4-5 mm silicone elastomer capsules packed with oestradiol-17β were prepared, preincubated, and then implanted subcutaneously (2.2.5) into recipient female hpg or normal mice. In the rat, capsules of this size produced plasma concentrations of oestradiol-17β of about 30-50 pg/ml and when left implanted for 12-48 h produced a marked and sustained increase in

pituitary responsiveness to LHRH (Henderson, Baker and Fink, 1977). Insufficient blood remained after assay for LH for estimation of plasma oestradiol-17β in the present study, but in both normal and hpg mice implanted with oestradiol-containing capsules the weights of the uteri were significantly increased (Table 7-A). Control groups of animals received similarly constructed capsules without the oestradiol. In normal mice the capsules were implanted mostly two days, but in a few three days, after the last cornified vaginal smear. At the time of capsule implantation the smear was leucocytic. Pituitary responsiveness to LHRH was tested 48 h later at which time the smear was comprised of cornified cells in all animals bearing oestradiol-containing capsules and either cornified or cornified plus nucleated epithelial cells in animals bearing empty capsules. In the hpg mice the vagina had opened and the vaginal smear cornified at 48 h after implantation of an oestradiol-containing capsule in most animals. In hpg mice implanted with an empty capsule the vagina was closed in about 50% and the smear leucocytic when the vagina was open.

To test the pituitary responsiveness to LHRH, the mice were anaesthetized with urethane (120 mg/100 g body weight, administered i.p. as a 10% solution in 0.9% NaCl (w/v) solution) and, after withdrawal of an initial blood sample (0.2 ml) from the external jugular vein, the mice were injected with 400 ng synthetic LHRH (ICI Pharmaceuticals, Cheshire) (about 1.2 μg/100 g body weight) i.v. in 0.2 ml 0.9% NaCl. A second injection of the same dose of LHRH was administered one hour later. This relatively high dose of LHRH was used because at the time that these studies were begun, preliminary

experiments had shown that with the sampling regime used in this study, 400 ng LHRH/mouse were required to produce clear-cut and reproducible LH release. Venous blood samples were withdrawn from the external jugular vein at 30, 59, 90 and 120 min after the first injection of LHRH. At the end of the experiment, animals were killed and the anterior pituitary gland rapidly removed, weighed and homogenized in ice cold 0.09% NaCl (w/v) solution (2.2.6). The uterus was dissected out, freed from all adhering tissue, blotted and weighed using a torsion balance. Pituitary and plasma samples were stored at -40°C until assayed for LH (2.2.5). Previous studies (Cattanach, Iddon, Charlton, Chiappa and Fink, 1977) showed that in this assay, saline extracts of mouse pituitary gland inhibited binding in parallel with LH standard. The plasma samples were assayed in duplicate usually in volumes of 50 or 20 μl. For a 50 μl volume the lower limit of the sensitivity of the assay was usually 0.8 ng LH/ml but in one assay the sensitivity was 2 ng/ml. The significance of differences between groups was assessed by the Mann Whitney U test.

7.2.2 Assay of the biological activity of LH in the hpg pituitary

The anterior pituitary glands from 10 normal and 30 hpg mice were separately homogenized in 2 or 6 ml ice-cold 0.9% saline respectively and freeze-dried. The testes and seminal vesicles of each animal were checked carefully before the pituitary gland was added to the pool in the homogenizer tube to ensure that the pituitary glands had been placed in the correct category. The freeze-dried extracts were subsequently redissolved in assay buffer to provide stock solutions containing 1 normal or 10 hpg pituitary

glands/ml; the solutions were stored at -40°C until dispatched (frozen) to Dr. Tony Plant (University of Pittsburgh) for assay of LH bioactivity by the *in vitro* production of testosterone by mouse Leydig cells (Van Damme, Robertson and Diczfaluzy, 1974). A total of four assays were carried out in which serial dilutions of 1-11 μl of the diluted pituitary extracts were assayed in triplicate. In the absence of a readily available standard preparation of murine LH, a rhesus LH standard (WP-XV-20:NICHD-rLH) was included in three of the assays mainly to check that the characteristics of the assays were similar to those previously carried out in Pittsburgh. The potency of the WP-XV-20 was approximately 0.5 NIH-LH-S1 (Plant, 1982; Monroe, Peckham, Neill and Knobil, 1970; Karsch, Weick, Butler *et al*, 1973).

Testosterone released into the incubation medium was estimated by radioimmunoassay (Plant, Hess, Hotchkiss and Knobil, 1978) of 50 μl samples of the unextracted medium. The specificity of the antiserum (S250, provided by Dr. G.D. Niswender) and the accuracy of the assay were as described in detail by Plant *et al*. (1978). The sensitivity of the assay was 2 pg/tube and the intra-assay coefficients of variation of the two assays used in this study were 9% and 11% respectively. The statistical analyses of the slopes and relative potencies were calculated according to Bliss (1952).

7.3 RESULTS

7.3.1 The priming effect of LHRH and the effect of oestrogen implants

The plasma concentrations of LH are shown in Figure 7-1 and the mean maximal increments, pituitary LH contents and concentrations

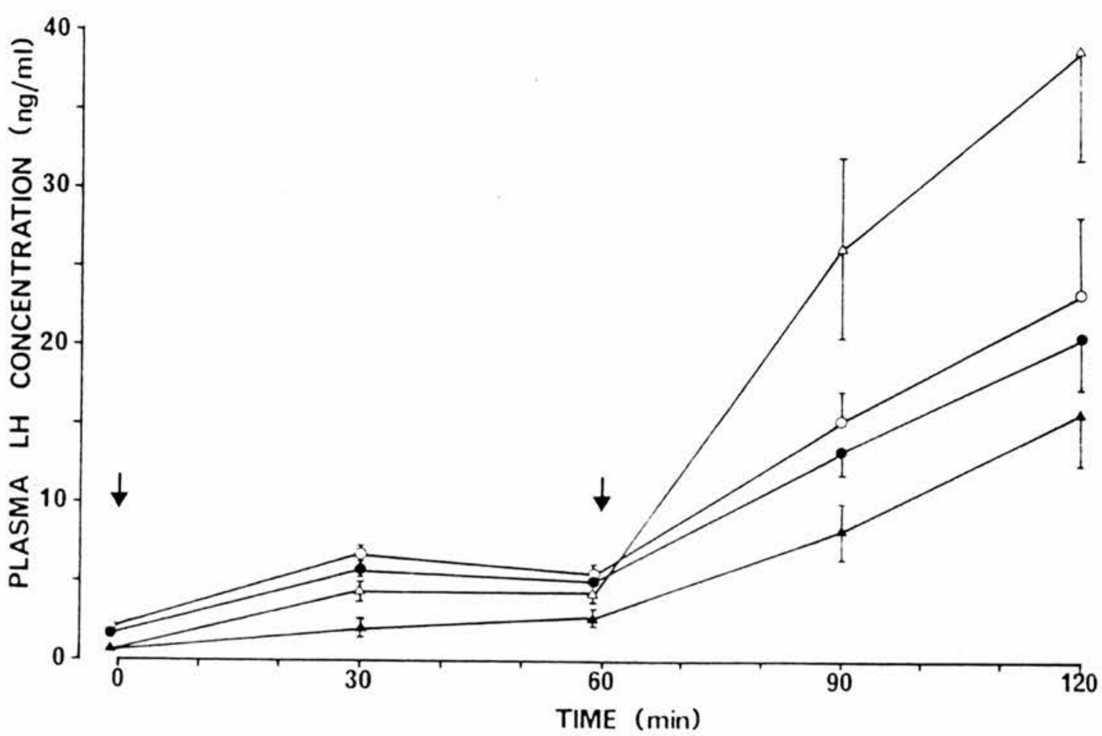


FIGURE 7-1: Mean plasma concentrations of LH before and after the i.v. injection of LH releasing hormone (LHRH; arrows) in normal mice bearing either empty (△) or oestradiol-containing silicone elastomer capsules (▲) or in hypogonadal (hpg) mice bearing either empty (○) or oestradiol-containing capsules (●). The vertical lines indicate S.E.M. These are not shown for the concentrations of LH in some samples taken before the first injection of LHRH because some of these values were below the limit of detection of the assay. For the purpose of calculating the mean LH concentrations in the preinjection samples of plasma, the undetectable samples were mostly assigned values of 0.8 ng/ml, the lower limit of detection of most of the assays for 50 µl plasma samples. A few samples were assigned a value of 2.0 ng/ml, the lower limit of detection of one assay. Nine to eleven animals were included in each treatment group.

Table 7-A Mean maximal increments of plasma LH after LHRH injection, pituitary gland LH content and concentration and uterine weight in hypogonadal (*hpg*) and normal mice implanted with either oestradiol-containing or empty silicone-elastomer capsules. Values are means ± S.E.M.; nine to eleven animals per group.

Treatment		Maximal increments in plasma LH concentration (ng/ml) after:		Pituitary LH		Uterine weight (mg)
Mice	Capsule	first injection of LHRH	second injection of LHRH	Content (ng/gland)	Concentration (ng/mg)	
Normal	Empty	4.4±0.6	34.8±6.5**	468.3±55.9	333.3±54.4	89.2±14.0
Normal	Oestradiol	2.1±0.5	13.5±3.1**	340.2±37.1	218.3±32.5	170.7±15.4
hpg	Empty	5.0±0.5	19.4±4.4*	76.0±7.8	63.3±6.0	5.9±1.2
hpg	Oestradiol	4.4±0.2	15.9±3.2*	110.1±16.7	79.3±14.9	31.8±3.0

* $P < 0.01$, ** $P < 0.001$ compared with increment after first LHRH injection.

and uterine weights are shown in Table 7-A. The maximal increments were taken as the differences between the plasma LH concentrations before and the maximal LH concentrations after the injection of LHRH. In all treatment groups the mean maximal increments after the second injection of LHRH were greater than after the first injection. In animals implanted with an empty capsule the maximal increments after the second LHRH injection in the hpg mice were lower, but not significantly compared with those in normal mice. Implantation of an oestradiol-containing capsule significantly ($P < 0.01$) reduced the LH response to both the first and second injection of LHRH in normal mice, but had no significant effect on the responses in the hpg mice. Pituitary LH contents in the hpg mice were significantly ($P < 0.001$) lower than in the normal mice. Implantation of an oestradiol-containing capsule had no significant effect on pituitary LH content or concentration in either hpg or normal mice.

7.3.2 The bioactivity of LH

Assays 1-3 (Table 7-B) were performed to determine the optimal starting dilutions of the pituitary extracts for potency comparisons; these proved to be 0.01 pituitary/ml for normal glands and 0.1 pituitary/ml for glands from hpg mice. The linear parts of the dose response curves for normal and hpg pituitary extracts did not diverge significantly (assay 3, Table 7-B; assay 4, Fig. 7-2 and Table 7-B). Normal mouse pituitary gland was about 20-times as potent as hpg pituitary gland (Table 7-B) a value similar to that obtained by radioimmunoassay (Cattanach et al. 1977; Iddon et al. 1980; McDowell, Morris and Charlton, 1982a). The divergence in

Table 7-B Analysis of dose-response curves and relative potency of the testosterone production assays

Assay	Substance	Slope	Significance			Precision (λ)	Divergence of slopes			Potency (95% confidence limits)
			N	F	P		Comparison	F	P	
1	(a) WP-XV-20	3.62	15	241	< 0.001	0.076	a *vs* b	56	< 0.001	ND
	(b) Normal pituitary	8.22	15	198	< 0.001	0.036				
2	(a) WP-XV-20	7.29	15	1091	< 0.001	0.025	a *vs* b	33	< 0.001	ND
	(b) Normal pituitary	4.61	15	119	< 0.001	0.068				
3	(a) Normal pituitary	7.52	15	105	< 0.001	0.039	a *vs* b	< 1	NS	(a) relative to (b) 20.5 (19.2-22.0)
	(b) *hpg* pituitary	6.86	15	91	< 0.001	0.038				
4	(a) WP-XV-20	3.05	18	103	< 0.001	0.108	a *vs* b	7.3	< 0.05	
	(b) Normal pituitary	4.87	18	57	< 0.001	0.107	a *vs* c	10.9	< 0.01	
	(c) *hpg* pituitary	4.66	24	174	< 0.001	0.053	b *vs* c	< 1	NS	(b) relative to (c) 19.1 (16.7-21.4)

Calculations and notations are according to Bliss (1952) using the linear parts of the dose-response curves.
N = total number of observations; Precision λ = slope $\div$ standard deviation; F = variance ratio.
NS = not significant; ND = not determined. WP-XV-20 is the Rhesus LH standard.

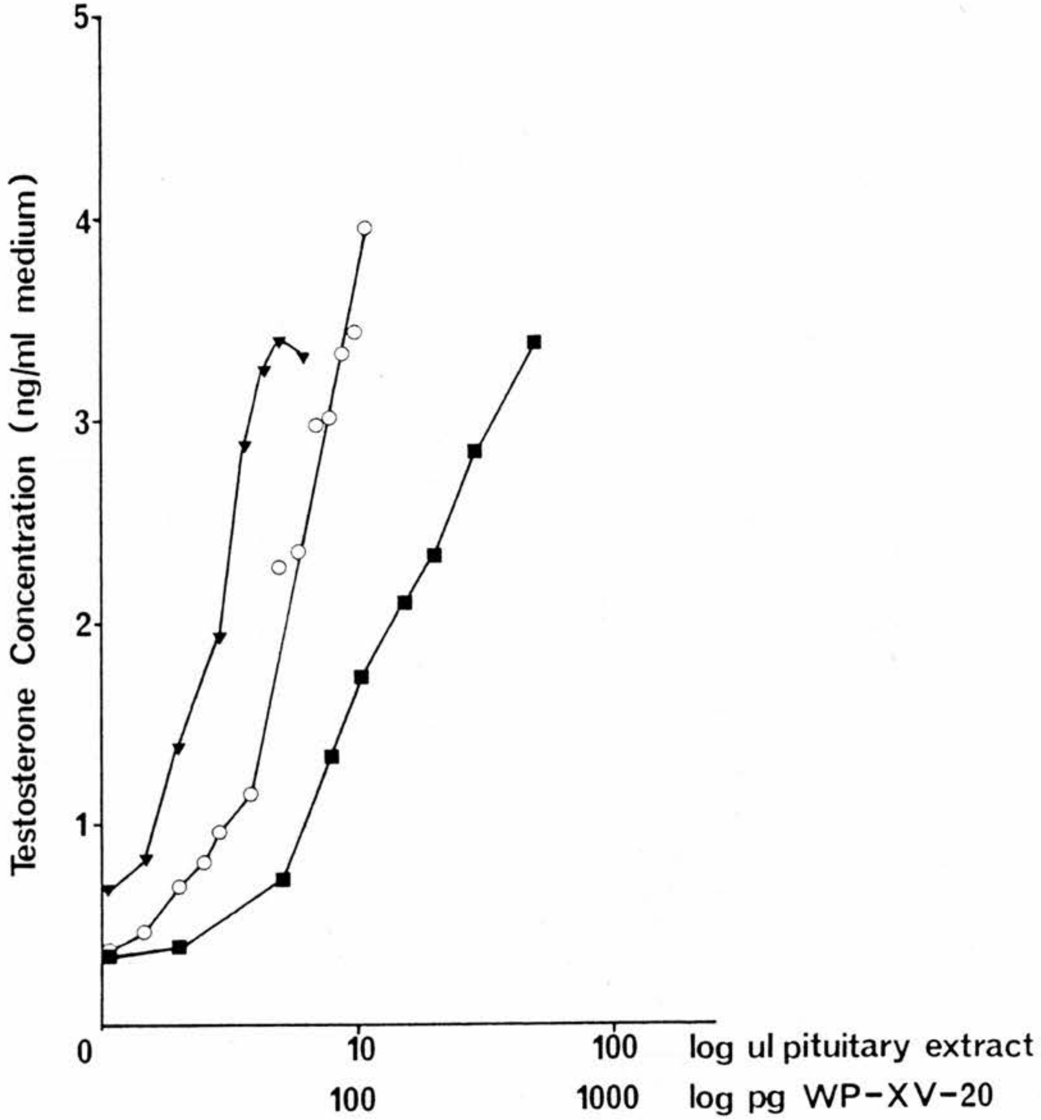


FIGURE 7-2: Testosterone production assay (number 4 in Table 7-B) showing log dose-response curves produced by extracts of pituitary glands from normal (▼) and hpg (○) mice and the WP-XV-20 LH standard (■). Each point is the mean of 3 estimations. The amount of testosterone produced in the absence of pituitary extracts or LH standard was 0.431 ng/ml medium.

slopes between the WP-XV-20 preparation and the mouse pituitary extracts made it impossible to determine relative potency of mouse pituitary gland in terms of a known standard preparation of LH.

7.4 DISCUSSION

Whilst the pituitary content of LH of hpg mice implanted with empty capsules was only 16% of that of normal animals (Table 7-A), the hpg animals have the capacity to respond to the injection of LHRH by releasing LH, as shown previously (Iddon, Charlton and Fink, 1980; McDowell, Morris, Charlton and Fink, 1982b). Furthermore, the data contained in Table 7-A and Figure 7-1 demonstrate that the priming effect in hpg mice was similar to that in normal animals and also that the magnitude of the priming effect is not directly proportional to pituitary LH content. In the rat, plasma LH concentrations reach a peak 20-30 minutes after each injection of LHRH and decline subsequently (Aiyer et al, 1974); in mice, the response to the second injection of LHRH was slower and consequently the plasma concentration of LH was greater 60 minutes after this injection than at 30 minutes (Fig. 7-1). It was not possible to carry out further experiments to examine the release of LH over a longer time period because of a lack of sufficient animals. More recently, in vitro studies have confirmed that the priming effect of LHRH can be elicited from pituitary glands from both normal and hpg mice (Lewis et al, 1985; Lewis, Morris, Fink and Johnson, 1986). The present data also showed that the primed response to LHRH was similar in magnitude in hpg and normal mice, and this was confirmed in vitro by Lewis et al (1986). In order to economize on animals in

the present experiments, control groups in which animals were injected with LHRH followed by saline, and vice versa, were not included because previous studies in mice showed that sequential blood sampling either in untreated or saline injected animals did not significantly alter plasma LH concentrations (Iddon, Charlton and Fink, 1980), and, in rats, the priming effect of LHRH could not be elicited by injecting LHRH followed by saline or vice versa (Aiyer et al. 1974; Fink and Pickering, 1980). The dose of LHRH used by Iddon et al (1980) to evoke LH release was lower than that used in the present study, but Iddon et al. used male mice and removed the first blood sample after LHRH injection 5 minutes after the injection.

Since the primary deficit in the hpg animals is related to the lack of hypothalamic LHRH (Chapter 6), it is clear that neither the releasing, nor the priming, actions of LHRH depend upon previous exposure to LHRH. The LHRH receptor density in hpg male and female mice is about 30% of that of normal mice (Young, Speight, Charlton and Clayton, 1983) and this shows that LHRH receptors are present in the pituitary gland in the absence of endogenous LHRH, albeit at lower concentrations. The present findings add to the evidence cited in the introduction to this chapter that LHRH receptor density may not play a critical role in the priming effect of LHRH.

The dependence of the priming effect upon the integrity of microfilaments was shown by in vitro studies in which the priming but not the releasing action of LHRH was blocked by cytochalasin B (Pickering and Fink, 1979; Lewis et al, 1985). Ultrastructural studies of the gonadotrophs of normal mice demonstrated that the

priming effect was associated with an increase in the length of the microfilaments and a change in their orientation, together with a migration of secretory granules towards the marginal zone of the cytoplasm adjacent to the cell membrane (Lewis et al, 1985). Although the total number of secretory granules was less in the gonadotrophs of hpg than normal mice, the number of granules present in the outer 500 nm wide band of cytoplasm closest to the cell membrane was similar in both strains of mice and was increased by exposure to LHRH (Lewis et al, 1986).

The increase in plasma oestradiol-17β concentrations on the morning of pro-oestrus is a major factor in the development of the enhanced responsiveness of the anterior pituitary gland to LHRH which precedes and accompanies the pre-ovulatory surge of LH in the rat. This action of oestradiol may be exerted, in part, by a direct effect on the gonadotroph, as is suggested by studies of the pituitary responsiveness to exogenous LHRH in vivo, in animals in which the pituitary stalk has been sectioned (Greeley, Allen and Mahesh, 1975; Fink and Henderson, 1977a; Greeley, Volcan and Mahesh, 1978), and in vitro, using dispersed cell systems (Drouin, Lagace and Labrie, 1976; Speight and Fink, 1981). Other data, however, indicate that a functionally intact hypothalamic-pituitary system is necessary for oestrogen to exert its full effect. Henderson, Baker and Fink (1977), for example, showed that the increased pituitary responsiveness to LHRH was apparent in rats 12 h after the implantation of an oestradiol-17β containing capsule, an effect which was blocked by sodium pentobarbitone administered 8 h after capsule implantation (Henderson et al, 1977; Speight, Popkin, Watts

and Fink, 1981); the barbiturate-induced blockade could be overcome by the infusion of LHRH at a low dose which, by itself, was insufficient to evoke a large release of LH (Speight et al, 1981). Furthermore, the increased concentrations of LHRH in rat hypophysial portal blood which follow immediately after ovariectomy (Sarkar and Fink, 1980) were maintained for 12 h in animals implanted with oestradiol capsules, whereas LHRH concentrations declined in control animals with empty capsules (Speight et al, 1981). From the foregoing studies it is apparent that in the rat a major component of the augmentation of pituitary responsiveness to LHRH by oestradiol involves either an increase in or a maintenance of LHRH release into hypophysial portal blood at a rate which is not sufficiently high to produce a surge of LH. In relation to the regulation of pituitary LRHH receptors and the control of the release of pituitary gonadotrophins, the mouse, however, differs markedly from the rat (Bronson, 1981; Naik, Young, Charlton and Clayton, 1984a,b). In the normal mice of the present experiment continuous exposure to oestradiol-17β reduced rather than facilitated gonadotrophin function. Previous studies (Bronson and vom Saal, 1979) showed that in contrast to female rats and hamsters, long-term implantation of oestradiol-17β-containing capsules did not produce spontaneous daily LH surges. In contrast to the effect of oestradiol on the priming effect of LHRH in normal mice, oestradiol-17β had no significant effect on the priming effect in the hpg mice suggesting that a normal LHRH mechanism may be necessary for the inhibitory effect of oestradiol seen in normal mice. A direct inhibitory effect of oestradiol on the gonadotroph

has been demonstrated by Charlton et al (1983) in experiments which showed that implantation of capsules containing oestradiol benzoate into hpg mice abolished the large increase in pituitary FSH content which was induced by daily administration of LHRH.

LHRH stimulates the synthesis of gonadotrophins as well as stimulating their release. This could be inferred from the results of early experiments in which it was noted with the aid of cytological staining techniques that there was a progressive loss in ability to detect gonadotrophs in pituitary glands which had been transplanted from the hypothalamus to the kidney capsule (Nikitovitch-Winer and Everett, 1958, 1959); normal pituitary cytology was restored by the retransplantation of the pituitaries to the median eminence (Nikitovitch-Winer and Everett, 1959) or by the infusion of hypothalamic extracts (Evans and Nikitovitch-Winer, 1969). Direct evidence for the stimulation of the synthesis of bioactive LH in response to LHRH was provided by studies of LH synthesis by cell cultures or pituitary gland fragments in vitro (e.g. Redding, Schally, Arimura and Matsuo, 1972; Ishikawa and Nagayama, 1973; Liu and Jackson, 1979) and LHRH may also stimulate the glycosylation of LH (Azhar, Reel, Pastushok and Menon, 1978; Liu and Jackson, 1978). The data shown in Figure 7-2 and Table 7-B show that the small amount of LH in the pituitary gland of the hpg mouse is capable of stimulating testosterone production from Leydig cells and is therefore biologically active. McDowell et al (1982a) found that gonadotrophs which were stained by an antiserum raised against the β subunit of LH were present in the pituitary gland of the hpg mouse and that the frequency of occurrence of the gonadotrophs was

72% of that in the pituitary glands of normal mice, though the LH-β staining cells in hpg animals were smaller and possessed fewer and smaller granules. The present results taken together with those of McDowell et al (1982a) show that both differentiation of the gonadotrophs and the synthesis of some bioactive LH can occur in the absence of LHRH, even though LHRH is essential for the full synthesis of normal amounts of pituitary LH. Treatment of hpg animals with either single or multiple daily injections of LHRH administered over a period of 10-20 days resulted in increased pituitary content of both LH and FSH, although the effect was most marked in the case of FSH for which there was a several-fold increase to amounts greater than those in normal animals (Charlton et al, 1983). Long-term LHRH administration also resulted in increases in the number and size of the pituitary cells which reacted with anti-LH-β serum, and an increase in the size of their granules to values within the normal range (McDowell, Morris, Charlton and Fink, 1982b).

CHAPTER 8

Effect of Δ^1-Tetrahydrocannabinol, Naloxone, or the Sucking Stimulus on the Release of LHRH into Hypophysial Portal Vessel Blood.

8.1 INTRODUCTION

The following series of experiments were carried out to examine the secretion of LHRH and LH during suckling in lactating rats and after the administration of naloxone or tetrahydrocannabinol to female rats at different times during the oestrous cycle.

8.1.1. Effects of Δ^1-tetrahydrocannabinol on hormone secretion

A number of studies have indicated that following the administration of Δ^1-tetrahydrocannabinol (THC) the principal psychoactive component of marijhuana (Mechoulam, Shani, Edery and Grunfeld, 1970), the secretion of a number of pituitary hormones is altered. These endocrine effects include an increase in ACTH secretion (Dewey, Peng and Harris, 1970; Kokka and Garcia, 1974) and decreased secretion of growth hormone (Kokka and Garcia, 1974), prolactin (Kramer and Ben-David, 1974; Steger, Silverman, Johns and Asch, 1981; Chakravarty, Sheth and Ghosh, 1975) and LH (Marks, 1973; Collu, Letarte, Leboeuf and Ducharme, 1975). The high serum concentrations of LH measured in ovariectomized rats or monkeys were reduced by THC (Steger, Silverman, Siler-Khodr and Asch, 1980; Smith, Besch, Smith and Besch, 1979), and Tyrey (1978) showed this reduction was a consequence of a central inhibition of pulsatile LH release. When administered to rats early in the afternoon of pro-oestrus, THC blocked the spontaneous surge of LH and inhibited subsequent ovulation (Nir, Ayalon, Tsafriri, Cordova and Lindner, 1973; Ayalon, Nir, Cordova _et al_, 1977); similarly, pretreatment of rabbits with THC also blocks the reflex ovulation following mating (Asch, Fernandez, Smith and Pauerstein, 1979). Although in many of the above studies the anti-ovulatory effects of THC were suggested

to be centrally mediated, there is no proof that THC inhibits the release of LHRH. Therefore, the effect of THC, administered at a dosage which blocked ovulation, was determined on the release of LHRH into hypophysial portal vessel blood during the period of the pro-oestrous LHRH surge.

8.1.2 Effect of continuous infusion of naloxone on the release of LHRH

The inhibition of gonadotrophin release by morphine has long been known (Barraclough and Sawyer, 1955) and there is much evidence in support of a functional role of the endogenous opioid peptides in the regulation of LHRH secretion (Kalra and Kalra, 1983, 1984; Bicknell, 1985). Thus, the administration of morphine or opioid peptides in vivo inhibited the basal secretion of LH (Bruni, Van Vugt, Marshall and Meites, 1977; Cicero, Badger, Wilcox, Bell and Meyer, 1977; Cicero, Schainker and Meyer, 1979) and the pro-oestrous LH surge (Muraki, Nakadate, Tokunaga, Kato and Makino, 1979; Ieiri, Chen, Campbell and Meites, 1980) and the surge of LH induced in long-term ovariectomized rats by the administration of oestrogen and progesterone (Adler and Crowley, 1984; Akabori and Barraclough, 1986b). Conversely in many of the foregoing preparations the administration of the opiate receptor antagonist, naloxone, resulted in enhanced LH secretion.

In a number of physiological situations, LH is secreted from the pituitary gland in a pulsatile manner. In the rat these pulses are most clearly demonstrated following gonadectomy (Gay and Sheth, 1972; Gallo, 1980), but regular pulses of LH have been shown to occur with varying frequency and amplitude throughout the oestrous

cycle (Gallo, 1981a,b). Several lines of evidence indicate that pulsatile LH release is mediated by central mechanisms involving the pulsatile release of LHRH. Although in the rat it is not possible to measure simultaneously LH and LHRH output in the same animal, discrete pulses of LHRH (which were consistent with the frequency of LH pulses) have been detected in hypophysial portal blood (Sarkar and Fink, 1980) and pulsatile LH secretion was suppressed after passive immunoneutralization with anti-LHRH serum (Snabes and Kelch, 1979). Gallo (1981a) reported that the low plasma concentrations of LH measured on the morning of oestrus were associated with the slowest frequency of pulsatile LH release when compared with other times of the oestrous cycle. Fox and Smith (1985) were unable to detect measurable pulses of LH during the day of oestrus, but demonstrated that regular pulses could be induced by the continuous infusion of naloxone. In the second series of experiments described below, LHRH secretion into portal blood was measured in rats anaesthetized with alphaxalone and infused with naloxone or saline on either the afternoon of pro-oestrus or the morning of oestrus. In each case an attempt was made to correlate LHRH secretion with the pattern of LH release during the hour of infusion which preceded the section of the pituitary stalk for the collection of portal blood.

8.1.3 Effect of the suckling stimulus on the release of LHRH in lactating rats

The plasma and pituitary concentrations of the gonadotrophins are reduced during lactation and the oestrous cycle suppressed (Minaguchi and Meites, 1967; Lu, Chen, Huang et al, 1976a; Smith and

Neill, 1977). The magnitude of the reduction of plasma LH and FSH concentrations is directly related to the intensity of the suckling stimulus (Hammonds, Velasco and Rothchild, 1973; Ford and Melampy, 1973; Smith, 1978a). Suckling also prevents the increased plasma concentration of LH observed after ovariectomy (Ford and Melampy, 1973; Hammonds et al, 1973; Harms, Fleeger and Owens, 1977). The suppressive effect of suckling on plasma gonadotrophin concentration and ovulation may be mediated directly by a neural pathway activated by suckling which inhibits LHRH release, or may be a secondary consequence of the effects, at the hypothalamus and/or pituitary gland, of the increased plasma prolactin concentration. The aims of the final series of experiments which are described in this chapter were two-fold. Firstly, LHRH secretion was compared in suckled and non-suckled lactating rats. Secondly, since oxytocin was shown to be released into hypophysial portal blood from nerve terminals in the external layer of the median eminence (Gibbs, 1984; Sarkar and Gibbs, 1984; Horn, Robinson and Fink, 1985c) and since synthetic oxytocin is reported to release prolactin from the anterior pituitary gland both in vivo and in vitro (Salisbury, Krieg and Seibel, 1980; Lumpkin, Samson and McCann, 1983) the effect of the suckling stimulus on the secretion of oxytocin into portal blood was also investigated.

8.1.4 Rationale for the use of alphaxalone as an anaesthetic

In contrast to the effects of many other anaesthetics, the steroidal anaesthetic, alphaxalone (Saffan, formerly available as Althesin, 2.2.1) does not block (although it does reduce) either the spontaneous pro-oestrous surge of LH and LHRH in adult rats (Sarkar,

Chiappa, Fink and Sherwood, 1976; Sherwood, Chiappa, Sarkar and Fink, 1980; Ching, 1982), or the surges of LH and LHRH induced by the administration of pregnant mare serum gonadotrophin in immature rats (Sarkar and Fink, 1979b). Similarly, unlike sodium pentobarbitone, alphaxalone did not block the pulsatile release of LH in ovariectomized rats (Watts and Fink, 1981) or the diurnal rhythm of LH secretion in long-term ovariectomized rats treated with oestrogen (Sarkar and Fink, 1980). Thus, alphaxalone proved to be the anaesthetic of choice for studies of the regulation of LHRH and LH release and was therefore used in the following investigations of some aspects of the pharmacological and physiological control of the release of LHRH into hypophysial portal vessel blood.

8.2 MATERIALS AND METHODS

Samples of hypophysial and peripheral blood were obtained and assayed for LHRH (using the HC6 antiserum), LH and prolactin respectively, using the methods and assays described in Chapter 2. Samples of hypophysial portal blood from lactating rats were sent to Dr. I.C.A.F. Robinson at Mill Hill for determination of oxytocin concentration by radioimmunoassay using a specific antiserum (R_3) raised in rabbits against oxytocin conjugated to porcine thyroglobulin (Robinson, 1980). The significance of differences between means was determined using the Mann-Whitney U test.

8.2.1 Effect of Δ^1-tetrahydrocannabinol on LHRH and LH release

Tetrahydrocannabinol was purchased from Sigma, U.K. as a solution in ethanol containing 10 or 25 mg/ml. The oily residue which remained after evaporating the alcohol under a stream of

nitrogen gas was suspended in vehicle [0.9% saline, propylene glycol (Sigma) and polyoxyethylene sorbitan mono-oleate (Tween 80; Sigma) (78:20:2 v/v)] to produce a 5 mg/ml solution. Adult female rats were injected at 13.30 h on the afternoon of pro-oestrus with THC (10 mg/kg) or with vehicle. At 15.30 h (Group A) or 17.00 h (Group B) animals were anaesthetized with Saffan (5 ml/kg body wt) and the hypophysial portal vessels exposed (2.2.3). Samples (0.2 ml) of peripheral blood were withdrawn (for plasma LH determination) from the EJV at the time of induction of anaesthesia; further blood samples were withdrawn an hour later (0.5 ml, for LH and LHRH determination). Immediately after removing the second peripheral sample, animals were injected with heparin, and after cutting the pituitary stalk, hypophysial portal vessel blood was collected for four 30 min collection periods. At the end of the final collection period, a further sample of peripheral blood (0.5 ml) was removed for LHRH determination.

8.2.2 Effect of naloxone infusions

Female rats were anaesthetized with alphaxalone (5 ml/kg b.w.) at 17.00 h on the afternoon of pro-oestrous at 10.00 h on the morning of oestrus. After the withdrawal of an initial sample of peripheral blood (0.2 ml) animals were injected with 1 mg naloxone hydrochloride (Sigma UK; 2 mg/ml solution in 0.9% saline, i.v.) and for the remainder of the experiment naloxone hydrochloride was infused into the contralateral EJV at a rate of 0.7 mg/h. The infusions were made through a 25 gauge hypodermic needle which was connected to a 2 ml syringe by way of a length of polythene tubing (Portex PP20). The piston of the syringe was driven by a six-speed

continuous slow injection pump (Model 6130, C.F. Palmer Ltd., London) to give an infusion rate of 0.54 ml/h. Control animals were injected and infused with saline. Further peripheral blood samples were removed at 15 min intervals during the first hour of the infusion, while the hypophysial portal vessels were exposed. Hypophysial blood was collected for four 30 min periods and at the end of the last collection period a 0.5 ml sample of peripheral blood was withdrawn for determination of LHRH.

8.2.3 Effect of the suckling stimulus on LHRH release

Lactating female rats, 9-11 days post partum, were separated overnight from all of their pups (6.2.2). The following morning the mother was anaesthetized with alphaxalone (7 ml/kg b.w.) and the dura overlying the portal vessels exposed, but not cut (2.2.3). Animals were maintained under anaesthesia for 7 h during which the rectal temperature was closely monitored and maintained at $37^\circ \pm 1^\circ C$ with the aid of a warming lamp. During the first 3 h, supplementary doses of anaesthetic (0.5 ml) were administered at approximately hourly intervals as required. Three hours after the exposure of the dura, peripheral blood samples were withdrawn and 10 pups attached to the mother's teats in the suckled group; no pups were returned to the control group. After a further hour a further peripheral blood sample was taken, the dura cut and hypophysial portal vessel blood collected for four consecutive 30 min collection periods. A final peripheral sample was taken at the end of the last collection period.

In a parallel series of experiments in which all the animals were suckled, the portal vessels were exposed, but not cut, a series of samples (0.5 ml) of peripheral blood were withdrawn from the EJV at 30 min intervals throughout the period of suckling.

8.3 RESULTS

8.3.1 Effects of tetrahydrocannabinol

The concentration of LH in the plasma of rats which had been injected with THC at 13.30 h and anaesthetized with alphaxalone at 15.30 h did not differ significantly from the plasma concentrations in vehicle treated rats (Table 8-A; group A). There was a small, but significant increase in plasma LH concentration ($P < 0.05$) in vehicle treated rats only, between 15.30 and 16.30 h. In the second group of rats which were injected at 13.30 h with either THC or with vehicle and anaesthetized at 17.00 h (Table 8-A, group B), vehicle treated rats showed a significant pro-oestrous surge of LH ($P < 0.05 - 0.01$ compared with plasma LH concentrations in group A) which was completely blocked in THC treated animals. Figures 8-1 and 8-2 show the concentration (pg/ml) and content (pg released/30 min collection period) of LHRH in samples of portal blood obtained from the rats in groups A and B respectively. In both groups of animals the LHRH content and concentration in hypophysial portal blood was lower in blood samples collected from THC treated animals though these differences were significant ($P < 0.05$) only during the first 30 min collection period. A progressive decline in LHRH release over the four successive collection periods occurred over the 2 h of blood collection. The concentration of LHRH in all the samples of peripheral plasma (including samples from the naloxone-treated and lactating rats, described below) was below the limit of detection of the assay, i.e. less than 30 pg/ml.

8.3.2 Effects of continuous naloxone infusion

During the first hour of the infusion of naloxone there was no

Table 8-A Mean ± SEM concentrations of LH in peripheral plasma of female rats, injected at 13.30 h on the afternoon of pro-oestrus with Δ^1-tetrahydrocannabinol (THC) or with vehicle and anaesthetized with alphaxalone at either 15.30 h (Group A) or 17.00 h (Group B). 6-9 animals in each treatment group. The hypophysial portal vessels were exposed for the subsequent collection of portal blood, commencing at either 16.30 (Group A; see Figure 8.1) or 18.00 h (Group B; see Figure 8.2).

Time of Sampling		Plasma LH concentration (ng/ml)	
		THC	Vehicle
Group A	15.30	2.9 ± 1.1	1.8 ± 0.2
	16.30	2.5 ± 0.3	2.5 ± 0.2
Group B	17.00	2.1 ± 0.3*	31.3 ± 9.4
	18.00	1.6 ± 0.1*	23.7 ± 5.3

* P < 0.025 compared with corresponding vehicle-treated values (Mann-Whitney U test).

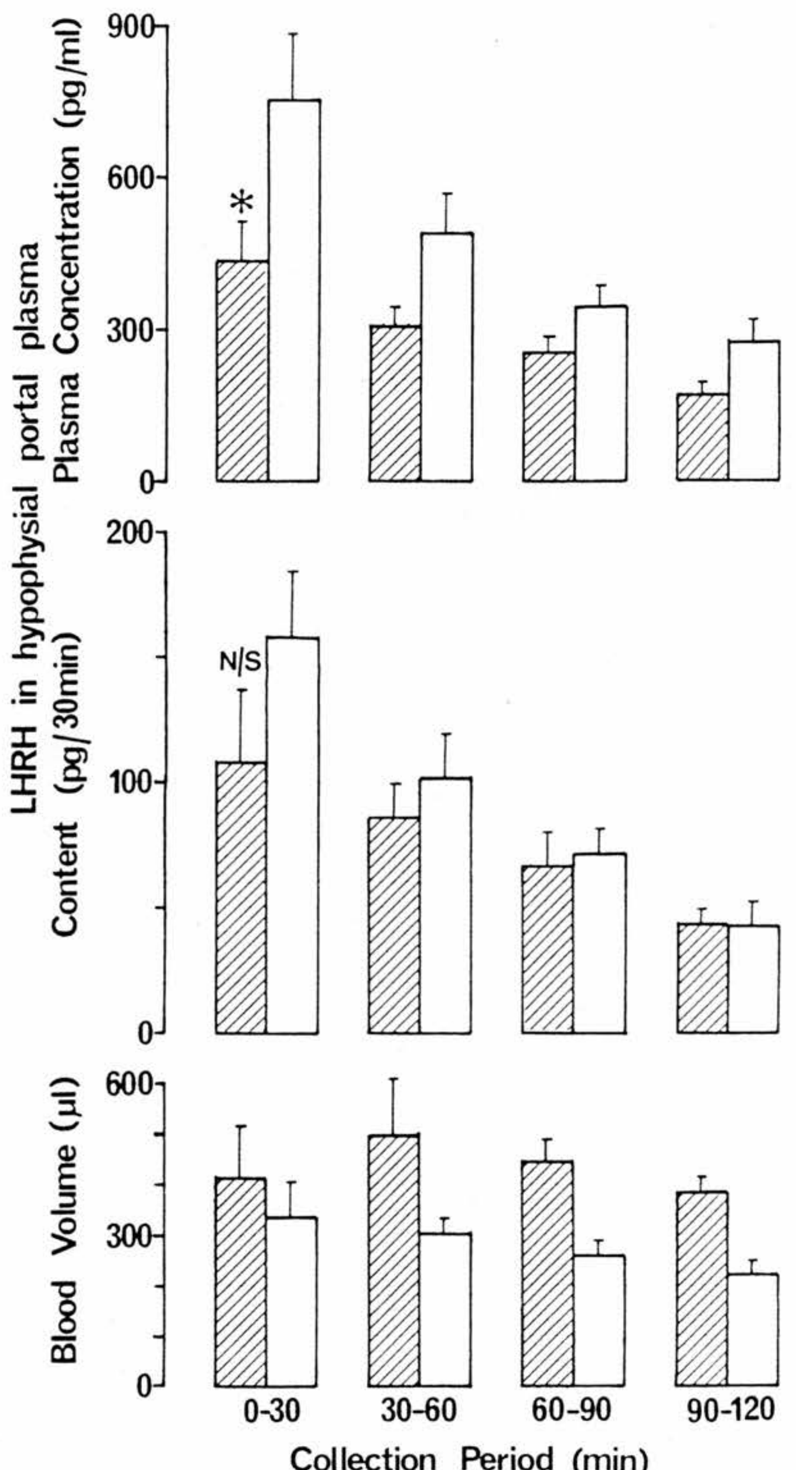


FIGURE 8-1: Mean (± SEM) concentration and content of LHRH in hypophysial portal vessel plasma, together with volume of blood collected during four successive 30 min periods of collection from female rats injected with either 10 mg/kg of tetrahydrocannabinol (▨) or vehicle (□) at 13.30 h on the afternoon of pro-oestrus. Rats were later anaesthetized with alphaxalone and collection of blood commenced at 16.30 h. 6-8 animals in each treatment group. *$P < 0.05$; N/S, not significant: significance of difference between means determined by Mann-Whitney U test.

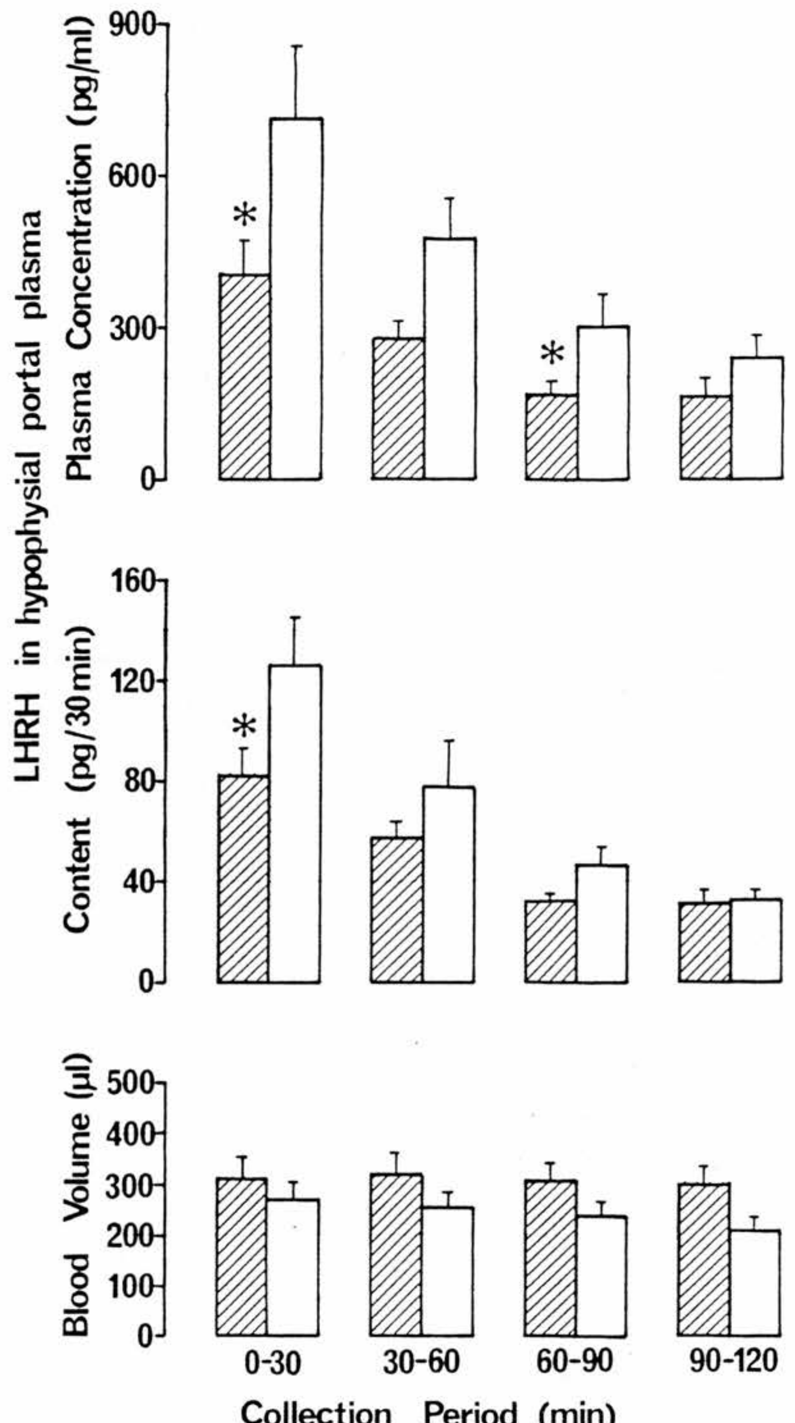


FIGURE 8-2: Mean (± SEM) concentration and content of LHRH in hypophysial portal vessel plasma, together with volume of blood collected during four successive 30 min periods of collection from female rats injected with either 10 mg/kg of tetrahydrocannabinol (hatched) or vehicle (open) at 13.30 h on the afternoon of pro-oestrus. Rats were later anaesthetized with alphaxalone and collection of blood commenced at 18.00 h. 7-9 animals in each treatment group. *P < 0.05: significance of difference between means determined by Mann-Whitney U test.

change in the magnitude of the LH surge which occurred on the afternoon of pro-oestrus (Table 8-B) and no differences were apparent between the profiles of LH secretion in individual rats infused with either naloxone or saline (Table 8-C). After the withdrawal of the final sample of peripheral blood the infusions were continued for a further 2 h period during which time hypophysial portal blood was collected. The infusion of naloxone did not result in significant changes in the release of LHRH (Fig. 8-3). Over the 2 h period there was a progressive decrease in both the content ($P < 0.001$) and concentration ($P < 0.01-0.02$) of LHRH in portal blood from both naloxone and saline infused animals; the pattern of LHRH secretion in individual rats is summarized in Table 8-D).

In rats infused during the morning of oestrus the mean plasma concentrations of LH did not differ between the naloxone and saline infused groups (Table 8-B), but in both these treatment groups, plasma LH increased significantly after the start of the infusion. Concentrations of LH in the plasma of individual rats during the first hour of the infusions are shown in Table 8-E, and the data suggest that in the majority of both naloxone and saline infused rats a small amplitude pulse of LH was secreted during the sampling period. For LHRH release in these animals, there were no significant differences between naloxone and saline treated groups, however, in both groups there was a decrease in LHRH content ($P < 0.001$) and concentration ($P < 0.005-0.001$) over the 2 h period of portal blood collection (Fig. 8-4). A pulsatile pattern of LHRH secretion into portal vessel blood was not seen in any of the individual animals

Table 8-B Mean ± SEM concentrations of LH in peripheral plasma and the peak plasma LH concentration in pro-oestrous or oestrous rats injected or infused with either naloxone or saline. Animals were anaesthetized with alphaxalone and samples were withdrawn from the EJV at 15 min intervals from 17.00 (sample 0) to 18.00 h (sample 60) on the afternoon of pro-oestrus or from 10.00 to 11.00 h on the morning of oestrus. The injections and infusions were begun immediately after the first blood sample was withdrawn. 7-10 animals per treatment group.

		Duration of Infusion (min)					Peak LH
		0	15	30	45	60	
Pro-oestrus	Naloxone	46.6 ± 13.2	50.7 ± 12.2	48.1 ± 9.7	38.9 ± 9.5	23.5 ± 5.7	59.1 ± 11.1
	Saline	31.4 ± 11.6	32.0 ± 8.8	39.6 ± 10.4	27.4 ± 8.7	15.9 ± 4.5	45.6 ± 11.5
Oestrus	Naloxone	1.6 ± 0.3	3.7 ± 0.8*	4.3 ± 0.7**	2.7 ± 0.4	2.1 ± 0.4	--
	Saline	0.9 ± 0.1	2.4 ± 0.7*	4.2 ± 1.2**	3.3 ± 0.5***	2.5 ± 0.3**	--

Significance of differences of mean LH concentration from pre-infusion (0 min) value; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$

Table 8-C Plasma concentrations of LH (ng/ml) in individual female rats anaesthetized with alphaxalone at 17.00 h on the afternoon of pro-oestrus. Peripheral blood samples were withdrawn before (0 min sample), and at 15 min intervals during, the intravenous infusion of either naloxone or saline. The hypophysial portal vessels were also exposed for the subsequent collection of portal blood (see Figure 8-3).

Infusion	Animal Number	Duration of Infusion (min)				
		0	15	30	45	60
Saline	1	12.9	20.0	30.4	19.9	15.0
	2	3.1	3.4	5.8	3.3	2.6
	3	27.5	37.1	47.4	95.3	14.0
	4	125.2	98.4	113.3	33.6	47.8
	5	27.0	44.0	61.4	48.8	33.3
	6	23.0	27.6	43.1	15.8	10.4
	7	1.6	1.5	2.5	2.9	2.8
	8	29.0	21.4	26.3	16.4	10.9
	9	8.0	21.2	11.9	11.6	6.4
	10	56.7	44.9	54.0	26.8	16.1
Naloxone	11	8.1	13.0	29.4	35.9	7.2
	12	21.3	26.2	18.3	9.4	7.5
	13	26.3	56.4	36.9	49.0	36.7
	14	107.0	106.5	77.3	63.6	25.2
	15	6.7	9.4	16.3	10.9	7.6
	16	69.6	41.0	50.3	24.5	18.5
	17	85.3	80.6	87.4	88.1	50.6
	18	48.3	72.9	69.0	29.5	34.9

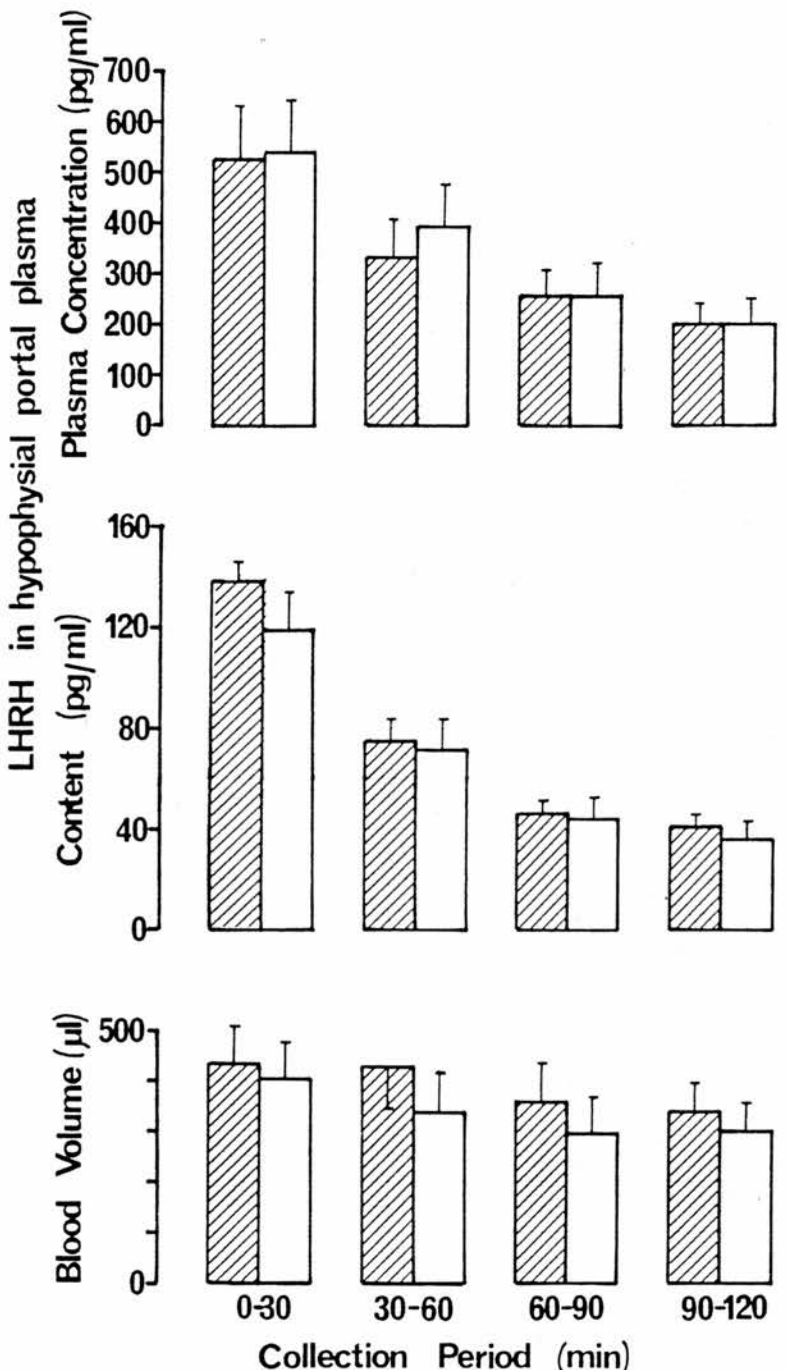


FIGURE 8-3: Mean (± SEM) concentration and content of LHRH in hypophysial portal vessel plasma, together with volume of blood collected during four successive 30 min periods of collection from female rats anaesthetized with alphaxalone at 17.00 h on the afternoon of pro-oestrus and injected and infused with either naloxone (▨) or saline (☐). The infusions commenced at 17.00 h and the collection of portal blood started at 18.00 h.

Table 8-D LHRH content of hypophysial portal vessel blood collected during 4 consecutive 30 min periods from individual female rats anaesthetized with alphaxalone on the afternoon of pro-oestrus. The animals were infused with naloxone or saline for 1 h before the pituitary stalk was cut and the infusion was continued throughout the blood collection period. When the rate of flow of blood from the cut stalk was sufficiently high, the 30 min collection period was subdivided into two 15 min periods (denoted by the subscripts a and b). Peripheral plasma concentrations of LH in these animals during the first hour of infusion are shown in Table 8-C.

Infusion	Animal Number	Collection Period 1			2			3			4		
			a	b		a	b		a	b		a	b
Saline	1	39			27			10			--		
	2	115			40			29			13		
	3	119			55			--			--		
	4	92			68			41			28		
	5	169			127			85			69		
	6	163				58	54		40	31	55		
	7		57	44		20	26		21	16		14	12
	8		41	53		29	29		19	19		19	13
	9		104	92		68	45	45			31		
	10		65	40	---			--			--		
Naloxone	11	162			86			39			47		
	12	97			50			34			25		
	13	174			98			65			58		
	14	78				37	31			23		26	12
	15		68	62		27	21		21	17		14	15
	16		70	39		31	30		27	19		21	19
	17		59	48		42	34		20	--	--		
	18		111	84		71	44	55			53		

Table 8-E Plasma concentrations of LH (ng/ml) in individual female rats anaesthetized with alphaxalone at 10.00 h on the morning of oestrus. Peripheral blood samples were withdrawn before, and at 15 min intervals during, the intravenous infusion of either naloxone or saline. The hypophysial portal vessels were also exposed for the subsequent collection of portal blood (see Figure 8-4).

Infusion	Animal Number	Duration of Infusion (min)				
		0	15	30	45	60
Saline	21	1.2	1.5	2.9	2.1	2.0
	22	1.0	2.9	3.3	2.5	2.6
	23	0.6	0.7	1.6	1.6	1.2
	24	0.7	6.0	6.5	4.2	2.7
	25	0.9	3.5	10.5	4.9	3.4
	26	1.0	0.8	1.1	3.3	2.8
	27	0.7	1.6	3.5	4.2	2.7
Naloxone	31	1.5	0.6	3.0	1.3	1.2
	32	2.8	8.1	4.1	2.5	3.7
	33	1.4	3.1	3.6	2.6	1.8
	34			3.9	2.6	2.6
	35	1.3	4.4	6.2	3.7	3.0
	36	2.5	3.9	2.6	1.6	1.0
	37	1.6	1.1	1.3	1.3	0.5
	38	0.8	5.3	6.9	4.1	2.9
	39	0.7	2.7	6.5	4.3	2.8

Applying the criteria of Watts and Fink (1984) an LH pulse is indicated by a rise in plasma LH concentration which exceeded 4 x standard deviation of the appropriate quality control assay pool (i.e. for present data, with a 50 μl assay sample a $>$ 0.8 ng/ml increase indicated an LH pulse).

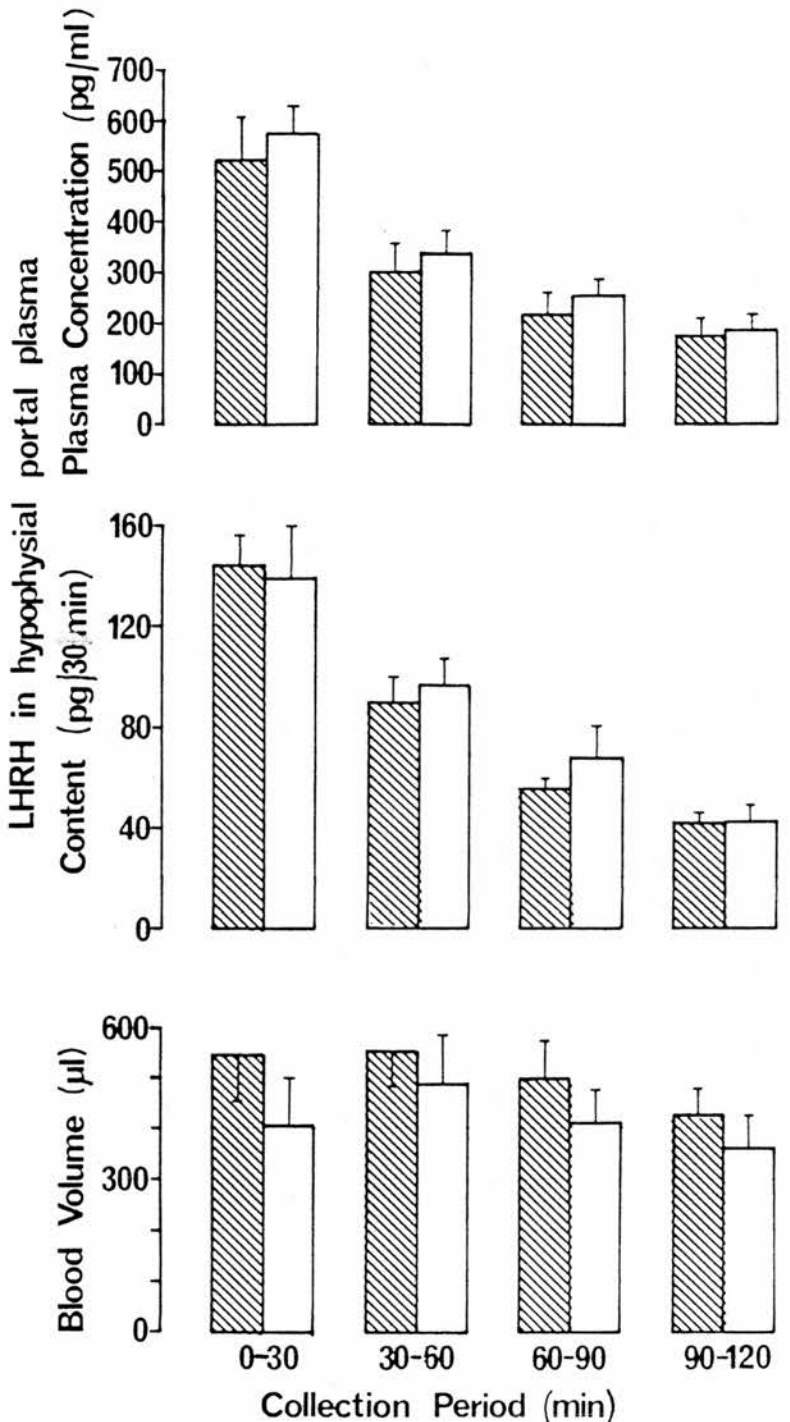


FIGURE 8-4: Mean (± SEM) concentration and content of LHRH in hypophysial portal vessel plasma, together with the volume of blood collected, during four consecutive 30 min periods of collection from female rats anaesthetized with alphaxalone at 10.00 h on the morning of oestrus and injected and infused with either naloxone (▨) or saline (☐). The infusions commenced at 10.00 h and collection of portal blood started at 11.00 h.

(Table 8-F).

8.3.3 Effects of the suckling stimulus

In lactating rats in which the ME and pituitary were exposed ready for the subsequent collection of portal blood, a 1 h period of suckling did not result in a significant increase in the plasma concentration of prolactin (Table 8-G). The concentration of LH in the plasma of lactating rats was low and no decrease in plasma LH concentration could be detected after a period of suckling (Table 8-G). Immediately after the second sample of peripheral blood had been withdrawn, both suckled and non-suckled animals were injected with heparin and the pituitary stalk sectioned for the collection of portal blood. There were no significant differences between either the content or the concentration of LHRH in hypophysial portal plasma from suckled and non-suckled animals except that the concentration of LHRH in the plasma collected during the final collection periods was lower ($P < 0.05$) in non-suckled compared with suckled rats (Fig. 8-5). For both groups of rats there was a significant decrease in LHRH concentration ($P < 0.02$) and content ($P < 0.005$) over the two hours of blood collection. Similarly, for oxytocin measured in hypophysial portal blood, there were no differences in either content or concentration between suckled and non-suckled rats, but in both groups the concentration of oxytocin decreased over the 2 h period and, for the suckled group only, there was also a significant decrease in plasma content of oxytocin ($P < 0.001$) (Fig. 8-6). Oxytocin was undetectable (< 5 pg/ml) in samples of peripheral blood withdrawn from suckled and non-suckled rats either before or after cutting the pituitary stalk.

Table 8-F LHRH content of hypophysial portal vessel blood collected during 4 consecutive 30 min periods from individual female rats anaesthetized with alphaxalone on the morning of oestrus. The animals were infused with naloxone or saline for 1 h before the pituitary stalk was cut and the infusion was then continued throughout the blood collection period. When the rate of flow of blood from the cut stalk was sufficiently high, the 30 min collection period was subdivided into two 15 min periods (denoted by the subscripts a and b). Peripheral plasma concentrations of LH in these animals during the first hour of infusion are shown in Table 8-5.

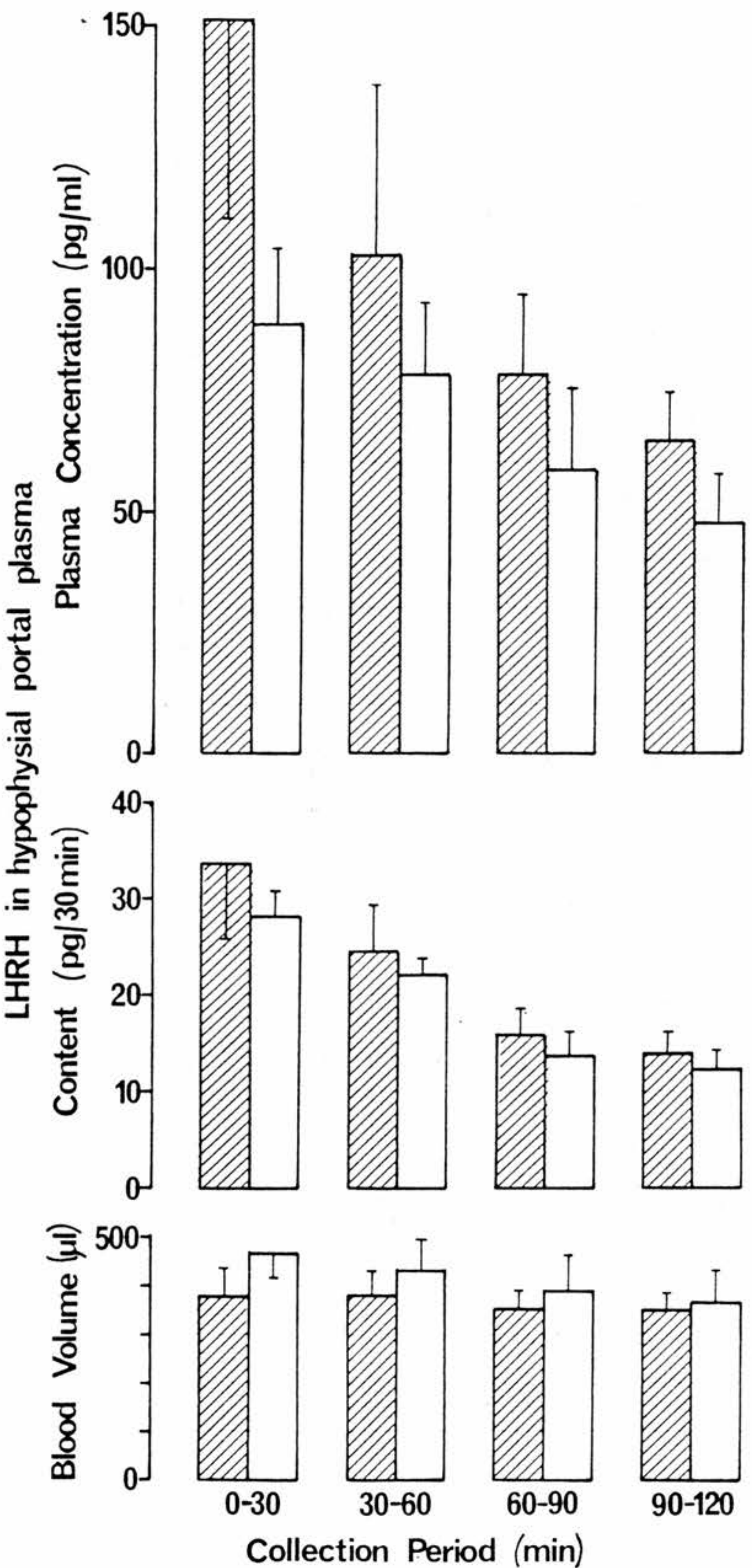
Infusion	Animal Number	Collection Period											
			1			2			3			4	
		a		b	a		b	a		b	a		b
Saline	21		96			56			40			30	
	22		119			86			58			44	
	23		122			111		35		26	22		19
	24		84		42		29	19		16	9		7
	25		137		57		47	52		40		51	
	26	88		78	62		46		56			40	
	27	132		116	71		70	72		59	34		40
Naloxone	31		104			73			58			46	
	32		133			65			62			47	
	33		160		51		48		55			41	
	34		136		46		38	31		24	21		15
	35		150		57		43	38		28	25		32
	36	75		44	36		32	29		16	12		16
	37	72		36	24		24	16		14	8		10
	38	89		74	78		55	32		29	22		20
	39	114		108	83		53	35		32	37		21

Table 8-G Mean (± SEM) concentrations of LH and prolactin in the peripheral plasma of lactating female rats which were anaesthetized with alphaxalone and the portal vessels exposed for the subsequent collection of hypophysial portal blood. Rats were maintained under anaesthesia for 3 h before the removal of 0.5 ml of blood from the EJV (sample I), after which, 10 pups were applied to the nipples (suckled group). A second sample of peripheral blood (II) was withdrawn 1 h later, following which, heparin was injected and the pituitary stalk cut for the collection of portal blood. 8-10 rats in each treatment group.

	Plasma LH concentration (ng/ml)		Plasma prolactin concentration (ng/ml)	
	I	II	I	II
Lactating, suckled	0.41 ± 0.01	0.40 ± 0.07	168 ± 83	198 ± 76*
Lactating, non-suckled	0.34 ± 0.36	0.34 ± 0.05	82 ± 27	76 ± 18

*$P < 0.05$ compared with non-suckled group (Mann-Whitney U test).

Figure 8-5. Mean (± S.E.M.) plasma concentration and content of LHRH in hypophysial portal vessel plasma together with the volume of blood collected during four consecutive 30 min periods of collection from lactating female rats either suckled (▨) or non-suckled (□). Rats were separated from all but one of their litter overnight and were anaesthetized with alphaxalone for 4 h before commencement of the collection of portal blood. In the group of suckled animals, 10 pups were applied to the nipples 1 h before the start of portal blood collection and suckling then continued throughout the 2 h collection period.


LHRH in hypophysial portal plasma
Plasma Concentration (pg/ml)
150
100
50
0
Content (pg/30 min)
40
30
20
10
0
Blood Volume (µl)
500
0
0-30
30-60
60-90
90-120
Collection Period (min)

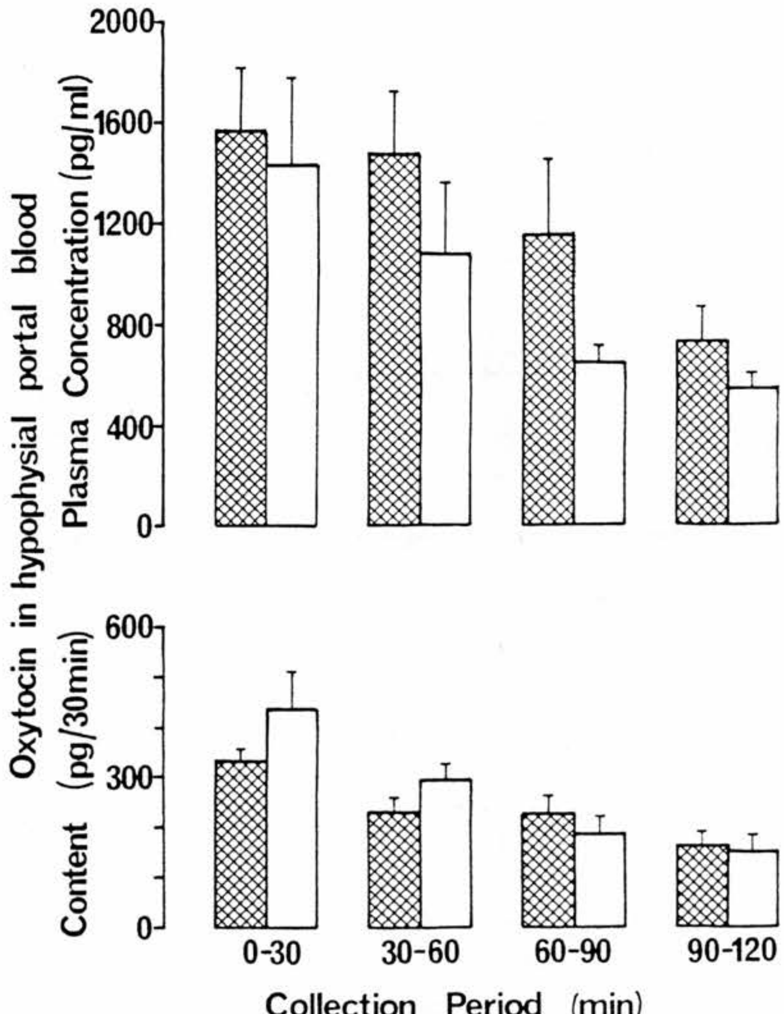


FIGURE 8-6: Mean (± SEM) plasma concentration and content of oxytocin in hypophysial portal vessel plasma collected during four consecutive 30 min periods of collection from lactating female rats either suckled (▨) or non-suckled (□). Rats were separated from all but one of their litter overnight and were anaesthetized with alphaxalone for 4 h before commencement of the collection of portal blood. In the group of suckled animals, 10 pups were applied to the nipples 1 h before the start of portal blood collection and suckling then continued throughout the 2 h collection period.

Samples of peripheral blood were also withdrawn, throughout a 3.5 h period of suckling, from a small number of lactating rats in which the pituitary stalk was not sectioned. Two (of three) rats anaesthetized with alphaxalone, but not subjected to surgical manipulation, showed an increased plasma concentration of prolactin in response to the suckling stimulus (Table 8-H). An increase in plasma prolactin concentration was observed after 3.5 h of suckling in one (number 45) of the three rats in which the ME and pituitary had been exposed. In all animals the plasma concentrations of LH were low throughout the experiment and no further decrease could be detected in response to suckling (Table 8-H).

8.4 DISCUSSION

In confirmation of previously reported observations (Nir *et al*, 1973; Ayalon *et al*, 1977), the administration of THC, to female rats at 13.30 h on the afternoon of pro-oestrus, blocked the pro-oestrous LH surge (Table 8-A). In addition, Ayalon *et al* (1977) showed that when THC was administered between 12.00 and 16.00 h on the afternoon of pro-oestrus, the subsequent ovulation was delayed by 24 h; earlier or later administration resulted in only a partial block of ovulation. As well as these effects on the pro-oestrous LH surge and ovulation, THC has also been shown to inhibit the progesterone-induced LH surge in long-term ovariectomized rats primed with oestrogen (Steger, De Paolo, Asch and Silverman, 1983). These effects of THC might be mediated by an action within the hypothalamus to inhibit the secretion of LHRH and/or a reduction in the pituitary responsiveness to LHRH. Figures 8-1 and 8-2 show that

Table 8-H Concentrations (ng/ml) of LH and prolactin in the peripheral plasma of individual lactating female rats which were anaesthetized with alphaxalone. Three rats (41-43) were not subjected to surgery (except for exposure of the EJV) whereas in the remainder (44-46) the hypophysial portal vessels were exposed but the pituitary stalk was left intact throughout the experiment. All animals were maintained under anaesthesia for 3 h before the removal of 0.5 ml of peripheral blood (sample I) after which 10 pups were applied to the nipples. Further blood samples (II-VIII) were removed at 30 min intervals during the period of suckling.

	Plasma LH Concentration							
	I	II	III	IV	V	VI	VII	VIII
41	0.55	0.35	0.60	0.45	0.40	0.44	0.82	< 0.25
42	0.78	0.46	0.45	0.46	0.45	0.40	0.70	N/A
43	0.55	0.61	0.60	0.30	N/A	0.45	0.42	0.60
44	0.57	0.60	0.46	0.48	0.46	0.46	0.45	0.50
45	0.37	0.33	0.29	0.35	0.37	< 0.25	0.35	0.49
46	0.22	0.19	0.22	< 0.25	0.22	< 0.25	0.17	0.34

	Plasma Prolactin Concentration							
	I	II	III	IV	V	VI	VII	VIII
41	458	288	408	460	508	1162	1108	907
42	489	496	399	877	641	543	480	N/A
43	48	58	59	61	N/A	92	65	59
44	352	126	168	125	133	119	152	188
45	110	119	132	159	149	248	320	521
46	< 50	< 50	< 50	< 50	< 50	< 50	< 50	< 50

N/A, not assayed.

the secretion of LHRH was lower in the rats injected with THC than those treated with vehicle and taken together with several other data these results are consistent with a central inhibition of LHRH release by THC. In rats, the blockade of ovulation may be overcome by the administration of exogenous LHRH (Ayalon _et al_, 1977) and similarly, in rabbits in which the reflex ovulation in response to mating was blocked by pre-treatment with THC, the later administration of LHRH or human chorionic gonadotrophin resulted in ovulation (Asch _et al_, 1979). Normal pituitary responsiveness to exogenous LHRH has been shown in THC-treated rats in several studies (e.g. Ayalon _et al_, 1977; Tyrey, 1978). In long term ovariectomized rats primed with oestrogen, 4 h after progesterone administration, the LHRH content of the medio-basal hypothalamus was higher in THC-treated than in vehicle-treated rats (Steger _et al_, 1983). Since there was no difference in the release of LH in response to LHRH in the vehicle or THC-treated rats, this was interpreted as suggesting that the effect of THC on steroid-induced LH surges might be mediated through inhibition of LHRH release. In this context, Sarkar and Fink (1980) could not demonstrate a surge of LHRH in hypophysial stalk plasma in steroid primed ovariectomized rats and concluded that in this model the LHRH surge is brought about mainly by a large increase in pituitary responsiveness to LHRH.

Early experiments showed that dibenamine or atropine blocked ovulation if administered before 14.00 h (but not after 16.00 h) on the day of pro-oestrus (Everett, Sawyer and Markee, 1949). This and similar observations led to the development of the concept of a critical period during the afternoon of pro-oestrus when the

administration of a number of drugs (e.g. barbiturates and opiates) blocks the gonadotrophin surge and subseqent ovulation (Everett, 1964). As shown also for barbiturates (Barraclough and Wise, 1982; Kalra and Simpkins, 1981), the turnover of noradrenaline in the mediobasal and anterior hypothalamus was reduced by THC administration (Steger et al, 1983) and ovulation was delayed by 24 h (Nir et al, 1973). In contrast to the barbiturates however, THC was reported still to be partially effective in blocking ovulation when the drug was given after the end of the critical period (Ayalon et al, 1977).

A direct action of THC upon the ovary may contribute to the ovulation blocking effect. This was suggested by the observation that THC was still partially effective in blocking ovulation when administered at the end of the critical period (Ayalon et al, 1977) and the finding that higher doses of LH were required to induce ovulation in THC-blocked than in pentobarbitone-blocked rats (Nir et al, 1973; Ayalon et al, 1977). Progressively higher doses of LH were also shown to reverse the reduction in ovarian prostaglandin (E series) content which occurred following administration of THC (Ayalon et al, 1977).

Data from many studies have suggested that the release of LHRH is controlled by central stimulatory NA neurons and inhibitory DA and opioid (principally the β-endorphin neurons in the arcuate nucleus which project to the preoptic area) neurons (see reviews by Bicknell, 1985; Ferin, Van Vugt and Wardlaw, 1984; Kalra and Kalra, 1983, 1984; Ramirez, Feder and Sawyer, 1984). Interactions between central opioid and CA neurons may result in the generation of

steroid induced LH surges in ovariectomized rats (Adler and Crowley, 1984; Akabori and Barraclough, 1986a,b) and opioid-containing neurons may also mediate some of the feedback effects of gonadal steroids on LHRH release (Van Vugt, Sylvester, Aylesworth and Meites, 1982; Bhanot and Wilkinson, 1984). Whilst LHRH neurons themselves do not appear to possess oestrogen receptors (Shivers, Harlan, Morrell and Pfaff, 1983), studies using combined immunocytochemical and autoradiographic techniques have shown that a small proportion (4-10%) of β-endorphin- or dynorphin-immunoreactive cells in the mediobasal hypothalamus concentrate oestradiol (Morrell, McGinty and Pfaff, 1985). Some NA neurons in the lower brainstem and arcuate DA neurons also appear to concentrate oestradiol (Sar, 1984; Sar and Stumpf, 1981). Some of the possible interactions between opioid, CA and steroids in the control of LHRH release in the female rat are summarized in Figure 8-7.

Continuous infusion of naloxone during the time of the pro-oestrous surge of LH did not affect either the plasma concentration of LH measured during the first hour of infusion (Tables 8-B and 8-C) or the release of LHRH into portal blood during the following two hours (Figure 8-3). These results suggest that at the time of the infusion the inhibitory influence of the opioid system was already completely suppressed and it was therefore impossible to augment further the magnitude of the LH surge or the output of LHRH. This is supported by the data of Sarkar and Yen (1985a) who showed that in the rats anaesthetized with alphaxalone the concentration of immunoreactive β-endorphin in hypophysial portal plasma fell sharply during the afternoon of pro-oestrus,

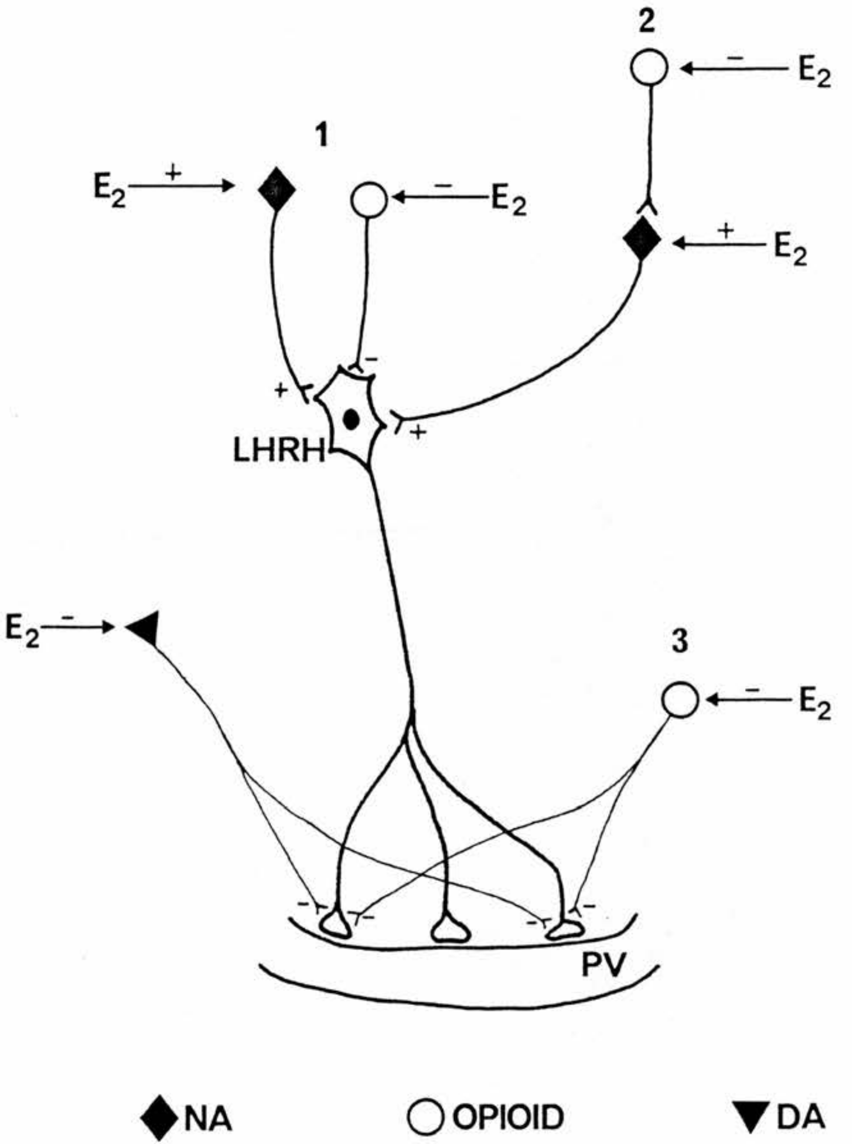


FIGURE 8-7: Schematic diagram to illustrate possible opioid-catecholaminergic interactions and their effects on the activity of LHRH neurons in the female rat. The possible sites of the feedback effects of oestradiol are arrowed and + and - signs indicate stimulatory and inhibitory effects, respectively, of oestradiol, opioids and amines; PV denotes the hypophysial portal vessels.

1. Oestradiol stimulates LHRH release either by the stimulation of noradrenergic neurons or by inhibition of opioid neurons.
2. Oestradiol inhibition of opioid neurons resulting in the disinhibition of NA neurons which then facilitate LHRH release.
3. Inhibitory actions of dopamine and opioids exerted at the terminals of the LHRH neurons in the median eminence.

secretion being minimal at the time of the LHRH surge. The inability of naloxone infusion to elevate plasma LH concentrations in oestrous rats (see below) cannot be explained in terms of an inhibition of the opioid system at this stage of the cycle. Gabriel, Simpkins and Kalra (1983) reported that naloxone stimulated LH secretion at all stages of the oestrous cycle including during the pro-oestrous surge. The LH responses to naloxone treatment were similar in dioestrous and in pro-oestrous rats at the onset of the preovulatory LH surge, and Gabriel et al (1983) argued that, since the pituitary sensitivity to LHRH is lower on the day of dioestrus, naloxone had a greater stimulatory effect on LHRH release on the day of dioestrus. Muraki et al (1979) showed that, in conscious rats, whilst naloxone reversed the morphine-induced blockade of the pro-oestrous surge, the administration of naloxone at 14.00 h by itself did not alter the magnitude of the surge at 18.00 h. Similarly, a single intravenous injection of naloxone at 14.00 h on the afternoon of pro-oestrus suppressed the prolactin surge, but did not alter the magnitude of the LH surge although the duration of the surge was prolonged (Ieiri, Chen, Campbell and Meites, 1980; Gabriel et al, 1983; Akabori and Barraclough, 1986a,b). Naloxone administration did not affect either the magnitude or the time of the onset of the LHRH surge induced in immature female rats by the administration of pregnant mare serum gonadotrophin (Blank, Panerai and Friesen, 1980).

Pulses of LH were shown in the plasma at 50-60 minute intervals on the days of met- and dioestrus and on the morning of pro-oestrus (Gallo, 1981a) and higher frequency pulses, occurring approximately

every 15-30 minutes, were detected during the surge of LH on the afternoon of pro-oestrus (Gallo, 1981b); Gallo (1981a) also showed pulses of LH occurring about once every two hours on the day of oestrus. Fox and Smith (1985) were not able to detect pulses of LH secreted during the day of oestrus, but showed that a rapid release of LH could be induced on that day by a single injection of naloxone, and that continuous intravenous infusion of naloxone initiated a pulsatile pattern of LH release in 12 out of 17 oestrous rats. The frequency of the pulses induced by naloxone infusion was similar to that observed during the other days of the oestrous cycle (Fox and Smith, 1985). In the present experiments, in which peripheral blood samples were withdrawn from anaesthetized oestrous rats at 15 minute intervals during the infusion of naloxone (compared with a 10 minute sampling interval from conscious rats, Fox and Smith, 1985), the pattern of LH secretion was unchanged by the naloxone treatment (Tables 8-B and 8-E). Similarly, LHRH output during the subsequent two hours of infusion also remained unaltered by naloxone (Figure 8-4) and there was no evidence of pulsatile release of LHRH during this period (Table 8-F). Using a RIA for LHRH which used the R-42 antiserum (Nett, Akbar, Niswender, Hedlund and White, 1973) and 15 minute blood collection periods, Sarkar and Fink (1980) were able to demonstrate the pulsatile release of LHRH in ovariectomized rats. It is possible that if in the present experiments naloxone had induced pulsatile release of LHRH, the amplitude of the pulses may have been smaller than those apparent in ovariectomized rats and thus not detected or that the HC-6 antiserum used here is less sensitive than the R-42 antiserum in

discriminating small fluctuations in LHRH concentration. In this context, although the profiles of LHRH-IR recovered after HPLC of hypothalamic or portal plasma extracts are similar when assayed by each of the two anti-LHRH sera (Chapter 6), there are several qualitative and quantitative differences in the results obtained by direct measurement of portal plasma LHRH concentration by the two assays. In comparison with the results obtained in previous studies which used the R-42 antiserum (Sarkar et al, 1976; Sherwood et al, 1980), the present results obtained with the HC-6 antiserum showed much higher concentrations of LHRH in portal plasma, and did not show a difference between the LHRH concentrations measured in portal plasma collected from pro-oestrous or oestrous rats (Figs. 8-3 and 8-4). For example, data taken from Sarkar et al (1974) show concentrations of 120 pg/ml (during the pro-oestrous surge) and 30 pg/ml (oestrous) compared with the 500 pg/ml measured here in both pro-oestrous and oestrous rats. Finally, in contrast to the data of Sherwood et al (1980) in which in some animals the portal blood content of LHRH increased during successive collection periods during the time of the expected pro-oestrous surge, in the present studies LHRH content declined in all animals after cutting the pituitary stalk.

In lactating animals there were no significant differences between LHRH output in suckled or non-suckled animals (Fig. 8-5). LHRH concentrations were lower in portal plasma from lactating compared with pro-oestrous or oestrous rats. However, since in comparison with cycling rats the lactating animals were maintained under anaesthesia for an extended period before the pituitary stalk

was cut, the data from these animals may not be comparable. Plasma concentrations of LH were low in lactating rats and were not reduced in response to suckling (Table 8-G) and such a decrease may have been more readily apparent if ovariectomized animals had been used. As shown for urethane-anaesthetized rats (Chapter 5, Burnet and Wakerley, 1976; Isherwood and Cross, 1980) anaesthesia with alphaxalone delayed the onset of the prolactin response to suckling (Table 8-G). At the time of cutting the pituitary stalk for the collection of portal blood, plasma concentrations of prolactin were higher in suckled than non-suckled rats (though this reflected largely the higher initial prolactin concentration in the suckled group), but prolactin concentration at this time was low compared with the high concentrations measured after longer periods of suckling in animals anaesthetized with urethane (Chapter 5) or in other hyperprolactinaemic conditions (see below).

Other than lactation, there are many physiological and experimental situations in which the plasma concentrations of gonadotrophins are reduced in the presence of a high prolactin concentration; for example, pseudopregnancy (Smith, Freeman and Neill, 1975), after grafting anterior pituitary gland tissue under the kidney capsule (McNeilly, Sharpe and Fraser, 1980) or injection of prolactin (Cohen-Becker, Selmanoff and Wise, 1986). However, this inverse relationship between the secretion of prolactin and gonadotrophins does not always apply as is shown by the concurrent release of LH and prolactin during the afternoon of pro-oestrus (e.g. Horn, Fraser and Fink, 1985b) or in ovariectomized rats injected with oestradiol (e.g. Horn and Fink, 1985a).

The suppression of LH release during lactation may be mediated by the direct inhibition of LHRH release by the suckling stimulus or may be secondary to the hyperprolactinaemia induced by suckling. Elevated plasma concentrations of prolactin may decrease the pituitary responsiveness to LHRH and/or reduce LHRH output either by direct inhibition of the LHRH neurons or by increased release of DA from the tuberoinfundibular DA system (Fig. 8-7).

Since in conscious rats the secretion of LH was apparently suppressed virtually instantaneously at the start of the period of suckling, Isherwood and Cross (1980) suggested that the suckling stimulus alone, rather than the associated hyperprolactinaemia, accounted for the inhibition of LH secretion. In support of this, plasma LH concentrations were also suppressed in lactating rats when prolactin secretion was inhibited pharmacologically by the administration of dopamine-agonists (Lu, Chen, Huang et al, 1976a; Smith, 1978b). Smith (1978b) suggested that although in the early days of lactation the suckling stimulus alone was principally responsible for blocking the post-ovariectomy increase in plasma LH concentrations, in the later stages of lactation (around day 12 and onwards) it is the hyperprolactinaemia (in the presence of the suckling stimulus) which accounts for the inhibition of gonadotrophin secretion.

The long-term hyperprolactinaemia induced by the implantation of anterior pituitary gland tissue under the kidney capsule results in the suppression of plasma concentrations of LH and FSH (McNeilly, et al, 1980). However, Brar, McNeilly and Fink (1985b) showed that LHRH output was unchanged in this model suggesting that at least in

the male rat, the effects of hyperprolactinaemia on gonadotrophin secretion are mediated largely by an action at the level of the pituitary gland e.g. by a reduction in either pituitary responsiveness to LHRH or LHRH receptor number. In male rats bearing a tumour which secreted both prolactin and adrenocorticotrophin (ACTH) the secretion of LH and FSH was reduced by 45% and 70%, respectively, the concentration of LHRH in hypophysial portal blood reduced by approximately 50%, and the portal blood concentration of dopamine increased three-fold (Weber, de Greef, de Koning, Vreeburgh, 1983). However after adrenalectomy only the change in dopamine output was apparent, suggesting that the altered secretion of LHRH and the gonadotrophins was related to the hypersecretion of ACTH rather than prolactin. A four-fold decrease in the portal blood concentration of LHRH was reported in ovariectomized rats which had pituitary tissue implanted under the kidney capsule (Sarkar and Yen, 1985b). Sarkar and Yen (1985b) also suggested that this inhibitory effect of prolactin on LHRH secretion may be mediated by an opioid mechanism, since the concentration of β-endorphin in portal blood was raised in hyperprolactinaemic rats and the administration of naloxone resulted in increased portal blood LHRH and peripheral blood LH concentrations in hyperprolactinaemic but not in control rats.

In vivo and in vitro evidence suggests that in lactating rats the pituitary responsiveness to LHRH is decreased (Lu, Chen, Grandison, Huang and Meites, 1976b; Smith, 1985). Fox and Smith (1984) showed that the suckling stimulus which was provided by two pups was sufficient to inhibit pulsatile LH secretion without a

reduction in pituitary responsiveness to LHRH, though pituitary responsiveness was reduced when the size of the suckling stimulus was increased to 8 pups. In contrast, in ovariectomized, non-suckling rats, a series of injections of ovine prolactin was sufficient to decrease both the frequency and amplitude of the LH pulses without decreasing pituitary responsiveness (Cohen-Becker *et al*, 1986). A direct effect of prolactin on the anterior pituitary gland to decrease its responsiveness to LHRH has been demonstrated *in vitro* (Cheung, 1983).

Immunohistochemical studies have shown an oxytocin-containing fibre system which originates in the PVN and terminates in the external layer of the ME, adjacent to the portal vessels (Vandesande, Dierickx and de Mey, 1977; Burlet, Chateau and Czernichow, 1979). Synthetic oxytocin can stimulate prolactin secretion from the anterior pituitary gland *in vivo* and *in vitro* (Lumpkin *et al*, 1983) and may be a factor involved in the regulation of prolactin secretion. The present results (Fig. 8-6) confirm previous findings which showed that oxytocin is present in higher concentrations in hypophysial portal blood than in peripheral blood (Gibbs, 1984; Sarkar and Gibbs, 1984; Horn, Robinson and Fink, 1985c). It is unlikely that a significant amount of the oxytocin content of portal blood was due to contamination by the backflow of blood from the posterior pituitary gland as the concentration of oxytocin measured in portal blood was not altered by the removal of the pituitary gland before the start of blood collection (Sarkar and Gibbs, 1984; Horn *et al*, 1985c). Similarly, the concentration of oxytocin in C.S.F. was too low to account for the oxytocin in portal

blood (Horn _et al_, 1985c).

In the present study there were no significant differences between the concentration of oxytocin in hypophysial portal blood collected from suckled or non-suckled lactating rats (Fig. 8-6). However these results must be interpreted with caution since in urethane anaesthetized male rats, Horn _et al_ (1985c) were unable to increase oxytocin output by electrical stimulation of the ME and concluded that the secretion of oxytocin was maximal following the exposure and section of the pituitary stalk. Nonetheless, using female rats anaesthetized with alphaxalone, Sarkar and Gibbs (1984) showed that oxytocin release into portal blood varied during the oestrous cycle; the highest concentrations were measured during the afternoon of pro-oestrus and corresponded with the prolactin surge.

CHAPTER 9

The Effects of Intravenously Administered 6-Hydroxydopamine on the Content of Monoamines in the Median Eminence and Neurointermediate Lobe, and on Pituitary Hormone Secretion in the Male Rat

9.1 INTRODUCTION

The neurotoxic drug, 6-hydroxydopamine (6-OHDA) has been widely used in studies of the anatomy and function of catecholaminergic (CA) neurons in both the peripheral and central nervous systems. The neurodegenerative action of 6-OHDA depends upon both its accumulation in CA containing terminals as a result of its uptake by the high affinity amine uptake mechanism (hence the specificity for CA neurons) and on its auto-oxidation to form quinone derivatives, and oxidation products such as hydrogen peroxide, which may be responsible for the cytotoxic effects (see reviews by Breese, 1975; Jonsson, 1983). As it does not readily penetrate the blood-brain barrier (Sachs, 1973; Garver, Cedarbaum and Mass, 1975), for studies of the function of central CA pathways, 6-OHDA has usually been administered by intraventricular or intracisternal injection or applied locally to specific brain areas (e.g. Iversen and Uretsky, 1970; Uretsky and Iversen, 1970; Breese and Traylor, 1971; Ungerstedt, 1971; Breese, Smith, Cooper and Grant, 1973). Since the median eminence lies outside the blood-brain barrier (Wislocki and King, 1936), in the majority of such studies, no marked effect on the CA content of the median eminence was reported. In addition to producing a lesion of the sympathetic neurons in the peripheral nervous system, systemic administration of 6-OHDA results in a depletion of CA in brain areas outside the blood-brain barrier including the area postrema (Klara, Kostrezewa and Brizzee, 1976) and the median eminence (Cuello, Shoemaker and Ganong, 1974; Smith and Helme, 1974; Smith Courtney, Wreford and Walker, 1982). The spinal cord is also affected by systemic 6-OHDA (Wong and Tan, 1974)

and since a small amount of 6-OHDA may penetrate the blood-brain barrier (Garver et al. 1975) some changes in whole brain CA turnover have also been reported (Clark, Corrodi and Masuoka, 1971; Kawa, Kamisaki, Ariyama et al, 1979). As assessed by fluorescence histochemistry, the intravenous injection of 150 mg/kg 6-OHDA resulted in a complete depletion of CA in the median eminence within 24 h, whilst the remainder of the hypothalamus is relatively unaffected (Smith et al. 1982); although there was some depletion of CA in the mediobasal hypothalamus at 24 h, the fluorescence appearance of this region appeared normal 48 h after injection whereas the return of fluorescence to the median eminence was slower and occurred over a 5 week period.

The following experiments were carried out to determine the effect of lesions of the CA neurons of the ME and NIL produced by intravenous 6-OHDA administration on the plasma concentrations of prolactin, thyrotrophin, LH, FSH and corticosterone. In the initial experiments the extent of the depletion of CA was monitored by fluorescence histochemistry, in a later series of experiments, NA, DA, DOPAC, 5-HT and 5-HIAA were measured directly, with the aid of HPLC with electrochemical detection, in the median eminence and neurointermediate lobe of control animals, 6-OHDA lesioned animals and of animals in which the lesion had been modified by pretreatment with desipramine (DMI) in order to produce a more selective depletion of DA (Smith, Cooper and Breese, 1973; Kelly and Iversen, 1976).

9.2 MATERIALS AND METHODS

The animals used were adult male Wistar rats maintained as described in Chapter 2 and which had had at least 3 weeks to become accustomed to the conditions of the animal house before the time of the experiment.

9.2.1 Effects of 6-OHDA on pituitary hormone secretion

The animals were lightly anaesthetized with anaesthetic ether and slowly injected intravenously with either 150 mg/kg 6-OHDA-HCl (Labkemi, Stockholm; 50 mg/ml in 0.1% ascorbic acid in 0.9% saline [vehicle]) or with vehicle alone. The 6-OHDA solutions were freshly prepared immediately before the time of injection. All injections were administered between 17.00 and 18.00 h. One group of animals (untreated control) were not injected. At 1, 2, 7, 14, 21 and 36 days after the injection the animals were killed by decapitation at 17.30 h in batches of six at one time. No more than two minutes elapsed between the time that a cage of six animals were removed quietly from the animal housing room and the time that the last animal in the group was decapitated. The trunk blood was collected into ice-cooled 10 ml heparinized plastic tubes with the aid of a glass filter funnel which had also previously been heparinized. After centrifugation of the blood samples, the plasma was stored at -40°C until the time of assay. The anterior pituitary gland was removed from each animal, weighed and homogenized (2.2.6). The plasma concentration of corticosterone and plasma and pituitary concentrations of LH, FSH, prolactin and thyrotrophin were determined as described in Chapter 2. The significance of the differences between groups was determined by unpaired t-tests.

9.2.2 Fluorescence histochemistry

Two animals from each of the experimental groups described above were perfused with fixative, the brains removed and processed for catecholaminergic fluorescence histochemistry using the 'FAGLU' technique (2.3).

9.2.3 Assay of tissue monoamines

Male rats were injected with 150 mg/kg 6-OHDA (Sigma, U.K.) or vehicle as described above. A third group of rats was also injected with 30 mg/kg DMI-HCl (Geigy, Macclesfield; 12 mg/ml w/v in 0.9% saline i.p.) which was administered 30 min before the injection of 6-OHDA. As in the previous experiments a group of untreated, control animals was not injected with either vehicle or drugs. Forty-eight hours after drug treatment the animals were killed by decapitation and the brain rapidly removed, placed on an ice-cold glass microscope slide and the stalk median eminence (SME) and the neurointermediate lobe of the pituitary (NIL) dissected (2.2.6). The mean (± S.E.M.) weights of the ME and NIL samples taken were 0.44 ± 0.07 mg and 1.39 ± 0.39 mg respectively.

NIL and SME samples were stored in liquid nitrogen until the contents of amines were determined by LCED (2.4.1). The significance of differences between means was calculated using unpaired t-tests.

9.3 RESULTS

9.3.1 Pituitary hormone secretion

Figure 9-1 shows that in the group injected with 6-OHDA the plasma concentrations of prolactin were not significantly different

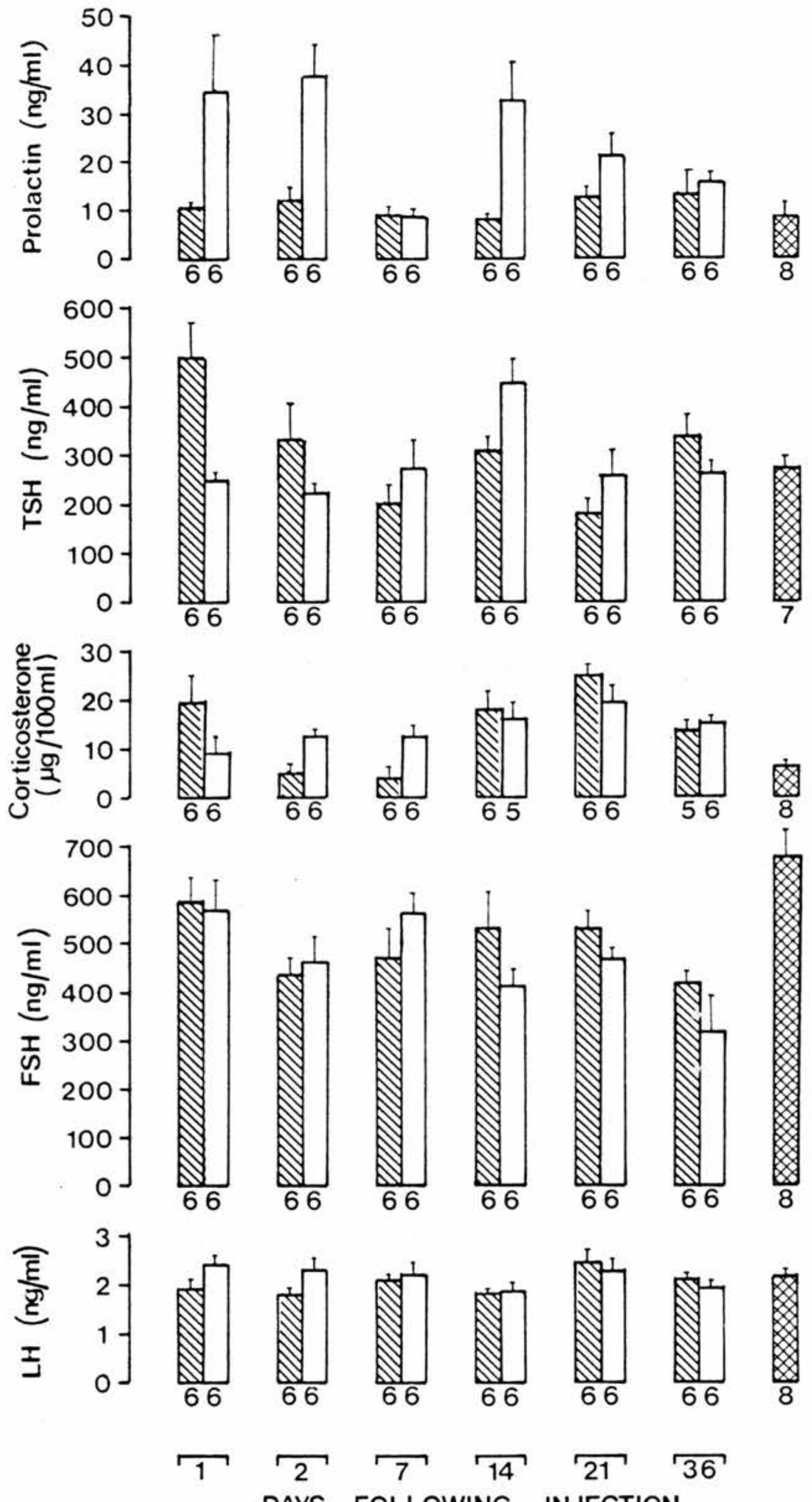


FIGURE 9-1: Mean (±SEM) plasma concentrations of prolactin, thyrotrophin (TSH), corticosterone, LH and FSH in male rats killed by decapitation 1-36 days after the i.v. injection of either 6-OHDA ▨ or vehicle □. Also shown ▩ are hormone concentrations in animals which had not been injected with either 6-OHDA or vehicle alone. The number of animals in each group are shown at the base of each column.

from those in the untreated control group. However, in animals injected with vehicle alone the concentrations of prolactin were significantly elevated above the values in the untreated control group at 1 and 2 days and at 14 and 21 days after treatment ($P < 0.05$, 0.001, 0.01 and 0.025, respectively). The plasma concentration of thyrotrophin was significantly ($P < 0.01$) elevated 24 h after injecting 6-OHDA compared with the concentrations in either untreated control animals or animals injected with vehicle alone. The plasma concentrations of corticosterone were significantly elevated ($P < 0.02$) 24 h after injecting 6-OHDA compared with those in untreated control animals. On days 2 and 7 the concentration of corticosterone in animals injected with 6-OHDA were lower ($P < 0.01$ and 0.02, respectively) than in vehicle-treated animals. In animals treated with vehicle the concentrations of corticosterone were higher ($P < 0.05$-0.001) than in untreated control animals for all days except day 1 after injection. Treatment with either 6-OHDA or vehicle had no significant effect on the plasma concentrations of LH or FSH, except that at day 36 plasma FSH concentrations in both of these treatment groups were lower than those in the untreated control group ($P < 0.005$ and $P < 0.001$, respectively).

For LH, FSH and thyrotrophin there were no major changes in pituitary content (Fig. 9-2) or concentration (Fig. 9-3) in the treated animals compared with those in the untreated control animals other than reduced values for LH and FSH on day 36 ($P < 0.005$-0.001). The pituitary content and concentration of prolactin in the treated animals were mostly lower than in the untreated, control

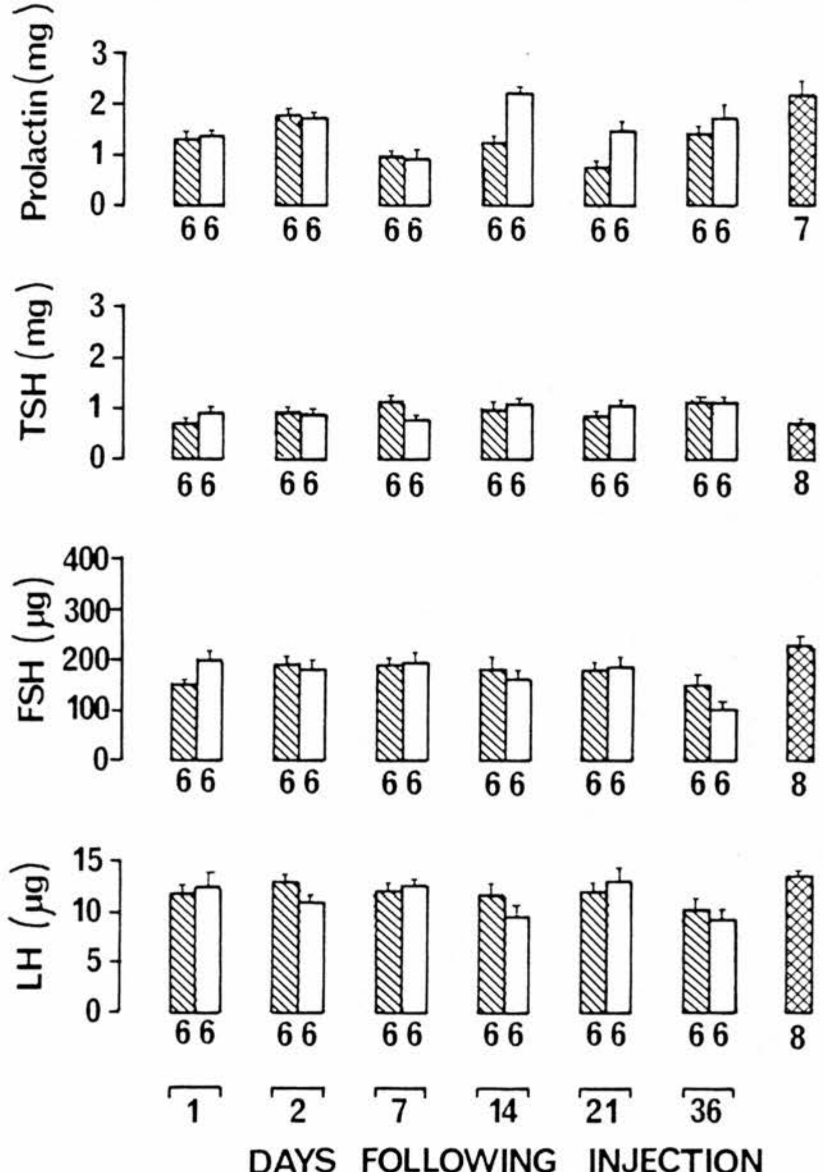


FIGURE 9-2: Mean (±SEM) pituitary hormone content in male rats killed by decapitation 1-36 days after i.v. injection of either 6-OHDA ▧, or ascorbic acid vehicle □. Values from untreated rats ▩ are also shown. Number of animals per group given at the base of each column.

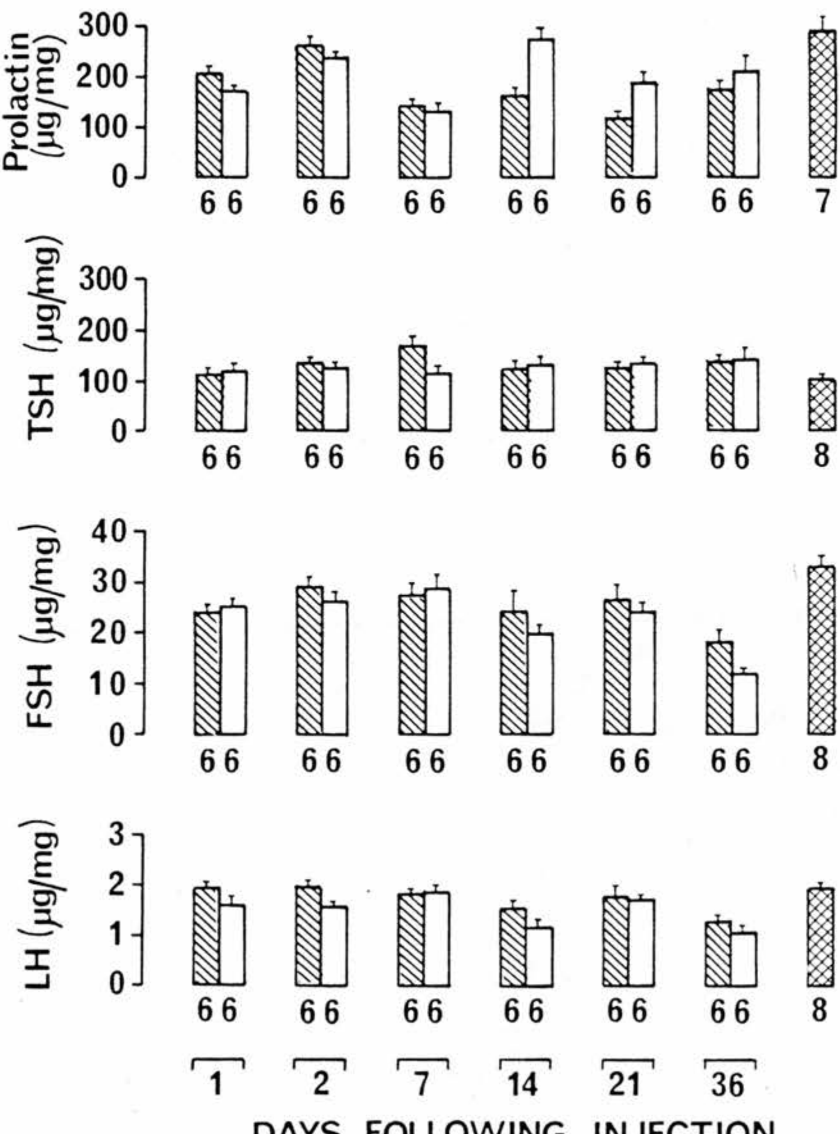


FIGURE 9-3: Mean (±SEM) pituitary hormone concentration in male rats killed by decapitation 1-36 days after i.v. injection of either 6-OHDA , or ascorbic acid vehicle alone. Values from untreated rats are also shown. Number of animals per group given at the base of each column.

animals, significantly on days 1 and 7 ($P < 0.025$–0.005). The pituitary content and concentrations of prolactin were lower ($P < 0.02$–0.005) at 14 and 21 days in the animals treated with 6-OHDA compared with those in animals treated with vehicle alone.

Full details of all the statistical comparisons of plasma and pituitary gland hormone concentrations, and ME and NIL amine and metabolite contents (9.3.3), in 6-OHDA treated and control group animals are shown in Table 9-C at the end of the chapter.

9.3.2 Fluorescence histochemistry

The fluorescence histochemical findings, which are illustrated in Figures 9-4 to 9-6 and are summarized in Table 9-A, showed that terminals in the ME-NIL were depleted of CA fluorescence 24 h after injection of 6-OHDA. The adjacent mediobasal hypothalamus was moderately depleted at 24 h, but was of normal appearance at 48 h as was the rest of the hypothalamus at all times studied. Regenerating terminals in the median eminence were seen by day 7 after injecting 6-OHDA, and by day 36, the fluorescence appearance of this region could not be distinguished from that in untreated animals or animals treated with vehicle alone.

9.3.3 Monoamine content of neurointermediate lobe and median eminence

Table 9-B shows the amine and metabolite content of the SEM and NIL after drug treatment; typical LCED traces of SME samples from drug-treated or control animals are shown in Figures 9-7a-d. Forty-eight hours after 6-OHDA administration there was a significant decrease in the contents of both NA ($P < 0.005$ and $P < 0.02$, SME and NIL, respectively) and DA ($P < 0.001$ and $P < 0.005$)

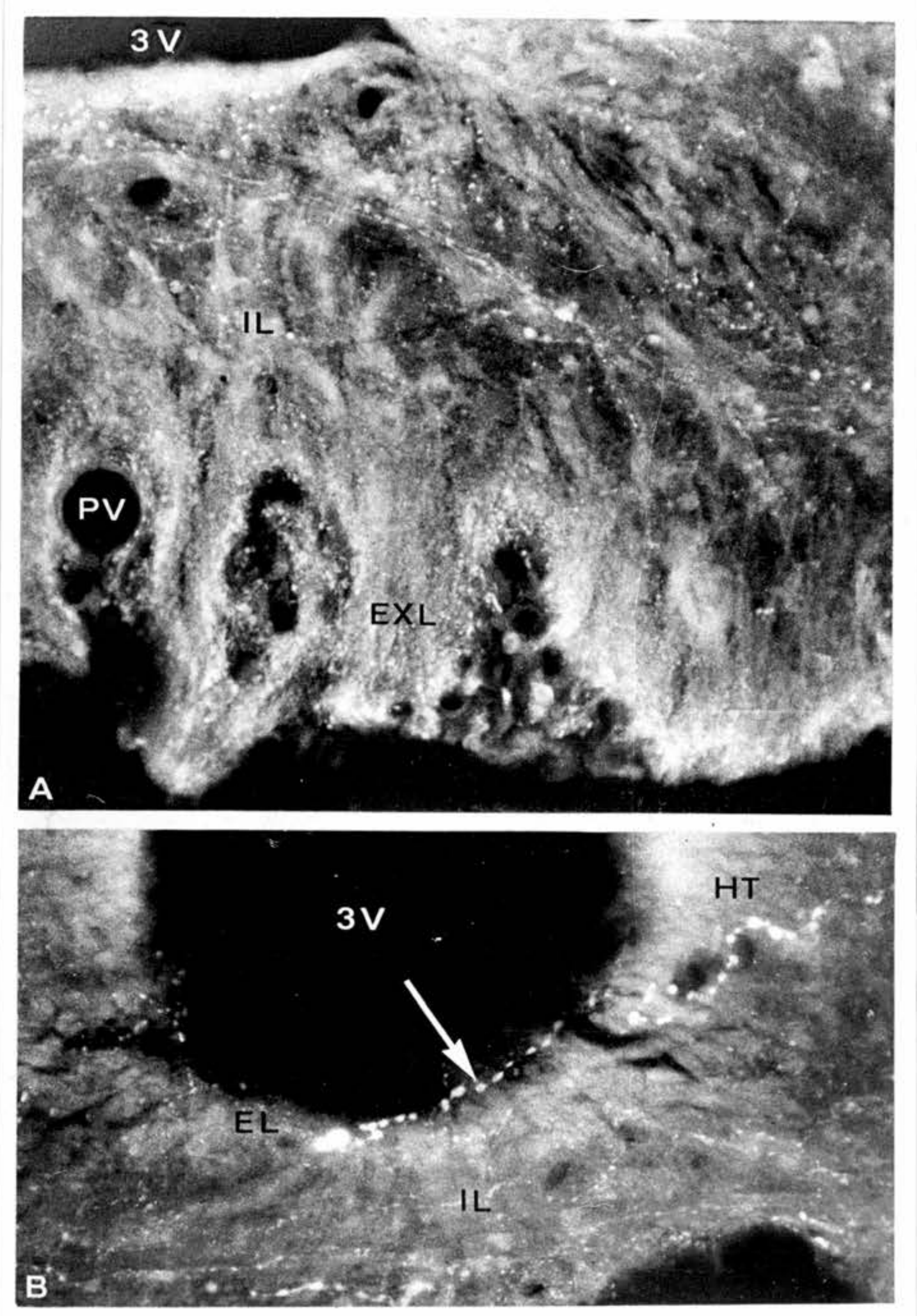


FIGURE 9-4: Fluorescence photomicrographs of coronal sections through the median eminence of adult male Wistar rats treated with ascorbic acid 24 h previously and prepared by the FAGLU perfusion technique. The fluorescence histochemical appearance is indistinguishable from that of untreated control rats prepared similarly.
A: Mid POI*, showing the fine varicose nature of terminals of catecholamine-containing fibres in the external layer (EXL) and the larger varicose fibres of the intermediate layer (IL). 3V, third ventricle; PV, portal vessel.
B: Anterior POI, showing a distinct varicose fluorescent fibre (arrowed) passing from the arcuate nucleus of the hypothalamus through the ependymal layer (EL) of the median eminence.

*pars oralis infundibuli

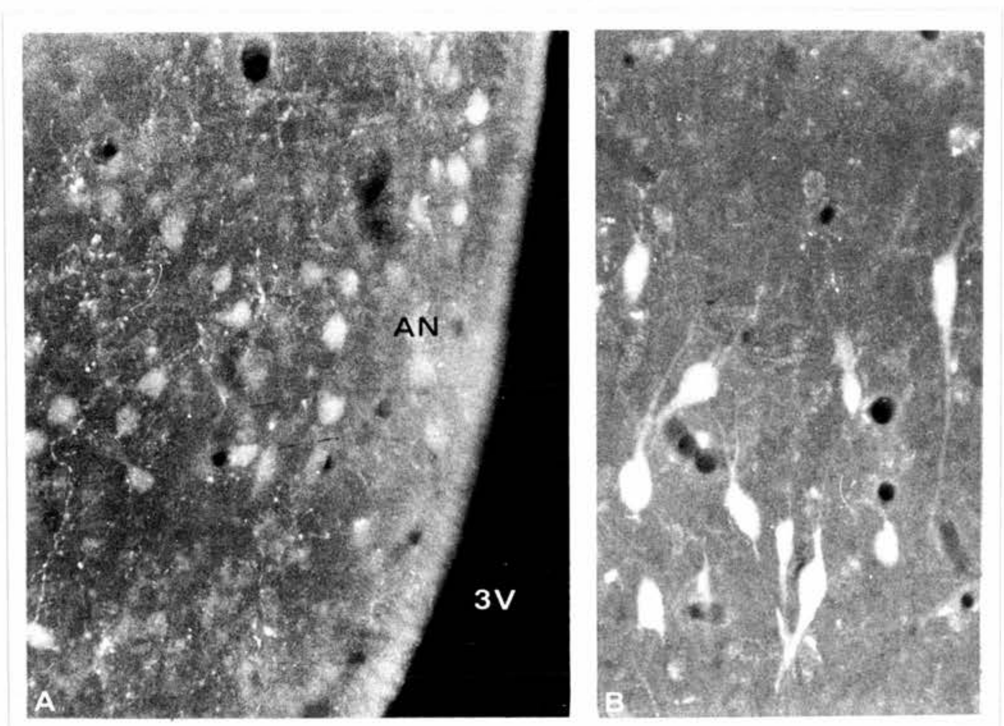


FIGURE 9-5: Fluorescence photomicrographs of sections from the diencephalon of male Wistar rats previously injected with ascorbic acid.
(A) Arcuate nucleus demonstrating numerous dopamine-containing cell bodies of the A12 group and showing the presence of fine varicose fluorescent fibres. 3V, third ventricle.
(B) Dopamine-containing neuronal cell bodies of the A11 group.

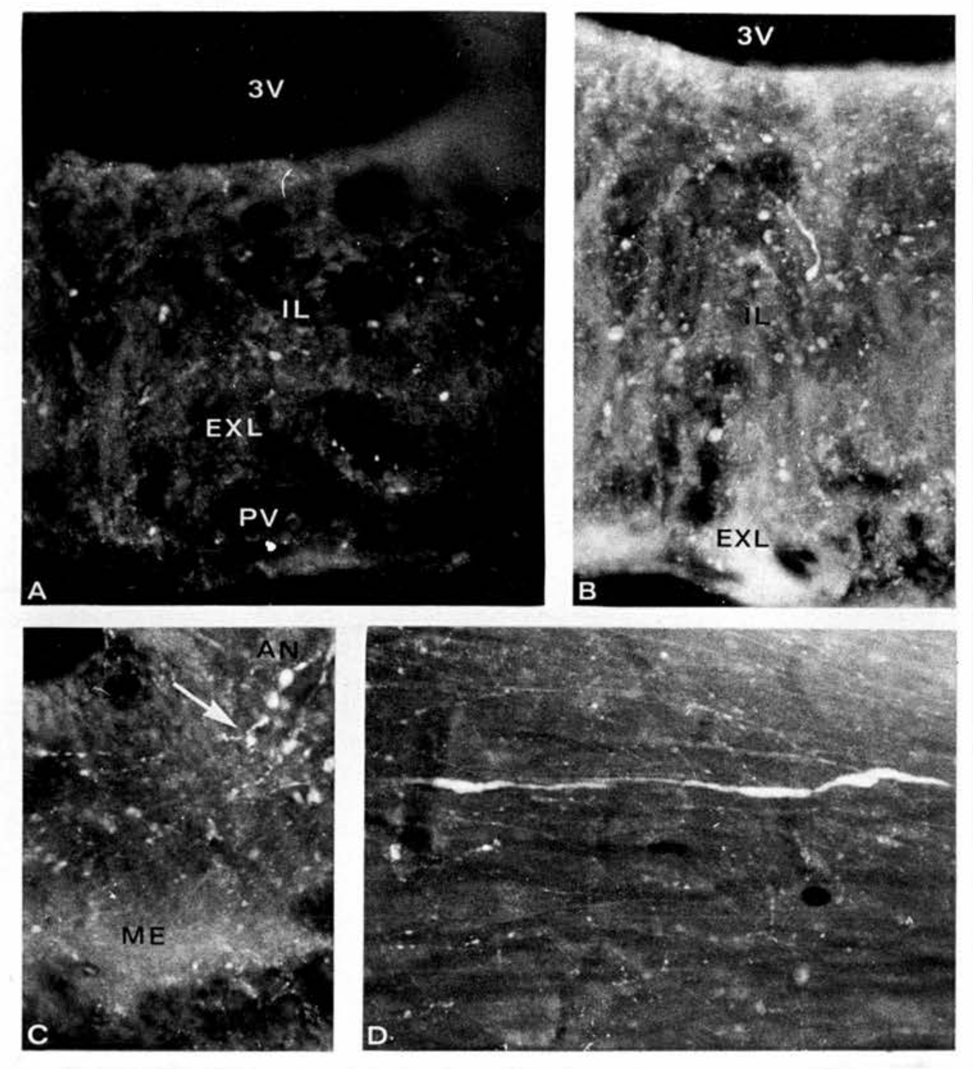


FIGURE 9-6: Fluorescence photomicrographs of coronal sections from male rats injected previously with 6-hydroxydopamine and prepared by the FAGLU technique. (A) Median eminence of a male rat treated 24 h previously with 6-OHDA (150 mg/kg i.v.). Specific catecholamine fluorescence is absent, except for occasional fluorescent masses in the intermediate layer (IL). Compare with Fig. 9-4A. 3V, third ventricle; EXL, external layer; PV, portal vessel. (B) Median eminence of an adult male rat treated 7 days previously with 6-OHDA (150 mg/kg i.v.). A large number of fluorescent masses, produced by accumulations of catecholamines in fibres proximal to degeneration, are seen in the IL and EXL. Some re-innervation of the EXL has occurred. (C) Junction of the arcuate nucleus (AN) and median eminence (ME), from a Wistar rat treated 48 h previously with 6-OHDA (150 mg/kg i.v.). Accumulation of catecholamine induced fluorescence (arrowed) in axons proximal to degeneration marks the boundary of the lesion. ME fluorescence is still depleted. (D) Swollen preterminal axon in the lateral hypothalamus of the same animal as seen in Fig. C.

Table 9-A Changes in catecholamine fluorescence intensity at different times followng injection of 6-OHDA.

Region	Control	Time after injection (days) 1	2	7	14	21	35
SON	++++	+++(+)	+++	++++	++++	++++	++++
PVN	++++	+++(+)	+++	+++	++++	++++	++++
Hypothalamus	++++	++(+)	++	+++(+)	++++	++++	++++
Median eminence:							
(a) Anterior (i) IL	++++	0	0	++(+)	++++	+++(+)	++++
(ii) EL (lateral)	++++	0	0(+)	++++	++++	++++	++++
(midline)	++++	0	0	+++	+++	+++(+)	++++
(b) Mid (i) IL	++++	0	0	++	++	++	++++
(ii) EL (lateral)	++++	0	0(+)	++++	+++	++++	++++
(midline)	++++	0	0	+	0	+	++++
(c) Distal (i) IL	++++	0	0	+	++	+	+++
(ii) EL (lateral)	++++	0	0	NE	+	NE	++++
(midline)	++++	0	0	NE	+	NE	++++

IL, Intermediate layer; EL, external layer; NE, not examined; 0, no fluorescence;
+, markedly reduced fluorescence; ++/+++ reduced fluorescence; ++++ normal fluorescence.

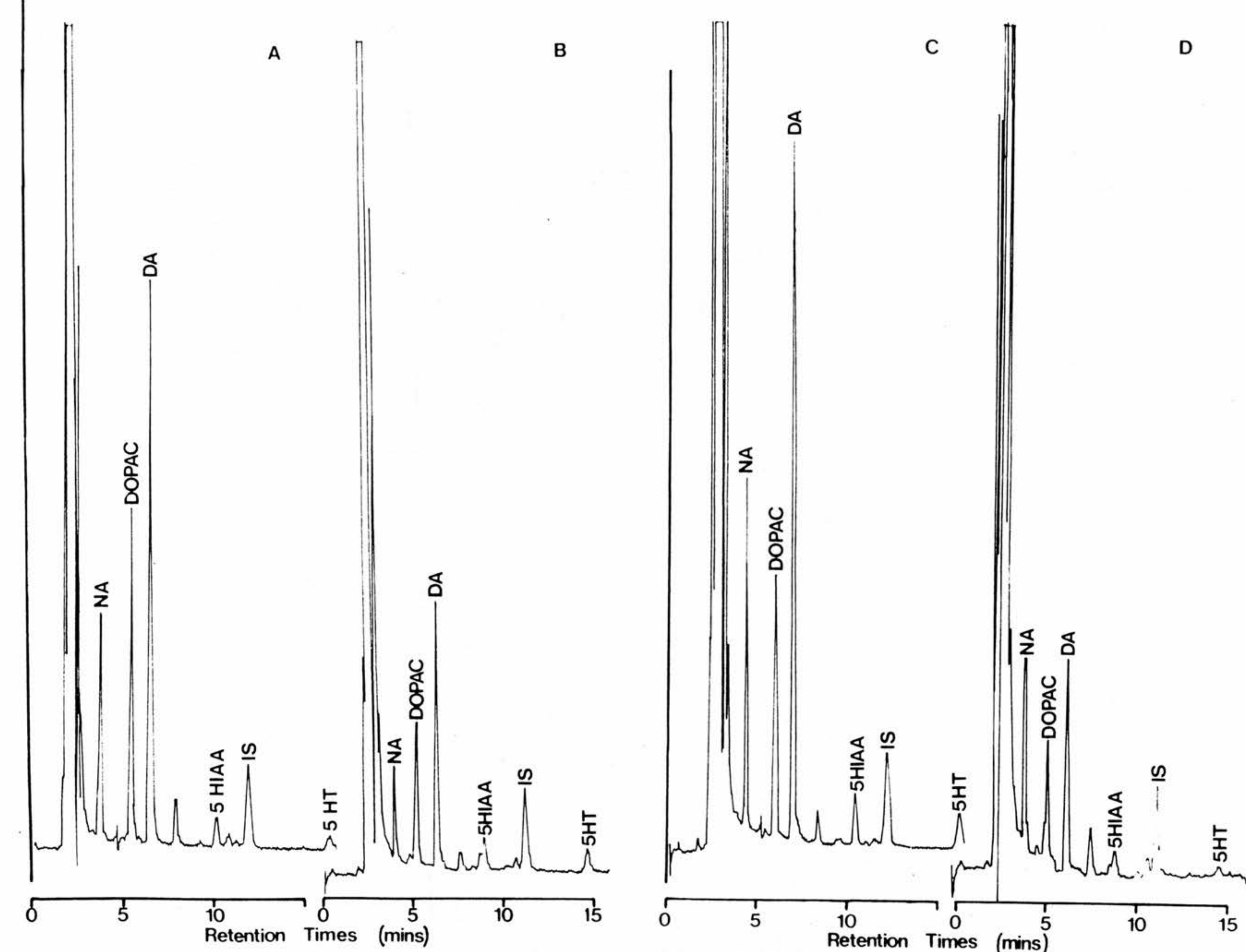
Table 9-B Content of amines and metabolites in stalk median eminence (SME) and neurointermediate lobe (NIL) 48 h after drug treatment (pg/area dissected. Mean ± S.E.M., n = number of animals per treatment group).

	Treatment group	n	NA	DA	DOPAC	5-HT	5-HIAA
NIL 48 h after injections							
	Untreated	6	229±25	2558±157	530±111	632±246	251±56
	Ascorbic acid	6	204±20	2407±130	785±100	385±32	248±52
	6-OHDA	6	124±17	1479±177	ND	403±83	219±51
	6-OHDA+DMI	7	265±34	1457±153	ND	420±24	314±65
SME 48 h after injections							
	Untreated	6	1161±141	5600±289	1860±237	483±172	356±86
	Ascorbic acid	5	913±70	5042±375	2088±500	280±58	301±50
	6-OHDA	6	584±38	2397±191	899±89	369±97	235±44
	6-OHDA+DMI	7	1130±206	2314±281	1085±90	275±28	239±49

ND - not determined

FIGURE 9-7: (A-D) Typical LCED chromatograms of extracts of SME obtained from male rats killed by decapitation 2 days after i.v. injection of (A) ascorbic acid, (B) 6-OHDA, (C) uninjected controls and (D) 6-OHDA and DMI. The identified peaks are as follows:

NA	Noradrenaline
DA	Dopamine
DOPAC	Dihydroxyphenylacetic acid
5-HT	5-hydroxytryptamine
5-HIAA	5-hydroxyindole acetic acid
IS	internal standard (n-acetyl dopamine)


A
B
C
D
NA
DOPAC
DA
5 HIAA
5HIAA
IS
5 HT
5HT
0
5
10
15
Retention Times (mins)

compared with the contents in untreated or vehicle-treated control groups. In animals treated with DMI 30 min before 6-OHDA administration there was also a significant depletion of DA after 48 h ($P < 0.001$, both areas) but NA content remained similar to the values in the control animals. DOPAC was reduced in the SME in both 6-OHDA and DMI plus 6-OHDA treated groups compared with the values in controls ($P < 0.05$ - $P < 0.005$). 5-HT and 5-HIAA content remained unchanged in all treatment groups.

9.4 DISCUSSION

The data obtained with the aid of fluorescence histochemistry (Table 9.A) were similar to those previously published (Smith and Helme, 1974; Smith et al. 1982) and showed a maximum depletion of CA fluorescence in the ME 1-2 days after the injection of 6-OHDA with a gradual recovery of fluorescence intensity to that of control animals over a subsequent 4-5 week period. The CA terminals in the NIL were not examined with the FAGLU technique in the present study, but Smith et al. (1982) have shown that in the first 2 days after the 6-OHDA treatment there was a depletion of fluorescent varicosities in the intermediate lobe of the pituitary gland, and also showed that only a few fluorescent fibres remained in the neural lobe.

Fluorescence histochemistry only yields semi-quantitative data and does not distinguish between NA and DA containing structures, and, therefore, the second series of experiments were carried out to measure the amine content of the ME and NIL directly. The results obtained with the aid of LCED confirmed the depletion of both

catecholamines from the ME and NIL of lesioned animals (Table 10.B). The magnitude of depletion (≃ 50% of the catecholamine content of the control animals) was, however, much lower than suggested by the apparent completeness of the lesion at 48 h after 6-OHDA revealed by histochemistry. The most likely explanation for the discrepancy in these results is that in the assay of catecholamines the extent of the lesion had been underestimated as a result of the inclusion in the dissected areas of regions in which, as a result of continued synthesis and axoplasmic transport, catecholamines had accumulated behind the lesioned nerve terminals (Ungerstedt, 1971a). Cuello *et al*. (1974) showed a 37% reduction in the hypothalamic content of NA, but no change in DA content 24 h after 6-OHDA treatment. However, the latter study differs in two respects from the present investigation in which amine concentration was measured in more discrete areas and a higher dose of 6-OHDA was administered (in the study of Cuello *et al*, the amine content of the whole hypothalamus was measured after the intraperitoneal injection of two, 50 mg/kg doses of 6-OHDA). The NA containing nerve terminals are reported to be more susceptible than DA terminals to the neurotoxic action of 6-OHDA (Ungerstedt, 1971b), but with the high dose used in the present study the relative magnitude of the depletion appeared similar for both types of nerve terminals.

The uptake mechanism for 5-HT has some affinity for catecholamines and a few studies have noted a degree of depletion of 5-HT after 6-OHDA administration in some species (e.g. Laguzzi, Petitjean, Pujol and Jouvet, 1971; de Montigny, Wang, Reader and Aghajanian, 1980). The present study, in agreement with the results

of the majority of previous studies of the effects of 6-OHDA in the rat (see review by Breese, 1975) showed that 5-HT-containing terminals in the ME or NIL were not affected.

DMI blocks the uptake mechanism in NA terminals and, therefore, pretreatment with DMI prevents the subsequent uptake of 6-OHDA into these terminals, thus resulting in a more specific lesion of the DA terminals (Kelly and Iversen, 1976). Within the ME, the depletion of fluorescence after 6-OHDA and DMI administration has been shown to be confined to the midline of the external layer; fluorescence intensity was reduced in the NIL and appeared unaffected in the mediobasal hypothalamus, and in the intermediate layer and lateral parts of the external layer of the ME (Sarkar, Smith and Fink, 1981; Smith et al. 1982). The turnover of dopamine in the tuberoinfundibular neurons which project to the lateral palisade zone is slower than in those neurons which project to the portal vessels in the midline of the external layer (Lofstrom, Jonsson, Wiesel and Fuxe, 1976b) and this may result in an apparent difference in the sensitivity of the DA terminals to 6-OHDA in these two regions. The data obtained in the present study with the aid of LCED confirmed that a more selective lesion of DA terminals occurred when 6-OHDA was administered after pretreatment with DMI.

In sum, the data obtained with fluorescence histochemistry or by HPLC, confirm that treatment with 6-OHDA depleted terminals in the ME of DA and NA but a 'masking' effect by the accumulation of amines proximal to the lesion made it impossible to assess fully the magnitude of the NA and DA deficit in these animals. From this arises one of several difficulties in attempting to interpret the

effects of this lesion on hormone release as the data give no indication of the extent to which the functioning of the CA terminals are affected by the lesion (i.e. the amount of transmitter which may still be released by any undamaged neurons is not known). The administration of 6-OHDA alone, results in lesions of both DA and NA terminals, possibly to differing degrees and, as will be discussed below, these two transmitters often have opposing actions in the central control of hormone release (Weiner and Ganong, 1978). Although histochemistry reveals the loss of fluorescence, there may be residual functioning in CA terminals which are depleted of their amine content, but not damaged by the 6-OHDA (Day and Willoughby, 1980). A further complication arises from the possibility of the development of receptor supersensitivity following the denervation induced by 6-OHDA. The release of amines from a few remaining undamaged CA neurons combined with an increased sensitivity of the receptors on either the pituitary cells or the terminals of peptidergic neurons in the ME, may be sufficient to maintain a relatively unchanged basal release of the pituitary hormones. It is also possible that, once developed, the receptor supersensitivity may outlast the time required for regeneration of the damaged CA terminals resulting in a period when the inhibitory or facilitatory effects of the amines are potentiated.

The plasma corticosterone concentrations (Fig. 9-1) in the untreated control animals were similar to those reported by Chiappa and Fink (1977) and suggest that the plasma samples from these animals were obtained under relatively unstressed conditions. That is, in agreement with the results of studies on the prolactin

response to stress (Mattheij and Van Pijkeren, 1977; Seggie and Brown, 1976) the time interval between the removal of the animals from the room in which they were housed and their subsequent decapitation was too short for a significant endocrine response to stress. The increases in the plasma concentration of prolactin which occurred in animals injected with vehicle alone may, however, have been due to the long term effects of the stress of the injection as several studies have indicated a similar effect in animals subjected to surgical manipulations (Ajika, Kalra, Fawcett, Krulich and McCann, 1972; Fenske and Wuttke, 1976; Harms, Langlier and McCann, 1975; Neill, 1972). Such a long term effect may require the functional integrity of ME-NIL CA terminals since, as shown in Figure 9-1, injection of 6-OHDA prevented the increase in the plasma concentration of prolactin which occurred in animals injected with vehicle alone. In support of this there is some evidence to suggest that NA neurons may be involved in the stress-induced (and also oestrogen-induced) release of prolactin (Weiner and Ganong, 1978). For example, Fenske and Wuttke (1976) showed the absence of a prolactin response to stress in animals in which 6-OHDA had been injected into the lateral ventricles 12 days previously; the basal secretion of prolactin in these animals was elevated. If, as discussed earlier (Chapter 5), DA is the principal prolactin inhibitory factor (PIF) then an increase in prolactin secretion would have been expected in the animals injected with 6-OHDA in the present experiment. That this was not observed suggests that either the normal inhibition of prolactin was maintained by an alternative PIF or that the lesion of the DA terminals was incomplete. This

question could partly be resolved by the direct measurement of DA concentrations in hypophysial portal blood.

The stress of injection may also explain the increased concentrations of plasma corticosterone in animals treated with vehicle compared with those in untreated animals, although in this case 6-OHDA prevented the increase only on days 2 and 7. The findings of an increase in the plasma concentration of corticosterone 24 h after injection of 6-OHDA is in agreement with the data obtained by Cuello et al. (1974) who interpreted this as indicating that ACTH secretion is inhibited by the influence of NA terminals within the ME. However, the data from a number of studies in both rats and dogs, whilst indicating an inhibition of ACTH by central NA neurons, suggest that these inhibitory neurons terminate not in the ME but in a hypothalamic site inside the blood-brain barrier (Weiner and Ganong, 1978). In the dog intraventricular, but not systemic administration of NA results in the inhibition of the release of ACTH in response to stress (Van Loon, Hilger, King, Boryczka and Ganong, 1971; Van Loon, Scapagnini, Cohen and Ganong, 1971) and data from pharmacological studies suggest that α-adrenergic receptors in the dorsal hypothalamus are involved in this inhibitory mechanism (Ganong, Kramer, Salmon et al, 1976). Earlier studies (Halasz, Slusher and Gorski, 1967) also demonstrated that plasma corticosterone concentrations were elevated after the removal of the NA input to the rat hypothalamus as a result of total hypothalamic deafferentation.

Central CA neurons appear to play an important role in the mechanism of the spontaneous (Fink and Geffen, 1978; Ramirez, Feder

and Sawyer, 1984) and steroid-induced gonadotrophin surge (Wise, Rance and Barraclough, 1981; Rance, Wise and Barraclough, 1981a). In studies in which amine turnover was assessed by the disappearance of CA fluorescence after the inhibition of tyrosine hydroxylase it was shown that DA turnover in the lateral palisade zone (LPZ) of the ME was lower on pro-oestrus than at all other times during the oestrous cycle; NA turnover in the subependymal layer was increased during pro-oestrus (Lofstrom, 1977). Similar results were obtained using a radioenzymatic assay (Rance, Wise, Selmanoff and Barraclough, 1981b), although Rance et al. (1981b) also showed a small but significant increase in DA turnover before the surge. The turnover rates of DA and NA were shown to decrease and increase respectively in the median eminence of pre-pubertal rats in which an LH surge was induced by the administration of pregnant mare serum gonadotrophin [PMSG] (Agnati, Fuxe, Lofstrom and Hokfelt, 1977); the intravenous injection of 6-OHDA after pretreatment with DMI resulted in an enhancement of the LH surge in PMSG-treated animals (Sarkar et al. 1981). Thus while there is considerable evidence that in the female rat the tuberoinfundibular DA system inhibits gonadotrophin secretion, the present data indicate that the terminals in the ME-NIL may have little to do with controlling the basal secretion of the gonadotrophins in the male, though transient changes in the median eminence content of NA and DA may occur during the first 8 h after orchidectomy (Chiocchio, Negro-Vilar and Tramezzani, 1976).

The central control of thyrotrophin secretion may also be mediated by facilitatory NA and inhibitory DA neurons (Krulich, 1979). Following thyroidectomy, tyrosine hydroxylase activity was

significantly increased in the periventricular, arcuate and dorsomedial nuclei, the medial forebrain bundle and in the ME (Kizer, Muth and Jacobowitz, 1976). In the studies of Kizer *et al*. (1976), bilateral lesion of the ventral NA tracts prevented the increase in tyrosine hydroxylase in all of the areas examined with the exception of the ME and, since this lesion had resulted in a greater than 85% fall in dopamine β-hydroxylase activity in the ME, it was concluded that the majority of the endocrine responsive neurons in the ME were dopaminergic. However, in another study, no change in DA turnover in the ME was observed 4 weeks after thyroidectomy (Fuxe, Hokfelt, Andersson *et al*, 1978). The physiological significance of the direct effects of DA and NA on the basal and TRH-stimulated release of thyrotrophin seen in some (Peters, Foord, Dieguez, Scanlon and Hall, 1983; Dieguez, Foord, Peters, Hall and Scanlon, 1984), but not all, *in vitro* studies (Price, Grossman, Besser and Rees, 1983) remains to be elucidated. The marked increase in the plasma concentration of thyrotrophin seen in the present studies 24 h after 6-OHDA administration suggest that the ME-NIL CA system does moderate the secretion of thyrotrophin.

Although several studies have shown that, under stress, the secretion of thyrotrophin is inversely related to the secretion of ACTH (Brown-Grant, Harris and Reichlin, 1954; Brown-Grant, 1960; Guillemin, 1968), the release of the two hormones is not always inversely related (Pamenter and Hedge, 1980) and this is illustrated by the present results in which the secretion of both thyrotrophin and corticosterone were increased 24 h after 6-OHDA administration. Similarly, although stress stimulates the release of both prolactin

and ACTH (Harms, Langlier and McCann, 1975) the present data indicate that under relatively unstressed conditions the secretion of these two hormones is dissociated.

Table 9-C Summary of statistical analysis. All comparisons not shown were non-significant (unpaired t test)

Plasma hormone concentrations

Hormone	Day	Comparison	P
Prolactin	1	Vehicle vs untreated	< 0.05
	2	Vehicle vs untreated	< 0.001
	14	Vehicle vs untreated	< 0.01
	21	Vehicle vs untreated	< 0.025
TSH	1	6-OHDA vs untreated	< 0.01
	1	6-OHDA vs vehicle	< 0.01
Corticosterone	1	6-OHDA vs untreated	< 0.02
	2	6-OHDA vs vehicle	< 0.01
	2	Vehicle vs untreated	< 0.01
	7	6-OHDA vs vehicle	< 0.02
	7	Vehicle vs untreated	< 0.05
	14	Vehicle vs untreated	< 0.01
	21	Vehicle vs untreated	< 0.001
	36	Vehicle vs untreated	< 0.001
FSH	36	6-OHDA vs untreated	< 0.005
	36	Vehicle vs untreated	< 0.001

Pituitary hormone content

Hormone	Day	Comparison	P
Prolactin	1	6-OHDA vs untreated	< 0.05
	1	Vehicle vs untreated	< 0.05
	7	6-OHDA vs untreated	< 0.005
	7	Vehicle vs untreated	< 0.005
	14	6-OHDA vs vehicle	< 0.001
	21	6-OHDA vs vehicle	< 0.01
FSH	36	6-OHDA vs untreated	< 0.005
	36	Vehicle vs untreated	< 0.001
LH	36	6-OHDA vs untreated	< 0.005
	36	Vehicle vs untreated	< 0.005

Pituitary hormone concentrations

Hormone	Day	Comparison	P
Prolactin	1	6-OHDA vs untreated	< 0.025
	1	Vehicle vs untreated	< 0.005
	7	6-OHDA vs untreated	< 0.001
	7	Vehicle vs untreated	< 0.001
	14	6-OHDA vs vehicle	< 0.005
	21	6-OHDA vs vehicle	< 0.02
FSH	36	6-OHDA vs untreated	< 0.001
	36	Vehicle vs untreated	< 0.001
LH	36	6-OHDA vs untreated	< 0.005
	36	Vehicle vs untreated	< 0.001

Table 9-C (cont.) Summary of statistical analysis. All comparisons not shown were non-significant.

Amine (or metabolite) content of the median eminence or neurointermediate lobe.

Median eminence

	Day	Comparison	P
Noradrenaline	2	6-OHDA vs vehicle	< 0.005
	2	6-OHDA vs untreated	< 0.005
	2	6-OHDA vs 6-OHDA + DMI	< 0.05
Dopamine	2	6-OHDA vs vehicle	< 0.001
	2	6-OHDA vs untreated	< 0.001
	2	6-OHDA + DMI vs vehicle	< 0.001
	2	6-OHDA + DMI vs untreated	< 0.001
DOPAC	2	6-OHDA vs vehicle	< 0.05
	2	6-OHDA vs untreated	< 0.005
	2	6-OHDA + DMI vs vehicle	< 0.05
	2	6-OHDA + DMI vs untreated	< 0.01

Neurointermediate lobe

	Day	Comparison	P
Noradrenaline	2	6-OHDA vs vehicle	< 0.02
	2	6-OHDA vs untreated	< 0.01
	2	6-OHDA vs 6-OHDA + DMI	< 0.005
Dopamine	2	6-OHDA vs vehicle	< 0.005
	2	6-OHDA vs untreated	< 0.005
	2	6-OHDA + DMI vs vehicle	< 0.001
	2	6-OHDA + DMI vs untreated	< 0.001

CHAPTER 10

Summary and Conclusions

Many biologically active peptides are synthesized as components of a larger precursor molecule. These precursors may contain multiple copies of the same peptide (e.g. TRH and met-enkephalin) or, as a result of enzymatic cleavage at different sites within the molecule, may generate extended forms of the peptide (e.g. somatostatin-28) which may also show biological activity. The main aims of this thesis were to investigate (i) the immunoreactive forms of somatostatin (SS), TRH and LHRH which are released into hypophysial portal vessel blood, (ii) the role of TRH in the control of thyrotrophin and prolactin release, (iii) some aspects of the mechanism of action of LHRH on the pituitary gland of hypogonadal (*hpg*) mice, (iv) some aspects of the effects of cannabinoids, opioids and suckling on the release of LHRH and LH, and (v) some aspects of the role of catecholamines in the control of pituitary hormone secretion.

The application of an electrical stimulus to the ME of male rats anaesthetized with urethane resulted in a 6 to 7-fold increase in both the concentration and the total amount of somatostatin released into portal blood. High performance liquid chromatography (HPLC) followed by radioimmunoassay using an antiserum directed against the mid-region of the SS-14 molecule showed the presence of two peaks of immunoreactivity in extracts of portal plasma, and these peaks corresponded with SS-14 and SS-28 respectively. The amount of SS-28 in portal plasma was, on a molar basis, similar to or greater than that of SS-14. These findings are supported by *in vitro* studies showing the release of both forms of SS from the median eminence in response to depolarizing stimuli (Kewley *et al*, 1981; Pierotti *et al*, 1985).

However, in contrast to the ME, SS-14 was the predominant form of SS released from blocks of hypothalamic tissue *in vitro* suggesting the possibility of at least two types of SS-containing neurons which process the SS-precursor in a different manner (Pierotti *et al*, 1985). The fact that both SS-14 and SS-28 are released into portal blood, and SS-28 is as or more potent than SS-14 in inhibiting the release of growth hormone, insulin and glucagon and the different pituitary receptor affinities for the two peptides suggests that SS-28 as well as SS-14 is an hormonally active peptide. Further studies are necessary to define the physiological functions of SS-14 and SS-28 and to correlate variations in the release of these peptides with varying secretion rates of growth hormone (and thyrotrophin) under different experimental conditions.
Radioimmunoassay of extracts of portal plasma using an antiserum which recognizes the C-terminus of SS-28(1-12) but which does not recognize either SS-14 or SS-28, showed the presence of SS-28(1-12)-IR in portal blood in higher concentrations than were present in peripheral blood. The release of SS-28(1-12) into portal blood was also increased 4 to 5-fold by electrical stimulation of the ME. No biological activity of SS- 28(1-12) has yet been found and possibly it may be a non-functional fragment which is co-secreted with SS-14.

High performance liquid chromatography revealed the presence of a single peak IR form of TRH in acid or ethanolic extracts of rat hypothalami which corresponded in retention time to synthetic TRH. In contrast, chromatography resolved the TRH-IR in ethanol extracts of rat hypophysial portal vessel blood into three components, the

first of which corresponded in retention time to the authentic tripeptide. The additional peaks of IR material did not correspond to the major metabolites of TRH since the anti-TRH serum used for the RIA does not cross-react with either diketopiperazine or with the free acid form of TRH. Two synthetic analogues of TRH, which showed high cross-reactivity with the antiserum, were also excluded as potential sources of the additional IR material. The additional IR peaks did not originate from the pituitary gland since HPLC of pituitary gland extracts revealed the presence of only a single IR peak corresponding to TRH, and all three IR components were present in extracts of hypophysial portal blood collected from rats from which the pituitary gland had been removed. The concentration of TRH-IR in peripheral blood was generally too low to be detected in the RIA, however when present in measurable quantities, traces of all three IR peaks were detected. Only a single IR peak was obtained after addition of either synthetic TRH or hypothalamic extracts to peripheral blood and no additional peaks of IR material were generated by incubating hypothalamic extracts with peripheral blood. It is possible that a precursor of TRH, which itself is not immunoreactive, could on release into portal blood generate intermediates of TRH biosynthesis which are immunoreactive, or alternatively on release TRH may undergo some minor modification by reactions which might alter its retention time on HPLC. However, in the light of the above observations of the effect of incubating hypothalamic extracts with peripheral blood it appears unlikely that the additional IR material is formed by the action of blood-borne enzymes; rather these conversions may occur during the release

process itself.

Using the same HPLC system as used for the TRH studies and RIAs with two highly specific anti-LHRH sera, only a single peak of LHRH-IR was present in extracts of hypophysial portal blood. The LHRH-IR peak corresponded in retention time to synthetic LHRH. This may suggest that LHRH is processed to completion before entering the portal vessels, but a possiblity remains that due to losses during the extraction and chromatography of LHRH-IR, small quantities of a precursor or intermediate form of LHRH may not have been detected.

The nature of the IR forms of LHRH in extracts of hypothalami from rats and from 'normal' and hypogonadal (*hpg*) mice was also examined, and as shown for rat hypophysial portal blood a single IR peak was present in hypothalamic extracts from both rats and 'normal' mice. This peak corresponded in retention time to synthetic LHRH. No LHRH-IR was detected in hypothalamic extracts from the *hpg* strain of mice which confirms the results of previous studies of crude hypothalamic extracts (Cattanach *et al*, 1977). In spite of the total LHRH deficit in the *hpg* mice a small amount of gonadotrophin is synthesized in the anterior pituitary gland of the mutant. Saline extracts of pituitary glands from normal and *hpg* mice were assayed for LH by the testosterone production assay. The extract of *hpg* pituitary tissue stimulated testosterone production by mouse Leydig cells in a dose-dependent manner which paralleled that of normal mouse pituitary. Extracts of normal mouse pituitary were about 20 times as potent as those of *hpg* pituitary, a relative potency similar to that determined by RIA. The *hpg* gonadotrophes have receptors for LHRH, albeit in reduced numbers (Young *et al*,

1983) and the mutant is capable of releasing LH in response to exogenous LHRH. Both the releasing action and self-priming effect of LHRH were not significantly different in hpg compared with normal mice. Raised plasma concentrations of oestradiol-17β reduced the pituitary responsiveness to LHRH in normal, but not in hpg mice. Taken together, these data showed that LHRH is not essential for the biosynthesis of some biologically active and immunoreactive LH and that in the mouse neither long-term exposure to normal levels of LHRH nor a normal pituitary content of LH are necessary for either the releasing or the priming action of LHRH.

The secretion of LHRH into hypophysial portal blood was studied in female rats anaesthetized with a steroidal anaesthetic (alphaxolone; 'Saffan') which when given i.p., in contrast to other anaesthetics, does not block the pro-oestrous surge of LHRH. The administration of Δ^1-tetrahydrocannabinol (THC) during the critical period (c.a. 14.00-16.00 h) of pro-oestrus blocks the pre-ovulatory surge of gonadotrophins and ovulation (Ayalon et al, 1977). The present results confirmed that injection of THC at 13.30 h on the afternoon of pro-oestrus blocked the expected surge of LH and showed that this was a consequence of a central action of THC in reducing the output of LHRH into portal blood. The endogenous opioid peptides also inhibit the secretion of LH and may play a functional role in the regulation of LHRH secretion (Kalra and Kalra, 1983, 1984). Pulsatile release of LH is infrequent on the day of oestrus, but Fox and Smith (1985) showed that regular pulses of LH secretion could be induced by continuous infusion of the opioid antagonist, naloxone. However, in the present experiments no

difference between the secretion of LHRH or LH was apparent in oestrous rats infused with naloxone and saline and similarly naloxone did not alter LHRH output or the magnitude of the pro-oestrous surge of LH.

The plasma and pituitary concentrations of LH and FSH are suppressed during lactation therefore the release of LHRH was examined in suckled and non-suckled lactating rats. Plasma concentrations of LH were low in both groups of rats and no difference in the secretion of LHRH into portal vessel blood was apparent. Oxytocin was shown to be present in hypophysial portal blood in greater concentrations than in peripheral blood confirming previous reports of its secretion from nerve terminals in the external layer of the median eminence (Gibbs, 1984; Sarkar and Gibbs, 1984; Horn, Robinson and Fink, 1985c). As with LHRH, there was no difference in the concentration of oxytocin in portal blood between suckled and non-suckled rats.

In contrast to the large increase in the release of LHRH (Chiappa et al, 1977) and SS in response to electrical stimulation of the ME, stimulation of either the terminals of the TRH-containing neurons in the ME, or the cell bodies of these neurons in the PVN, did not increase the release of either authentic TRH or the associated TRH-IR material. The amount of TRH released into portal blood during a 1 h collection period was high relative to the hypothalamic TRH content. In this respect the secretion of TRH differs from both SS and LHRH; although authentic TRH constituted about 37% of the total TRH-IR in portal blood, the amount released during a 1 h collection period (1.7 ng) in relation to hypothalamic

content (~ 5 ng) was high (~ 34%) compared with that of LHRH (~ 0.6%) and SS (~ 0.3%). The high secretion rate of TRH did not result in a depletion of its hypothalamic content, which suggests that the turnover rate for this tripeptide is correspondingly higher. The capacity for more rapid synthesis, processing and release of TRH than LHRH or SS may be related to the presence of multiple copies of TRH in the precursor molecule compared with the single copies of the LHRH and SS peptides contained in their respective precursors. As shown here for TRH, previous studies showed that stimulation of the ME or PVN did not result in the increased release into portal blood of VIP (Brar et al, 1985a) or oxytocin and vasopressin (Horn et al, 1985c). It is likely that the apparent lack of any effect of hypothalamic stimulation on the release of these three peptides is due to their maximal secretion into portal blood following sectioning of the pituitary stalk which thus renders it impossible to drive the system further by electrical stimulation.

In order to examine the thyrotrophin and prolactin responses to electrical stimulation of the ME or PVN, the ME and pituitary gland was exposed but the pituitary stalk not cut. Samples of peripheral blood were withdrawn from the external jugular vein at regular intervals before and after the period of stimulation. Stimulation of either the PVN or the ME resulted in a massive increase in the plasma concentration of prolactin, however possibly due to the trauma of surgery, increased secretion of thyrotrophin in response to stimulation was not seen unless a 4 h delay period was interposed between the completion of surgery and the onset of stimulation.

This delay of 4 h between surgery and blood collection was used to permit plasma concentrations of thyrotrophin and prolactin to return to steady baseline after the stress of surgery and also to allow comparison of the electrical stimulation studies with the suckling model referred to below. As an alternative to direct measurements of TRH secretion, passive immunization with anti-TRH serum was used to investigate the role of TRH in mediating the thyrotrophin and prolactin response to electrical stimulation of the hypothalamus and the prolactin response to suckling in lactating rats. Injection of sheep anti-TRH serum. but not control non-immune or anti-bovine serum albumin, blocked the rise in plasma thyrotrophin concentration in response to stimulation of either the ME or PVN, but did not block the rise in plasma prolactin concentration. In anaesthetized lactating female rats, the suckling stimulus produced a significant increase in the plasma prolactin concentration, but did not alter the plasma thyrotrophin concentration. Injection of anti-TRH serum, but not control serum, significantly decreased the basal release of thyrotrophin, but did not abolish the prolactin response to suckling. These results showed that TRH is the principal mediator of the neural control of thyrotrophin release in the rat, but is not crucial for the release of prolactin in response to either hypothalamic stimulation or suckling.

The secretion of several anterior pituitary hormones was examined in male rats in which the CA-containing neurons of the ME and NIL had been lesioned by the intravenous injection of a high dose of 6-OHDA. The changes in the CA contents of both the hypothalamus and ME-NIL were monitored by CA-fluorescence

histochemistry and also by HPLC with electrochemical detection. Fluorescence histochemistry revealed a complete depletion of NA and DA from nerve terminals in the ME and NIL (whilst the remainder of the hypothalamus remained relatively unaffected) 24-48 h after the injection of 150 mg/kg 6-OHDA. When measured directly by HPLC, the magnitude of the depletion (~ 50%) appeared less than was suggested by histochemistry, probably as a result of the accumulation of amines behind the lesioned terminals. There was a progressive return to a normal fluorescent appearance of the ME over a period of several weeks. Regenerating nerve terminals were visible in the ME after 7 days, and by 36 days after injection the fluorescent appearance of the ME could not be distinguished from untreated or ascorbic acid injected control animals. Studies of plasma and pituitary hormone concentrations measured at various time intervals after injection of either 6-OHDA or ascorbic acid (vehicle) showed that:

1. Plasma concentrations of prolactin were similar in 6-OHDA and untreated control animals, but were increased in ascorbic acid injected animals. This increased secretion of prolactin in ascorbic acid treated rats was possibly due to a long term stress effect following the injection which might be mediated by way of facilitatory NA terminals in the ME-NIL which were lesioned in the 6-OHDA treated animals.

2. Plasma corticosterone concentrations were increased in rats killed 24 h after 6-OHDA injection supporting previously reported findings (Cuello _et al_, 1974) that the secretion of ACTH is inhibited by hypothalamic CA neurons which were depleted by 6-OHDA.

3. The CA neurons of the ME-NIL may also inhibit the secretion of thyrotrophin.

4. Plasma and pituitary concentrations of LH and FSH were relatively unaltered in lesioned rats showing that the CA neurons terminating in the ME-NIL are not important in the control of basal secretion of these hormones in the male rat, although in the female rats these neurons may be important in the control of LH secretion.

Measurements of CA content in the ME and NIL of animals which had been pretreated with desipramine (to block the uptake of 6-OHDA into NA terminals) provided direct confirmation of the fact that a selective lesion of DA neurons could be produced by this treatment. As shown by studies of gonadotrophin secretion in female rats (Sarkar et al, 1981) this model may be of further use for studying aminergic-peptidergic interactions in the hypothalamic control of hormone release from the anterior pituitary gland.

In conclusion, this thesis has described and illustrated some of the interactions which occur between peptidergic and aminergic neurons in the regulation of anterior pituitary hormone secretion. In comparison with techniques such as passive immunization, the technique for collection of hypophysial portal blood has several advantages for studies of these complex interactions between a number of inhibitory and stimulatory factors in that, with the aid of appropriate, sensitive assay systems, variations in the secretion of a number of amine, amino acid and peptide neurotransmitters may be studied simultaneously. However, collection of portal blood requires anaesthesia and entails surgical trauma and therefore may be more suited to studies of the release of some peptides (e.g. LHRH

and SS) than other peptides (e.g. TRH and VIP). The studies of the immunoreactive forms of LHRH, SS and TRH present in portal blood show that, in addition to facilitating studies of their physiological function, the measurement of peptides in hypophysial portal blood provides a useful approach for determining which of the hypothalamic peptides derived from a precursor are secreted and which are not, and also for examining the possibilities of minor modifications of these peptides during or after their release from the nerve terminals in the median eminence.

REFERENCES

ABE, H., ENGLER, D., MOLITCH, M.E., BOLLINGER-GRUBER, J. and REICHLIN, S. (1985) Vasoactive intestinal peptide is a physiological mediator of prolactin release in the rat. Endocrinology 116, 1383-1390.

ABRAHAM, G.N. and PODELL, D.N. (1981) Pyroglutamic acid. Mol. Cell. Biochem. 38, 181-190.

ADAMS, K. (1969) Electrochemistry of solid electrodes. New York: Marcel Dekker.

ADLER, B.A. and CROWLEY, W.R. (1984) Modulation of luteinizing hormone release and catecholamine activity by opiates in the female rat. Neuroendocrinology 38, 248-253.

AGNATI, L., FUXE, K., LOFSTROM, A. and HOKFELT, T. (1977) Dopaminergic drugs and ovulation: studies on PMS-induced ovulation and changes in median eminence DA and NE turnover in immature female rats. In Advances in biochemical pharmacology. Vol. 16, pp. 159-168, (Eds) Costa, E. and Gessa, G.L. New York: Raven Press.

AIYER, M.S. (1974) Changes in the sensitivity of the anterior pituitary gland to gonadotrophin-releasing factors during the oestrous cycle of the rat. D. Phil. Thesis: Oxford University.

AIYER, M.S., CHIAPPA, S.A. and FINK, G. (1974) A priming effect of luteinizing hormone releasing factors on the anterior pituitary gland in the female rat. J. Endocrinol. 62, 573-588.

AIYER, M.S. and FINK, G. (1974) The role of sex steroid hormones in modulating the responsiveness of the anterior pituitary gland to luteinizing hormone releasing factor in the female rat. J. Endocrinol. 62, 553-572.

AIYER, M.S., FINK, G. and GREIG, F. (1974) Changes in the sensitivity of the pituitary gland to luteinizing hormone releasing factor (LRF) during the oestrous cycle of the rat. J. Endocrinol. 60, 47-64.

AIZAWA, T. and GREER, M.A. (1981) Delineation of the hypothalamic area controlling thyrotropin secretion in the rat. Endocrinology 109, 1731-1738.

AJIKA, K. and HOKFELT, T. (1975) Projections to the median eminence and the arcuate nucleus with special reference to monoamine systems: effects of lesions. Cell Tissue Res. 158, 15-35.

AJIKA, K.S., KALRA, S.P., FAWCETT, C.P., KRULICH, L. and McCANN, S.M. (1972) The effect of stress and Nembutal on plasma levels of gonadotropin and prolactin in ovariectomized rats. Endocrinology 90, 707-715.

AKABORI, A. and BARRACLOUGH, C.A. (1986a) Gonadotropin responses to naloxone may depend upon spontaneous activity in noradrenergic neurons at the time of treatment. Brain Res. 362, 55-62.

AKABORI, A. and BARRACLOUGH, C.A. (1986b) Effects of morphine on luteinizing hormone secretion and catecholamine turnover in the hypothalamus of estrogen-treated rats. Brain Res. 362, 221-226.

ALBERTI, K.G.M.M., CHRISTENSEN, S.E., IVERSEN, J., SEYER-HANSEN, K., CHRISTENSEN, N.J., HANSEN, A.P., LUNDBACK, K. and ORSKOV, H. (1973) Inhibition of insulin secretion by somatostatin. Lancet ii, 1299-1301.

AMBACH, G., PALKOVITS, M. and SZENTAGOTHAI, J. (1976) Blood supply of the rat hypothalamus. IV. Retrochiasmatic area, median eminence, arcuate nucleus. Acta Morphol. Hung. 29, 93-119.

AMENOMORI, Y., CHEN, C.L. and MEITES, J. (1970) Serum prolactin levels during the different reproductive states. Endocrinology 86, 506-510.

ANDERSON, R.A. and MITCHELL, R. (1986) Effects of γ-aminobutyric acid receptor agonists on the secretion of growth hormone, luteinizing hormone, adrenocorticotrophic hormone and thyroid-stimulating hormone from the rat pituitary gland in vitro. J. Endocrinol. 108, 1-8.

ARANCIBIA, S., TAPIA-ARANCIBIA, L., ASSENMACHER, I. and ASTIER, H. (1983) Direct evidence of short-term cold-induced TRH release in the median eminence of unanesthetized rats. Neuroendocrinology 37, 225-228.

ARIMURA, A., SATO, H., DUPONT, A., NISHI, N. and SCHALLY, A.V. (1975) Somatostatin: abundance of immunoreactive hormone in rat stomach and pancreas. Science 189, 1007-1009.

ARIMURA, A. and SCHALLY, A.V. (1976) Increase in basal and thyrotropin-releasing hormone (TRH)-stimulated secretion of thyrotropin (TSH) by passive immunization with antiserum to somatostatin in rats. Endocrinology 98, 1069-1072.

ASCH, R.H., FERNANDEZ, E.O., SMITH, C.G., PAUERSTEIN, C.J. (1979) Precoital single doses of Δ^9-tetrahydrocannabinol block ovulation in the rabbit. Fertil. Steril. 31, 331-334.

AVERILL, R.L.W. (1969) Depression of thyrotropin releasing factor induction of thyrotropin release by thyroxine in small doses. Endocrinology 85, 67-71.

AVERILL, R.L.W., SALAMAN, D.F. and WORTHINGTON, W.C. Jr. (1966) Thyrotrophin releasing factor in hypophyseal portal blood. Nature (Lond.) 211, 144-145.

AYALON, D., NIR, I., CORDOVA, T., BAUMINGER, S., PUDER, M., NAOR, Z., KASHI, R., ZOR, U., HARELL, A. and LINDNER, H.R. (1977) Acute effect of Δ^1-tetrahydrocannabinol on the hypothalamo-pituitary-ovarian axis in the rat. Neuroendocrinology 23, 31-42.

AZHAR, S., REEL, J.R., PASTUSHOK, C.A. & MENON, K.M.J. (1978) LH biosynthesis and secretion in rat anterior pituitary cell cultures: stimulation of LH glycosylation and secretion by GnRH and an agonistic analogue and blockade by an antagonistic analogue. Biochem. Biophys. Res. Commun. 80, 659-666.

BAKER, B.L. and YEN, Y.-Y. (1976) The influence of hypophysectomy on the stores of somatostatin in the hypothalamus and pituitary stem. Proc. Soc. Exp. Biol. Med. 151, 599-602.

BAKHIT, C., BENOIT, R. and BLOOM, F.E. (1983a) Release of somatostatin-28(1-12) from rat hypothalamus in vitro. Nature (Lond.) 301, 524-526.

BAKHIT, C., BENOIT, R. and BLOOM, F.E. (1983b) Effects of cysteamine on pro-somatostatin related peptides. Regul. Pept. 6, 169-177.

BAKHIT, C., KODA, L., BENOIT, R., MORRISON, J.H. and BLOOM, F.E. (1984) Evidence for selective release of somatostatin-14 and somatostatin-28(1-12) from rat hypothalamus. J. Neurosci. 4, 411-419.

BARAM, T., KOCH, Y. and HAZUM, E. (1977) Gonadotropin-releasing hormone in milk. Science 198, 300-301.

BARNEA, A. and PORTER, J.C. (1975) Demonstration of a macromolecule cross-reacting with antibodies to luteinizing hormone releasing hormone and its tissue distribution. Biochem. Biophys. Res. Commun. 67, 1346-1352.

BARRACLOUGH, C.A. and SAWYER, C.H. (1955) Inhibition of the release of pituitary ovulating hormone in the rat by morphine. Endocrinology 57, 329-337.

BARRACLOUGH, C.A. and WISE, P.M. (1982) The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion. Endocr. Rev. 3, 91-119.

BARRY, J., DUBOIS, M.P. and POULAIN, P. (1973) LRF-producing cells of the mammalian hypothalamus. A fluorescent antibody study. Z. Zellforsch. mikrosk. Anat. 146, 351-366.

BAUER, K. and LIPMAN, S. (1976) Attempts toward biosynthesis of the thyrotropin-releasing hormone and studies on its breakdown in hypothalamic tissue preparations. Endocrinology 99, 230-242.

BAXTER, J.D., EBERHARDT, N.L., APRILETTI, J.W., JOHNSSON, L.K., IVARIE, R.D., SCHACHTER, B.S., MORRIS, J.A., SEEBURG, P.H., GOODMAN, H.M., LATHAM, K.R., POLANSKY, J.R. and MARTIAL, J.A. (1979) Thyroid hormone receptors and responses. Recent Prog. Horm. Res. 35, 97-147.

BEAUDET, A. and DESCARRIES, L. (1979) Radioautographic characterization of a serotonin-accumulating nerve cell group in adult rat hypothalamus. Brain Res. 160, 231-243.

BELANGER, A., LABRIE, F., BORGEAT, P., SAVARY, M., COTE, J., DROUIN, J., SCHALLY, A.V., COY, D.H., COY, E.J., IMMER, M., SESTAN, J.K., NELSON, V. and GOTZ, M. (1974) Inhibition of growth hormone and thyrotropin release by growth hormone-release inhibiting hormone. Mol. Cell. Endocrinol. 1, 329-339.

BELCHETZ, P.E., GREDLEY, G., BIRD, D. and HIMSWORTH, R.L. (1978) Regulation of thyrotrophin secretion by negative feedback of tri-iodothyronine on the hypothalamus. J. Endocrinol. 76, 439-448.

BEN-JONATHAN, N. (1980) Catecholamines and pituitary prolactin release. J. Reprod. Fertil. 58, 501-512.

BEN-JONATHAN, N., NEILL, M.A., ARBOGAST, L.A., PETERS, L.L. and HOEFER, M.T. (1980) Dopamine in hypophysial portal blood: relationship to circulating prolactin in pregnant and lactating rats. Endocrinology 106, 690-696.

BEN-JONATHAN, N., OLIVER, C., WEINER, H.J., MICAL, R.S. and PORTER, J.C. (1977) Dopamine in hypophysial portal plasma of the rat during the estrous cycle and throughout pregnancy. Endocrinology 100, 452-458.

BENOIT, R., BOHLEN, P., ESCH, F. and LING, N. (1984) Neuropeptides derived from prosomatostatin that do not contain the somatostatin-14 sequence. Brain Res. 311, 23-29.

BENOIT, R., BOHLEN, P., LING, N., BRISKIN, A., ESCH, F., BRAZEAU, P., YING, S.Y and GUILLEMIN, R. (1982a) Presence of somatostatin-28 (1-12) in hypothalamus and pancreas. Proc. Natl. Acad. Sci. U.S.A. 79, 917-921.

BENOIT, R., LING, N., ALFORD, B. and GUILLEMIN, R. (1982b) Seven peptides derived from prosomatostatin in rat brain. Biochem. Biophys. Res. Commun. 107, 944-950.

BENOIT, R., LING, N., BAKHIT, C., MORRISON, J.M., ALFORD, B. and GUILLEMIN, R. (1982c) Somatostatin-28(1-12)-like immunoreactivity in the rat. Endocrinology 111, 2149-2151.

BERELOWITZ, M., FIRESTONE, S.L. and FROHMAN, L.A. (1981) Effects of growth hormone excess and deficiency on hypothalamic somatostatin content and release and on tissue somatostatin distribution. Endocrinology 109, 714-719.

BERELOWITZ, M., MAEDA, K., HARRIS, S. and FROHMAN, L.A. (1980) The effect of alterations in the pituitary-thyroid axis on hypothalamic content and in vitro release of somatostatin-like immunoreactivity. Endocrinology 107, 24-29.

BERELOWITZ, M., SZABO, M., FROHMAN, L.A., FIRESTONE, S., CHU, L. and HINTZ, R.L. (1981) Somatostatin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. Science 212, 1279-1281.

BERGLAND, R.M. and PAGE, R.B. (1978) Can the pituitary secrete directly to the brain? (Affirmative anatomical evidence). Endocrinology 102, 1325-1338.

BERGLAND, R.M. and PAGE, R.B. (1979) Pituitary-brain vascular relations: a new paradigm. Science 204, 18-24.

BHANOT, R. and WILKINSON, M. (1984) The inhibitory effect of opiates on gonadotrophin secretion is dependent upon gonadal steroids. J. Endocrinol. 102, 133-141.

BICKNELL, R.J. (1985) Endogenous opioid peptides and hypothalamic neuroendocrine neurones. J. Endocrinol. 107, 437-446.

BICKNELL, R.J. and LENG, G. (1981) Relative efficiency of neural firing patterns for vasopressin release in vitro. Neuroendocrinology 33, 295-299.

BIGGINS, J.A., DAS, S., DODD, P.R., EDWARDSON, J.A., HARDY, J.A., McDERMOTT, J.R. and SMITH, A.I. (1983) Studies on the release, degradation and presynaptic actions of thyrotropin-releasing hormone. In Thyrotropin-Releasing Hormone. pp. 241-251 (Eds) Griffiths, E.C. and Bennet, G.W. New York: Raven Press.

BJORKLUND, A., FALCK, B., HROMEK, F., OWMAN, C. and WEST, K.A. (1970) Identification and terminal distribution of the tuberohypophyseal monoamine systems in the rat by means of stereotaxic and microfluorimetric techniques. Brain Res. 17, 1-23.

BJORKLUND, A., FALCK, B., NOBIN, A. and STENEVI, V. (1973a) Organization of the dopamine and noradrenaline innervations of the median eminence-pituitary region in the rat. In Neurosecretion - The Final Neuroendocrine Pathway. (Eds) Knowles, F. and Vollrath, L. New York: Springer.

BJORKLUND, A. and LINDVALL, O. (1984) Dopamine-containing systems in the CNS. In Handbook of Chemical Neuroanatomy. Vol. 2, Part 1, pp. 55-122 (Eds) Bjorklund, A. and Hokfelt, T. Amsterdam: Elsevier.

BJORKLUND, A., LINDVALL, O. and NOBIN, A. (1975) Evidence of an incerto-hypothalamic dopamine neurone system in the rat. Brain Res. 89, 29-42.

BJORKLUND, A., MOORE, R.Y., NOBIN, A. and STENEVI, V. (1973b) The organization of tubero-hypophyseal and reticulo-infundibular catecholamine neuron systems in the rat brain. Brain Res. 51, 171-191.

BLAKE, C.A. (1974). Stimulation of pituitary prolactin and TSH release in lactating and proestrous rats. Endocrinology 94, 503-508.

BLAKE, C.A. (1976) Simulation of the proestrous luteinizing hormone surge after infusion of LH-releasing hormone in phenobarbitol-blocked rats. Endocrinology 98, 451-460.

BLAKE, C.A., SCARAMUZZI, R.J., NORMAN, R.L., HILLIARD, J. and SAWYER, C.H. (1973) Effects of hypothalamic deafferentation on circulating levels of LH, FSH, prolactin and testosterone in the male rat. Endocrinology 92, 1419-1425.

BLANK, C.L. (1976) Dual electrochemical detector for liquid chromatography. J. Chromatogr. Sci. 14, 213-219.

BLANK, M.S., PANERAI, A.E. and FRIESEN, H.G. (1980) Effects of naloxone on luteinizing hormone and prolactin in serum of rats. J. Endocrinol. 85, 307-315.

BLISS, C.I. (1952) The Statistics of Bioassay: with Special Reference to the Vitamins. New York: Academic Press.

BLOOM, S.R., MORTIMER, C.H., THORNER, M.O., BESSER, G.M., HALL, R., GOMEZ-PAN, A., ROY, V.M., RUSSELL, R.C.G., COY, D.H., KASTIN, A.J. and SCHALLY, A.V. (1974) Inhibition of gastrin and gastric acid secretion by growth-hormone release inhibiting hormone. Lancet ii, 1106-1109.

BOER, K., CRANSBERG, K. and DOGTEROM, J. (1980) Effect of low-frequency stimulation of the pituitary stalk on neurohypophysial hormone release in vivo. Neuroendocrinology 30, 313-318.

BOLER, J., ENZMANN, F., FOLKERS, K., BOWERS, C.Y. and SCHALLY, A.V. (1969) The identity of chemical and hormonal properties of the thyrotropin releasing hormone and pyroglutamyl-histidyl-prolineamide. Biochem. Biophys. Res. Commun. 37, 705-710.

BOWERS, C.Y., FRIESEN, H.G., HWANG, P., GUYDA, H.J. and FOLKERS, K. (1971) Prolactin and thyrotropin release in man by synthetic pyroglutamyl-histidyl-prolinamide. Biochem. Biophys. Res. Commun. 45, 1033-1041.

BOWERS, C.Y., SCHALLY, A.V., ENZMANN, F., BOLER, J. and FOLKERS, K. (1970) Porcine thyrotropin releasing hormone is (pyro)Glu-His-Pro (NH_2). Endocrinology 86, 1143-1153.

BOWERS, C.Y., SCHALLY, A.V. REYNOLDS, G.A. and HAWLEY, W.D. (1967) Interactions of L-thyroxine or L-triiodothyronine and thyrotropin-releasing factor on the release and synthesis of thyrotropin from the anterior pituitary gland of mice. Endocrinology 81, 741-747.

BOYD III, A.E., SPENCER, E., JACKSON, I.M.D. and REICHLIN, S. (1976) Prolactin releasing factor (PRF) in porcine hypothalamic extract distinct from TRH. Endocrinology 99, 861-871.

BRADBURY, A.F., FINNIE, M.D.A. and SMYTHE, D.G. (1982) Mechanism of C-terminal amide formation by pituitary enzymes. Nature (Lond.) 298, 686-688.

BRAR, A.K., FINK, G., MALETTI, M. and ROSTENE, W. (1985a) Vasoactive intestinal peptide in rat hypophysial portal blood: effects of electrical stimulation of various brain areas, the oestrous cycle and anaesthetics. J. Endocrinol. 106, 275-280.

BRAR, A.K., McNEILLY, A.S. and FINK, G. (1985b) Effects of hyperprolactinaemia and testosterone on the release of LH-releasing hormone and the gonadotrophins in intact and castrated rats. J. Endocrinol. 104, 35-43.

BRAZEAU, P., LING, N., ESCH, F., BOHLEN, P., BENOIT, R. and GUILLEMIN, R. (1981) High biological activity of the synthetic replicates of somatostatin-28 and somatostatin-25. Regul. Pept. 1, 255-264.

BRAZEAU, P., VALE, W., BURGUS, R., LING, N., BUTCHER, M., RIVIER, J. and GUILLEMIN, R. (1973) Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science 179, 77-79.

BREESE, G.R. (1975) Chemical and immunochemical lesions by specific neurotoxic substances and antisera. In Handbook of Pharmacology. Vol. 1, pp. 137-189 (Eds) Iversen, L.L., Iversen, S.D. and Snyder, S.H. New York: Plenum Press.

BREESE, G.R., SMITH, R.D., COOPER, B.R. and GRANT, L.D. (1973) Alterations in consummatory behaviour following intracisternal injection of 6-hydroxydopamine. Pharmacol. Biochem. Behav. 1, 319-328.

BREESE, G.R. and TRAYLOR, T.D. (1971) Depletion of brain noradrenaline and dopamine by 6-hydroxydopamine. Br. J. Pharmacol. 42, 88-99.

BROLIN, S.E. (1946) A study of the structural and hormonal reactions of the pituitary body of rats exposed to cold. Acta Anat. (Basel) 2, suppl. no. 3.

BRONSON, F.H. (1981) The regulation of luteinizing hormone secretion by estrogen: relationship among negative feedback, surge potential, and male stimulation in juvenile, peripubertal, and adult female mice. Endocrinology 108, 506-516.

BRONSON, F.H. and vom SAAL, F.S. (1979) Control of the preovulatory release of luteinizing hormone by steroids in the mouse. Endocrinology 104, 1247-1255.

BROWN-GRANT, K. (1960) The hypothalamus and the thyroid gland. Br. Med. Bull. 16, 165-169.

BROWN-GRANT, K. (1969) Regulation and control of the thyroid-pituitary system. J. Basic Eng. 91, 313-320.

BROWN-GRANT, K., HARRIS, G.W. and REICHLIN, S. (1954) The effect of emotional and physical stress on thyroid activity in the rabbit. J. Physiol. (Lond.) 126, 29-40.

BROWNSTEIN, M.J., ARIMURA, A., SCHALLY, A.V. PALKOVITS, M. and KIZER, J.S. (1976) The effect of surgical isolation of the hypothalamus on its luteinizing hormone-releasing hormone content. Endocrinology 98, 662-665.

BROWNSTEIN, M.J., ARIMURA, A., FERNANDEZ-DURANGO, R., SCHALLY, A.V., PALKOVITS, M. and KIZER, J.S. (1977) The effect of hypothalamic deafferentation on somatostatin-like activity in the rat brain. Endocrinology 100, 246-249.

BROWNSTEIN, M.J., PALKOVITS, M., SAAVEDRA, J.M., BASSIRI, R.M. and UTIGER, R.D. (1974) Thyrotropin-releasing hormone in specific nuclei of rat brain. Science 185, 267-269.

BROWNSTEIN, M.J., PALKOVITS, M., TAPPAZ, M.L., SAAVEDRA, J.M. and KIZER, J.S. (1976) Effect of surgical isolation of the hypothalamus on its neurotransmitter content. Brain Res. 117, 287-295.

BRUNI, J.F., VAN VUGT, D., MARSHALL, S. and MEITES, J. (1977) Effects of naloxone, morphine and methionine enkephalin on serum prolactin, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone and growth hormone. Life Sci. 21, 461-466.

BURGUS, R., BUTCHER, M., AMOSS, M., LING, N., MONAHAN, M., RIVIER, J., FELLOWS, R., BLACKWELL, R., VALE, W. and GUILLEMIN, R. (1972) Primary structure of the ovine hypothalamic luteinizing hormone releasing factor (LRF). Proc. Natl. Acad. Sci. U.S.A. 69, 278-282.

BURGUS, R., DUNN, T.F., DESIDERIO, D. and GUILLEMIN, R. (1969) Structure moleculaire du facteur hypothalamique hypophysiotrope TRF d'origine ovine: mise en evidence par spectrometrie de masse de la sequence PCA-His-Pro-NH_2. C.R. Acad. Sci.[D] Paris 269, 1870-1873.

BURGUS, R., DUNN, T.F., DESIDERIO, D., WARD, D.N., VALE, W. and GUILLEMIN, R. (1970) Characterization of ovine hypothalamic hypophysiotropic TSH-releasing factor. Nature (Lond.) 226, 321-325.

BURGUS, R., DUNN, T.F., WARD, D.N., VALE, W., AMOSS, M. and GUILLEMIN, R. (1969) Derives polypeptidiques de synthese doues d'activitie hypophysiotrope TRF. C.R. Acad. Sci. [D] (Paris) 268, 2116-2118.

BURLET, A., CHATEAU, M. and CZERNICHOW, P. (1979) Infundibular localization of vasopressin, oxytocin and neurophysins in the rat: its relationships with corticotrope function. Brain Res. 168, 275-286.

BURNET, F.R. and WAKERLEY, J.B. (1976). Plasma concentrations of prolactin and thyrotrophin during suckling in urethane-anaesthetized rats. J. Endocrinol. 70, 429-437.

BUSBY, W.H. Jr., YOUNGBLOOD, W.W., HUMM, J. and KIZER, J.S. (1981a) A review of the methods used for the measurement of thyrotropin-releasing hormone (TRH). J. Neurosci. Methods 4, 305-314.

BUSBY, W.H. Jr., YOUNGBLOOD, W.W., HUMM, J. and KIZER, J.S. (1981b) A reliable method for the quantification of thyrotropin-releasing hormone (TRH) in tissue and biological fluids. J. Neurosci. Methods. 4, 315-328.

CALABRO, M.A. and MacLEOD, R.M. (1978) Binding of dopamine to bovine anterior pituitary gland membranes. Neuroendocrinology 25, 32-46.

CALAS, A., ALONSO, G., ARNAULD, E. and VINCENT, J.D. (1974) Demonstration of indolaminergic fibres in the median eminence of the duck, rat and monkey. Nature (Lond.) 250, 241-243.

CAMPBELL, H.J., FEUER, G. and HARRIS, G.W. (1964) The effect of intra-pituitary infusion of median eminence and other brain extracts on anterior pituitary gonadotrophic secretion. J. Physiol. (Lond.) 170, 474-486.

CATTANACH, B.M., IDDON, C.A., CHARLTON, H.M., CHIAPPA, S.A. and FINK, G. (1977) Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism, Nature (Lond.) 269, 338-340.

CHAKRAVARTY, I., SHETH, A.R. and GHOSH, J.J. (1975) Effect of acute Δ^9-tetrahydrocannabinol treatment on serum luteinizing hormone and prolactin levels in adult female rats. Fertil. Steril. 26, 947-948.

CHANG, N.G. and NIKITOVITCH-WINER, M.B. (1976) Correlation between suckling-induced changes in the ultrastructure of mammotrophs and prolactin release. Cell Tissue Res. 166, 399-406.

CHAN-PALAY, V. (1977) Indoleamine neurons and their processes in normal rat brain and in chronic diet-induced thiamine deficiency demonstrated by uptake of ^{3}H serotonin. J. Comp. Neurol. 176, 467-494.

CHARLTON, H.M., HALPIN, D.M.G., IDDON, C., ROSIE, R., LEVY, G., McDOWELL, I.F.W., MEGSON, A., MORRIS, J.F., BRAMWELL, A., SPEIGHT, A., WARD, B.J., BROADHEAD, J., DAVEY-SMITH, G. and FINK, G. (1983) The effects of daily administration of single and multiple injections of gonadotropin-releasing hormone on pituitary and gonadal function in the hypogonadal (hpg) mouse, Endocrinology, 113, 535-544.

CHARLTON, H.M., SPEIGHT, A., HALPIN, D.M.G., BRAMWELL, A., SHEWARD, W.J. and FINK, G. (1983) Prolactin measurements in normal and hypogonadal mice: developmental and experimental studies. Endocrinology 113, 545-548.

CHEUNG, C.Y. (1983) Prolactin suppresses luteinizing hormone secretion and pituitary responsiveness to luteinizing hormone-releasing hormone by a direct action at the anterior pituitary. Endocrinology 113, 632-638.

CHIAPPA, S.A. (1976) Measurement of gonadotrophin and corticotrophin releasing activity of pituitary stalk blood and brain extracts under various physiological and experimental conditions. D. Phil. Thesis: Oxford University.

CHIAPPA, S.A. and FINK, G. (1977) Hypothalamic luteinizing hormone releasing factor and corticotrophin releasing activity in relation to pituitary and plasma hormone levels in male and female rats, J. Endocrinol. 72, 195-210.

CHIAPPA, S.A., FINK, G. and SHERWOOD, N.M. (1977) Immunoreactive luteinizing hormone releasing factor (LRF) in pituitary stalk plasma from female rats: effects of stimulating diencephalon, hippocampus and amygdala, J. Physiol. (Lond.) 267, 625-640.

CHIHARA, K., ARIMURA, A., CHIHARA, M. and SCHALLY, A.V. (1978) Studies on the mechanism of growth hormone and thyrotropin responses to somatostatin antiserum in anesthetized rats. Endocrinology 103, 1916-1923.

CHIHARA, K., ARIMURA, A., KUBLI-GARFIAS, C. and SCHALLY, A.V. (1979a) Enhancement of immunoreactive somatostatin release into hypophysial portal blood by electrical stimulation of the preoptic area in the rat. Endocrinology 105, 1416-1418.

CHIHARA, K., ARIMURA, A. and SCHALLY, A.V. (1979b) Immunoreactive somatostatin in rat hypophyseal portal blood: effects of anesthetics. Endocrinology 104, 1434-1441.

CHIHARA, K., MINAMITANI, N., KAJI, H., ARIMURA, A. and FUJITA, T. (1981) Intraventricularly injected growth hormone stimulates somatostatin release into rat hypophysial portal blood. Endocrinology 109, 2279-2281.

CHING, M. (1982) Correlative surges of LHRH, LH and FSH in pituitary stalk plasma and systemic plasma of rat during proestrus. Effect of anesthetics. Neuroendocrinology 34, 279-285.

CHING, M.C-H. and UTIGER, R.D. (1983) Hypothalamic portal blood immunoreactive TRH in the rat: lack of effect of hypothyroidism and thyroid hormone treatment. J. Endocrinol. Invest. 6, 347-352.

CHIOCCHIO, S.R., NEGRO-VILAR, A. and TRAMEZZANI, J.H. (1976) Acute changes in norepinephrine content in the median eminence induced by orchidectomy or testosterone replacement. Endocrinology 99, 629-635.

CICERO, T.J., BADGER, T.M., WILCOX, C.E., BELL, R.D. and MEYER, E.R. (1977) Morphine decreases luteinizing hormone by an action on the hypothalamic-pituitary axis. J. Pharmacol. Exp. Ther. 203, 548-555.

CICERO, T.J., SCHAINKER, B.A. and MEYER, E.R. (1979) Endogenous opioids participate in the regulation of the hypothalamic-pituitary-luteinizing hormone axis and testosterone's negative feedback control of luteinizing hormone. Endocrinology 104, 1286-1291.

CLARK, W.G., CORRODI, H. and MASUOKA, D.T. (1971) The effects of peripherally administered 6-hydroxydopamine on rat brain monoamine turnover. Eur. J. Pharmacol. 15, 41-44.

CLAYTON, R.N., SOLANO, A.R., GARCIA-VELA, A., DUFAU, M.L. and CATT, K. (1980) Regulation of pituitary receptors for gonadotropin releasing hormone during the rat estrous cycle. Endocrinology 107, 699-706.

CODY, W.L., WILKES, B.C. and HRUBY, V.J. (1984) Reversed-phase high-performance liquid chromatography studies of α-MSH fragments. J. Chromatogr. 314, 313-321.

COHEN-BECKER, I.R., SELMANOFF, M. and WISE, P.M. (1986) Hyper-prolactinaemia alters the frequency and amplitude of pulsatile luteinizing hormone secretion in the ovariectomized rat. Neuroendocrinology 42, 328-333.

COLLU, R., LETARTE, Y., LEBOEUF, G. and DUCHARME, J.R. (1975) Endocrine effects of chronic administration of psychoactive drugs to prepubertal male rats. I: Δ^9-tetrahydrocannabinol. Life Sci. 16, 533-542.

CONDON, T.P., HEBER, D., STEWART, J.M., SAWYER, C.H. and WHITMOYER, D.I. (1984) Differential gonadotropin secretion: blockade of peri-ovulatory LH but not FSH secretion by a potent LHRH antagonist. Neuroendocrinology 38, 357-361.

CONNORS, J.M. and HEDGE, G.A. (1981) Effects of continuous thyroxine administration on thyrotropin secretion in thyroidectomized rats. Endocrinology 108, 2098-2102.

CONVEY, E.M., TUCKER, H.A., SMITH, V.G. and ZOLMAN, J. (1973) Bovine prolactin, growth hormone, thyroxine and corticoid response to thyrotropin-releasing hormone. Endocrinology 92, 471-476.

CORKER, C.S., NAFTOLIN, F. and RICHARDS, M.R. (1971) An ultra-micro protein binding method for the assay of corticosteroids in small volumes of plasma. J. Physiol. (Lond.) 218, 28-29P.

CORRODI, H. and JONSSON, G. (1976) The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines. A review of the methodology. J. Histochem. Cytochem. 15, 65-78.

CRONIN, M.J. and WEINER, R.I. (1979) [^{3}H] Spiroperidol (spiperone) binding to a putative dopamine receptor in sheep and steer pituitary and stalk median eminence. Endocrinology 104, 307-312.

CROSS, B.A., DYBALL, R.E.J., DYER, R.G., JONES, C.W., LINCOLN, D.W., MORRIS, J.F. and PICKERING, B.T. (1975) Endocrine neurons. Recent Progr. Horm. Res. 31, 243-286.

CROWLEY, W.R. and TERRY, L.C. (1980) Biochemical mapping of somato-statinergic systems in rat brain: effects of periventricular hypo-thalamic and medial basal amygdaloid lesions on somatostatin-like immunoreactivity in discrete brain nuclei. Brain Res. 200, 283-291.

CUELLO, A.C., HORN, A.J., MACKAY, A.V.P. and IVERSEN, L.L. (1973) Catecholamines in the median eminence: new evidence for a major noradrenergic input. Nature (Lond.) 243, 465-467.

CUELLO, A.C., SHOEMAKER, W.J. and GANONG, W.F. (1974) Effect of 6-hydroxydopamine on hypothalamic norepinephrine and dopamine content, ultrastructure of the median eminence, and plasma corticosterone. Brain Res. 78, 57-69.

CULLER, M.D. and NEGRO-VILAR, A. (1986) Evidence that pulsatile follicle-stimulating hormone secretion is independent of endogenous luteinizing hormone-releasing hormone. Endocrinology 118, 609-612.

CURTIS, A. and FINK, G. (1983) A high molecular weight precursor of luteinizing hormone releasing hormone from rat hypothalamus. Endocrinology 112, 390-392.

CURTIS, A., LYONS, V. and FINK, G. (1983) The human hypothalamic LHRH precursor is the same size as that in rat and mouse hypothalamus. Biochem. Biophys. Res. Commun. 117, 872-877.

CURTIS, A., LYONS, V. and FINK, G. (1985) The priming effect of LH-releasing hormone: effects of cold and involvement of new protein synthesis. J. Endocrinol. 105, 163-168.

DAANE, T.A. and PARLOW, A.F. (1971) Periovulatory patterns of rat serum follicle-stimulating hormone and luteinizing hormone during the normal estrous cycle: effects of pentobarbital. Endocrinology 88, 653-663.

DAHLSTROM, A. and FUXE, K. (1964) Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. Acta Physiol. Scand. suppl. 247, 64, 1-36.

DAY, T.A. and WILLOUGHBY, J.O. (1980) Noradrenergic afferents to median eminence: inhibitory role in rhythmic growth hormone secretion. Brain Res. 202, 335-345.

DEIS, R.P. and ALONSO, N. (1975) Effect of synthetic thyrotrophin releasing factor on prolactin and luteinizing hormone secretion in male and female rats during various reproductive states. J. Endocrinol. 67, 425-430.

DENEF, C., MANET, D. and DEWALS, R. (1980) Dopaminergic stimulation of prolactin release. Nature (Lond.) 285, 243-246.

DEUBEN, R.R. and MEITES, J. (1964) Stimulation of pituitary growth hormone release by a hypothalamic extract in vitro. Endocrinology 74, 408-414.

DEWEY, W.L., PENG, T.C. and HARRIS, L.S. (1970) The effect of 1-trans-Δ^9-tetrahydrocannabinol on the hypothalamo-hypophyseal adrenal axis of rats. Eur. J. Pharmacol. 12, 382-384.

DIEFENBACH, P.W., CARMEL, P.W., FRANTZ, A.G. and FERIN, M. (1976) Suppression of prolactin secretion by L-dopa in the stalk-sectioned rhesus monkey. J. Clin. Endocrinol. Metab. 43, 638-642.

DIEGUEZ, C., FOORD, S.M., PETERS, J.R., HALL, R. and SCANLON, M.F. (1984) Interactions among epinephrine, thyrotropin (TSH)-releasing hormone, dopamine, and somatostatin in the control of TSH secretion in vitro. Endocrinology 114, 957-961.

DROUIN, J., LAGACE, L. and LABRIE, F. (1976) Estradiol-induced increase of the LH responsiveness to LH releasing hormone (LHRH) in rat anterior pituitary cells in culture. Endocrinology 99, 1477-1481.

DROUVA, S.V., LAPLANTE, E. and KORDON, C. (1983) Effects of ovarian steroids on in vitro release of LHRH from mediobasal hypothalamus. Neuroendocrinology 37, 336-341.

DUCOMMUN, P., VALE, W., SAKIZ, E. and GUILLEMIN, R. (1967) Reversal of the inhibition of TSH secretion due to acute stress. Endocrinology 80, 953-956.

DULAR, R., LABELLA, F., VIVIAN, S. and EDDIE, L. (1974) Purification of prolactin-releasing and inhibiting factors from beef. Endocrinology 94, 563-567.

EDWARDSON, J.A. and GILBERT, D. (1976) Application of an in-vitro perifusion technique to studies of luteinizing hormone release by rat anterior hemipituitaries: self potentiation by luteinizing hormone releasing hormone. J. Endocrinol. 68, 197-207.

EKINS, R.P. (1974) Basic principles and theory. Br. Med. Bull. 30, 3-11.

EMERSON, C.H., ALEX, S., BRAVERMAN, L.E. and SAFFAN, M.S. (1981) A study of the effect of the thyrotropin-releasing hormone metabolite, histidyl-proline diketopiperazine, on prolactin secretion in vivo. Endocrinology 109, 1375-1379.

EMERSON, C.H., FROHMAN, L.A., SZABO, M., THAKKAR, I. (1977) TRH immunoreactivity in human urine: evidence for dissociation from TRH. J. Clin. Endocrinol. Metab. 45, 392-399.

EMSON, P.C., BENNETT, G.W. and ROSSOR, M.N. (1981) The distribution and characterization of thyrotrophin releasing hormone (TRH) in the human brain. Neuropeptides 2, 115-122.

ENGLER, D., SCANLON, M.F and JACKSON, I.M.D. (1981) Thyrotropin-releasing hormone in the systemic circulation of the neonatal rat is derived from pancreas and other extraneuronal tissues. J. Clin. Invest. 67, 800-808.

ENJALBERT, A., ARANCIBIA, S., RUBERG, M., PRIAM, M., BLUET-PAJOT, M.T., ROTSZTEJN, W.H. and KORDON, C. (1980) Stimulation of in vitro prolactin release by vasoactive intestinal peptide. Neuroendocrinology 31, 200-204.

ENJALBERT, A., RUBERG, M., ARANCIBIA, S., FIORE, L., PRIAM, M. and KORDON, C. (1979a) Independent inhibition of prolactin secretion by dopamine and γ-aminobutyric acid in vitro. Endocrinology 105, 823-826.

ENJALBERT, A., RUBERG, M., ARANCIBIA, S., PRIAM, M. and KORDON, C. (1979b) Inhibition of in vitro prolactin secretion by histidyl-proline-diketopiperazine, a degradation product of TRH. Eur. J. Pharmacol. 58, 97-98.

EPELBAUM, J., TAPIA-ARANCIBIA, L., HERMAN, J.P., KORDON, C. and PALKOVITS, M. (1981) Topography of median eminence somatostatin-ergic innervation. Brain Res. 230, 412-416.

EPELBAUM, J., WILLOUGHBY, J.O., BRAZEAU, P. and MARTIN, J.B. (1977) Effects of brain lesions and hypothalamic deafferentation on somatostatin distribution in the rat brain. Endocrinology 101, 1495-1502.

ESCH, F., BOHLEN, P., LING, N., BENOIT, R., BRAZEAU, P. and GUILLEMIN, R. (1980) Primary structure of ovine hypothalamic somatostatin-28 and somatostatin-25. Proc. Natl. Acad. Sci. U.S.A. 77, 6827-6831.

ESKAY, R.L., MICAL, R.S. and PORTER, J.C. (1977) Relationship between luteinizing hormone releasing hormone concentration in hypophysial portal blood and luteinizing hormone release in intact castrated and electrochemically-stimulated rats. Endocrinology 100, 263-270.

ESKAY, R.L., OLIVER, C., BEN-JONATHAN, N. and PORTER, J.C. (1975) Hypothalamic hormones in portal and systemic blood. In Hypothalamic Hormones: Chemistry, Physiology and Clinical Uses. pp. 125-137 (Eds) Motta, M., Martini, L. and Crosignani, P.C. New York: Academic Press.

EVANS, J.S. and NIKITOVITCH-WINER, M.B. (1969) Functional reactivation and cytological restoration of pituitary grafts by continuous local intravascular infusion of median eminence extracts. Neuroendocrinology 4, 83-100.

EVERETT, J.W. (1964) Central neural control of reproductive functions of the adenohypophysis. Physiol. Rev. 44, 373-431.

EVERETT, J.W. and SAWYER, C.H. (1950) A 24-hour periodicity in the 'LH-release apparatus' of female rats, disclosed by barbiturate sedation. Endocrinology 47, 198-218.

EVERETT, J.W., SAWYER, C.H. and MARKEE, J.E. (1949) A neurogenic timing factor in control of the ovulatory discharge of luteinizing hormones in the cyclic rat. Endocrinology 44, 234-250.

FALCK, B., HILLARP, N.-A., THIEME, G. and TORP, A. (1962) Fluorescence of catecholamines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. 10, 348-354.

FINK, G. (1985) Has the prolactin inhibitory peptide at last been found? Nature (Lond.) 316, 487-488.

FARQUHAR, M.G., REID, J.J. and DANIELL, L.W. (1976) Intracellular transport and packaging of prolactin: a quantitative electron microscope autoradiographic study of mammotrophs dissociated from rat pituitaries. Endocrinology 102, 296-311.

FAWCETT, C.P., BEEZLEY, A.E. and WHEATON, J.E. (1975) Chromatographic evidence for the existence of another species of luteinizing hormone-releasing factor (LRF). Endocrinology 96, 1311-1314.

FEDER, H.H., BROWN-GRANT, K. and CORKER, C.S. (1971) Pre-ovulatory progesterone, the adrenal cortex and the 'critical period' for luteinizing hormone release in rats. J. Endocrinol. 50, 29-39.

FELL, L.R., FINDLAY, J.K., CUMMING, I.A. and GODING, J.R. (1973) Effect of synthetic TRF on prolactin release in the sheep. Endocrinology 93, 487-491.

FENSKE, M. and WUTTKE, W. (1976) Effects of intraventricular 6-hydroxydopamine injections on serum prolactin and LH levels: absence of stress-induced prolactin release. Brain Res. 104, 63-70.

FERIN, M., TEMPONE, A., ZIMMERING, P.E. and VAN de WIELE, R.L. (1969) Effect of antibodies to 17β-estradiol and progesterone on the estrous cycle of the rat. Endocrinology 85, 1070-1078.

FERIN, M., VAN VUGT, D. and WARDLAW, S. (1984) The hypothalamic control of the menstrual cycle and the role of endogenous opioid peptides. Recent Prog. Horm. Res. 40, 441-480.

FERLAND, L., LABRIE, F., JOBIN, M., ARIMURA, A. and SCHALLY, A.V. (1976) Physiological role of somatostatin in the control of growth hormone and thyrotropin secretion. Biochem. Biophys. Res. Commun. 68, 149-154.

FERNANDEZ-DURANGO, R., ARIMURA, A., FISHBACK, J. and SCHALLY, A.V. (1978) Hypothalamic somatostatin and LH-RH after hypophysectomy, in hyper- or hypothyroidism, and during anesthesia in rats. Proc. Soc. Exp. Biol. Med. 157, 235-240.

FINK, G. (1976) The development of the releasing factor concept. Clin. Endocrinol. Suppl. 5, 245-260.

FINK, G. (1977) Hypothalamic pituitary ovarian axis. In Recent Advances in Obstetrics and Gynaecology. No. 12, pp. 3-54 (Eds) Stallworthy, J. and Bourne, G. Edinburgh: Churchill Livingstone.

FINK, G. (1979a) Neuroendocrine control of gonadotrophin secretion. Br. Med. Bull. 35, 155-160.

FINK, G. (1979b) Feedback actions of target hormones on hypothalamus and pituitary with special reference to gonadal steroids. Annu. Rev. Physiol. 41, 571-585.

*FINK, G. (1985) See foot of p. 179.

FINK, G. and AIYER, M.S. (1974) Gonadotrophin secretion after electrical stimulation of the preoptic area during the oestrous cycle of the rat. J. Endocrinol. 62, 589-604.

FINK, G., AIYER, M., CHIAPPA, S., HENDERSON, S., JAMIESON, M., LEVY-PEREZ, V., PICKERING, A., SARKAR, D., SHERWOOD, N., SPEIGHT, A. and WATTS, A. (1982) Gonadotropin-releasing hormone. Release into hypophysial portal blood and mechanism of action. In Hormonally Active Brain Peptides. pp. 397-426 (Eds) McKerns, K. and Pantic, V. New York: Plenum Press.

FINK, G., CHIAPPA, S.A. and AIYER, M.S. (1976) Priming effect of luteinizing hormone releasing factor elicited by preoptic stimulation and by intravenous infusion and multiple injections of the synthetic decapeptide. J. Endocrinol. 69, 359-372.

FINK, G. and GEFFEN, L.B. (1978) The hypothalamo-hypophysial system: model for central peptidergic and monoaminergic transmission. In International review of physiology, neurophysiology III. Vol. 17, pp. 1-48 (Ed) Porter, R. Baltimore: University Park Press.

FINK, G. and HARRIS, G.W. (1970) The luteinizing hormone releasing activity of extracts of blood from the hypophysial portal vessels of rats. J. Physiol. (Lond.) 208, 221-241.

FINK, G. and HENDERSON, S.R. (1977a) Steroids and pituitary responsiveness in female, androgenized female and male rats. J. Endocrinol. 73, 157-164.

FINK, G. and HENDERSON, S.R. (1977b) Site of modulatory action of oestrogen and progesterone on gonadotrophin response to luteinizing hormone releasing factor. J. Endocrinol. 73, 165-170.

FINK, G. and JAMIESON, M.G. (1976) Immunoreactive luteinizing hormone releasing factor in rat pituitary stalk blood: effects of electrical stimulation of the medial preoptic area, J. Endocrinol. 68, 71-87.

FINK, G., KOCH, Y. and BEN AROYA, N. (1982) Release of thyrotropin releasing hormone into hypophysial portal blood is high relative to other neuropeptides and may be related to prolactin release. Brain Res. 243, 186-189.

FINK, G., KOCH, Y. and BEN AROYA, N. (1983) TRH in hypophysial portal blood: characteristics of release and relationship to thyrotropin and prolactin secretion. In Thyrotropin-releasing hormone. pp. 127-143 (Eds) Griffiths, E.C. and Bennett, G.W. New York: Raven Press.

FINK, G., NALLAR, R. and WORTHINGTON, W.C. JR. (1967) The demonstration of luteinizing hormone releasing factor in hypophysial portal blood of pro-oestrous and hypophysectomized rats. J. Physiol. (Lond.) 191, 407-416.

FINK, G. and PICKERING, A. (1980) Modulation of pituitary responsiveness to gonadotropin-releasing hormone. In Synthesis and Release of Adenohypophyseal Hormones. pp. 617-638 (Eds) Jutisz, M. and McKerns, K.W. New York: Plenum Press.

FINK, G., STANLEY, H.F. and WATTS, A.G. (1983) Central nervous control of sex and gonadotropin release: peptide and nonpeptide transmitter interactions. In Brain Peptides. pp. 413-435 (Eds) Krieger, D., Brownstein, M. and Martin, J. New York: John Wiley and Sons, Inc.

FOLKERS, K., ENZMANN, F., BOLER, J., BOWERS, C.Y. and SCHALLY, A.V. (1969) Discovery of modification of the synthetic tripeptide-sequence of the thyrotropin releasing hormone having activity. Biochem. Biophys. Res. Commun. 37, 123-126.

FORD, J.J. and MELAMPY, R.M. (1973) Gonadotropin levels in lactating rats: effect of ovariectomy. Endocrinology 93, 540-547.

FOX, S.R. and SMITH, M.S. (1984) The suppression of pulsatile luteinizing hormone secretion during lactation in the rat. Endocrinology 115, 2045-2051.

FOX, S.R. and SMITH, M.S. (1985) Changes in the pulsatile pattern of luteinizing hormone secretion during the rat estrous cycle. Endocrinology 116, 1485-1492.

FRASER, H.M. and McNEILLY, A.S. (1982) Effect of chronic immunoneutralization of thyrotropin-releasing hormone on the hypothalamic-pituitary-thyroid axis, prolactin, and reproductive function in the ewe. Endocrinology 111, 1964-1973.

FRASER, H.M. and McNEILLY, A.S. (1983) Inhibition of thyrotropin-releasing hormone by antibodies. In Thyrotropin-releasing Hormone. pp. 179-190, (Eds) Griffiths, E.C. and Bennett, G.W. New York: Raven Press.

FRAWLEY, L.S., LEONG, D.A. and NEILL, J.D. (1985) Oxytocin attenuates TRH-induced TSH release from rat pituitary cells. Neuroendocrinology 40, 201-204.

FRAWLEY, L.S. and NEILL, J.D. (1981) Stimulation of prolactin secretion in rhesus monkeys by vasoactive intestinal polypeptide. Neuroendocrinology 33, 79-83.

FREEMAN, M.E. and BANKS, J.A. (1980) Hypothalamic sites which control the surges of prolactin secretion induced by cervical stimulation. Endocrinology 106, 668-673.

FREEMAN, M.C., DUPKE, K.C. and CROTEAU, C.M. (1976) Extinction of the estrogen-induced daily signal for LH release in the rat: a role for the proestrous surge of progesterone. Endocrinology 99, 223-229.

FREEMAN, M.E., REICHERT, L.E. Jr. and NEILL, J.D. (1972) Regulation of the proestrous surge of prolactin secretion by gonadotropins and estrogen in the rat. Endocrinology 90, 232-238.

FRIEDMAN, E., KRIEGER, D.T., MEZEY, E., LERANTH, C.S., BROWNSTEIN, M.J. and PALKOVITS, M. (1983) Serotonergic innervation of the rat pituitary intermediate lobe: decrease after stalk section. Endocrinology 112, 1943-1947.

FUKUDA, H. and GREER, M.A. (1977) The effect of basal hypothalamic isolation on pituitary-thyroid activity and the response to propylthiouracil. Endocrinology 100, 911-917.

FURNESS, J.B., COSTA, M. and BLESSING, W.W. (1977) Simultaneous fixation and production of catecholamine fluorescence in central nervous tissue by perfusion with aldehydes. Histochem. J. 9, 745-750.

FURNESS, J.B., HEATH, J.W. and COSTA, M. (1978) Aqueous aldehyde (FAGLU) methods for the fluorescence histochemical localization of catecholamines and for ultrastructural studies of central nervous tissue. Histochemistry 57, 285-295.

FUXE, K. (1965) Evidence for the existence of monoamine neurons in the central nervous system. IV Distribution of monoamine terminals in the central nervous system. Acta Physiol. Scand. 64, suppl. 247, 39-85.

FUXE, K., HOKFELT, T., ANDERSSON, K., FERLAND, L., JOHANSSON, O., GANTEN, D., ENEROTH, P., GUSTAFSSON, J.-A., SKETT, P., SAID, S.I. and MUTT, V. (1978) The transmitters of the hypothalamus. In Pharmacology of the hypothalamus. pp. 31-59 (Eds) Cox, B., Morris, I.D. and Weston, A.H. London: MacMillan Press.

GABRIEL, S.M., SIMPKINS, J.W. and KALRA, S.P. (1983) Modulation of endogenous opioid influence on luteinizing hormone secretion by progesterone and estrogen. Endocrinology 113, 1806-1811.

GALLO, R.V. (1980) Neuroendocrine regulation of pulsatile luteinizing hormone release in the rat. Neuroendocrinology 30, 122-131.

GALLO, R.V. (1981a) Pulsatile LH release during periods of low level LH secretion in the rat estrous cycle. Biol. Reprod. 24, 771-777.

GALLO, R.V. (1981b) Pulsatile LH release during the ovulatory LH surge on proestrus in the rat. Biol. Reprod. 24, 100-104.

GANONG, W.F., KRAMER, N., SALMON, J., REID, I.A., LOVINGER, R., SCAPAGNINI, U., BORYCZKA, A.T. and SHACKELFORD, R. (1976) Pharmacological evidence for inhibition of ACTH secretion by a central adrenergic system in the dog. Neuroscience 1, 167-174.

GARD, T.G., BERNSTEIN, B. and LARSEN, P.R. (1981) Studies on the mechanism of 3,5,3'-triiodothyronine-induced suppression of secretagogue-induced thyrotropin release in vitro. Endocrinology 108, 2046-2053.

GARVER, D.L., CEDARBAUM, J. and MASS, J.W. (1975) Blood-brain barrier to 6-hydroxydopamine: uptake by heart and brain. Life Sci. 17, 1081-1084.

GAUTRON, J.P., PATTOU, E. and KORDON, C. (1981) Occurrence of higher molecular forms of GnRH in fractionated extracts from rat hypothalamus, cortex and placenta. Mol. Cell. Endocrinol. 24, 1-15.

GAUTVIK, K.M., WEINTRAUB, B.D., GRAEBER, C.T., MALOOF, F., ZUCKERMAN, J.E. and TASHJIAN, A.H.Jr. (1973) Serum prolactin and TSH: effects of nursing and pyro Glu-His-ProNH_2 administration in postpartum women. J. Clin. Endocrinol. Metab. 36, 135-139.

GAY, V.L. and SHETH, N.A. (1972) Evidence for periodic release of LH in castrated male and female rats. Endocrinology 90, 158-162.

GIBBS, D.M. (1984) High concentrations of oxytocin in hypophysial portal plasma. Endocrinology 114, 1216-1218.

GIBBS, D.M. and NEILL, J.D. (1978) Dopamine levels in hypophysial stalk blood are sufficient to inhibit prolactin secretion in vivo. Endocrinology 102, 1895-1900.

GIBSON, M.J., CHARLTON, H.M., PERLOW, M.J., ZIMMERMAN, E.A., DAVIES, T.F. and KRIEGER, D.T. (1984a) Preoptic area brain grafts in hypogonadal (hpg) female mice abolish effects of congenital hypothalamic gonadotropin-releasing hormone (GnRH) deficiency. Endocrinology 114, 1938-1940.

GIBSON, M.J., KRIEGER, D.T., CHARLTON, H.M., ZIMMERMAN, E.A. and SILVERMAN, A-J. (1984b) Mating and pregnancy can occur in genetically hypogonadal mice with preoptic area brain grafts. Science 225, 949-951.

GILLIOZ, P., GIRAUD, P., CONTE-DEVOLX, B., JAQUET, P., CODACCIONI, J.L. and OLIVER, C. (1979) Immunoreactive somatostatin in rat hypophysial portal blood. Endocrinology 104, 1407-1410.

GOODMAN, R.L. (1978) The site of the positive feedback action of estradiol in the rat. Endocrinology 102, 151-159.

GOODMAN, R.H., ARON, D.C. and ROOS, B.A. (1983) Rat-prepro-somatostatin. Structure and processing by microsomal membranes. J. Biol. Chem. 258, 5570-5573.

de GREEF, W.J., KLOOTWIJK, W., KARELS, B. and VISSER, T.J. (1985) Levels of dopamine and thyrotrophin-releasing hormone in hypophysial stalk blood during an oestrogen-stimulated surge of prolactin in the ovariectomized rat. J. Endocrinol. 105, 107-112.

de GREEF, W.J., PLOTSKY, P.M. and NEILL, J.D. (1981) Dopamine levels in hypophysial stalk plasma and prolactin levels in peripheral plasma of the lactating rat: effects of a simulated suckling stimulus. Neuroendocrinology 32, 229-233.

de GREEF, W.J. and VISSER, T.J. (1981) Evidence for the involvement of hypothalamic dopamine and thyrotrophin-releasing hormone in suckling-induced release of prolactin. J. Endocrinol. 91, 213-223.

GREELEY, G.H. Jr., ALLEN, M.B. Jr. and MAHESH, V.B. (1975) Potentiation of luteinizing hormone release by estradiol at the level of the pituitary. Neuroendocrinology 18, 233-247.

GREELEY, G.H., VOLCAN, I.J. Jr. and MAHESH, V.B. (1978) Direct effects of estradiol benzoate and testosterone on the response of the male rat pituitary to luteinizing hormone releasing hormone (LHRF). Biol. Reprod. 18, 256-263.

GREEN, J.D. (1947) Vessels and nerves of amphibian hypophyses. Anat. Rec. 99, 21-53.

GREEN, J.D. and HARRIS, G.W. (1947) The neurovascular link between the neurohypophysis and adenohypophysis. J. Endocrinol. 5, 136-146.

GREEN, J.D. and HARRIS, G.W. (1949) Observation of the hypophysio-portal vessels of the living rat. J. Physiol. (Lond.) 108, 359-361.

GREENWOOD, F.C., HUNTER, W.M. and GLOVER, J.S. (1963) The preparation of ^{131}I labelled human growth hormone of high specific radioactivity. Biochem. J. 89, 114-123.

GRIFFITHS, E.C. and MILLAR, R.P. (1983) TRH biosynthesis: a review. In Thyrotropin-releasing hormone. pp. 45-50. (Eds) Griffiths, E.C. and Bennett, G.W. New York: Raven Press.

GRIMM-JORGENSEN, Y. and McKELVY, J.F. (1974) Biosynthesis of thyrotropin releasing factor by newt (Triturus viridescens) brain in vitro. Isolation and characterization of thyrotropin releasing factor. J. Neurochem. 23, 471-478.

DE GROOT, J. (1959) The rat hypothalamus in stereotaxic coordinates. J. Comp. Neurol. 113, 389-400.

GROSVENOR, C.E. and MENA, F. (1980) Evidence that thyrotropin-releasing hormone and a hypothalamic prolactin-releasing factor may function in the release of prolactin in the lactating rat. Endocrinology 107. 863-868.

GROSVENOR, C.E., MENA, F. and WHITWORTH, N.S. (1979a) The secretion rate of prolactin in the rat during suckling and its metabolic clearance rate after increasing intervals of nonsuckling. Endocrinology 104, 372-376.

GROSVENOR, C.E., MENA, F. and WHITWORTH, N.S. (1979b) Ether releases large amounts of prolactin from rat pituitaries previously 'depleted' by short term suckling. Endocrinology 105, 884-887.

GROSVENOR, C.E., MENA, F. and WHITWORTH, N.S. (1980) Evidence that the dopaminergic prolactin-inhibiting factor mechanism regulates only the depletion-transformation phase and not the release phase of prolactin secretion during suckling in the rat. Endocrinology 106, 481-485.

GUBLER, U., SEEBURG, P., HOFFMANN, B.J., GAGE, L.P. and UDENFRIEND, S. (1982) Molecular cloning establishes proenkephalin as precursor of enkephalin-containing peptides. Nature (Lond.) 295, 206-208.

GUILLEMIN, R. (1968) Hypothalamic control of concomitant secretion of ACTH and TSH. Mem. Soc. Endocr. 17, 19-28.

GUILLEMIN, R., BRAZEAU, P., BOHLEN, P., ESCH, F., LING, N. and WEHRENBERG, W.B. (1982) Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. Science 218, 585-587.

GUNNET, J.W. and FREEMAN, M.E. (1983) The mating-induced release of prolactin: a unique neuroendocrine response. Endocr. Rev. 4, 44-61.

GUNNET, J.W. and FREEMAN, M.E. (1985) The interaction of the medial preoptic area and the dorsomedial-ventromedial nuclei of the hypothalamus in the regulation of the mating-induced release of prolactin. Neuroendocrinology 40, 232-237.

HALASZ, B., SLUSHER, M.A. and GORSKI, R.A. (1967) Adrenocorticotrophic hormone secretion in rats after partial or total deafferentation of the medial basal hypothalamus. Neuroendocrinology 2, 43-55.

HAMMONS, J., VELASCO, M. and ROTHCHILD, I. (1973) Effect of the sudden withdrawal or increase of suckling on serum LH levels in ovariectomized postparturient rats. Endocrinology 92, 206-211.

HARMS, P.G., FLEEGER, J.L. and OWENS, R.E. (1977) A suppressive effect of suckling on plasma luteinizing hormone independent of plasma prolactin. Fed. Proc. 36, 277.

HARMS, P.G., LANGLIER, P. and McCANN, S.M. (1975) Modification of stress-induced prolactin release by dexamethasone or adrenalectomy. Endocrinology 96, 475-478.

HARRIS, G.W. (1950) Oestrous rhythm, pseudopregnancy and the pituitary stalk in the rat. J. Physiol. (Lond.) 111, 347-360.

HARRIS, G.W. (1955) Neural control of the pituitary gland. London: Edward Arnold Ltd.

HARRIS, G.W. (1972) Humours and hormones. J. Endocrinol. 53, ii-xxiii.

HARRIS, A.R.C., CHRISTIANSON, D., SMITH, M.S., FANG, S-L., BRAVERMAN, L.E. and VAGENAKIS, A.G. (1978) The physiological role of thyrotropin-releasing hormone in the regulation of thyroid-stimulating hormone and prolactin secretion in the rat. J. Clin. Invest. 61, 441-448.

HARRIS, G.W. and JACOBSOHN, D. (1952) Functional grafts of the anterior pituitary gland. Proc. R. Soc. Lond. B 139, 263-267.

HARRIS, G.W. and WOODS, J.W. (1958) The effect of electrical stimulation of the hypothalamus or pituitary gland on thyroid activity. J. Physiol. (Lond.) 143, 246-274.

HEFCO, E., KRULICH, L. and ASCHENBRENNER, J.E. (1975) Effect of hypothalamic deafferentation on the secretion of thyrotropin in resting conditions in the rat. Endocrinology 97, 1226-1233.

HEIMAN, M.L. and BEN-JONATHAN, N. (1982) Dopaminergic receptors in the rat anterior pituitary change during the estrous cycle. Endocrinology 111, 37-41.

HENDERSON, S.R., BAKER, C. and FINK, G. (1977) Oestradiol-17β and pituitary responsiveness to luteinizing hormone releasing factor in the rat: a study using rectangular pulses of oestradiol-17β monitored by non-chromatographic radioimmunoassay. J. Endocrinol. 73, 441-453.

HERMAN, K., LANG, R.E., UNGER, Th., BAYER, C. and GANTEN, D. (1984) Combined high performance liquid chromatography-radioimmunoassay for the characterization and quantitative measurement of neuropeptides. J. Chromatogr. 312, 273-284.

HINKLE, P.M., PERRONE, M.H. and SCHONBRUNN, A. (1981) Mechanism of thyroid hormone inhibition of thyrotropin-releasing hormone action. Endocrinology 108, 199-205.

HOFFMAN, D.L. and BAKER, B.L. (1977) Effect of treatment with growth hormone on somatostatin in the median eminence of hypophysectomized rats. Proc. Soc. Exp. Biol. Med. 156, 265-271.

HOFFMAN, G.E. and GIBBS, F.P. (1982) LHRH pathways in the rat brain: 'deafferentation' spares a sub-chiasmatic LHRH projection to the median eminence. Neuroscience 7, 1979-1993.

HOKFELT, T., EFENDIC, S., HELLERSTROM, C., JOHANSSON, O., LUFT, R. and ARIMURA, A. (1975) Cellular localization of somatostatin in endocrine-like cells and neurons of the rat with special reference to the A_1-cells of the pancreatic islets and to the hypothalamus. Acta Endocrinol. 80, Suppl. 200, 1-41.

HOKFELT, T., FAHRENKRUG, J., TATEMOTO, K., MUTT, V. and WERNER, S. (1982) PHI, a VIP-like peptide, is present in rat median eminence. Acta Physiol. Scand. 116, 469-471.

HOKFELT, T., FAHRENKRUG, J., TATEMOTO, K., MUTT, V., WERNER, S., HULTING, A.-L., TERENIUS, L. and CHANG, K.J. (1983) The PHI (PHI-27)/corticotropin-releasing factor/enkephalin immunoreactive neuron: possible morphological basis for integrated control of prolactin, corticotropin and growth hormone secretion. Proc. Natl. Acad. Sci. (USA) 80, 895-898.

HOKFELT, T. and FUXE, K. (1972) Brain endocrine interactions: on the morphology and the neuro-endocrine role of hypothalamus catecholamine neurons. In Median Eminence. Structure and Function. (Eds.) Knigge, K.M., Scott, E.E. and Weindl, A. Basel: Karger.

HOKFELT, T., JOHANSSON, O., EFENDIC, S., LUFT, R. and ARIMURA, A. (1975) Are there somatostatin-containing nerves in the rat gut? Immunohistochemical evidence for a new type of peripheral nerves. Experientia 31, 852-854.

HORN, A.M. and FINK, G. (1985a) Effects of 5-hydroxytryptamine uptake blockers on the release of LH and prolactin in several different experimental steroid models in the rat. J. Endocrinol. 104, 397-406.

HORN, A.M., FRASER, H.M. and FINK, G. (1985b) Effects of antiserum to thyrotrophin-releasing hormone on the concentrations of plasma prolactin, thyrotrophin and LH in the pro-oestrous rat. J. Endocrinol. 104, 205-209.

HORN, A.M., ROBINSON, I.C.A.F. and FINK, G. (1985c) Oxytocin and vasopressin in rat hypophysial portal blood: experimental studies in normal and Brattleboro rats. J. Endocrinol. 104, 211-224.

HOUSSAY, B.-A., BIASOTTI, A. and SAMMARTINO, R. (1935) Modifications fonctionelles de l'hypophyse apres les lesions infundibulo-tuberienne chez le crapaud. C.R. Soc. Biol. (Paris) 120, 725-727.

IDDON, C.A., CHARLTON, H.M. and FINK, G. (1980) Gonadotrophin release in hypogonadal and normal mice after electrical stimulation of the median eminence or injection of luteinizing hormone releasing hormone. J. Endocrinol. 85, 105-110.

IERI, T., CHEN, H.T., CAMPBELL, G.A. and MEITES, J. (1980) Effects of naloxone and morphine on the proestrous surge of prolactin and gonadotropins in the rat. Endocrinology 106, 1568-1570.

ISHERWOOD, K.M. and CROSS, B.A. (1980) Effects of the suckling stimulus on secretion of prolactin and luteinizing hormone in conscious and anaesthetized rats. J. Endocrinol. 87, 437-444.

ISHIKAWA, K., KAKEGAWA, T. and SUZUKI, M. (1984) Role of the hypothalamic paraventricular nucleus in the secretion of thyrotropin under adrenergic and cold-stimulated conditions in the rat. Endocrinology 114, 352-358.

ISHIKAWA, H. and NAGAYAMA, T. (1973) The mechanism of the stimulation of LH production by synthetic LH-releasing hormone (LH-RH) in tissue culture. Biochem. Biophys. Res. Commun. 55, 492-498.

IVERSEN, L.L. and URETSKY, N.J. (1970) Regional effects of 6-hydroxydopamine on catecholamine containing neurons in rat brain and spinal cord. Brain Res. 24, 364-367.

JACKSON, I.M.D. (1980) TRH in the rat nervous system: identity with synthetic TRH on high performance liquid chromatography following affinity chromatography. Brain Res. 201, 245-248.

JACKSON, R. and BLOBEL, G. (1977) Post-translational cleavage of presecretory proteins with an extract of rough microsomes from dog pancreas containing signal peptidase activity. Proc. Natl. Acad. Sci. USA 74, 5598-5602.

JACKSON, I.M.D., PAPAPETROU, P.D. and REICHLIN, S. (1979) Metabolic clearance of thyrotropin-releasing hormone in the rat in hypothyroid and hyperthyroid states: comparison with serum degradation in vitro. Endocrinology 104, 1292-1298.

JACKSON, I.M.D. and REICHLIN, S. (1974) Thyrotropin-releasing hormone (TRH): distribution in hypothalamic and extrahypothalamic brain tissues of mammalian and submammalian chordates. Endocrinology 95, 854-862.

JACKSON, I.M.D. and REICHLIN, S. (1977a) Brain thyrotrophin-releasing hormone is independent of the hypothalamus. Nature (Lond.) 267, 853-854.

JACKSON, I.M.D. and REICHLIN, S. (1977b) Thyrotropin releasing hormone: abundance in the skin of the frog, Rana pipiens. Science 198, 414-415.

JACKSON, I.M.D. and REICHLIN, S. (1979) Thyrotropin releasing hormone in the blood of the frog, Rana pipiens: its nature and possible derivation from regional locations in the skin. Endocrinology 104, 1814-1821.

JACKSON, I.M.D., WU, P. and LECHAN, R.M. (1985) Immunohistochemical localization in the rat brain of the precursor for thyrotropin-releasing hormone. Science 229, 1097-1099.

JACOBS, L.S., SNYDER, P.J., WILBER, J.F., UTIGER, R.D. and DAUGHADAY, W.H. (1971) Increased serum prolactin after administration of synthetic thyrotropin releasing hormone (TRH) in man. J. Clin. Endocrinol. Metab. 33, 996-999.

JAFFE, B.M. and BEHRMAN, H.R. (1979) Methods of hormone radioimmuno-assay. New York: Academic Press.

JAMIESON, M.G. and FINK, G. (1976) Parameters of electrical stimulation of the medial preoptic area for release of gonadotrophins in male rats. J. Endocrinol. 68, 57-70.

JEPPSSON, S., NILSSON, K.O., RANNEVIK, G. and WIDE, L. (1976) Influence of suckling and of suckling followed by TRH or LH-RH on plasma prolactin, TSH, GH and FSH. Acta Endocrinol. 82, 246-253.

JEW, J.Y., LERANTH, C., ARIMURA, A. and PALKOVITS, M. (1984) Preoptic LH-RH and somatostatin in the rat median eminence. An experimental light and electron microscopic immunocytochemical study. Neuroendocrinology 38, 169-175.

JOHANSSON, O., HOKFELT, T. and ELDE, R.P. (1984) Immunohistochemical distribution of somatostatin-like immunoreactivity in the central nervous system of the adult rat. Neuroscience 13, 265-339.

JONES, B.E. and MOORE, R.Y. (1977) Ascending projections of the locus coeruleus in the rat. II. Autoradiographic study. Brain Res. 127, 23-53.

JONSSON, G. (1983) Chemical lesioning techniques: monoamine neurotoxins. In Handbook of chemical neuroanatomy. Methods in chemical neuroanatomy. Vol. 1, pp. 463-507 (Eds) Bjorklund, A. and Hokfelt, T. Amsterdam: Elsevier.

JONSSON, G., FUXE, K. and HOKFELT, T. (1972) On the catecholamine innervation of the hypothalamus with special reference to the median eminence. Brain Res. 40, 271-281.

JOSEPH-BRAVO, P., CHARLI, J.L., PALACIOS, J.M. and KORDON, C. (1979) Effect of neurotransmitters on the in vitro release of immunoreactive thyrotropin-releasing hormone from rat mediobasal hypothalamus. Endocrinology 104, 801-806.

KAJI, H., CHIHARA, K., ABE, H., KITA, T., KASHIO, Y., OKIMURA, Y. and FUJITA, T. (1985b) Effect of passive immunization with antisera to vasoactive intestinal polypeptide and peptide histidine isoleucine amide on 5-hydroxy-L-tryptophan-induced prolactin release in rats. Endocrinology 117, 1914-1919.

KAJI, H., CHIHARA, K., ABE, H., MINAMITANI, N., KODAMA, H., KITA, T., FUJITA, T. and TATEMOTO, K. (1984) Stimulatory effects of peptide histidine isoleucine amide 1-27 on prolactin release in the rat. Life Sci. 35, 641-647.

KAJI, H., CHIHARA, K., KITA, T., KASHIO, Y., OKIMURA, Y. and FUJITA, T. (1985a) Administration of antisera to vasoactive intestinal polypeptide and peptide histidine isoleucine attenuates ether-induced prolactin secretion in rats. Neuroendocrinology 41, 529-531.

KALRA, S.P. and KALRA, P.S. (1983) Neural regulation of luteinizing hormone secretion in the rat. Endocr. Rev. 4, 311-351.

KALRA, S.P. and KALRA, P.S. (1984) Opioid-adrenergic-steroid connection in regulation of luteinizing hormone secretion in the rat. Neuroendocrinology 38, 418-426.

KALRA, S.P. and SIMPKINS, J.W. (1981) Evidence for noradrenergic mediation of opioid effects on luteinizing hormone secretion. Endocrinology 109, 776-782.

KANEMATSU, S. and SAWYER, C.H. (1973) Elevation of plasma prolactin after hypophysial stalk section in the rat. Endocrinology 93, 238-241.

KARDON, F., MARCUS, R.J., WINOKUR, A. and UTIGER, R.D. (1977) Thyrotropin-releasing hormone content of rat brain and hypothalamus: results of endocrine and pharmacologic treatments. Endocrinology 100, 1604-1609.

KARSCH, F.J., DIERSCHKE, D.J., WEICK, R.F., YAMAJI, T., HOTCHKISS, J. and KNOBIL, E. (1973) Positive and negative feedback control by estrogen of luteinizing hormone secretion in the rhesus monkey. Endocrinology 92, 799-804.

KARSCH, F.J., WEICK, R.F., BUTLER, W.R., DIERSCHKE, D.J., KREY, L.C., WEISS, G., HOTCHKISS, J., YAMAJI, T. and KNOBIL, E. (1973) Induced LH surges in the rhesus monkey: strength-duration characteristics of the estrogen stimulus. Endocrinology 92, 1740-1747.

KATO, Y., IWASAKI, Y., IWASAKI, J., ABE, H., YANAIHARA, N. and IMURA, H. (1978) Prolactin release by vasoactive intestinal polypeptide in rats. Endocrinology 103, 554-558.

KAWA, A., KAMISAKI, T., ARIYAMA, T., KAWABATA, T., MAEDA, Y., OKAMOTO, O. and KANEHISHA, T. (1979) The effects of intraperitoneal injection of 6-hydroxydopamine on the turnover and the levels of the brain catecholamines and the levels of plasma corticosterone in rats. Clin. Exp. Pharmacol. Physiol. 6, 123-128.

KAWANO, H. and DAIKOKU, S. (1981) Immunohistochemical demonstration of LHRH neurons and their pathways in the rat hypothalamus. Neuroendocrinology 32, 179-186.

KAWANO, H., DAIKOKU, S. and SAITO, S. (1982) Immunohistochemical studies of intrahypothalamic somatostatin-containing neurons in rat. Brain Res. 242, 227-232.

KELLOKUMPU, S., VUOLTEENAHO, O. and LEPPALUOTO, J. (1980) Behaviour of rat hypothalamic and extrahypothalamic immunoreactive TRF in thin-layer chromatography (TLC) and high pressure liquid chromatography (HPLC). Life Sci. 26, 475-480.

KELLY, P.A., BEDIRIAN, K.N., BAKER, R.D. and FRIESEN, H.G. (1973) Effect of synthetic TRH on serum prolactin, TSH and milk production in the cow. Endocrinology 92, 1289-1293.

KELLY, P.H. and IVERSEN, S.D. (1976) Selective 6-OHDA-induced destruction of mesolimbic dopamine neurons: abolition of psychostimulant-induced locomotor activity in rats. Eur. J. Pharmacol. 40, 45-46.

KERDELHUE, B., JUTISZ, M., GILLESSEN, D. and STUDER, R.O. (1973) Obtention of antisera against a hypothalamic decapeptide (luteinizing hormone/follicle stimulating hormone releasing hormone) which stimulates the release of pituitary gonadotropins and development of its radioimmunoassay. Biochim. Biophys. Acta 297, 540-548.

KEWLEY, C.F., MILLAR, R.P., BERMAN, M.C. and SCHALLY, A.V. (1981) Depolarization and ionophore-induced release of octacosa somatostatin from stalk median eminence synaptosomes. Science 213, 913-915.

KING, J.C. and GERALL, A.A. (1976) Localization of luteinizing hormone-releasing hormone. J. Histochem. Cytochem. 24, 829-845.

KIRKHAM, K.E. and HUNTER, W. (1971) Radioimmunoassay methods. London: Churchill Livingstone.

KIZER, J.S., BUSBY, W.H., COTTLE, C. and YOUNGBLOOD, W.W. (1984) Glycine-directed peptide amidation: presence in rat brain of two enzymes that convert p-Glu-His-Pro-Gly-OH into p-Glu-His-Pro-NH_2 (thyrotropin-releasing hormone). Proc. Natl. Acad. Sci. USA 81, 3228-3232.

KIZER, J.S., MUTH, E. and JACOBOWITZ, D.M. (1976) The effect of bilateral lesions of the ventral noradrenergic bundle on endocrine-induced changes of tyrosine hydroxylase in the rat median eminence. Endocrinology 98, 886-893.

KIZER, J.S., PALKOVITS, M. and BROWNSTEIN, M.J. (1976) The projections of the A8, A9 and A10 dopaminergic cell bodies: evidence for a nigro-hypothalamic-median eminence dopaminergic pathway. Brain Res. 108, 363-370.

KLARA, P.M., KOSTREZEWA, R.M. and BRIZZEE. K.R. (1976) Destructive action of systemically administered 6-hydroxydopamine on the rat area postrema. Brain Res. 104, 187-192.

KNOBIL, E. (1974) On the control of gonadotropin secretion in the rhesus monkey. Recent Prog. Horm. Res. 30, 1-36.

KOCH, Y., GOLDHABER, G., FIREMAN, I., ZOR, U., SHANI, J. and TAL, E. (1977) Suppression of prolactin and thyrotropin secretion in the rat by antiserum to thyrotropin-releasing hormone. Endocrinology 100, 1476-1478.

KOKKA, N. and GARCIA, J.F. (1974) Effects of Δ^9-THC on growth hormone and ACTH secretion in rats. Life Sci. 15, 329-338.

DE KONING, J., van DIETEN, J.A.M.J. and van REES, G.P. (1976) LH-RH-dependent synthesis of protein necessary for LH release from rat pituitary glands in vitro. Mol. Cell. Endocrinol. 5, 151-160.

KRAGT, C.L. and MEITES, J. (1967) Dose-response relationships between hypothalamic PIF and prolactin release by rat pituitary tissue in vitro. Endocrinology 80, 1170-1173.

KRAMER, J. and BEN-DAVID, M. (1974) Suppression of prolactin secretion by acute administration of Δ^9-THC in rats. Proc. Soc. exp. Biol. Med. 147, 482-484.

KREIDER, M.S., WINOKUR, A. and UTIGER, R.D. (1979) TRH-like immunoreactivity in rat hypothalamus and brain: assessment by gel filtration and thin layer chromatography. Brain Res. 171, 161-165.

KREY, L.C. and SILVERMAN, A.J. (1983) Luteinizing hormone releasing hormone (LHRH). In Brain Peptides. pp. 687-709 (Eds) Krieger, D., Brownstein, M. and Martin, J. New York. John Wiley and Sons Inc.

KRIEGER, D.T., PERLOW, M.J., GIBSON, M.J., DAVIES, T.F., ZIMMERMAN, E.A., FERIN, M. and CHARLTON, H.M. (1982) Brain grafts reverse hypogonadism of gonadotropin releasing hormone deficiency. Nature (Lond.) 298, 468-471.

KRISCH, B. (1978) Hypothalamic and extrahypothalamic distribution of somatostatin-immunoreactive elements in the rat brain. Cell Tissue Res. 195, 499-513.

KRULICH, L. (1979) Central neurotransmitters and the secretion of prolactin, GH, LH and TSH. Annu. Rev. Physiol. 41, 603-615.

KRULICH, L., DHARIWAL, A.P.S. and McCANN, S.M. (1968) Stimulatory and inhibitory effects of purified hypothalamic extracts on growth hormone release from rat pituitary in vitro. Endocrinology 83, 783-790.

LAGUZZI, L., PETITJEAN, F., PUJOL, J.F. and JOUVET, M. (1971) Effets de l'injection intraventriculaire de 6-hydroxydopamine sur les etats de sommeil et les monoamines cerebrales du chat. C.R. Soc. Biol. (Paris) 165, 1649-1653.

LAND, H., SCHUTZ, G., SCHMALE, H. and RICHTER, D. (1982) Nucleotide sequence of cloned cDNA encoding bovine vasopressin-neurophysin II precursor. Nature (Lond.) 295, 299-303.

LARSEN, P.R., DICK, T.E., MARKOVITZ, B.P., KAPLAN, M.M. and GARD, T. G. (1979) Inhibition of intrapituitary thyroxine to 3,5,3'-triiodothyronine conversion prevents the acute suppression of thyrotrophin release by thyroxine in hypothyroid rats. J. Clin. Invest. 64, 117-128.

LARSEN, P.R. and FRUMESS, R.D. (1977) Comparison of the biological effects of thyroxine and triiodothyronine in the rat. Endocrinology 100, 980-988.

LECHAN, R.M. and JACKSON, I.M.D. (1982) Immunohistochemical localization of thyrotropin-releasing hormone in the rat hypothalamus and pituitary. Endocrinology 111, 55-65.

LECHAN, R.M., NESTLER, J.L., JACOBSON, S. and REICHLIN, S. (1980) The hypothalamic tuberoinfundibular system of the rat as demonstrated by horseradish peroxidase (HRP) microiontophoresis. Brain Res. 195, 13-27.

LECHAN, R.M., WU, P., JACKSON, I.M.D., WOLF, H., COOPERMAN, S., MANDEL, G. and GOODMAN, R.H. (1986) Thyrotropin-releasing hormone precursor: characterization in rat brain. Science 231, 159-161.

LEGAN, S.J., COON, G.A. and KARSCH, F.J. (1975) Role of estrogen as initiator of daily LH surges in the ovariectomized rat. Endocrinology 96, 50-56.

LEGAN, S.J. and KARSCH, F.J. (1975) A daily signal for the LH surge in the rat. Endocrinology 96, 57-62.

LENGYEL, A.-M.J., KRUSEMAN, A.C.N., GROSSMAN, A., REES, L.H. and BESSER, G.M. (1984) Glucose-induced changes in somatostatin-14 and somatostatin-28 released from rat hypothalamic fragments 'in vitro'. Life Sci. 35, 713-719.

LEONG, D.A., FRAWLEY, S. and NEILL, J.D. (1983) Neuroendocrine control of prolactin secretion. Annu. Rev. Physiol. 45, 109-127.

LEPPALUOTO, J., KOIVULSALO, F. and KRAAMA, R. (1978) Thyrotropin-releasing factor: distribution in neural and gastrointestinal tissues. Acta Physiol. Scand. 104, 175-179.

LEWIS, C.E., MORRIS, J.F. and FINK, G. (1985) The role of micro-filaments in the priming effect of LH-releasing hormone: an ultrastructural study using cytochalasin B. J. Endocrinol. 106, 211-218.

LEWIS, C.E., MORRIS, J.F., FINK, G. and JOHNSON, M. (1986) Changes in the granule population of gonadotrophes of hypogonadal (hpg) and normal female mice associated with the priming effect of LH-releasing hormone in vitro. J. Endocrinol. 109, 35-44.

LINCOLN, D.W., HILL, A. and WAKERLEY, J.B. (1973) The milk-ejection reflex of the rat: an intermittent function not abolished by surgical levels of anaesthesia. J. Endocrinol. 57, 459-476.

LINDVALL, O. and BJORKLUND, A. (1974) The glyoxylic acid fluorescence histochemical method: A detailed account of the methodology for the visualization of central catecholamine neurons. Histochemistry 39, 97-127.

LINDVALL, O., BJORKLUND, A., MOORE, R.Y. and STENEVI, U. (1974) Mesencephalic dopamine neurons projecting to neocortex. Brain Res. 81, 325-331.

LIU, T-C. and JACKSON, G.L. (1978) Modifications of luteinizing hormone biosynthesis and release by gonadotropin-releasing hormone, cycloheximide and actinomycin D. Endocrinology 103, 1253-1263.

LIU, T-C. and JACKSON, G.L. (1979) Comparison of the biosynthesis and release of luteinizing hormone by rat pituitaries in vitro in response to gonadotropin-releasing hormone analogues. Endocrinology 104, 962-966.

LOFSTROM, A. (1977) Catecholamine turnover alterations in discrete areas of the median eminence of the 4- and 5-day cyclic rat. Brain Res. 120, 113-131.

LOFSTROM, A., JONSSON, G. and FUXE, K. (1976a) Microfluoremetric quantitation of catecholamine fluorescence in rat median eminence. I. Aspects of the distribution of dopamine and noradrenaline nerve terminals. J. Histochem. Cytochem. 24, 415-429.

LOFSTROM, A., JONSSON, G., WIESEL, F-A. and FUXE, K. (1976b) Microfluorimetric quantitation of catecholamine fluorescence in rat median eminence: II. Turnover changes in hormonal states. J. Histochem. Cytochem. 24, 430-442.

LU, K.H., CHEN, H.T., HUANG, H.H., GRANDISON, L., MARSHALL, S. and MEITES, J. (1976a) Relation between prolactin and gonadotrophin secretion in post-partum lactating rats. J. Endocrinol. 68, 241-250.

LU, K.H., CHEN, H.T., GRANDISON, L., HUANG, H.H. and MEITES, J. (1976b) Reduced luteinizing hormone release by synthetic luteinizing hormone-releasing hormone (LHRH) in postpartum lactating rats. Endocrinology 98, 1235-1240.

LUMPKIN, M.D. and McCANN, S.M. (1984) Effect of destruction of the dorsal anterior hypothalamus on follicle-stimulating hormone secretion in the rat. Endocrinology 115, 2473-2480.

LUMPKIN, M.D., SAMPSON, W.K. and McCANN, S.M. (1983) Hypothalamic and pituitary sites of action of oxytocin to alter prolactin secretion in the rat. Endocrinology 112, 1711-1717.

McCANN, S.M. (1962) Effect of progesterone on plasma luteinizing hormone activity. Am. J. Physiol. 202, 601-604.

McCANN, S.M., TALEISNIK, S. and FRIEDMAN, H.M. (1960) LH-releasing activity in hypothalamic extracts. Proc. Soc. Exp. Biol. Med. 104, 432-434.

McDOWELL, I.F.W., MORRIS, J.F. and CHARLTON, H.M. (1982a) Characterization of the pituitary gonadotroph cells of hypogonadal (hpg) male mice: comparison with normal mice. J. Endocrinol. 95, 321-330.

McDOWELL, I.F.W., MORRIS, J.F., CHARLTON, H.M. and FINK, G. (1982b) Effects of luteinizing hormone releasing hormone on the gonadotrophs of hypogonadal (hpg) mice. J. Endocrinol. 95, 331-340.

McKELVY, J.F. (1974) Biochemical neuroendocrinology. I. Biosynthesis of thyrotropin releasing hormone (TRH) by organ culture of mammalian hypothalamus. Brain Res. 65, 489-502.

McKELVY, J.F. (1983) Thyrotropin-releasing hormone biosynthesis. In Thyrotropin-releasing hormone. pp. 51-59 (Eds) Griffiths, E.C. and Bennett, G.W. New York: Raven Press.

McKELVY, J.F., SHERIDAN, M., JOSEPH, S., PHELPS, C.H. and PERRIE, S. (1975) Biosynthesis of thyrotropin-releasing hormone in organ cultures of the guinea-pig median eminence. Endocrinology 97, 908-918.

McNEILLY, A.S., SHARPE, R.M. and FRASER, H.M. (1980) Effect of adrenalectomy or castration on the inhibition of gonadotrophin secretion induced by hyperprolactinaemia in the adult male rat. J. Endocrinol. 85, 83-92.

MAKARA, G.B., PALKOVITS, M., ANTONI, F.A. and KISS, J.Z. (1983) Topography of the somatostatin-immunoreactive fibers to the stalk-median eminence of the rat. Neuroendocrinology 37, 1-8.

MALLIK, T.K., WILBER, J.F. and PEGUES, J. (1982) Measurements of thyrotropin-releasing hormone-like material in human peripheral blood by affinity chromatography and radioimmunoassay. J. Clin. Endocrinol. Metab. 54, 1194-1198.

MALVERN, P.V. (1975) Immediate release of prolactin and biphasic effects on growth hormone release following stimulation of the median eminence. Endocrinology 97, 808-815.

MARKS, B.H. (1973) Δ^1-tetrahydrocannabinol and luteinizing hormone secretion. Prog. Brain Res. 39, 331-338.

MARTIN, J.B., BOSHANS, R. and REICHLIN, S. (1970) Feedback regulation of TSH secretion in rats with hypothalamic lesions. Endocrinology 87, 1032-1040.

MARTIN, J.B. and REICHLIN, S. (1972) Plasma thyrotropin (TSH) response to hypothalamic electrical stimulation and to injection of synthetic thyrotropin releasing hormone (TRH). Endocrinology 90, 1079-1085.

MASON, W.T. (1983) Excitation by dopamine of putative oxytocinergic neurones in the rat supraoptic nucleus in vitro: evidence for two classes of continuously firing neurones. Brain Res. 267, 113-121.

MATSUO, H., BABA, Y., NAIR, R.M.G., ARIMURA, A. and SCHALLY, A.V. (1971) Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochem. Biophys. Res. Commun. 43, 1334-1339.

MATTHEIJ, J.A.M. and VAN PIJKEREN, T.A. (1977) Plasma prolactin in undisturbed cannulated male rats: effects of perphenazine, frequent sampling, stress and castration plus oestrone treatment. Acta Endocrinol. 84, 51-61.

MAYER, G.S. and SHOUP, R.E. (1983) Simultaneous multiple electrode liquid chromatographic/electrochemistry assay for catecholamines, indoleamines, and metabolites in brain tissue. J. Chromatogr. 255, 533-544.

MECHOULAM, R., SHANI, A., EDERY, H. and GRUNFELD, Y. (1970) Chemical basis of hashish activity. Science 169, 611-612.

MEFFORD, I.N. (1981) Application of high performance liquid chromatography with electrochemical detection to neurochemical analysis: measurement of catecholamines, serotonin and metabolites in rat brain. J. Neurosci. Methods 3, 207-227.

MEIDAN, R., FINK, G. and KOCH, Y. (1981) Ontogeny of the sensitizing effect of oestradiol and luteinizing hormone releasing hormone on the anterior pituitary gland of the female rat. J. Endocrinol. 91, 347-351.

MEITES, J., LU, K.H., WUTTKE, W., WELSCH, C.W., NAGASAWA, H. and QUADRI, S.K. (1972) Recent studies on functions and control of prolactin secretion in rats. Recent Prog. Horm. Res. 28, 471-516.

MENA, F., PACHECO, P., AGUAYO, D., CLAPP, C. and GROSVENOR, C.E. (1978) A rise in intramammary pressure follows electrical stimulation of mammary nerve in anaesthetized rats. Endocrinology 103, 1929-1936.

MENA, F., PACHECO, P. and GROSVENOR, C.E. (1980) Effect of electrical stimulation of mammary nerve upon pituitary and plasma prolactin concentrations in anaesthetized lactating rats. Endocrinology 106, 458-462.

MEYERS, C.A., MURPHY, W.A., REDDING, T.W., COY, P.H. and SCHALLY, A.V. (1980) Synthesis and biological actions of prosomatostatin. Proc. Natl. Acad. Sci. USA 77, 6171-6174.

MEZEY, E. and KISS, J.Z. (1985) Vasoactive intestinal peptide containing neurons in the paraventricular nucleus may participate in regulating prolactin secretion. Proc. Natl. Acad. Sci. (USA) 82, 245-247.

MIDGLEY, A.R. Jr., NISWENDER, G.D. and REBAR, R.W. (1969) Principles for the assessment of the reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity). Acta Endocrinol. Suppl. 142, 163-180.

MILLAR, R.P., AEHNELT, C. and ROSSIER, G. (1977) Higher molecular weight immunoreactive species of luteinizing hormone releasing hormone: possible precursors of the hormone. Biochem. Biophys. Res. Commun. 74, 720-731.

MILLAR, R.P., KLAFT, L.J., BARRON, J., LEVITT, N.S. and LING, N. (1982) Somatostatin-28 inhibits GnRH-stimulated gonadotropin secretion in man. Clin. Endocrinol. 17, 103-107.

MILLAR, R.P., WEGENER, I. and SCHALLY, A.V. (1981) Putative prohormone of luteinizing hormone-releasing hormone. In Neuropeptides: Biochemical and Physiological Studies. pp. 111-130 (Ed) Millar, R.P. New York: Churchill-Livingstone.

MILLARD, W.J., SAGAR, S.M., BADGER, T.M. and MARTIN, J.B. (1983) Cysteamine effects on growth hormone secretion in the male rat. Endocrinology 112, 509-517.

MINAGUCHI, H. and MEITES, J. (1967) Effect of suckling on hypothalamic LH-releasing factor and prolactin inhibiting factor, and on pituitary LH and prolactin. Endocrinology 80, 603-607.

MITCHELL, R., GRIEVE, G., DOW, R. and FINK, G. (1983) Endogenous GABA receptor ligands in hypophysial portal blood. Neuroendocrinology 37, 169-176.

MITNICK, M. and REICHLIN, S. (1972) Enzymatic synthesis of thyrotropin-releasing hormone (TRH) by hypothalamic 'TRH synthetase'. Endocrinology 91, 1146-1153.

MIZUNUMA, H., SAMSON, W.K., LUMPKIN, M.D., MOLTZ, J.H., FAWCETT, C.P. and McCANN, S.M. (1983) Purification of a bioactive FSH-releasing factor (FSHRF). Brain Res. Bull. 10, 623-629.

MONROE, S.E., PECKHAM, W.D., NEILL, J.D. and KNOBIL, E. (1970) A radioimmunoassay for rhesus monkey luteinizing hormone (RhLH). Endocrinology 86, 1012-1018.

de MONTIGNY, C., WANG, R.Y., READER, T.A. and AGHAJANIAN, G.K. (1980) Monoaminergic denervation of the rat hippocampus: microiontophoretic studies on pre- and postsynaptic supersensitivity to norepinephrine and serotonin. Brain Res. 200, 363-376.

MOORE, R.Y. and BLOOM, F.E. (1978) Central catecholamine neuron systems: anatomy and physiology of the dopamine systems. Annu. Rev. Neurosci. 1, 129-169.

MOORE, R.Y. and CARD, J.P. (1984) Noradrenaline-containing neuron systems. In Handbook of Chemical Neuroanatomy. Vol. 2, Part 1, pp. 123-156 (Eds) Bjorklund, A. and Hokfelt, T. Amsterdam: Elsevier.

MOOS, F. and RICHARD, P. (1982) Excitatory effect of dopamine on oxytocin and vasopressin reflex releases in the rat. Brain Res. 241, 249-260.

MORLEY, J.E. (1981) Neuroendocrine control of thyrotropin secretion. Endocr. Rev. 2, 396-436.

MORLEY, J.E., GARVIN, T.J., PEKARY, A.E. and HERSHMAN, J.M. (1977) Thyrotropin-releasing hormone in the gastrointestinal tract. Biochem. Biophys. Res. Commun. 79, 314-318.

MORLEY, J.E., MEYER, N., PEKARY, A.E., MELMED, S., CARLSON, H.E., BRIGGS, J.E. and HERSHMAN, J.M. (1980) A prolactin inhibitory factor with immunocharacteristics similar to thyrotropin releasing factor (TRH) is present in rat pituitary tumors (GH3 and W5), testicular tissue and a plant material, alfalfa. Biochem. Biophys. Res. Commun. 96, 47-53.

MORRELL, J.I., McGINTY, J.F. and PFAFF, D.W. (1985) A subset of β-endorphin- or dynorphin-containing neurons in the medial basal hypothalamus accumulates estradiol. Neuroendocrinology 41, 417-426.

MORRISON, J.H., BENOIT, R., MAGISTRETTI, P.J., LING, N. and BLOOM, F.E. (1982) Immunohistochemical distribution of prosomatostatin-related peptides in hippocampus. Neurosci. Lett. 34, 137-142.

MORRISON, J.H., BENOIT, R., MAGISTRETTI, P.J. and BLOOM, F.E. (1983) Immunohistochemical distribution of pro-somatostatin-related peptides in cerebral cortex. Brain Res. 262, 344-351.

MORTIMER, C.H., CARR, D., LIND, T., BLOOM, S.R., MALLINSON, C.N., SCHALLY, A.V., TUNBRIDGE, W.M.G., YEOMANS, L., COY, D.H., KASTIN, A., BESSER, G.M. and HALL, R. (1974) Effects of growth-hormone release-inhibiting hormone on circulating glucagon, insulin and growth hormone in normal, diabetic, acromegalic, and hypo-pituitary patients. Lancet i, 697-701.

MUNEMURA, M., ESKAY, R.L., KEBANINA, J.W. and LONG, R. (1980) Release of α-melanocyte-stimulation hormone from dispersed cells of the intermediate lobe of the rat pituitary gland: involvement of catecholamines and adenosine 3',5'-monophosphate. Endocrinology 106, 1795-1803.

MURAKI, T., NAKADATE, H., TOKUNAGA, Y., KATO, R. and MAKINO, T. (1979) Effect of narcotic analgesics and naloxone on proestrous surges of LH, FSH and prolactin in rats. Neuroendocrinology 28, 241-247.

NAIK, S.I., YOUNG, L.S., CHARLTON, H.M. and CLAYTON, R.N. (1984a) Pituitary gonadotropin-releasing hormone receptor regulation in mice. I: Males. Endocrinology 115, 106-113.

NAIK, S.I., YOUNG, L.S., CHARLTON, H.M. and CLAYTON, R.N. (1984b) Pituitary gonadotropin-releasing hormone receptor regulation in mice. II: Females. Endocrinology 115, 114-120.

NEILL, J.D. (1972) Comparison of plasma prolactin levels in cannulated and decapitated rats. Endocrinology 90, 568-572.

NEILL, J.D, FREEMAN, M.E. and TILLSON, S.A. (1971) Control of the pro-oestrous surge of prolactin and luteinizing hormone secretion by oestrogens in the rat. Endocrinology 89, 1448-1453.

NETT, T.M., AKBAR, A.M., NISWENDER, G.D., HEDLUND, M.T. and WHITE, W.F. (1973) A radioimmunoassay for gonadotropin-releasing hormone (Gn-RH) in serum. J. Clin. Endocrinol. Metab. 36, 880-885.

NIKITOVITCH-WINER, M.B. and EVERETT, J.W. (1958) Functional restitution of pituitary grafts re-transplanted from kidney to median eminence. Endocrinology 63, 916-930.

NIKITOVITCH-WINER, M.B. and EVERETT, J.W. (1959) Histocytologic changes in grafts of rat pituitary on the kidney and upon re-transplantation under the diencephalon. Endocrinology 65, 357-368.

NIKOLICS, K., MASON, A.J., SZONYI, E., RAMACHANDRAN, J. and SEEBURG, P.H. (1985) A prolactin-inhibiting factor within the precursor for human gonadotropin-releasing hormone. Nature (Lond.) 316, 511-517.

NIR, I., AYALON, D., TSAFRIRI, A., CORDOVA, T. and LINDNER, H.R. (1973) Suppression of cyclic surge of luteinizing hormone secretion and of ovulation in the rat by Δ^1-tetrahydro-cannabinol. Nature (Lond.) 243, 470-471.

NISWENDER, G.D., MIDGLEY, A.R. Jr., MONROE, S.E. and REICHERT, L.E. Jr. (1968) Radioimmunoassay for rat luteinizing hormone with anti-ovine LH serum and ovine LH-^{131}I. Proc. Soc. Exp. Biol. Med. 128, 807-811.

NORDMANN, J.J. (1976) Evidence for calcium inactivation during hormone release in the rat neurohypophysis. J. exp. Biol. 65, 669-683.

ODELL, W.D. and DAUGHADAY, W.H. (1971) Principles of competitive protein-binding assays. Philadelphia: Lippencott.

O'HARE, M.J. and NICE, E.C. (1979) Hydrophobic high-performance liquid chromatography of hormonal polypeptides and proteins on alkylsilane-bonded silica. J. Chromatogr. 171, 209-226.

OLIVER, C., CHARVET, J.P., CODACCIONI, J-L. and VAGUE, J. (1974) Radioimmunoassay of thyrotropin-releasing hormone (TRH) in human plasma and urine. J. Clin. Endocrinol. Metab. 39, 406-409.

OLIVER, C., MICAL, R.S. and PORTER, J.C. (1977) Hypothalamic-pituitary vasculature: evidence for retrograde blood flow in the pituitary stalk. Endocrinology 101, 598-604.

OPPENHEIMER, J.H. (1979) Thyroid hormone action at the cellular level. Science 203, 971-979.

ORTOWSKI, H., RICHMAN, P.G. and MEISTER, A. (1969) Isolation and properties of γ-L-glutamylcyclotransferase from human brain. Biochemistry 8, 1048-1055.

PAGE, R.B. (1983) Directional pituitary blood flow: a microcine-photographic study. Endocrinology 112, 157-165.

PALKOVITS, M. (1973) Isolated removal of hypothalamic or other brain nuclei of the rat. Brain Res. 59, 449-450.

PALKOVITS, M. (1981) Catecholamines in the hypothalamus: an anatomical review. Neuroendocrinology 33, 123-128.

PALKOVITS, M., MEZEY, E., CHIUEH, C.G., KRIEGER, D.T., GALLATZ, K. and BROWNSTEIN, M.J. (1986) Serotonin-containing elements of the rat pituitary intermediate lobe. Neuroendocrinology 42, 522-525.

PALKOVITS, M., SAAVEDRA, J.M., JACOBOWITZ, D.M., KIZER, J.S., ZABORSZKY, L. and BROWNSTEIN, M.J. (1977) Serotonergic innervation of the forebrain: effect of lesions on serotonin and tryptophan hydroxylase levels. Brain Res. 130, 121-134.

PAMENTER, R.W. and HEDGE, G.A. (1980) Inhibition of thyrotropin secretion by physiological levels of corticosterone. Endocrinology 106, 162-166.

DE PAOLO, L.V. (1985) Differential regulation of pulsatile luteinizing hormone (LH) and follicle-stimulating hormone secretion in ovariectomized rats disclosed by treatment with a LH-releasing hormone antagonist and Phenobarbital. Endocrinology 117, 1826-1833.

PARENT, A., DESCARRIES, L. and BEAUDET, A. (1981) Organization of ascending serotonin systems in the adult rat brain. A radioautographic study after intraventricular administration of [^{3}H] 5-hydroxytryptamine. Neuroscience 6, 115-138.

PARKER, C.R. Jr. (1981) Characterization of immunoreactive thyrotropin releasing hormone in human fetal cerebellum. J. Neurochem. 37, 1266-1271.

PATEL, Y.C. and PIERZCHALA, I. (1985) Cysteamine induces a loss of tissue somatostatin-28 when measured as somatostatin-$28_{(15-28)}$-like immunoreactivity but not when assessed as somatostatin-$28_{(1-14)}$-like immunoreactivity: evidence for the importance of the disulphide bond for cysteamine action. Endocrinology 116, 1699-1702.

PATEL, Y.D. and WHEATLEY, T. (1983) In vivo and in vitro plasma disappearance and metabolism of somatostatin-28 and somatostatin-14 in the rat. Endocrinology 112, 220-225.

PEKARY, A.E., MEYER, N.V., VAILLANT, C. and HERSHMAN, J.M. (1980) Thyrotropin-releasing hormone and a homologous peptide in the male rat reproductive system. Biochem. Biophys. Res. Commun. 95, 993-1000.

PEKARY, A.E,, MORLEY, J.E. and HERSHMAN, J.M. (1978) Absence of pyroglutamyl-N^{3im}-methyl-histidyl-prolineamide (methyl-thyrotrophin releasing hormone) in the rat hypothalamus. J. Endocrinol. 77, 405-408.

PEKARY, A.E., RICHKIND, M. and HERSHMAN, J.M. (1983) Thyrotrophin releasing hormone and related peptides in canine tissues. J. Endocrinol. 98, 299-306.

PETERS, J.R., FOORD, S.M., DIEGUEZ, C., SCANLON, M.F. and HALL, R. (1983) α_1-adrenoreceptors on intact rat anterior pituitary cells: correlation with adrenergic stimulation of thyrotropin secretion. Endocrinology 113, 133-140.

PICKERING, A.J.M.C. (1978) Aspects of the mechanism of action of the gonadotrophin releasing hormone. D. Phil. Thesis Oxford University.

PICKERING, A.J.M.C. and FINK, G. (1976a) Priming effects of luteinizing hormone releasing factor: in-vitro and in-vivo evidence consistent with its dependence upon protein and RNA synthesis. J. Endocrinol. 69, 373-379.

PICKERING, A.J.M.C. and FINK, G. (1976b) Priming effect of luteinizing hormone releasing factor: in vitro studies with raised potassium ion concentrations. J. Endocrinol. 69, 453-454.

PICKERING, A.J.M.C. and FINK, G. (1977) A priming effect of luteinizing hormone releasing factor with respect to release of follicle-stimulating hormone in vitro and in vivo. J. Endocrinol. 75, 155-159.

PICKERING, A.J.M.C. and FINK, G. (1979a) Priming effect of luteinizing hormone releasing factor in vitro: role of protein synthesis, contractile elements, Ca^{2+} and cyclic AMP. J. Endocrinol. 81, 223-234.

PICKERING, A.J.M.C. and FINK, G. (1979b) Do hypothalamic regulatory factors other than luteinizing hormone releasing factor exert a priming effect? J. Endocrinol. 81, 235-238.

PIEROTTI, A.R. and HARMAR, A.J. (1985) Multiple forms of somatostatin-like immunoreactivity in the hypothalamus and amygdala of the rat: selective localization of somatostatin-28 in the median eminence. J. Endocrinol. 105, 383-389.

PIEROTTI, A.R., HARMAR, A.J., TANNAHILL, L.A. and ARBUTHNOTT, G.W. (1985) Different patterns of molecular forms of somatostatin are released by the rat median eminence and hypothalamus. Neurosci. Lett. 57, 215-220.

PLANT, T.M. (1982) Pulsatile luteinizing hormone secretion in the neonatal male rhesus monkey (Macaca mulatta). J. Endocrinol. 93, 71-74.

PLANT, T.M., HESS, D.L., HOTCHKISS, J. and KNOBIL, E. (1978) Testosterone and the control of gonadotrophin secretion in the male rhesus monkey (Macaca mulatta). Endocrinology 103, 535-541.

PLOTSKY, P.M., GIBBS, D.M. and NEILL, J.D. (1978) Liquid chromatographic-electrochemical measurement of dopamine in hypophysial stalk blood of rats. Endocrinology 102, 1887-1894.

PLOTSKY, P.M. and NEILL, J.D. (1982a) The decrease in hypothalamic dopamine secretion induced by suckling: comparison of voltametric and radioisotopic methods of measurement. Endocrinology 110, 691-696.

PLOTSKY, P.M. and NEILL, J.D. (1982b) Interactions of dopamine and thyrotropin-releasing hormone in the regulation of prolactin release in lactating rats. Endocrinology 111, 168-173.

PLOTSKY, P.M. and VALE, W. (1985) Patterns of growth hormone-releasing factor and somatostatin secretion into the hypophysial-portal circulation of the rat. Science 230, 461-463.

POPA, G. and FIELDING, U. (1930) A portal circulation from the pituitary to the hypothalamic region. J. Anat. 65, 88-91.

POPA, G. and FIELDING, U. (1933) Hypophysio-portal vessels and their colloid accompaniment. J. Anat. 67, 227-232.

PRADAYROL, L., JORNVALL, H., MUTT, V. and RIBET, A. (1980) N-terminally extended somatostatin: the primary structure of somatostatin-28. FEBS Lett. 109, 55-58.

PRASAD, C., WILBER, J.F., AKERSTROM, V. and BANERJI, A. (1980) Cyclo (His Pro): a selective inhibitor of rat prolactin secretion *in vitro*. Life Sci. 27, 1979-1983.

PRICE, J., GROSSMAN, A., BESSER, G.M. and REES, L.H. (1983) Dopaminergic control of the rat thyrotroph. Neuroendocrinology 36, 125-129.

RAMIREZ, V.D., FEDER, H.H. and SAWYER, C.H. (1984) The role of brain catecholamines in the regulation of LH secretion: a critical enquiry. In Frontiers in Neuroendocrinology. Vol. 8, pp. 27-84 (Eds) Martini, L. and Ganong, W.F. New York: Raven Press.

RANCE, N., WISE, P.M. and BARRACLOUGH, C.A. (1981a) Negative feed-back effects of progesterone correlated with changes in hypothalamic norepinephrine and dopamine turnover rates, median eminence luteinizing hormone-releasing hormone, and peripheral plasma gonadotropins. Endocrinology 108, 2194-2199.

RANCE, N., WISE, P.M., SELMANOFF, M.K. and BARRACLOUGH, C.A. (1981b) Catecholamine turnover rates in discrete hypothalamic areas and associated changes in median eminence luteinizing hormone-releasing hormone and gonadotropins of pro-oestrus and diestrus day 1. Endocrinology 108, 1795-1802.

REDDING, T.W., SCHALLY, A.V., ARIMURA, A. and MATSUO, H. (1972) Stimulation of release and synthesis of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in tissue cultures of rat pituitaries in response to natural and synthetic LH and FSH releasing hormone. Endocrinology 90, 764-770.

REICHLIN, S., MARTIN, J.B., BOSHANS, R.L., SCHALCH, D.S., PIERCE, J.G. and BOLINGER, J. (1970) Measurement of TSH in plasma and pituitary of the rat by radioimmunoassay utilizing bovine TSH: effect of thyroidectomy or thyroxine administration on plasma TSH levels. Endocrinology 87, 1022-1031.

REICHLIN, S., MARTIN, J.B., MITNICK, M.A., BOSHANS, R.L., GRIMM, Y., BOLLINGER, J., GORDON, J. and MALACARA, J. (1972) The hypothalamus in pituitary-thyroid regulation. Recent Prog. Horm. Res. 28, 229-277.

REICHLIN, S., SAPERSTEIN, R., JACKSON, I.M.D., BOYD, A.E. III, and PATEL, Y. (1976) Hypothalamic hormones. Annu. Rev. Physiol. 38, 389-424.

RETHELYI, M., VIGH, S., SETALO, G., MERCHENTHALER, I., FLERKO, B. and PETRUSZ, P. (1981) The luteinizing hormone releasing hormone-containing pathways and their co-termination with tanycyte processes in and around the median eminence and in the pituitary stalk of the rat. Acta Morphol. Acad. Sci. Hung. 29, 259-283.

RICHTER, K., KAWASHIMA, E. EGGER, R. and KREIL, G. (1984) Biosynthesis of thyrotropin releasing hormone in the skin of Xenopus laevis: partial sequence of the precursor deduced from cloned cDNA. EMBO J. 3, 617-621.

RISKIND, P.N., MILLARD, W.J. and MARTIN, J.B. (1984) Evidence that thyrotropin-releasing hormone is not a major prolactin-releasing factor during suckling in the rat. Endocrinology 115, 312-316.

RIVIER, J., SPIESS, J., THORNER, M. and VALE, W. (1982) Characterization of a growth hormone-releasing factor from a human pancreatic islet tumour. Nature (Lond.) 300, 276-278.

RIVIER, C. and VALE, W. (1974) In vivo stimulation of prolactin secretion in the rat by thyrotropin releasing factor, related peptides and hypothalamic extracts. Endocrinology 95, 978-983.

ROBERTS, J.L., PHILLIPS, M., ROSA, P.A. and HERBERT, E. (1978) Steps involved in the processing of common precursor forms of adrenocorticotropin and endorphin in cultures of mouse pituitary cells. Biochemistry 17, 3609-3618.

ROBINSON, I.C.A.F. (1980) The development and evaluation of a sensitive and specific radioimmunoassay for oxytocin in unextracted plasma. J. Immunoassay 1, 323-347.

RODBARD, D. (1971) Statistical aspects of radioimmunoassays. In Principles of competitive protein-binding assays. pp. 204-253 (Eds) Odell, W.D. and Daughaday, W.H. Philadelphia: Lippencott.

RODRIGUES-ARNO, M.S., GOMEZ-PAN, A., RAINBOW, S.J., WOODHEAD, S., COMARU-SCHALLY, A.M., SCHALLY, A.V., MEYERS, C.A., COY, D.H. and HALL, R. (1981) Effects of prosomatostatin on growth hormone and prolactin in response to arginine in man. Lancet 1, 353-356.

ROSTENE, W.H., LERANTH, C.S., MALETTI, M., MEZEY, E., BESSON, J., EIDEN, L.E., ROSSELIN, G. and PALKOVITS, M. (1982) Distribution of vasoactive intestinal peptide (VIP) following various brain transections in the rat by radioimmunoassay and electronmicroscopic immunochemistry. Neuropeptides 2, 337-350.

RUPNOW, J.H., HINKLE, P.M. and DIXON, J.E. (1979) A macromolecule which gives rise to thyrotropin releasing hormone. Biochem. Biophys. Res. Commun. 89, 721-728.

SAAVEDRA, J.M. (1985) Central and peripheral catecholamine innervation of the rat intermediate and posterior lobe. Neuroendocrinology 40, 281-284.

SAAVEDRA, J.M., PALKOVITS, M., BROWNSTEIN, M.J. and AXELROD, J. (1974) Serotonin distribution in the nuclei of the rat hypothalamus and preoptic region. Brain Res. 77, 157-165.

SAAVEDRA, J.M., PALKOVITS, M., KIZER, J.S., BROWNSTEIN, M. and ZIVIN, J.A. (1975) Distribution of biogenic amines and related enzymes in the rat pituitary gland. J. Neurochem. 25, 257-260.

SACHS, C. (1973) Development of the blood-brain barrier for 6-hydroxydopamine. J. Neurochem. 20, 1753-1760.

SAGAR, S.M., LANDRY, D., MILLARD, W.J., BADGER, T.M., ARNOLD, M.A. and MARTIN, J.B. (1982) Depletion of somatostatin-like immunoreactivity in the rat central nervous system by cysteamine. J. Neurosci. 2, 225-231.

SAID, S.I. and PORTER, J.C. (1978) Vasoactive intestinal peptide: release into hypophysial portal blood. Life Sci. 24, 227-230.

SALISBURY, R.L., KRIEG, R.J. and SEIBEL, H.R. (1980) Effects of arginine vasotocin, oxytocin and arginine vasopressin on steroid-induced surges of luteinizing hormone and prolactin in ovariectomized rats. Acta Endocrinol. 94, 166-173.

SAMSON, W.K. and McCANN, S.M. (1979) Effects of lesions in the organum vasculosum lamina terminalis on the hypothalamic distribution of luteinizing hormone-releasing hormone and gonadotropin secretion in the ovariectomized rat. Endocrinology 105, 939-946.

SAR, M. (1984) Estradiol is concentrated in tyrosine hydoxylase-containing neurons of the hypothalamus. Science 223, 938-940.

SAR, M. and STUMPF, W.E. (1981) Central noradrenergic neurones concentrate ^{3}H-oestradiol. Nature (Lond.) 289, 500-502.

SARKAR, D.K. (1979) The regulation of gonadotrophin release in the adult and developing female rat. D. Phil. Thesis Oxford University.

SARKAR, D.K., CHIAPPA, S.A., FINK, G. and SHERWOOD, N.M. (1976) Gonadotropin-releasing hormone surge in pro-oestrous rats. Nature (Lond.) 264, 461-463.

SARKAR, D.K. and FINK, G. (1979a) Effects of gonadal steroids on output of luteinizing hormone releasing factor into pituitary stalk blood in the female rat. J. Endocrinol. 80, 303-313.

SARKAR, D.K. and FINK, G. (1979b) Mechanism of the first spontaneous gonadotrophin surge and that induced by pregnant mare serum and effects of neonatal androgen in rats. J. Endocrinol. 83, 339-354.

SARKAR, D.K. and FINK, G. (1980) Luteinizing hormone releasing factor in pituitary stalk plasma from long-term ovariectomized rats: effects of steroids. J. Endocrinol. 86, 511-524.

SARKAR, D.K. and FINK, G. (1981) Gonadotropin-releasing hormone surge: possible modulation through postsynaptic α and two pharmacologically distinct dopamine receptors. Endocrinology 108, 862-867.

SARKAR, D.K. and GIBBS, D.M. (1984) Cyclic variation of oxytocin in the blood of pituitary portal vessels of rats. Neuroendocrinology 39, 481-483.

SARKAR, D.K., GOTTSCHALL, P.E., MEITES, J., HORN, A., DOW, R.C., FINK, G. and CUELLO, A.C. (1983) Uptake and release of [^{3}H] dopamine by the median eminence: evidence for presynaptic dopaminergic receptors and for dopaminergic feedback inhibition. Neuroscience 10, 821-830.

SARKAR, D.K., SMITH, G.C. and FINK, G. (1981) Effect of manipulating central catecholamines on puberty and the surge of luteinizing hormone and gonadotropin releasing hormone induced by pregnant mare serum gonadotropin in female rats. Endocrinology 213, 335-349.

SARKAR, D.K. and YEN, S.S.C. (1985a) Changes in β-endorphin-like immunoreactivity in pituitary portal blood during the estrous cycle and after ovariectomy in rats. Endocrinology 116, 2075-2079.

SARKAR, D.K. and YEN, S.S.C. (1985b) Hyperprolactinaemia decreases the luteinzing hormone-releasing hormone concentration in pituitary portal plasma: a possible role for β-endorphin as a mediator. Endocrinology 116, 2080-2084.

SCANLON, M.F., ROBBINS, R.J., BOLAFFI, J.L., JACKSON, I.M.D. and REICHLIN, S. (1983) Characterisation of somatostatin and TRH release by rat hypothalamic and cerebral cortical neurons maintained on a capillary membrane perfusion system. Neuroendocrinology 37, 269-274.

SCHAEFFER, J.M., BROWNSTEIN, M.J. and AXELROD, J. (1977) Thyrotropin-releasing hormone-like material in the rat retina: changes due to environmental lighting. Proc. Natl. Acad. Sci. (USA) 74, 3579-3581.

SCHALLY, A.V., ARIMURA, A. and KASTIN, A.J. (1973) Hypothalamic regulatory hormones. Science 179, 341-350.

SCHALLY, A.V. and BOWERS, C.Y. (1964) The purification of luteinizing hormone releasing factor from bovine hypothalamus. Endocrinology 75, 608-614.

SCHALLY, A.V., BOWERS, C.Y., REDDING, T.W. and BARRETT, J.F. (1966) Isolation of thyrotropin releasing factor (TRF) from porcine hypothalamus. Biochem. Biophys. Res. Commun. 25, 165-169.

SCHALLY, A.V., DUPONT, A., ARIMURA, A., REDDING, T.W., NISHI, N., LINTHICUM, G.L. and SCHLESINGER, D.H. (1976) Isolation and structure of somatostatin from porcine hypothalami. Biochemistry 15, 509-514.

SCHALLY, A.V., HUANG, W.Y., CHANG, R.C.C., ARIMURA, A., REDDING, T.W., MILLAR, R.P., HUNKAPILLER, M.W. and HOOD, L.E. (1980) Isolation and structure of prosomatostatin: a putative somatostatin precursor from pig hypothalamus. Proc. Natl. Acad. Sci. U.S.A. 77, 4489-4493.

SCHALLY, A.V., REDDING, T.W., ARIMURA, A., DUPONT, A. and LINTHICUM, G.L. (1977) Isolation of gamma-aminobutyric acid from pig hypothalami and demonstration of its prolactin release-inhibiting (PIF) activity in vivo and in vitro. Endocrinology 100, 681-691.

SEEBURG, P.H. and ADELMAN, J.P. (1984) Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. Nature (Lond.) 311, 666-668.

SEGAL, M., PICKEL, V. and BLOOM, F.E. (1974) The projections of the nucleus locus coeruleus: an autoradiographic study. Life Sci. 13, 817-821.

SEGGIE, J.A. and BROWN, G.M. (1976) Twenty-four-hour resting prolactin levels in male rats: the effect of septal lesions and order of sacrifice. Endocrinology 98, 1516-1522.

SETALO, G., VIGH, S., SCHALLY, A.V., ARIMURA, A. and FLERKO, B. (1976) Immunohistological study of the origin of LH-RH-containing nerve fibers of the rat hypothalamus. Brain Res. 103, 597-602.

SHAAR, C.J., CLEMENS, J.A. and DININGER, N.B. (1979) Effect of vasoactive intestinal polypeptide on prolactin release in vitro. Life Sci. 25, 2071-2074.

SHERWOOD, N.M., CHIAPPA, S.A. and FINK, G. (1976) Immunoreactive luteinizing hormone releasing factor in pituitary stalk blood from female rats; sex steroid modulation of response to electrical stimulation of preoptic area or median eminence. J. Endocrinol. 70, 501-511.

SHERWOOD, N.M., CHIAPPA, S.A., SARKAR, D.K. and FINK, G., (1980) Gonadotropin-releasing hormone (GnRH) in pituitary stalk blood from proestrous rats: effects of anesthetics and relationship between stored and released GnRH and luteinizing hormone, Endocrinology 107, 1410-1417.

SHIINO, M., WILLIAMS, G. and RENNELS, E.G. (1972) Ultrastructural observation of pituitary release of prolactin in the rat by suckling stimulus. Endocrinology 90, 176-187.

SHIN, S.H. (1979) Prolactin secretion in acute stress is controlled by prolactin releasing factor. Life Sci. 25, 1829-1836.

SHIVERS, B.D., HARLAN, R.E., MORRELL, J.I. and PFAFF, D.W. (1983) Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. Nature (Lond.) 304, 345-347.

SIEGEL, S. (1956) Non Parametric Statistics for the Behavioural Sciences. London: McGraw-Hill.

SMITH, M.S. (1978a) A comparison of pituitary responsiveness to luteinizing hormone-releasing hormone during lactation and the estrous cycle of the rat. Endocrinology 102, 114-120.

SMITH, M.S. (1978b) The relative contribution of suckling and prolactin to the inhibition of gonadotropin secretion during lactation in the rat. Biol. Reprod. 19, 77-83.

SMITH, M.S. (1985) Release of luteinizing hormone and follicle-stimulating hormone from pituitaries of lactating rats by gonadotropin-releasing hormone and high potassium concentration. Endocrinology 116, 1826-1834.

SMITH, C.G., BESCH, N.F., SMITH, R.G. and BESCH, P.K. (1979) Effects of tetrahydrocannabinol on the hypothalamic-pituitary axis in the ovariectomized rhesus monkey. Life Sci. 31, 335-339.

SMITH, R.D., COOPER, B.R. and BREESE, G.R. (1973) Growth and behavioural changes in developing rats treated intracisternally with 6-hydroxydopamine: Evidence for involvement of brain dopamine. J. Pharmacol. exp. Ther. 185, 609-619.

SMITH, G.C., COURTNEY, P.G., WREFORD, N.G.M. and WALKER, M.McD. (1982) Further studies on the effects of intravenously administered 6-hydroxydopamine on the median eminence of the rat. Brain Res. 234, 101-110.

SMITH, G.C. and FINK, G. (1972) Experimental studies on the origin of monoamine-containing fibres in the hypothalamo-hypophysial complex of the rat. Brain Res. 43, 37-51.

SMITH, M.S., FREEMAN, M.E. and NEILL, J.D. (1975) The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96, 219-226.

SMITH, S.W. and GALA, R.R. (1978) Rat plasma prolactin levels in response to electrical stimulation of the ventromedial nucleus of the hypothalamus: dependence upon stimulation parameters. Brain Res. 147, 390-395.

SMITH, G.C. and HELME, R.D. (1974) Ultrastructural and fluorescence histochemical studies on the effects of 6-hydroxydopamine on the rat median eminence. Cell Tissue Res. 152, 493-512.

SMITH, M.S. and NEILL, J.D. (1977) Inhibition of gonadotropin secretion during lactation in the rat: relative contribution of suckling and ovarian steroids. Biol. Reprod. 17, 255-261.

SNABES, M.C. and KELCH, R.P. (1979) Acute inhibitory effects of antiserum to GnRH in ovariectomized rats. Evidence for pulsatile secretion of GnRH. Neuroendocrinology 29, 34-41.

SPEIGHT, A. and FINK, G. (1981) Comparison of steroid and LHRH effects on the responsiveness of hemipituitary glands and dispersed pituitary cells. Mol. Cell. Endocrinol. 24, 267-281.

SPEIGHT, A., POPKIN, R., WATTS, A.G. and FINK, G. (1981) Oestradiol-17β increases pituitary responsiveness by a mechanism that involves the release and the priming effect of luteinizing hormone releasing factor. J. Endocrinol. 88, 301-308.

SPIESS, J., RIVIER, J. and VALE, W. (1983) Characterization of rat hypothalamic growth hormone-releasing factor. Nature (Lond.) 303, 532-535.

SPIESS, J., VILLARREAL, J. and VALE, W. (1981) Isolation and sequence analysis of a somatostatin-like polypeptide from ovine hypothalamus. Biochemistry 20, 1982-1988.

SPINDEL, E. and WURTMAN, R.J. (1980) TRH immunoreactivity in rat brain regions, spinal cord and pancreas: validation by high-pressure liquid chromatography and thin-layer chromatography. Brain Res. 201, 279-288.

SRIKANT, C.B. and PATEL, Y.C. (1981) Receptor binding of somatostatin-28 is tissue specific. Nature (Lond.) 294, 259-260.

STEGER, R.W., DE PAOLO, L., ASCH, R.H. and SILVERMAN, A.Y. (1983) Interactions of Δ^9-Tetrahydrocannabinol (THC) with hypothalamic neurotransmitters controlling luteinizing hormone and prolactin release. Neuroendocrinology 37, 361-370.

STEGER, R.W., SILVERMAN, A.Y., JOHNS, A. and ASCH, R. (1981) Interactions of cocaine and Δ^9-tetrahydrocannabinol (THC) with the hypothalamo-hypophysial axis of the female rat. Fertil. Steril. 35, 567-572.

STEGER, R.W., SILVERMAN, A.Y., SILER-KHODR, T.M. and ASCH, R.H. (1980) The effect of Δ^9-tetrahydrocannabinol on the positive and negative feedback control of luteinizing hormone release. Life Sci. 27, 1911-1916.

STEINBUSCH, H.W.M. (1981) Distribution of serotonin-immunoreactivity in the central nervous system of the rat - cell bodies and terminals. Neuroscience 6, 557-618.

STEINBUSCH, H.W.M. and NIEUWENHUYS, R. (1981) Localization of serotonin-like immunoreactivity in the central nervous system and pituitary of the rat, with special reference to the innervation of the hypothalamus. Adv. Exp. Med. Biol. 133, 7-35.

SUTHERLAND, R.C. and FINK, G. (1983) The milk ejection pathway in brain studied with the 2-deoxyglucose method. Brain Res. 273, 291-296.

SWANSON, L.W. and HARTMAN, B.K. (1975) The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. J. Comp. Neurol. 163, 467-506.

SZABO, M. and FROHMAN, L.A. (1976) Dissociation of prolactin-releasing activity from thyrotropin-releasing hormone in porcine stalk median eminance. Endocrinology 98, 1451-1459.

SZABO, M. and FROHMAN, L.A. (1977) Suppression of cold-stimulated thyrotropin secretion by antiserum to thyrotropin-releasing hormone. Endocrinology 101, 1023-1033.

SZABO, M., KOVATHANA, N., GORDON, K. and FROHMAN, L.A. (1978) Effect of passive immunization with an antiserum to thyrotropin (TSH)-releasing hormone on plasma TSH levels in thyroidectomized rats. Endocrinology 102, 799-805.

TALWALKER, P.K., RATNER, A. and MEITES, J. (1963) In vitro inhibition of pituitary prolactin synthesis and release by hypothalamic extracts. Am. J. Physiol. 205, 213-218.

TANNENBAUM, G.S. and LING, N. (1984) The interrelationship of growth hormone (GH)-releasing factor and somatostatin in generation of the ultradian rhythm of GH secretion. Endocrinology 115, 1952-1957.

TANNENBAUM, G.S., LING, N. and BRAZEAU, P. (1982) Somatostatin-28 is longer acting and more selective than somatostatin-14 on pituitary and pancreatic hormone release. Endocrinology 111, 101-107.

TANNENBAUM, G.S. and MARTIN, J.B. (1976) Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. Endocrinology 98, 562-570.

TAPPAZ, M.L., BROWNSTEIN, M.J. and KOPIN, I.J. (1977) Glutamate decarboxylase (GAD) and γ-aminobutyric acid (GABA) in discrete nuclei of hypothalamus and substantia nigra. Brain Res. 125, 109-121.

TASHJIAN, A.H., BAROWSKY, N.J. and JENSEN, D.K. (1971) Thyrotropin releasing hormone: direct evidence for stimulation of prolactin production by pituitary cells in culture. Biochem. Biophys. Res. Commun. 43, 516-523.

TATEMOTO, K., CARLQUIST, M., McDONALD, T.J. and MUTT, V. (1983) Isolation of a brain peptide identical to the intestinal PHI (peptide HI). FEBS Lett. 153, 248-252.

TERRY, L.C. and CROWLEY, W.R. (1980) The effect of hypophysectomy on somatostatin-like immunoreactivity in discrete hypothalamic and extrahypothalamic nuclei. Endocrinology 107, 1771-1775.

TERRY, L.C. and CROWLEY, W.R. (1986) Regulation of growth hormone and thyrotropin secretion by somatostatin systems in rat brain. Neuroendocrinology 42, 218-225.

TERRY, L.C. and MARTIN, J.B. (1981) The effects of lateral hypothalamic-medial forebrain stimulation and somatostatin antiserum on pulsatile growth hormone secretion in freely behaving rats: evidence for a dual regulatory mechanism. Endocrinology 109, 622-627.

TILDERS, F.J.H., van der WOUDE, H.A., SWAAB, D.F. and MULDER, A.H. (1979) Identification of MSH release-inhibiting elements in the neurointermediate lobe of the rat. Brain Res. 171, 425-435.

TINDAL, J.S. and KNAGGS, G.S. (1972) Pathways in the forebrain of the rabbit concerned with the release of prolactin. J. Endocrinol. 52, 253-262.

TINDAL, J.S. and KNAGGS, G.S. (1977) Pathways in the forebrain of the rat concerned with the release of prolactin. Brain Res. 119, 211-221.

TINDAL, J.S., KNAGGS, G.S. and TURVEY, A. (1969) The afferent path of the milk-ejection reflex in the brain of the rabbit. J. Endocrinol. 43, 663-671.

TOROK, B. (1964) Structure of the vascular connections of the hypothalamo-hypophysial region. Acta Anat. (Basel) 59, 84-99.

TRYGSTAD, O., FOSS, I., EDMINSON, P.D., JOHANSEN, J.H. and REICHELT, K.L. (1978) Humoral control of appetite: a urinary anorexigenic peptide. Chromatographic patterns of urinary peptides in anorexia nervosa. Acta Endocrinol. 89, 196-208.

TURGEON, J.L. and WARING, D.W. (1981) Acute progesterone and 17β-estradiol modulation of luteinizing hormone secretion by pituitaries of cycling rats superfused in vitro. Endocrinology 108, 413-419.

TYREY, L. (1978) Δ-9-Tetrahydrocannabinol suppression of episodic luteinizing hormone secretion in the ovariectomized rat. Endocrinology 102, 1808-1814.

UNGERSTEDT, U. (1971a) A stereotaxic mapping of the monoamine pathways in the rat brain. Acta Physiol. Scand. Suppl. 367, 1-48.

UNGERSTEDT, U. (1971b) Histochemical studies on the effect of intracerebral and intraventricular injections of 6-hydroxydopamine neurons in the rat brain. In 6-hydroxydopamine and catecholamine neurons. pp. 101-127 (Eds) Malmfors T. and Thoenen, H. Amsterdam: North-Holland.

UOTILA, U.U. (1939) On the role of the pituitary stalk in the regulation of the anterior pituitary with special reference to the thyrotropic hormone. Endocrinology 25, 605-614.

URBAN, I., MOSS, R.L. and CROSS, B.A. (1971) Problems in electrical stimulation of afferent pathways for oxytocin release. J. Endocrinol. 51, 347-358.

URETSKY, N.J. and IVERSEN, L.L. (1970) Effects of 6-hydroxydopamine on catecholamine containing neurones in rat brain. J. Neurochem. 17, 269-278.

UTIGER, R.D., PARKER, M.L. and DAUGHADAY, W.H. (1962) Studies on human growth hormone. I. A radioimmunoassay for human growth hormone. J. Clin. Invest. 41, 254-261.

VALE, W., BLACKWELL, R., GRANT, G. and GUILLEMIN, R. (1973) TRF and thyroid hormones on prolactin secretion by rat anterior pituitary cells in vitro. Endocrinology 93, 26-33.

VALE, W., RIVIER, C., BRAZEAU, P. and GUILLIMEN, R. (1974) Effects of somatostatin on the secretion of thyrotropin and prolactin. Endocrinology 95, 968-977.

VALE, W., RIVIER, C. and BROWN, M. (1977) Regulatory peptides of the hypothalamus. Am. Rev. Physiol. 39, 473-527.

VALE, W., RIVIER, C. and BROWN, M. (1980) Physiology and pharmacology of hypothalamic regulatory peptides. In Handbook of the hypothalamus. Vol 2, pp. 165-252 (Eds) Morgane, P.J. and Panksepp, J. New York and Basel: Marcel Dekker Inc.

VALVERDE-R.C., CHIEFFO, V. and REICHLIN, S. (1972) Prolactin-releasing factor in porcine and rat hypothalamic tissue. Endocrinology 91, 982-993.

VALVERDE-R.C., CHIEFFO, V. and REICHLIN, S. (1973) Failure of reserpine to block ether-induced release of prolactin: physiological evidence that stress-induced prolactin is not caused by acute inhibition of PIF secretion. Life Sci. 12, 327-335.

VAN DAMME, M.-P., ROBERTSON, D.M. & DICZFALUZY, E. (1974) An improved in vitro bioassay method for measuring luteinizing hormone (LH) activity using mouse Leydig cell preparations. Acta Endocrinol. 77, 655-671.

VANDESANDE, F., DIERICKX, K. and de MEY, J. (1977) The origin of the vasopressinergic and oxytocinergic fibres of the external region of the median eminence of the rat hypophysis. Cell Tissue Res. 180, 443-452.

VAN LOON, G.R., HILGER, L., KING, A.B., BORYCZKA, A.T. and GANONG, W.F. (1971) Inhibitory effects of L-dihydroxyphenylalanine on the adrenal venous 17-hydroxycorticosteroid response to surgical stress in dogs. Endocrinology 88, 1404-1414.

VAN LOON, G.R., SCAPAGNINI, U., COHEN, R. and GANONG, W.F. (1971) The effect of intraventricular administration of adrenergic drugs on the adrenal venous 17-hydroxycorticosteroid response to surgical stress in the dog. Neuroendocrinology 8, 257-272.

VAN VUGT, D.A., SYLVESTER, P.W., AYLESWORTH, C.F. and MEITES, J. (1982) Counteraction of gonadal steroid inhibition of luteinizing hormone release by naloxone. Neuroendocrinology 34, 274-278.

VINCENT, S.R., HOKFELT, T. and WU, J-Y. (1982) GABA neuron systems in hypothalamus and the pituitary gland: Immuno-histochemical demonstration using antibodies against glutamate decarboxylase. Neuroendocrinology 34, 117-125.

WAKERLEY, J.B. and LINCOLN, D.W. (1973) The milk-ejection reflex of the rat: a 20- to 40-fold acceleration in the firing of paraventricular neurones during oxytocin release. J. Endocrinol. 57, 477-493.

WAKERLEY, J.B., O'NEILL, D.S. and ter HAAR, M.B. (1978) Relationship between the suckling-induced release of oxytocin and prolactin in the urethane-anaesthetized lactating rat. J. Endocrinol. 76. 493-500.

WATTS, A.G. (1982) The neuroendocrine control of gonadotrophin secretion in the female rat with special emphasis on the effects of continuous exposure to light. D. Phil. Thesis Oxford University.

WATTS, A.G. and FINK, G. (1981) Constant light blocks diurnal but not pulsatile release of luteinizing hormone in the ovariectomized rat. J. Endocrinol. 89, 141-146.

WATTS, A.G. and FINK, G. (1984) Pulsatile luteinizing hormone release, and the inhibitory effect of estradiol-17β, in gonadectomized male and female rats: effects of neonatal androgen or exposure to constant light. Endocrinology 115, 2251-2259.

WEBER, R.F.A., de GREEF, W.J., de KONING, J. and VREEBURGH, J.T.M. (1983) LH-RH and dopamine levels in hypophysial stalk plasma and their relationship to plasma gonadotrophins and prolactin levels in male rats bearing a prolactin- and adrenocorticotrophin-secreting pituitary tumour. Neuroendocrinology 36, 205-210.

WEBER, S.G. & PURDY, W.C. (1978) The behaviour of an electrochemical detector used in liquid chromatography and continuous flow voltammetry. Part 1. Mass Transport limited current. Anal. Chim. Acta 100, 531-544.

WEHRENBERG, W.B., BRAZEAU, P., LUBEN, R., BOHLEN, P. and GUILLEMIN, R. (1982) Inhibition of the pulsatile secretion of growth hormone by monoclonal antibodies to the hypothalamic growth hormone releasing factor (GRF). Endocrinology 111, 2147-2148.

WEINER, R.I. and GANONG, W.F. (1978) Role of brain monoamines and histamine in regulation of anterior pituitary secretion. Physiol. Rev. 58, 905-976.

WEINER, R.I., PATTOU, E., KERDELHUE, B. and KORDON, C. (1975) Differential effects of hypothalamic deafferentation upon luteinizing hormone-releasing hormone in the median eminence and organum vasculosum of the lamina terminalis. Endocrinology 97, 1597-1600.

WIEGAND, S.J. and PRICE, J.L. (1980) The cells of origin of the afferent fibers to the median eminence in the rat. J. Comp. Neurol. 192, 1-19.

WILBER, J.F. and PORTER, J.C. (1970) Thyrotropin and growth hormone releasing activity in hypophysial portal blood. Endocrinology 87, 807-811.

WINOKUR, A. and UTIGER, R.D. (1974) Thyrotropin-releasing hormone. Regional distribution in rat brain. Science 185, 265-267.

WISE, P.M., RANCE, N. and BARRACLOUGH, C.A. (1981) Effects of estradiol and progesterone on catecholamine turnover rates in discrete hypothalamic regions in ovariectomized rats. Endocrinology 108, 2186-2193.

WISLOCKI, G.B. (1937) The meningeal relations of the hypophysis cerebri. II An embryological study of the meninges and blood vessels of the human hypophysis. Am. J. Anat. 61, 95-129.

WISLOCKI, G.B. (1938) The vascular supply of the hypophysis cerebri of the Rhesus monkey and man. Res. Publ. Assoc. Res. Nerv. Ment. Dis. 17, 48-68.

WISLOCKI, G.B. and KING, L.S. (1936) The permeability of the hypophysis and hypothalamus to vital dyes, with a study of the hypophysial vascular supply. Am. J. Anat. 58, 421-472.

WITKIN, J.W., PADEN, C.M. and SILVERMAN, A-J. (1982) The luteinizing hormone-releasing hormone (LHRH) systems in the rat brain. Neuroendocrinology 35, 429-438.

WONG, W.C. and TAN, C. (1974) Degeneration in the adult rat spinal cord following systemic treatment with 6-hydroxydopamine: electronmicroscopic study. Experientia 30, 1455-1459.

WORTHINGTON, W.C. Jr. (1966) Blood samples from the pituitary stalk of the rat; method of collection and factors determining volume, Nature (Lond.) 210, 710-712.

YALOW, R.S. (1980) Radioimmunoassay. Annu. Rev. Biophys. Bioeng. 9, 327-345.

YALOW, R.S. and BERSON, S.A. (1971) Introduction and general considerations. In Competitive Protein-Binding Assays. pp. 1-21 (Eds) Odel, W.D. and Daughaday, W.H. Philadelphia: Lippencott.

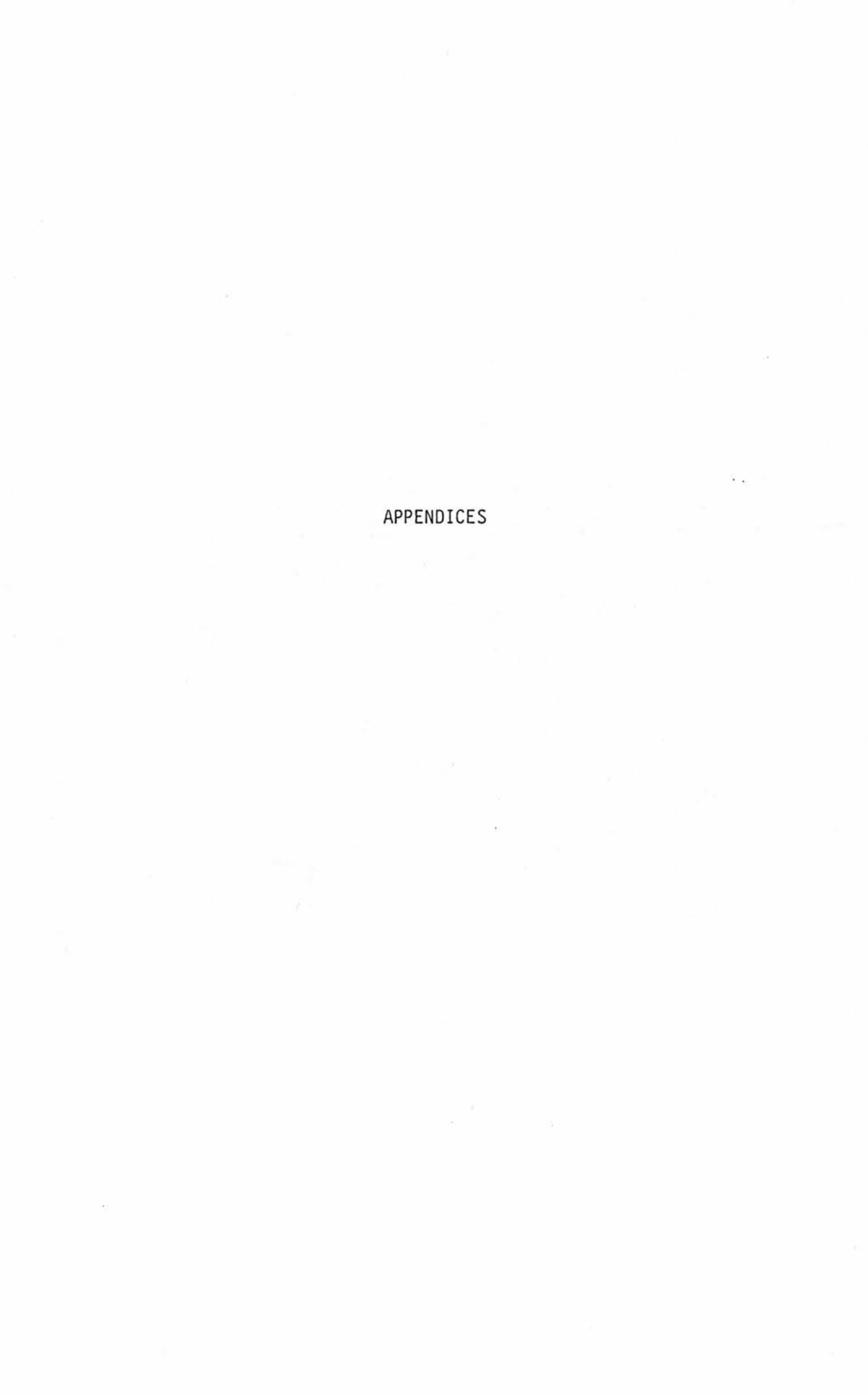
YASUDA, N., YASUDA, Y. and GREER, S.E. (1984) Heterogeneity of activity of the prolactin-releasing factor in the bovine hypothalamo-neurohypophysial complex. J. Endocrinol. 103, 243-249.

YOUNG, L.S., SPEIGHT, A., CHARLTON, H.M. and CLAYTON, R.N. (1983) Pituitary gonadotropin-releasing hormone receptor regulation in the hypogonadotrophic hypogonadal (hpg) mouse. Endocrinology 113, 55-61.

YOUNGBLOOD, W.W., HUMM, J. and KIZER, J.S. (1979) TRH-like immunoreactivity in rat pancreas and eye, bovine and sheep pineals, and human placenta: non-identity with synthetic pyroglu-His-Pro-NH_2 (TRH). Brain Res. 163, 101-110.

Youngblood W.W., HUMM, J., LIPTON, M.A. and KIZER, J.S. (1980) TRH-like bioactivity in placenta: evidence for the existence of substances other than p-glu-his-pro-NH_2 (TRH) capable of stimulating pituitary thyrotropin release. Endocrinology 106, 541-546.

ZWIZINSKI, C. and WICKNER, W. (1980) Purification and characterization of leader (signal) peptidase from escherichia coli. J. Biol. Chem. 255, 7973-7977.



APPENDICES

Appendix A

Buffers

0.01 M Phosphate buffered saline (PBS)	8.17 g sodium chloride 1.193 g disodium hydrogen phosphate (anhydrous) 0.25 g sodium dihydrogen orthophosphate ($2H_2O$) 0.1 g sodium merthiolate 990 ml distilled water pH adjusted to 7.6
0.05 M Phosphate buffer pH = 7.6	1.235 g disodium hydrogen phosphate 0.203 g sodium dihydrogen orthophosphate 200 ml distilled water
0.5 M Phosphate buffer pH = 7.6	12.35 g disodium hydrogen phosphate 2.03 g sodium dihydrogen orthophosphate 200 ml distilled water
Antiserum buffer	500 ml 0.01 M PBS 9.306 g EDTA 2 ml normal rabbit serum pH adjusted to 7.0
Borate buffer	0.01 M boric acid 0.01% sodium merthiolate pH adjusted to 8.6 with 0.1 M sodium hydroxide
TRIS buffer	100 ml 0.2 M TRIS (Tris [hydroxymethyl] aminomethane) 80 ml 0.1 M hydrochloric acid 170 g sucrose 0.58 g EDTA Volume made up to 2 l with distilled water and pH adjusted to 8.0
Charcoal suspension	5 mg dextran T-70 (Sigma UK) 500 mg Norit-A activated charcoal 200 ml TRIS buffer
'FAGLU' buffer	30.5 ml 0.2 M disodium hydrogen phosphate 19.5 ml 0.2 M sodium dihydrogen orthophosphate 10.5 ml formaldehyde (38% solution) 2.0 ml gluteraldehyde (25% solution) 37.5 ml distilled water

Appendix B

The Iodination and Chromatographic Purification of Tracers for Radioimmunoassays

The iodination reactions and subsequent chromatographic steps were all performed at room temperature. Iodinated products were either used immediately in the assay or were stored at -70°C until required. In some cases (TRH and LH) after storage for 2-3 weeks the iodinated product was re-chromatographed before use.

B.1 Thyrotrophin Releasing Hormone

Synthetic TRH (2.5 μg/25 μl of 0.05 M phosphate buffer)
^{125}Iodine (1 mCi/10 μl)
Reaction started by the addition of
Chloramine T (25 μl of a 2 mg/ml solution in 0.05 M phosphate buffer)
Reaction terminated 30 s later by the addition of
Sodium metabisulphite (10 μl of a 15 mg/ml solution in 0.05 M phosphate buffer)

Contents of reaction vial were applied to a 1 x 12 cm column of sephadex(R) G-10 (pre-coated with 0.05 M phosphate buffer/0.25 % BSA) and ^{125}I-TRH eluted with 0.05 M phosphate buffer/0.25% BSA. 1 ml fractions of the eluent collected; elution volume of ^{125}I-TRH was approximately 12 mls.

B.2 Luteinizing Hormone Releasing Hormone

Synthetic LHRH (2 μg/10 μl PBS)
0.5 M phosphate buffer, pH 7.5 (10 μl)
^{125}Iodine (1 mCi/10 μl)
Reaction started by the addition of
Chloramine T (20 μl of a 1 mg/1 ml solution in PBS)
Reaction stopped after 15 s by the addition of
Sodium metabisulphite (50 μl of a 1 mg/1 ml solution in PBS)

Contents of the reaction vial were applied to a 0.75 x 20 cm column of sephadex G-25, pre-coated and eluted with PBS/0.1% gelatine. The elution volume of ^{125}I-LHRH was approximately 16-19 ml.

B.3 Thyrotrophin

Purified TSH (5 μg/20 μl 0.25 M phosphate buffer, pH 7.5)
0.5 M phosphate buffer, pH 7.5 (25 μl)
^{125}Iodine (1 mCi/10 μl)
Reaction started by the addition of
Chloramine T (20 μl of a 1.5 mg/ml solution in 0.05 M phosphate buffer)
Reaction stopped 40 s later by the addition of
Sodium metabisulphite (50 μl of a 2.4 mg/ml solution in 0.05 M phosphate buffer)
and with the addition 40 s later of
Normal rabbit serum (100 μl)

The contents of the reaction vial were applied to the top of a 1 x 30 cm column of sephadex G-75 which had been pre-coated with 4 ml of PBS/2% BSA. The labelled TSH was eluted from the column with PBS. 0.5 ml fractions were collected into tubes which contained 0.5 ml of PBS/1% BSA.

B.4 Prolactin

Purified protein (5 μg/20 μl 0.01 M sodium bicarbonate)
0.5 M phosphate buffer, pH 7.5 (40 μl)
^{125}Iodine (1 mCi/10 μl)
Reaction started by the addition of
Chloramine T (15 μl of a 1 mg/ml solution in 0.05 M phosphate buffer)
Reaction stopped after 20 s by the addition of
Sodium metabisulphite (50 μl of a 2.4 mg/ml solution in 0.05 M phosphate buffer)
followed by the addition of
Potassium iodide-sucrose solution (100 μl of a solution containing 0.1 g potassium iodide and 0.8 g sucrose/10 ml distilled water)

The contents of the reaction vial (together with a further 100 μl of the potassium iodide-sucrose solution used to rinse the vial) were applied to a 1 x 25 cm column of sephadex G-50 which had previously been coated with 200 μl of a 4.5% solution of human serum albumin in PBS buffer. ^{125}I-Prolactin was eluted from the column using borate buffer. 0.5 ml fractions were collected into tubes which contained 0.5 ml of PBS/1% BSA. The iodinated material was freshly prepared for each radioimmunoassay as freezing resulted in subsequent loss of immunoreactivity.

B.5 Luteinizing Hormone

Purified ovine LH (5 μg/10 μl distilled water)
0.5 M phosphate buffer, pH 7.5 (25 μl)
^{125}Iodine (1 mCi/10 μl)
Reaction started by the addition of
Chloramine T (10 μl of a 5 mg/ml solution in PBS)
Reaction stopped after 2 m by the addition of
Sodium metabisulphite (25 μl of a 5 mg/ml solution in PBS)

Contents of the reaction vial were applied to a 1 x 12 cm column of Biogel$^{(R)}$ P-60 which had been coated with 1.5 ml of PBS/5% egg white. ^{125}I-labelled LH was eluted from the column with PBS and 0.5 ml fractions collected into tubes which contained 0.5 ml PBS/5% egg white.

B.6 Follicle Stimulating Hormone

Purified FSH (2 μg/20 μl distilled water)
0.5 M phosphate buffer, pH 7.5 (25 μl)
^{125}Iodine (2 mCi/20 μl)
Reaction started by the addition of
Chloramine T (10 μl of a 2.5 mg/ml solution in PBS)
Reaction terminated after 45 s by the addition of
Sodium metabisulphite (25 μl of a 2.5 mg/ml solution in PBS)

The contents of the reaction vial were applied to a 1 x 12 column of Biogel P-60 which had previously been coated with 1.5 ml of PBS/5% egg white and the iodinated hormone eluted from the column with PBS. 0.5 ml fractions of the eluate were collected into tubes which contained 0.5 ml of PBS/5% egg white.

Appendix C

List of Abbreviations

ACTH	Adrenocorticotrophin
AN	Arcuate nucleus
ARGG	Anti-rabbit gamma globulin
$B_{(o)}$	Bound (total)
BSA	Bovine serum albumin
CA	Catecholamine
CNS	Central nervous system
CV	Coefficient of variation
DA	Dopamine
DMI	Desipramine
DOPAC	Dihydroxyphenylacetic acid
EL	Ependymal layer
EXL	External layer
FSH	Follicle stimulating hormone
GABA	γ-aminobutyric acid
GAP	Gonadotrophin releasing hormone associated peptide
GH	Growth hormone
GHRH	Growth hormone releasing hormone
5-HIAA	5-hydroxyindole acetic acid
HMW	High molecular weight
hpg	Hypogonadal
HPLC	High performance liquid chromatography
5-HT	5-hydroxytryptamine
IL	Internal layer
IR	Immunoreactive/immunoreactivity
IS	Internal standard

LCED	High performance liquid chromatography with electrochemical detection
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
LPZ	Lateral palisade zone
MBH	Medial basal hypothalamus
ME	Median eminence
(m)RNA	(messenger) ribonucleic acid
NA	Noradrenaline
nAcDA	n-acetyl dopamine
NIADDK	National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases
NIL	Neurointermediate lobe
N/S	Not significant
6-OHDA	6-hydroxydopamine
OVLT	Vascular organ of the lamina terminalis
PBS	Phosphate buffered saline
PHI	Peptide histidine isoleucine amide
PIF	Prolactin inhibitory factor
PMSG	Pregnant mare serum gonadotrophin
POI	Pars oralis infundibuli
PRF	Prolactin releasing factor
PRL	Prolactin
PV	Portal vessel
PVN	Paraventricular nucleus
RER	Rough endoplasmic reticulum
RIA	Radioimmunoassay

SAPU	Scottish Antibody Production Unit
SME	Stalk (pituitary) median eminence
SS	Somatostatin
SS14	Somatostatin 14
SS28	Somatostatin 28
SS28(1-12)	Somatostatin 28(1-12)
T_3	Triiodothyronine
T_4	Thyroxine
TFA	Trifluoroacetic acid
THC	Tetrahydrocannabinol
TRH	Thyrotrophin releasing hormone
TSH	Thyrotrophin (thyroid stimulating hormone)
V	Ventricle
VIP	Vasoactive intestinal peptide