THE INFLUENCE OF HORMONAL STATUS ON THE PRODUCTION OF PITUITARY MINORITY PEPTIDES - INVESTIGATION OF AN AUTOCRINE-PARACRINE SYSTEM IN THE RAT ANTERIOR PITUITARY GLAND

A THESIS FOR THE DEGREE OF DOCTOR IN PHILOSOPHY

IN THE

UNIVERSITY OF LONDON

1990

PHILIP MELVYN JONES BSc(HONS)

ROYAL POSTGRADUATE MEDICAL SCHOOL

ABSTRACT

A variety of neuropeptides have been detected in the anterior pituitary gland where the significance of their presence is uncertain. There is evidence to suggest the existence of locally acting regulatory systems within the anterior pituitary and it is possible that locally synthesised neuropeptides function as autocrine or paracrine agents within this tissue. The secretion of hormones from the anterior pituitary is influenced by target gland and peripheral hormones and such factors would be expected to influence locally synthesised neuropeptides, possibly in part regulating pituitary hormone secretion through them. The programme of work described in this thesis is a study of the influence of endocrine status on 5 of these neuropeptides - galanin (GAL), neurotensin (NT), neuropeptide Y (NPY), substance (SP) and vasoactive intestinal peptide (VIP) in the rat hypothalamo-adenohypophyseal complex. Northern blot analysis has been used in conjunction with radioimmunoassay to estimate steady state levels of prohormone encoding mRNA and peptide. The results of the study demonstrate for the first time that the rat anterior pituitary gland contains mRNAs encoding NPY, NT and VIP. The study has also shown that alterations in physiological endocrine status affected all 5 peptides and their prohormone encoding mRNAs in the anterior pituitary gland but not in the hypothalamus. Thyroidectomy increased pituitary NPY, SP and VIP peptide and mRNA contents but decreased those of GAL and NT, whilst hyperthyroidism

decreased the peptide contents of SP and VIP and had no effect on those of GAL or NT. Adrenalectomy had no effect on the pituitary peptide and mRNA contents of NPY and NT, but increased VIP and GAL peptide contents and decreased that of SP. Chronic dexamethasone treatment had no effect on pituitary NPY peptide content but decreased the peptide contents of GAL, NT, SP and VIP. Ovariectomy decreased GAL and VIP mRNA and peptide contents, but increased those of NPY, NT and SP. Chronic oestrogen treatment increased GAL and VIP mRNA and peptide contents but decreased those of NT and SP. Castration of male rats had no effect on any pituitary neuropeptide whilst chronic testosterone treatment affected only VIP, which was decreased in both mRNA and peptide content. The effects of the manipulations on the hypothalamo-adenohypophyseal growth hormone axis were also examined with some interesting results observed in the thyroid hormone manipulation group. Hypothalamic SRIF mRNA and peptide content was unaltered in either hypo or hyperthyroid rats whilst GHRH content was decreased in both groups with GHRH mRNA being increased in the former but decreased in the latter group. In the hypothyroid group the structure of the GH mRNA was altered by an increase in the length of the poly(A) tail. The results of this study suggest that the synthesis of GAL, NPY, NT, SP and VIP in the rat anterior pituitary gland is influenced by endocrine status. The precise functional roles of these neuropeptides are as yet uncertain but they may well be involved in the regulation of hormone secretion or possess some other as yet unknown function.

ACKNOWLEDGEMENTS

I am grateful to my supervisor Professor S R Bloom for providing me with the opportunity to work on this study and for his guidance, direction and encouragement during the course of the study. I should also like to acknowledge Dr D J O' Halloran, Dr M A Ghatei and Dr J Domin for their invaluable help with the animal work and radioimmunoassay, and Dr S Legon for providing technical facilities and advice.

I am indebted to Dr M A Ghatei, Dr D J O' Halloran, Dr C J Marshall, Mrs K Davies and Dr D Smith for their time and help in the preparation and proof-reading of this thesis.

This thesis is dedicated to the memory of my father Frank Tudno Jones, 1925 - 1989.

TABLE OF CONTENTS

LIST OF PLATES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

The Anterior Pituitary Gland

The pituitary gland, a small organ situated at the base of the brain, consists of 3 lobes; the anterior lobe or adenohypophysis (also known as the pars distalis or pars glandularis), the intermediate lobe (pars intermedia) and the neural lobe (posterior pituitary or infundibular process) (Daughaday WH 1985). For 1,500 years, from the second century to the seventeenth, the pituitary gland was believed to be involved in the production of nasal mucus (pituita) formed from the waste products of chemical reactions in the brain. It is only relatively recently that the significance and function of the pituitary gland has been appreciated. Studies carried out in the first half of this century demonstrated that the pituitary played an important role in the regulation of growth, metabolism and endocrine function. Surgical removal of the pituitary gland (hypophysectomy) was found to result in atrophy of several of the body's main endocrine organs, namely the adrenals, gonads and thyroids. It was subsequently realised that the anterior pituitary regulated the functions of these tissues and that it was in effect the "conductor of the endocrine orchestra" (Cushing HW 1910). Experiments carried out by Aschner, Cushing and others demonstrated that the anterior pituitary exerted a critical influence on development, metabolism, sexual maturation and behaviour. Subsequent studies revealed that the anterior pituitary exerts its effects on its target tissues by means of substances released into the general circulation. We now know that

these substances are peptide hormones and that 6 of them are responsible for the observed effects on metabolism, development etc.

Elucidation of Anterior Pituitary Function

Surgical hypophysectomy (and also clamping of the pituitary stalk) in dogs was found to result in death preceded by loss of weight and atrophy of the ovaries and uterus (Crowe J et al 1910, Cushing HW 1910). Hypophysectomy in puppies resulted in cessation of growth of the long bones, thyroid atrophy and genital hypoplasia (Aschner B 1912). Atrophy of the adrenal cortex in adult dogs also resulted from hypophysectomy (Ascoli G and Legnani T 1912). Destruction of the pituitary body in tadpoles led to atrophy of the thyroid gland and failure to metamorphosise (Adler L 1914). Studies that followed demonstrated that the anterior pituitary contained substances which could prevent the effects of hypophysectomy. Injection of anterior pituitary gland extracts into hypophysectomised rats prevented the loss of growth normally produced by this treatment and demonstrated the existence of growth hormone or somatotrophin (GH or STH) (Evans HM and Long JA 1921). Administration of anterior pituitary gland extracts to tadpoles following removal of the pituitary bodies regenerated their atrophied thyroid glands and demonstrated the existence of thyrotrophin (TSH) (Smith PE and Smith IP 1922). Treatment of mice with anterior pituitary extracts demonstrated that there were 2 distinct gonadotrophic hormones. These were initially called

Prolan A, which governed follicle maturation and Prolan B which controlled luteinisation and are now respectively called follicle stimulating hormone (FSH) and luteinising hormone (LH) which is also known as interstitial cell stimulating hormone (ICSH) (Zondek B and Aschheim S 1927). The secretion of hormones from the adrenal cortex of the rat was shown to be regulated by a substance present in the anterior pituitary now known as adrenocorticotrophin (ACTH) (Smith PE 1930). The induction of lactation in ovariectomised rabbits following the injection of anterior pituitary extracts demonstrated the presence of a substance regulating lactation, now referred to as prolactin (PRL) (Stricker P and Grueter F, 1929).

Following isolation and purification the anterior pituitary hormones were found to be peptide in nature (Li CH 1972).

Proopiomelanocortin Derived Peptides

In addition to the anterior pituitary hormones described above, there are several other peptides known to be derived from the precursor molecule which gives rise to ACTH. These corticotrophin-related peptides are derived by selective proteolytic cleavage from a common translation product called proopiomelanocortin (POMC). As judged by secretory products, corticotrophs cleave POMC into ACTH, a carboxy-terminal peptide called beta-lipotrophin and an amino-terminal glycopeptide. Secondary cleavage of beta-lipotrophin to beta-endorphin

and gamma-lipotrophin also occurs in corticotrophs. Further cleavage can occur to give a 13 amino acid molecule derived from ACTH - alpha-melanotrophin, and beta-lipotrophin can be cleaved to give an 18 amino acid molecule - beta-melanotrophin, although it is unlikely that these 2 peptides are produced in the anterior pituitary (Daughaday WH 1985).

Anterior Pituitary Cell types

The anterior pituitary hormones are synthesised, stored and released from at least 5 different cell types. Growth hormone (GH), adrenocorticotrophin (ACTH), prolactin (PRL) and thyrotrophin (TSH) are found in separate cells of the adenohypophysis whilst follicle stimulating hormone (FSH) and luteinising hormone (LH) appear to be produced by one cell type (Nakane PK 1970).

TABLE 1.1 Anterior pituitary secretory cell types

HORMONE	CELL TYPE
Growth hormone	somatotroph
Adrenocorticotrophin	corticotroph
Prolactin	lactotroph
Thyrotrophin	thyrotroph
Luteinising hormone	gonadotroph
Follicle stimulating hormone	gonadotroph
beta-lipotrophin	corticotroph
beta-endorphin	corticotroph
gamma-lipotrophin	corticotroph

Regulation of Anterior Pituitary Function

Regulation of the release of the anterior pituitary hormones is complex and is determined by feedback effects of target gland hormones and the central nervous system (McCann SM, Porter JC 1969). The central nervous system regulates anterior pituitary function through the hypothalamus, which exerts stimulatory and inhibitory influences on pituitary secretions. Target gland hormones exert mainly inhibitory feedback influences on anterior pituitary function through direct effects on the gland and indirect effects via the hypothalamus (Fink G 1979). The hypothalamus and pituitary thus form an integrated unit, sometimes referred to as the hypothalamo-adenohypophyseal complex, through which the functions of the body's endocrine tissues are regulated. Early studies on the sympathetic nerve supply to the anterior pituitary revealed that some fibres passed to the gland but these were few in number and large areas of the pituitary were free of nerve fibres (Dandy WE 1913). In 1930 a portal circulation system linking the medium eminence of the hypothalamus to the anterior pituitary gland was described (Poppa GT and Fielding U 1930). However, the direction of blood flow in these vessels was at first uncertain and a matter of dispute. Following these observations humoral transmission of stimuli from the hypothalmus to the anterior pituitary was suggested (Friedgood HB 1936). Neurohumoral factors synthesised by hypothalamic nerve cells (neurosecretion) were thought to be released into the portal circulation system linking the median eminence of the hypothalamus to the pituitary. This theory of

hypothalamic control of hypophyseal secretion was firmly established by Geoffrey Harris who demonstrated that the direction of blood flow in this system was mainly from the hypothalamus to the pituitary (Green JD and Harris GW 1949). The anterior pituitary was thus characterised as being "a gland under nervous control but lacking a nervous supply" (Harris GW 1948). The neurohumoral factors released by the hypothalamus control the anterior pituitary by stimulating or inhibiting the release of anterior pituitary hormones (Fink G 1976). These hypothalamic factors were found to be the mono-amine, dopamine, and several peptide hormones which are known as hypophyseotropic hormones. Releasing hormones for LH (Matsuo H et al 1971), GH (Rivier J et al 1982), TSH (Nair RMG et al 1970) and ACTH (Furutani Y et al 1983) have been isolated and characterised as well as a growth hormone-release inhibiting hormone known as somatostatin (Brazeau P et al 1973). The secretion of PRL is regulated through the release of dopamine which exerts an inhibitory influence on prolactin release.

Minority pituitary peptides

In addition to GH, PRL, LH, FSH, TSH and the POMC derived peptides a variety of neuropeptides, regulatory peptides and growth factors have been shown to be present in the anterior pituitary. The majority of these peptides were not detected as a result of any observed biological effect, but were shown to be present by radioimmunoassay (RIA), immunocytochemistry, or detection of specific messenger ribonucleic acid (mRNA). These peptides are present in the anterior pituitary at much lower levels than the pituitary hormones and will be referred to in this text as minority pituitary peptides.

TABLE 1.2 Minority pituitary peptides

Angiotensin	(Steele MK et al 1982)
Atrial natriuretic peptide	(McKenzie JC et al 1985)
Calcitonin	(Deftos LJ et al 1978)
Calcitonin gene related peptide	(Skofitsch G and Jacobowitz DM 1985)
Cholecystokinin	(Rehfeld JF 1978a)
Chondrocyte growth factor	(Kasper S et al 1982)
Corticotropin-releasing factor	(Morel G et al 1982a)
Epidermal growth factor	(Kudlow JE et al 1984)
Fibroblast growth factor	(Ferrara N et al 1987)
Galanin	(Vrontakis ME et al 1987)
Gastrin	(Rehfeld JF 1978b)
Growth hormone-releasing hormone	(Morel G et al 1984)
Inhibin	(Meunier H et al 1988)
Insulin-like growth factor I	(Binoux M et al 1981)
Luteinising hormone-releasing hormone	(Duello TM 1978)
Motilin	(Korchak et al 1984)
Neurotensin	(Goedert M et al 1982)
Neuromedin B	(Steele JH et al 1988)
Neuromedin U	(Domin J et al 1987)
Neuropeptide Y	(Jones PM et al 1989)
Oxytocin	(Morel G et al 1988)
Secretin	(Samson WK et al 1984)
Somatostatin	(Morel G et al 1983)
Substance P	(Morel G et al 1982b)
Thyrotropin-releasing hormone	(Childs GV et al 1978)
Transforming growth factor-alpha	(Kobrin MS et al 1987)
Vasoactive intestinal peptide	(Morel G et al 1982c)
Vasopressin	(Chateau M et al 1979)

Hypophyseotropic Peptides in the Anterior Pituitary

A possible explanation for the detection of hypophyseotropic peptides in the anterior pituitary is suggested by the nature of hypothalamic regulation of pituitary hormone secretion. Peptide hormones, such as the hypophyseotropic hormones, function by binding to specific receptor proteins on the surfaces of target cells and generating intracellular signals. The receptor molecule is thought to undergo a conformational change upon the binding of such a ligand, leading to the activation of a plasma-membrane bound enzyme such as adenylate cyclase or the opening or closing of ion channels in the plasma membrane. Peptides bound to such receptors are then taken up into the target cell and degraded (Alberts B et al 1983). The hypophyseotropic hormones have been shown to be present in high concentrations in the hypothalamo-hypophyseal portal circulation and high affinity receptors for all have been demonstrated on anterior pituitary cell membranes.

TABLE 1.3a Hypophyseotropic peptides in the hypothalamo-hypophyseal portal circulation

Corticotropin-releasing factor	(Plotsky PM and Vale W 1984)
Growth hormone-releasing hormone	(Plotsky PM and Vale W 1985)
Luteinising hormone-releasing hormone	(Ching M 1982)
Somatostatin	(Chihara K et al 1979)
Thyrotrophin-releasing hormone	(Sheward WJ et al 1983)

TABLE 1.3b Adenohypophyseal receptors for hypophyseotropic peptides

Corticotropin-releasing factor	(Wynn PC et al 1983)
Growth hormone-releasing hormone	(Seifert H et al 1985)
Luteinising hormone-releasing hormone	(Hopkins CR and Gregory H 1977)
Somatostatin	(Srikant CB and Patel YC 1981)
Thyrotrophin-releasing hormone	(Labrie F et al 1978)

Thus the presence of hypophyseotropic hormones in the anterior pituitary may well result from uptake of peptide from the portal circulation by pituitary target cells. This explanation has been put forward to account for the presence in the anterior pituitary of corticotropin-releasing factor (CRF), luteinising hormone-releasing hormone (LHRH), somatostain (SRIF), and thyrotrophin-releasing hormone (TRH) (Morel G et al 1982a, Duello TM et al 1983, Morel G et al 1983, Morel G et al 1985). This hypothesis might also apply to other hypothalamic peptides which affect anterior pituitary function. Certain minority pituitary peptides are present in the hypothalamus and have been shown to affect anterior pituitary function. The presence of receptors for several minority pituitary peptides has also been demonstrated on anterior pituitary cell membranes.

TABLE 1.4 Minority pituitary peptides in hypothalamus

Angiotensin II	(Naruse M et al 1985)
Atrial natriuretic peptide	(Glembotski CC et al 1985)
Calcitonin	(Flynn JJ et al 1981)
Cholecystokinin	(Rehfeld JF 1978a)
Galanin	(Skofitsch G and Jacobowitz DM 1986)
Inhibin	(Ramasharma K and Li CH 1986)
Motilin	(O' Donohue TL et al 1981a)
Neuromedin B	(Steel JH et al 1988)
Neuromedin U	(Steel JH et al 1988)
Neuropeptide Y	(Lundberg JM et al 1984)
Neurotensin	(Carraway R and Leeman SE 1973)
Oxytocin	(Vandesande F and Dierickx K 1975)
Secretin	(O' Donohue TL 1981b)
Substance P	(Chang MM and Leeman SE 1971)
Vasoactive intestinal peptide	(Emson PC et al 1978)
Vasopressin	(Vandesande F and Dierickx K 1975)

TABLE 1.5 Minority pituitary peptides affecting anterior pituitary hormone release

PEPTIDE	HORMONE AFFECTED	
Angiotensin II	PRL	(Aguilera G et al 1982)
Calcitonin	PRL	(Shah GV et al 1988)
Cholecystokinin	PRL	(Matsumara M et al1985)
Galanin	GH	(Gabriel SM et al 1988)
Gastrin	TSH	(Vijayan E et al 1978)
Inhibin	FSH	(Vale W et al 1986)
Motilin	GH	(Samson WK et al 1984b)
Neuromedin B	TSH	(Rettori V et al 1989)
Neuropeptide Y	LH	(Crowley WR et al 1987)
Neurotensin	PRL	(Aronin N et al 1986)
Oxytocin	PRL	(Samson WK et al 1986)
Secretin	PRL	(Samson WK et al 1984b)
Substance P	PRL	(Aronin N et al 1986)
Vasoactive intestinal peptide	PRL	(Reichlin S 1988)
Vasopressin	ACTH	(Turkelson CM et al 1982)

TABLE 1.6 Minority pituitary peptide receptors in adenohypophyseal membranes

Angiotensin	(Hauger RL et al 1982)
Atrial natriuretic peptide	(Leitman DC and Murad F 1987)
Calcitonin	(Goltzman D and Mitchell J 1985)
Calcitonin gene related peptide	(Goltzman D and Mitchell J 1985)
Inhibin	(Seethalakshmi L 1984)
Substance P	(Kerdelhue B et al 1985)
Neurotensin	(Memo M et al 1986)
Oxytocin	(Antoni FA 1986)
Vasoactive intestinal peptide	(Robberecht P et al 1986)
Vasopressin	(Antoni FA et al 1984)

Thus it can be seen that these peptides might function in the same way as hypophyseotropic hormones, and this has been suggested to be the case for several including; - motilin (Samson WK et al 1984b), neuropeptide Y (Sutton SW et al 1988), neurotensin (Enjalbert A et al 1982), oxytocin (Morel G et al 1988), secretin (Samson WK et al 1984a), substance P (Vijayan E and McCann SM 1979) and vasoactive intestinal peptide (Shimatsu A et al 1981). It has not been possible to assign clear cut roles as releasing or release inhibiting hormones to these peptides but it is possible that they may function by modifying the actions of the known hypophyseotropic hormones.

Minority Peptide Synthesis in the Anterior Pituitary

The uptake of exogenous peptide by target cells may not, however, be sufficient explanation to account for the presence of these peptides in the anterior pituitary, as there is evidence that some of these peptides are synthesised within the gland itself. The presence of angiotensin (Saint-Andre JP et al 1986), cholecystokinin (Rehfeld JF 1987) and vasopressin (Loh YP et al 1988) prohormones have been demonstrated in rat pituitary, and cultured rat hemipituitaries were shown to incorporate 3-H labeled amino acids into immunoprecipitable vasoactive intestinal peptide (Arnaout MA et al 1986). Messenger RNA molecules (mRNAs) encoding several minority peptides have been shown to be present in the anterior pituitary.

TABLE 1.7 Minority peptide mRNAs in the anterior pituitary

Atrial Natriuretic Peptide	(Gardner DG et al 1986)
Angiotensin	(Hellmann W et al 1988)
Galanin	(Vrontakis ME et al 1987)
Gastrin	(Powell CT et al 1985)
Inhibin	(Meunier H et al 1988)
Insulin-Like Growth Factor I	(Fagin JA et al 1987)
Neuropeptide Y	(Jones PM et al 1989)
Neurotensin	(Jones PM et al 1989)
Preprotachykinin-A	(Jonassen JA et al 1987)
Somatostatin	(Pagesy P et al 1989)
Vasoactive Intestinal Peptide	(Jones PM et al 1989)
Vasopressin	(Loh YP et al 1988)

Significance of Minority Pituitary peptides

These peptides are found in the anterior pituitary in small amounts and are either undetectable in the general circulation or present at very low levels in comparison to the anterior pituitary hormones. This makes it unlikely that minority pituitary peptides affect distant target tissues in the same way as the anterior pituitary hormones. The low levels of the minority pituitary peptides suggests that the actions of these peptides are directed towards a target situated close to the site of peptide synthesis.

It is possible that these peptides may have no function within the anterior pituitary. In the rat anterior pituitary, atrial natriuretic peptide appears to be synthesised and internalised by gonadotrophs and yet exerts no known effects on hormone secretion (Morel G et al 1989). The direction of blood flow in the pituitary stalk may not be solely from the median eminence of the hypothalamus to the pituitary but may also occur in the opposite direction. Retrograde blood flow in the pituitary stalk has been observed (Oliver C et al 1977) and exogenously administered neuropeptides have been shown to reach the hypothalmus from the pituitary (Mezey E et al 1978). Thus peptides synthesised in the anterior pituitary could possibly be involved in a humoral feedback system within the hypothalamo-adenohypophsyeal complex. However, this is not a well-characterised phenomenon and requires further investigation. Many minority pituitary peptides have been shown to affect hormone secretion from

primary cultures of anterior pituitary cells suggesting that these peptides may be involved in regulating pituitary function. The most likely function of locally synthesised minority pituitary peptides would be as paracrine or autocrine agents. Paracrine regulation is a mechanism by which a substance is secreted by a group of cells within a tissue or organ and moves through the interstitial spaces to act on a group of neighbouring target cells (Feyrter F 1953). Autocrine regulation is a mechanism by which a substance influences the cell from which it is released (Sporn MB and Todaro GJ 1980). There is evidence from a number of indirect observations to suggest the existence of autocrine/paracrine interactions in the anterior pituitary.

Evidence of Autocrine-paracrine Systems in the Anterior Pituitary

A) Peptides involved in the local regulation of anterior pituitary function would be expected to be influenced by factors affecting the secretion of pituitary hormones such as target gland hormones and this has been demonstrated for certain minority peptides. Tissue specific changes in the anterior pituitary content of certain peptides have been demonstrated following alterations in endocrine status. Neurotensin in the rat anterior pituitary was shown to decrease after thyroidectomy but not after adrenalectomy or male castration (Goedert M et al 1982). Substance P in the rat anterior pituitary was shown to increase in thyroidectomised and ovariectomised animals (Coslovsky R et al 1984, Aronin N et al 1984) whilst

thyroxine or oestrogen treatment produced a decrease in substance P content (Coslovsky R et al 1984, Aronin N et al 1984). In the female rat anterior pituitary galanin mRNA content was shown to alter dramatically following alterations in oestrogen levels (Kaplan LM et al 1988a). Levels of insulin-like growth factor I mRNA in rat pituitary have been shown to be affected by circulating levels of GH (Fagin JA et al 1988). In heterotropic pituitaries transplanted beneath the kidney capsules of thyroidectoised rats, substance P again increased in content demonstrating that it could not be hypothalamic in origin (Aronin N et al 1988). The content of Neuromedin B in the rat anterior pituitary was shown to decrease in hypothyroid, and increase in hyperthyroid rats (Steel JH et al 1988). The anterior pituitary content of Neuromedin U in rats has been shown to increase following TRH treatment (Domin J et al 1989). Hyperprolactinaemia and thyroidectomy were reported to increase vasoactive intestinal peptide content in the rat anterior pituitary (Maletti M et al 1982, Lam KSL et al 1989). All these effects were specific for peptides in the anterior pituitary and were not seen in the hypothalamus.

B) The topographical arrangement of cell types within the anterior pituitary is highly organised. Within the pars tuberalis only gonadotrophs and thyrotrophs are found (Gross DS 1983, Gross DS et al 1984) whilst the dorsocephalic region (or sex-zone) is characterised by a high concentration of gonadotrophic cells (Nakane PK 1970). Thyrotrophs have been reported to occur mainly in

clusters in the centre of the gland (Nakane PK 1970). The reverse haemolytic plaque assay has been used to demonstrate that PRL secreting cells from different regions of the anterior pituitary are differentially responsive to hypothalamic stimuli (Bookfor FR and Frawley LS 1987).

The distribution of cell types suggests that the different cells may interact in some way, and this is further supported by observations that certain cell types are found in close juxtaposition to each other. Corticotrophs have been reported to be found frequently among clusters of somatotrophs (Nagata M et al 1980). A close juxtaposition of somatotrophs with thyrotrophs has also been reported (Yoshimura F and Nogami H 1980). Gonadotrophs and lactotrophs are frequently found in close association (Nakane PK 1970). Lactotrophs have frequently been observed to form cup shapes which embrace or completely surround and form junctional contacts with gonadotrophs (Nakane PK 1970, Sato S 1980, Horvath E et al 1977). No functional correlations of the associations between corticotrophs and somatotrophs or thyrotrophs and somatotrophs have been reported, but a direct inhibitory effect of PRL on LH release from cultured rat anterior pituitary cells has been demonstrated (Cheung CY 1983). Furthermore, hyperprolactinaemia was reported to weaken the LH response to LHRH (Smith S 1980) supporting the idea that such close physical associations underlie a possible functional relationship.

C) Denef and co-workers have used re-aggregation of semi-purified populations of anterior pituitary cells to demonstrate the existence of locally acting factors. Incubation of a lactotroph-enriched/gonadotroph-poor population of rat anterior pituitary cells in medium previously used to culture a gonadotroph rich cell population, increased PRL secretion several-fold implying that the gonadotrophs had released some PRL-releasing factor(s) into the medium (Denef C and Andries M 1983). A stimulatory effect of vasoactive intestinal peptide on GH secretion was demonstrated for enriched populations of somatotrophs reaggregated with populations of small-sized pituitary cells cultured in the presence of dexamethasone (Denef C et al 1985). The effect was largely ablated when populations of enriched somatotrophs were used on their own and restored when the small-sized cell population was added, indicating that the somatotroph's response was activated by an intercellular signal from the non-somatotroph cells (Denef C et al 1985). Coaggregates of enriched populations of folliculo-stellate (FS) cells with other pituitary cell populations resulted in an inhibition of the GH releasing effect of GHRH, the LH releasing effect of LHRH and the PRL releasing effects of TRH and angiotensin (Baes M et al 1987).
Folliculo-stellate cells are not believed to secrete hormones, and these observations suggest that their function may be as part of an intercellular messenger system which is not based on physical contact between interacting cells (Baes M et al 1987).

Criteria for Establishing Locally Acting Peptides

The above evidence is circumstantial and cannot be used to describe the function(s) of specific peptides, nor can it suggest the identity of peptides responsible for putative paracrine effects. In order to establish the probable paracrine nature of a peptide several criteria have been put forward by P Franchimont.

A) The peptide must be present in the tissue or organ under study.

B) It must be locally produced. Such local production should be demonstrated by incorporation of labelled amino acids into the molecule, identification of biochemical precursors such as prohormones and demonstration of the mRNA encoding the peptide.

C) It must exert a biological effect under physiological conditions. The demonstration of high affinity cell surface receptors is good evidence that the tissue is a direct target for a peptide.

D) The endogenous production and subsequent biological effect must be induced locally by hormonal stimulation. Conversely, immunoneutralization of endogenous parahormones with specific antibodies should lead to alterations in tissue or organ function.

E) Ablation of the gland normally secreting the peptide does not lead to the disappearance of the parahormone from the bloodstream or the tissue under study.

F) When the tissue or organ under study can be catheterised, there must exist a venoarterial gradient, the concentration of a parahormone being higher in the efferent vein than in the afferent artery. (Franchimont P 1986)

The above criteria for establishing the paracrine nature of a peptide are generalised guidlelines which cannot be applied to all peptides in all endocrine tissues. The minority pituitary peptides are an example of where such idealisations cannot easily be applied. The anterior pituitary has a complicated blood system as the result of its function as an endocrine gland and the nature of hypothalamic regulation. Consequently it would not be feasible to catheterise the gland and attempt to establish a venoarterial gradient. As mentioned above several minority pituitary peptides are present in the hypothalamus and may affect the pituitary in the same way as the hypophyseotropic hormones. Many of these peptides are synthesised in multiple sites around the mammalian body and it would be impossible to ablate the gland that normally secretes the peptide.

Several minority pituitary peptides appear to fulfil one or more of the above criteria and it was suggested

accordingly that they might function as autocrine/paracrine agents. These include vasoactive intestinal peptide (Arnaout MA et al 1986), substance P (Coslovsky R et al 1984), insulin-like growth factor I (Fagin JA et al 1987), angiotensin (Saint-Andre JP et al 1986), vasopressin (Loh YP et al 1988), inhibin (Meunier H et al 1988), and neurotensin (Goedert M et al 1984). These peptides are known to affect the secretion of anterior pituitary hormones and following demonstration of local synthesis were suggested to have local regulatory functions. In the investigation of putative parahormones it is important to show that they are locally synthesised and produce local effects. By these criteria the most well characterised locally acting peptide in the anterior pituitary is vasoactive intestinal peptide. Evidence for local synthesis comes from demonstration of the incorporation of labelled amino acids into immunoprecipitable vasoactive intestinal peptide (Arnaout MA et al 1986) and the dectection of vasoactive intestinal peptide mRNA (Jones PM et al 1989). The addition of a vasoactive intestinal peptide antiserum to dispersed rat anterior pituitary cells decreased PRL secretion from these cells (Hagen TC et al 1986). The reverse haemolytic plaque assay demonstrated not only that vasoactive intestinal peptide was released from lactotrophs, but also that treatment with a vasoactive intestinal peptide antiserum or antagonist could reversibly block PRL secretion from these cells (Nagy G et al 1988). The need to demonstrate that a minority peptide is synthesised within the anterior pituitary is particularly relevant as

it is necessary to distinguish between peptides which may be present as a result of uptake from the hypothalamo-hypophyseal circulation and those which are synthesised locally. It is possible that peptide immunoreactivity detected in a tissue may result from non-specificity or cross reactivity of anti-sera. Evidence of local synthesis is commonly provided by the detection of prohormone encoding mRNA (Hellman E et al 1988, Fagin JA et al 1987, Meunier H et al 1988, Jonassen JA et al 1987), and whilst this is not absolute proof of synthesis it is usually taken as strong evidence of such.

Aim of Study

The objective of this study has been to provide evidence of the local synthesis of 5 neuropeptides - galanin, neuropeptide Y, neurotensin, substance P and vasoactive intestinal peptide (hereafter referred to respectively as GAL, NPY, NT, SP and VIP) in the anterior pituitary gland and to examine the influence of alterations in endocrine status on the mRNA and peptide content of these 5 peptides in both the anterior pituitary gland and hypothalamus of the rat. With the possible exception of VIP, little is known at present about the identity and function of pituitary paracrine agents. Demonstration of local synthesis and changes in peptide and mRNA content following alterations in physiological endocrine status will provide evidence that these peptides are involved in regulating anterior pituitary function. The results of the study will act as a guide for future research and may also aid in indicating the functions of these peptides in the anterior pituitary.

The effects of certain endocrine manipulations on anterior pituitary NT, SP and VIP have been published making these peptides of interest and allowing comparison of the results of the present study with those of others. In addition to these 3 peptides NPY and GAL have been studied as these are recently discovered neuropeptides known to affect anterior pituitary hormone secretion.

Description of Peptides

The effects of these 5 peptides on the secretion of anterior pituitary gland hormones are discussed in detail in the relevant results chapters. Galanin, NPY, NT, SP and VIP are structurally unrelated peptides and are described briefly below. All 5 peptides show widespread tissue distributions, typically being found in the nervous system and various endocrine and peripheral tissues and producing a range of effects on these different tissues dependent on dosage and mode of administration.

Galanin, originally isolated from porcine intestine contains 29-amino acids and is structurally unrelated to any other known peptide (Tatemoto K et al 1983). Galanin-like immunoreactivity (GAL-LI) has been found throughout the central and peripheral nervous systems of various mammalian species and high concentrations have been detected in the hypothalamus, neurointermediate lobe of the pituitary and spinal cord (Skofitsch G and Jacobowitz DM 1986). In peripheral tissues GAL-LI has been found in the gastrointestinal tract, pancreas, adrenal medulla, genitourinary tract and respiratory tract of several species (Rokaeus A 1987). Injection of GAL into the third ventricle of rats has been shown to stimulate food intake (Kirkouli SE et al 1986) and induce an increase in the secretion of GH and PRL (Ottlecz A et al 1988). Galanin has been shown to inhibit the release of insulin and pancreatic polypeptide from perfused rat pancreas (Silvestre R et al 1987a, Silvestre R et al 1987b) and the intravenous injection of GAL into dogs

resulted in hyperglycaemia and glucose intolerance (Rokaeus A 1987).

Neuropeptide Y (NPY) is a 36-amino acid peptide which shows considerable sequence homologies with pancreatic polypeptide and peptide YY (Tatemoto K et al 1982) and is one of the most abundant and widespread peptides in the mammalian nervous system (Allen JM and Bloom SR 1986). Neuropeptide Y-like immunoreactivity has been found in high concetrations in nerve cell bodies in the arcuate and periventricular nucleii of the hypothalamus (Lundberg JM et al 1984, Chronwall BM et al 1985). Neuropeptide Y immunoreactive nerve fibres are widely distributed around the body and NPY mRNA has been detected in a variety of peripheral tissues such as heart, adrenal and spleen (Larhammar D et al 1987). Neuropeptide Y has been shown to be a potent vasoconstrictor (Edvinsson L et al 1983) and to enhance adrenergic vasoconstriction in vitro (Edvinsson L et al 1984). Injection of NPY into the third ventricle of rats induces feeding behaviour (Allen LG et al 1985) and modulates circulating levels of LH depending upon the sex hormone status of the animal (Kalra SP and Crowley 1984, Kerkerian L et al 1985, Sahu A et al 1987). Systemic administration of NPY has been shown to increase or decrease circulating LH levels, again depending on sex hormone status (Kerkerian L et al 1985, Rodriguez-Sierra JF et al 1987.

Neurotensin is a 13-amino acid peptide (Carraway R and Leeman SE 1973) which shows a widespread distribution

throughout the mammalian central nervous system (Emson PC et al 1982, Muraki K et al 1985) and is also found in the gastrointestinal tract (Muraki K et al 1985). Systemic administration of NT produces hypotension (Carraway R and Leeman SE 1973), gut contraction (Carraway R and Leeman SE 1975), hyperglycaemia (Brown M and Vale W 1976) and stimulation of the release of PRL, TSH, and GH (Aronin N et al 1986). Intraventricular injection of NT has been reported to decrease levels of circulating PRL (Aronin N et al 1986) and was reported by different groups to either increase or decrease circulating GH and LH levels (Aronin N et al 1986).

Substance P (SP), originally isolated from bovine hypothalamus (Chang MM and Leeman SE 1971) is an 11-amino acid member of the tachykinin family of peptides. Substance P is unevenly distributed throughout the central and peripheral nervous systems (Hokfelt T et al 1975) and is found in high concentrations in the hypothalamus (Ljungdahl A et al 1978). In peripheral tissues SP is found in the gastrointestinal tract (Pearse AGE and Polak JM 1975) and adrenal medulla (Role LW et al 1981). Peripheral effects of SP include modulation of gut contraction (Bury RW and Mashford ML 1976), salivation (Leeman SE and Hammershlag R 1967) and effects on the release of anterior pituitary hormones. Intraventricular injection of SP has been reported to elevate serum PRL, GH and LH (Aronin N et al 1986) whilst systemic administration of SP was reported to increase the release of GH and PRL (Aronin N et al 1986).

Vasoactive intestinal peptide is a 28-amino acid peptide originally isolated from porcine intestine (Said SI and Mutt V 1970) which is structurally related to several other peptides including secretin, glucagon and corticotropin releasing factor. Vasoactive intestinal peptide is widely distributed throughout the central nervous system and peripheral nervous system (Bryant MG et al 1976, Besson J et al 1979) and is found in high concentrations in the hypothalamus, particularly in the suprachiasmatic and paraventricular nuclei (Samson WK et al 1979). In peripheral tissues VIP is present in the gastro-intestinal (Bryant MG et al 1976) and genito-urinary (Larsson LI et al 1977) tracts as well as various endocrine tissues such as thyroid (Ahren B et al 1980) and ovary (Gozes I and Tsafriri A 1986). Peripheral effects of VIP include smooth muscle relaxation (Said SI 1984), vasodilation (Suzuki Y et al 1984) and modulation of the secretion of anterior pituitary hormones. Intracerebral administration of VIP was reported to increase PRL (Kato T et al 1978), LH and GH (Vijayan E et al 1978) secretion whilst systemic administration of vasoactive intestinal peptide effects only the release of PRL (Ottesen B et al 1981, Kato Y et al 1984). The potent PRL releasing action of vasoactive intestinal peptide and its presence in high concentrations in the hypothalamus and portal circulation (Shimatsu A, et al 1981) have led to speculation that it may be the putative hypothalamic PRL releasing factor (Abe H et al 1985).

Approach

Galanin, NPY, NT, SP and VIP are presumed to affect anterior pituitary function by local actions and so would be expected to alter following changes in pituitary hormone secretion. This has been investigated by altering the synthesis of the pituitary hormones regulating thyroid, adrenal and male and female gonad function and examining the effects on GAL, NPY, NT, SP and VIP. This approach has been followed by groups investigating anterior pituitary GAL, NT, SP and VIP (Kaplan LM et al 1988a, Goedert M et al 1982, Aronin N et al 1984, Coslovsky R et al 1984, DePalatis LR et al 1985, Lam KSL et al 1989). However, with the exception of the work on NT and SP, these observations are rather disparate and a thorough examination of the effects of alterations in hormonal status on these peptides will be an important contribution to the field of study. The 5 peptides have been examined in both the hypothalamus and anterior pituitary as these tissues form an integrated unit regulating physiological endocrine status. This approach has allowed the tissue specificity of effects to be assessed and provided information about the level at which regulation of pituitary function may occur. As part of this study growth hormone-releasing hormone (GHRH) and somatostatin (SRIF) have been studied in the hypothalamus for comparison as these are hypothalamic peptides known to affect anterior pituitary growth hormone function.

Northern blot analysis has been used in conjunction with radioimmunoassay to monitor the effects of the

manipulations on these peptides in the hypothalamus and anterior pituitary. This approach was employed to demonstrate the synthesis of these peptides within the anterior pituitary and to give a better overall picture of the effects of the manipulations on peptide production. The advent of molecular cloning techniques allows the isolation and characterisation of the nucleic acid coding sequence for any peptide for which a suitable screening procedure can be established. The nucleotide sequences of a number of minority pituitary peptide mRNAs have been determined and clones of these sequences have become available. These clones can be used as radioactive hybridisation probes to detect mRNA molecules encoding specific peptides and proteins in Northern blotting. This technique also allows quantitation of changes in steady state levels of mRNA. The hybridisation reactions used in Northern blotting can be carried out both under stringent conditions so that highly homologous sequences are detected and under less stringent conditions to detect related sequences. Deoxyribonucleic acid (DNA) clones for neuropeptide Y, substance P and vasoactive intestinal peptide were obtained from various laboratories and prepared for use as hybridisation probes. Probes for galanin and neurotensin have been synthesised using the polymerase chain reaction or PCR (Saiki R et al 1985). This technique has made it possible to amplify almost any nucleotide sequence up to several thousand base pairs in length, be it from a DNA or RNA source, purify and isolate the sequence. Thus it is now possible to isolate probes for any peptide sequence, providing the nucleotide

sequence is known, and to use these as hybridisation probes. The results show that all 5 peptides are expressed in the rat anterior pituitary gland and that changes in endocrine status can produce dramatic alterations in the production of these peptides.

The results of manipulating thyroidal, adrenal, female gonadal and male gonadal status are presented in chapters 3 to 6 respectively and the results of the manipulations on GHRH and SRIF are presented in chapter 7.

CHAPTER 2

MATERIALS AND METHODS

Animals

Intact or surgically operated male and female Wistar rats were supplied by either Charles Rivers Breeding Laboratories (Margate, Kent) or Interfauna Ltd (Huntingdon, Cambridgeshire). The treatment regimes for each series of manipulations are described in the appropriate chapters. Twenty four hours after the last day of treatment animals were sacrificed by cardiac puncture and exsanguination whilst under light ether anaesthesia.

Dissection

Following sacrifice the cranium was opened and the brain removed leaving the pituitary visible in its fossa within the skull. The whole pituitary gland was removed and the anterior lobe separated from the neurointermediate lobe by carefully pinching around the neurointermediate lobe with a pair of fine forceps. For Northern blot analysis anterior pituitary glands from each group were pooled and extracted, whilst for radioimmunoassay anterior pituitary glands were extracted individually.

The hypothalamus of each animal was dissected en bloc from a brain slice cut between the optic chiasm and the mammillary bodies. The hypothalamus was removed as a block from the brain slice by cutting vertically along the perihypothalamic sulci and then horizontally immediately below the anterior commisure. For Northern blot analysis whole hypothalamic blocks from each group were pooled and

extracted. For radioimmunoassay hypothalamic blocks were further subdivided into central and lateral portions which were extracted individually. This was carried out by Dr D J O' Halloran using a binocular dissecting microscope. The hypothalamic block, lying on its ventral surface, was divided into central and lateral portions by vertical longitudinal cuts through the two fibre bundles which can be seen lying on either side of the upper surface midline. The central hypothalamus contained most of the major hypothalamic nuclei namely; - periventricular, suprachiasmatic, arcuate, paraventricular, ventromedial, dorsomedial, posterior hypothalamic, premammillary and mammillary nuclei, together with the medial preoptic and anterior hypothalamic areas. The lateral portion consisted of the lateral pre-optic and lateral hypothalamic areas but also included the supraoptic nucleus.

Extraction and Quantitation of RNA

The method of RNA detection used in this study was Northern blotting which is a modification of the technique developed by E M Southern. The separation of DNA molecules by electrophoresis on neutral agarose gels, followed by immobilisation on nitrocellulose filters and hybridisation with radioactive probes produced a rapid and sensitive technique for the detection of specific DNA molecules which became known as Southern blotting (Southern EM 1975). When this technique was adapted and applied to the investigation of RNA through the use of denaturing agarose gels (Alwine JC et al 1979) it provided a means for detecting alterations in mRNA content and structure and aquired the name of Northern blotting. Dispersal of tissues in chaotropic solutions, such as salts of guanidinium, protects RNA from degradation by nucleases, allowing rapid extraction. Following purification RNA can be size separated by gel electrophoresis and fixed to a solid support where specific mRNAs can be detected by hybridisation with radioactively labelled DNA or RNA probes. Details of the methods of RNA extraction and of the preparation, labelling and use of probes are given below. The recipes for buffers and solutions are given in appendix A.

The extraction of RNA and all work involving the preparation and use of probes was carried out by the author in the laboratory of Dr S Legon.

Extraction of RNA (Chomczynski P and Sacchi N 1987)

Reagents

Solution D: 4M guanidinium isothiocyanate (Fluka)
25mM sodium citrate, pH7.0
0.5% sodium sarcosyl (Fluka)
0.1M 2-mercaptoethanol,

Phenol (water saturated)

Chlorophorm/iso-amyl alcohol mixture (49:1 vol:vol)

Method

Tissues were dispersed in solution D in a glass homogeniser with a teflon headed rotor. Homogenates were mixed sequentially with 0.1 volume of 2M sodium acetate pH 4.0, 1 volume of phenol and 0.2 volume of chlorophorm/iso-amyl alcohol, shaken for 20 seconds, chilled on ice for 15 minutes and then centrifuged for 20 minutes at 10,000g (8,000 rpm in a Sorvall RC-5B Refrigerated Superspeed Centrifuge using a Sorvall HB-4 rotor). After centrifugation the upper, aqueous layer was removed and the RNA precipitated by mixing it with an equal volume of isopropanol and chilling at -20^{o}C for 60 minutes. The RNA was then pelleted by centrifugation at 20,000g (12,000 rpm in a HB-4 rotor in a Sorvall RC-5B centrifuge) for 20 minutes, dissolved in one quarter of the original volume of solution D and precipitated with isopropanol at -20^{o}C. Following centrifugation the RNA was washed in 70% ethanol, dissolved in sterile TE and its concentration determined by measuring the optical density at 260 nm.

Visualisation of RNA

Reagents

Denaturing buffer: 100 ul formamide
30 ul formaldehyde
10 ul 20 X MOPS

Loading Buffer: 30% Ficoll
1 mM EDTA
0.25% Bromophenol blue

Method

Samples containing 5 ug of total RNA in 4 ul of distilled water (GDW) were mixed with 8 ul of denaturing buffer and heated at 65^{o}C for 5 minutes. After cooling to room temperature, 3 ul of loading buffer was added to each of the samples which were then loaded onto the formaldehyde gel (Appendix B) and electrophoresed at 100 V. When the samples had run approximately 30 mm the current was turned off and the gel placed in a plastic sandwich box on a rocker platform and stained for 30 minutes with TE containing 1.0 ug/ul of ethidium bromide followed by destaining in TE alone for at least one hour. Following destaining the RNA was visualised by illumination on a UV transilluminator (Chromato-vue 302 nm transilluminator, model TM-36, Ultra-violet products Inc., San Gabriel, California, USA) and the gel photographed using a Polaroid MP-3 Land Camera and Polaroid 667 film. Visualisation of RNA samples allowed the integrity and relative concentrations of samples to be assessed.

Northern Blotting (Maniatis T et al 1982, chapter 6)

Method

Samples containing up to 50 ug of total RNA in a 10 ul volume were mixed with 12 ul of denaturing buffer (page 54) and denatured by heating at 65°C. A formaldehyde gel (Appendix B) was submerged in the electrophoresis tank and the samples loaded and run at 100 V until the bromophenol blue dye had run 90 - 100 mm from the wells. A marker lane containing 5 ug of total RNA was stained and destained (as decribed on page 54) and photographed next to a ruler. The gel was placed inverted onto 2 pieces of Whatman 3MM paper soaked in 20 X SSC which were in contact with a reservoir of 20 X SSC. Cling film was placed around the gel which was then measured with a ruler. A piece of Hybond-N membrane cut to the size of the gel was placed on top of the gel and air bubbles were expelled by rolling a glass pipette over the gel. A piece of 3MM paper soaked in 20 X SSC and cut to the size of the membrane was placed onto the membrane followed by two dry pieces of cut 3MM paper followed by a stack of toilet roll tissues (5 - 10 mm thick) and then a stack of hand towels approximately 100 mm thick. A 200 g weight was placed on the stack which was left for 16 - 24 hours to allow transfer of RNA to occur. Following transfer, the membrane was air dried at room temperature for 5 minutes before the RNA was fixed to it by baking at 80°C for two hours, after which the membrane was sealed into a plastic bag for storage.

Probes

Probes for SRIF, NPY, PPT-A and VIP were kindly donated by Professors J Dixon (Purdue University, West Lafayette, USA), JE Krause (Washington Universtiy School of Medicine, St Louis, USA) and H Okamoto (Tohoku University, Tokyo, Japan). The rat TSH-beta subunit probe was obtained from Professor WW Chin, Brigham and Women's Hospital and Harvard Medical School, Boston, MA. The rat GHRH cDNA probe was kindly donated by Prof R Evans, Salk Institute, San Diego, USA. The rat GH probe was supplied by Dr F De Noto, Metabolic Research Unit, University of California and the rat PRL probe by Prof R A Maurer, Dept Physiology and Biophysics, University of Iowa, Iowa City. The POMC and LH-beta subunit probes were donated by Prof J L Roberts, College of Physicians and Surgeons of Columbia University. The human ubiquitin cDNA fragment was supplied by Professor K. Lund, School of Medicine, University of North Carolina at Chapel Hill, USA. Probes for GAL and NT were synthesised by amplifying cDNA fragments using the polymerase chain reaction. Single stranded cDNA prepared by reverse transcription provided a template for the polymerase chain reaction allowing amplification into double stranded cDNA fragments which were then isolated, ligated into plasid vectors and sequenced. Use of the plasmid bluescript allowed the selection of bacteria containing recombinant plasmids on the basis of inactivation of beta-galactosidase activity. Once the sequence and orientation of the insert had been determined the recombinant plasmid was grown up for the preparation of DNA and RNA probes.

Oligo(dT)-cellulose Chromatography

(Maniatis T et al, chapter 6)

Reagents

Oligo(dT)-cellulose (Sigma Chemical Co Ltd, Poole, Dorset)

Method

Both GAL and NT are known to be present at high levels in the rat hypothalamus and this was the tissue used in the preparation of RNA for reverse transcription for both probes. Total RNA (1 - 2 mg) was loaded dropwise onto the oligo(dT) column in 1 ml of TE containing 0.5 M NaCl and 0.1% SDS using a Pasteur pipette and the effluent kept. Following loading the column was washed twice with 1 ml of TE containing 0.5 M NaCl to remove unbound RNA (ie non-polyadenylated RNAs such as ribosomal, transfer and small nuclear species). The poly(A)+ purified RNA was then removed by the addition of TE to the column and the yield determined by spectrophotometry. Following quantitation, RNA was concentrated by adding sodium acetate (pH 5.0) to a final concentration of 0.3M, mixing with 2.5 volumes of absolute ethanol and chilling at -20^{o}C for one hour.

Synthesis of cDNA (Krug MS and Berger SL 1987)

Reagents

10 X Reaction buffer: 500 mM Tris pH 8.3
750 mM KCl
100 mM Dithiothreitol
30 mM MgCl

dNTP mixture (2.5 mM each dATP, dTTP, dCTP and dGTP; Pharmacia Ltd, Milton Keynes, Buckinghamshire)

poly(A)+ RNA (1 mg/ml)

oligo(dT) (1 mg/ml) (Sigma)

Moloney-Murine Leukaemia Virus (M-MLV) Reverse Transcriptase 200 u/ul (Gibco-BRL)

Method

Reactions were carried out in a total volume of 50 ul set up in the following way;

5 ul 10 X reaction buffer
2 ul rat hypothalamus poly(A)+ RNA
1 ul oligo(dT) (Sigma)
10 ul dNTP mixture
1 ul Reverse transcriptase (Gibco-BRL)
31 ul distilled water

The buffer, RNA, oligo(dT), nucleotides and distilled water were mixed together and heated at 65^{o}C for 3 minutes to denature the RNA. The reaction was initiated by the addition of 1 ul reverse transcriptase and incubated at 37^{o}C for 60 minutes before being frozen at -20^{o}C until use in the polymerase chain reaction.

Polymerase Chain Reaction (Saiki R et al 1985)

Reagents

10 X Taq polymerase buffer:

100 mM Tris pH 8.3

500 mM KCl

20 mM MgCl

0.1% gelatin

dNTP mixture (2.5 mM each dATP, dTTP, dCTP and dGTP)

Taq DNA polymerase, 2.5 u/ul (Amersham, Amersham, Buckinghamshire)

Oligonucleotides (synthesised on a Biosearch Cyclone DNA synthesiser, New Brunswick):

Galanin (Kaplan LM et al 1988b);

5'; - C T G A A C A G C G C T G G C T A C C T T C T G -

identical to nucleotides 250 - 273 of rat galanin mRNA

3'; - A G G A C A C A G G T G C A C A G T G G G T G T -

complementary to nucleotides 524 - 547 of rat galanin mRNA

Neurotensin (Kislauskis J et al 1988);

5'; - G G A A T G A A C C T T C A G C T G G T G -

identical to nucleotides 260 - 280 of rat neurotensin mRNA

3'; - C A T G A T C G A G G A T A T C C T C C T G

complementary to nucleotides 618 - 639 of rat neurotensin mRNA

Method

Taq polymerase is a heat stable DNA polymerase obtained from the thermophile bacterium Thermophilus aquaticus. The use of this heat stable DNA polymerase greatly facilitates the polymerase chain reaction by allowing denaturation of the DNA without inactivation of the enzyme making it unnecessary to add fresh enzyme to the reaction after every denaturation step as was the situation before this enzyme became available. One tenth of the reverse transcriptase reaction provided sufficient material to amplify DNA fragments. The reaction was prepared in the following way by mixing together:

5 ul the reverse transcriptase reaction
10 ul 10 X Taq polymerase buffer,
5 ul each primer (200 ng/ul)
20 ul dNTP mixture
59 ul distilled water

The mixture was overlaid with 100 ul parafin oil and heated in a water bath at 94^{o}C for 5 minutes to denature the DNA. It was then transferred to a 55^{o}C water bath for 3 minutes to allow the oligonucleotide primers to hybridise to the target sequence. The reaction was then initiated by adding 1 ul of Taq DNA polymerase to the reaction and incubating in a water bath at 72^{o}C for 5 minutes. Following this, the mixture was subjected to thirty cycles of repeated rounds of denaturation, primer annealing and DNA synthesis using a thermocycle profile of 2 minutes at 94^{o}C, 2 minutes at 55^{o}C and 3 minutes at

72°C. At the end of the last cycle the mixture was incubated for an extra 3 minutes at 72°C. One tenth (10 ul) of each reaction was then run out on a 1.0% neutral agarose gel in parallel with a ladder of DNA size markers, (a Hind III digest of phage lambda or a Hae III digest of phage PHI X) and visualised using a UV transilluminator. When a band of the correct size was observed it was excised with a scalpel blade, electroeluted and purified (page 64). The excised band was then ligated into plasmid Bluescript (Vector Cloning Systems, supplied through Northumbria Biologicals Ltd, Northumberland) which had been digested with Sma I to give a linear plasmid with blunt ends. When a bacterial colony containing a recombinant Bluescript plasmid had been isolated DNA sequencing was carried out to determine the identity and orientation of the inserted sequence.

Mini-Preparations of Plasmid DNA (Del Sal G et al 1988)

Reagents

STET buffer: 8% (w/v) sucrose
0.1 % Triton X-100
50 mM EDTA
50 mM Tris pH 8.0

2.0% (w/v in GDW) CTAB (cetyl trimethyl ammonium bromide, Sigma).

BCIG (5 bromo, 4 chloro 3 indolyl beta D galactoside, Sigma) 20 mg/ml in dimethyl formamide.

IPTG (Iso propyl beta D thio galactopyranoside, Sigma) 24 mg/ml in distilled water.

Method

Plasmids were introduced into E coli (Maniatis T et al 1982, chapter 8) and spread onto plates of L-broth containing 1.5% agar plus antibiotic. For isolation of recombinant bacteria the plates were coated with 20 ul each of BCIG and IPTG solutions. Single bacterial colonies were innoculated into 1.5 ml aliquots of L-Broth plus antibiotic in 30 ml universals and grown shaking at 37^{o}C overnight. Next day the cultures were transferred to 1.5 ml eppendorf tubes and centrifuged for two minutes. The supernatants were discarded and the pellets resuspended in 200 ul STET containing 1 mg/ml lysosyme and left at room temperature for 5 minutes before being boiled

for 90 seconds and then centrifuged for 15 minutes. The pellets were removed with wooden toothpicks and 20 ul 2% CTAB solution was added and the resulting precipitates centrifuged for 5 minutes. The pellets were resuspended in 300 ul 1.2 M NaCl by vigorous pipetting and then reprecipitated by the addition of 750 ul absolute alcohol. These were centrifuged for 10 minutes, the supernatants removed, the pellets rinsed with 200 ul 70% ethanol and then dried under vacuum. The pellets were redissolved in 20 ul TE and one tenth of each was then digested with the appropriate restriction enzymes (Maniatis T et al 1982, chapter 4). Plasmids containing inserts of the correct size and restriction pattern were then sequenced and/or re-introduced into bacteria for large-scale preparations of plasmid DNA (Maniatis T et al 1982, chapter 3).

Isolation of DNA Fragments

Method

The DNA sample from which the fragment was to be isolated (either a restriction enzyme digest or a PCR reaction) was electrophoresed on a neutral agarose gel (appendix B). When the desired band was observed to be separated from any other fragments by a satisfactory distance, it was excised by slicing through the agarose around it with a scalpel blade. The piece of agarose was placed in a length of dialysis tubing 5 - 8 cm in length with a small volume, usually 200 ul of 1 X TBE and electrophoresed at 200 V for 30 minutes, at the end of which time the direction of the current was reversed (keeping the voltage constant) for 45 seconds. The TBE was transferred to an eppendorf tube and the DNA precipitated by the addition of sodium acetate and ethanol to final concentrations of 0.3M and 70% respectively and chilling overnight at -20^{o}C. The DNA was then pelleted by centrifugation, dried and redissolved in a suitable volume of distilled water. The concentration was estimated by electrophoresis of one fiftieth and one tenth of an isolated fragment and comparing the intensity of fluorescence under UV light with 10, 20, 50 and 100 ng of uncut phage lambda DNA run on the same gel.

DNA Sequencing (Mierendorf RC, Pfeffer D 1987)

Reagents

10 X Klenow buffer:

100 mM Tris pH 7.5

500 mM NaCl

50 mM MgCl

Isotope/enzyme mixture: For 2 clones (8 reactions)

2 ul 0.1 mM DTT

1 ul 32-P dCTP

1 ul 10 uM dCTP

1 ul Klenow DNA polymerase (1 unit)

11 ul 10 mM Tris pH 8.0

Formamide dye: deionised formamide containing

20 mM EDTA

0.03 % Xylene cyanol

0.03 % Bromophenol blue

Deoxytriphosphate (dNTP) solutions (Pharmacia)

Dideoxytriphosphate (ddNTP) solutions (Pharmacia)

T7 oligonucleotide primer - AATACGACTCACTATAG

T3 oligonucleotide primer - ATTAACCCTCACTAAAGGGA

32-P CTP (Amersham; 3,000 Ci/mmol; 10 mmol/l)

Klenow fragment of DNA polymerase 1 u/ul, (GIBCO-BRL, Paisley, Scotland)

Deoxytriphosphate (dNTP) sequencing mixes:

	dTTP (0.5 mM)	dATP (0.5 mM)	dGTP (0.5 mM)	50 mM Tris (pH 8.0)
T mix	2 ul	40 ul	40 ul	10 ul
A mix	40 ul	2 ul	40 ul	10 ul
G mix	40 ul	40 ul	2 ul	10 ul
C mix	40 ul	40 ul	40 ul	10 ul

Sequencing solutions:

T - 5 ul T mix + 3 ul 0.5 mM ddTTP + 2 ul GDW

C - 5 ul C mix + 1 ul 0.1 mM ddCTP + 4 ul GDW

G - 5 ul G mix + 5 ul 0.1 mM ddGTP

A - 5 ul A mix + 3 ul 0.3 mM ddATP + 2 ul GDW

Method

The ratios of dideoxytriphosphate to deoxytriphosphate nucleotides used for DNA sequencing in this study were determined empirically from the method of Sanger in the laboratory of Dr S Legon (Sanger F et al 1977). One to 2 ug of plasmid DNA in 20 ul of distilled water was denatured by adding 2 ul 2 N NaOH, 2 mM EDTA and incubating at room temperature for 5 minutes. The solution was neutralised by the addition of 3 ul 2 M NaOAc and precipitated by adding 7 ul distilled water and 75 ul absolute alcohol and chilling at -70^{o}C for 5 minutes. Following this the solution was spun in a microcentrifuge for 10 minutes, the supernatant removed and the pellet washed with 70% ethanol and dryed under vacuum. The pellet was redissolved in 8 ul distilled water to which was then added 1 ul 10 X Klenow buffer and 1 ul of a

1 ng/ul solution of either oligonucleotide primer. This was then incubated at 37^{o}C for 15 - 20 minutes to allow the primer to anneal to the plasmid DNA. Whilst annealing was being carried out, 2 ul of each sequencing solution was aliquoted into appropriately labelled tubes (A, T, C or G) to which was then added 2 ul of annealed plasmid. The isotope/enzyme mixture was made up and 2 ul of this was added to each tube, the contents mixed thoroughly and then incubated at 37^{o}C for 15 minutes. After 15 minutes, 2 ul 0.5 mM dCTP was added to each reaction and the reactions were incubated at 37^{o}C for a further 15 minutes before being stopped by the addition of 4 ul formamide dye to each reaction. The reactions were denatured by boiling for 5 minutes and analysed by electrophoresis on a 6.0% acrilamide, 8.3 M urea gel (Ambrose BJB and Pless RC 1987).

Preparation of DNA Probes, Hybridisation and Washing

Reagents

DNA Labelling Buffer (Feinberg and Vogelstein 1983)
Prepared by mixing buffers A, B and C together in the ratios 100 : 250 : 150 (A : B : C)

Buffer A: To 1 ml 125 mM MgCl2/1.25 M Tris pH 8.0 was added:
18 ul beta mercaptoethanol
5 ul 100 mM dATP
5 ul 100 mM dTTP
5 ul 100 mM dGTP

Buffer B: 2 M HEPES pH 6.6 (titrated to pH with 4 M NaOH)

Buffer C: Hexameric oligonucleotides (6mers) random sequence (112 ug/ml, Pharmacia)

BSA 2 mg/ml in distilled water (GIBCO-BRL)

Alpha 32-P, dCTP (Amersham; 3000 Ci/mMol; 10 mmol/l)

Klenow (large) fragment of DNA polymerase I (GIBCO-BRL)

Method

Ten to 20 ng of DNA in a 15 ul volume of water was boiled for 5 minutes in a 0.5 ml eppendorf tube. Following boiling, the tube was centrifuged for 20 seconds in a bench microfuge and 5 ul of ABC buffer was added followed by 5 ul of BSA solution. To this was now added with care 10 uCi (1 ul) of alpha 32-P dCTP. One ul (1 unit) of

Klenow fragment DNA polymerase was added and the reaction placed at 37^{o}C for a minimum of one hour. Unincorporated nucleotide was removed from the reaction by passing the reaction mixture down a Sephadex G-50 column (Maniatis et al 1982, appendix A). A labelling reaction was used if >50% of labelled nucleotide was incorporated into newly synthesised DNA, the specific activity of probes prepared in this way was 1 - 2 X 10^{9} dpm/ug DNA. Membranes were prehybridised and hybridised in plastic bags. Prehybridisation was carried out for 2 hours at 42^{o}C in a solution containing 50 % formamide, 50 mM sodium phosphate pH 6.6, 5 X SSC, 1 x Denharts solution, 100 ug/ml sonicated, denatured herring sperm DNA. Hybridisation was carried out at 42^{o}C for 16 hours in prehybridisation solution to which had been added dextran sulphate and DNA probe at final concentrations of 10% w/v and 1 - 2 ng/ml respectively. Following hybridisation filters were washed twice with 2 X SSC/0.1%SDS at room temperature for 5 minutes and then twice with 0.1 X SSC/0.1%SDS in a shaking water bath at 60^{o}C for 30 minutes. After washing, filters were sealed into plastic bags and exposed to Kodak XAR-5 film at -70^{o}C with intensification screens for periods of a few hours in the case of Northern blots hybridised with anterior pituitary hormone probes or from 1 to 10 days for minority and hypothalamic peptides depending upon the intensity of the signal. After autoradiography bound probes were removed by heating at 80^{o}C for 20 minutes in a solution of 10 mM Tris pH 7.5, 1 mM EDTA plus 0.5% SDS. To ensure that bound probes had been removed filters were exposed to

XAR-5 film at -70^{o}C for 24 hours. Normalisation was carried out by rehybridisation with a human ubiquitin cDNA fragment, labelled and hybridised as described above but using less stringent conditions of washing. After 2 washes in 2 X SSC/0.1% SDS for 5 minutes each at room temperature ubiquitin probed filters were then washed for 2 hours in 2 X SSC/0.1% SDS at 65^{o}C followed by a 30 minute wash in 0.2 X SSC/0.1% SDS at 50^{o}C. Northern blots were analysed with a Joyce-Loebell chromoscan 3 scanning densitometer (Joyce-Loebell, Gateshead, UK). In chapters 3 to 7 for the results of Northern blot analysis the control groups have been given values of 100% and the values for manipulated groups expressed relative to this. In order to compensate for any errors in loading or transfer of RNA, membranes which had been hybridised with probes for GAL, GHRH, NPY, NT, PPT-A, SRIF and VIP mRNA were re-hybridised with a ubiquitin cDNA probe. The values for peptide mRNAs were then adjusted by comparing the amount of ubiquitin mRNA in manipulated groups relative to ubiquitin mRNA in the control group. It should be remembered that, unless otherwise stated, all values for mRNA are the results of single determinations, and as such may only be taken as estimates of alterations in mRNA level.

Preparation of RNA Probes, Hybridisation and Washing

Reagents

10 X reaction buffer (Melton DA et al 1984):

400 mmol/l Tris-HCl pH 8.0,

250 mmol/l NaCl

80 mmol/l MgCl

20 mmol/l spermidine

BSA/DTT mix 100 mmol/l dithiothreitol

1 mg/ml bovine serum albumin,

NTP mix (5 mM each of ATP, GTP and UTP)

32-P CTP (Amersham; 800 Ci/mmol; 20 mmol/l)

T7 RNA polymerase (BRL) 50 u/ul

SP6 RNA polymerase (Pharmacia) 20 u/ul

RNase-free DNase (Pharmacia) 1 u/ul

RNAse inhibitor 10 u/ul (Amersham)

DNA template 1 mg/ml

Method

Templates for riboprobe synthesis were prepared by digestion of plasmid DNA with suitable restriction enzymes. Templates for RNA probes were prepared by digesting plasmids containing rat cDNA inserts for GAL, NPY, PPT and VIP with Eco R1, Eco R1, Hind III and Bam HI respectively. Anti-sense RNA probes for GAL, NPY, PPT and VIP were synthesised by in vitro transcription using T7, SP6, T7 and SP6 RNA polymerases respectively. The reaction mixture was prepared in the following way;

DNA template	1 ul
NTP mix	1 ul
BSA/DTT mix	1 ul
RNAse inhibitor	1 ul
10 X buffer	1 ul
32-P CTP	5 ul
Enzyme	1 ul

Transcription was carried out at 40^{o}C for 60 minutes followed by digestion with 1u RNase-free DNase (Pharmacia) at 37^{o}C for 15 minutes. One twentieth of a reaction was passed down a Sephadex G-50 column to assess the efficiency of the reaction, the specific activity of RNA probes was approximately 5 - 8 X 10^{8} dpm/ug. Unincorporated nucleotides were removed by precipitating reactions twice with ammonium acetate and ethanol at final concentrations of 0.7 M and 70% respectively. Prehybridisation was carried out for 30 minutes at 55^{o}C in a solution of 50% formamide, 5 X Denharts solution, 5 X SSC, 50 mM Na PO4 (pH 6.6), 200 ug/ml yeast tRNA, 100 ug/ml denatured, sonicated salmon sperm DNA. Hybridisation was carried out at 55^{o}C for 16 hours in prehybridisation solution to which had been added dextran sulphate and RNA probe at final concentrations of 10% w/v and 10 ng/ml. Filters were washed at room tempertaure 3 times in a solution of 2 X SSC/0.1%SDS for 5 minutes then twice at 65^{o}C in a solution of 0.1 X SSC/0.1% SDS for 60 minutes. After washing, filters were sealed into plastic bags and exposed to Kodak XAR-5 film at -70^{o}C with intensification screens.

RNAse H Digestion (Carrazana EJ et al 1988)

Reagents

RNase H buffer:

60 mM KCl,

20 mM MgCl,

100 mM tris pH 7.5,

2 mM dithiothreitol

RNase H (Gibco BRL) 2 u/ul

Oligo dT 5 mg/ml (Sigma)

Method

Five ug of total RNA was precipitated from a solution of 0.3M sodium acetate pH 5.2 by addition of ethanol to a final concentration of 70% and chilling at -20^{o}C overnight. The RNA was pelleted by centrifugation, dried under vacuum, redissolved in 10ul 100 mM KCl/0.1 mM EDTA, mixed with 1ul oligo(dT) solution, heated at 65^{o}C for 3 minutes and then allowed to anneal at 22^{o}C for 30 minutes. Twelve ul of RNase H buffer containing 1 unit of RNAse H was added and the solution incubated at 37^{o}C for 30 minutes after which it was extracted with a mixture of phenol/chloroform, precipitated with sodium acetate and ethanol and analysed by Northern blot as described above.

Peptide Extraction

Peptides from individual central and lateral hypothalamic segments were extracted by placing the tissues in 0.5 ml of 0.5 M acetic acid and heating at 100°C for 10 minutes. Individual anterior pituitary glands were extracted in 0.3ml of 0.5M acetic acid held at 100°C for ten minutes. The extracts were stored at -20°C until assay.

Radioimmunoassay

The radioimmunoassay of a hormone depends on the competition between radioactively-labelled and unlabelled hormone for the binding of a specific antibody. The amounts of antibody and labelled hormone are fixed, the only variable being the unlabelled hormone concentration. The higher the concentration of unlabelled hormone, the less radioactively labelled hormone will be bound to antibody. After separation of the bound from the free hormone, a standard curve can be prepared with which unknown samples can be compared. The amount of antibody in the assay is adjusted to bind about 50% of the label added (in the absence of unlabelled hormone). This point being midway between the non-specific binding of the label and the maximal binding by excess antibody, gives the steepest standard curve slope and also optimal precision in counting. The development of the assays used in this study have previously been described in detail, galanin (Bauer FE et al 1986), growth hormone-releasing hormone (Jones PM et al 1990), neuropeptide Y (Allen JM et al 1984), neurotensin (Blackburn AM, Bloom SR 1983),

substance P (McGregor GP and Bloom SR 1983), somatostatin (O' Shaugnessy et al 1982) and vasoactive intestinal peptide (Mitchell SJ, Bloom SR 1978). Details of the development and sensitivities of the assays are given in table 2.1.

It should be noted that all the radioimmunoassays described in this thesis were carried out in Professor S R Bloom's laboratory by various members of his staff.

Assay Format

Tissue extracts were thawed and assayed in duplicate in 0.8 ml of 0.06 M phosphate buffer, pH 7.4 which contained 10 mM EDTA, 45 uM BSA and 20 kallikrein inhibiting units/ml of aprotinin. The sensitivity of an assay increases with incubation time reaching a maximum after about 5 to 6 days and all the radioimmunoassays carried out in this study were incubated for 5 days. Incubation was carried out at $4^{o}C$ to help prevent bacterial growth and minimalise proteolytic degradation and evaporation. After incubation antibody bound label was separated from free label by adding 250 ul of a suspension containing 4 - 8 mg charcoal (Norit GSX, Hopkins and Williams) coated with clinical grade dextran (1:10 g charcoal, average mol wt 70,000, Sigma). The tubes were centrifuged at 1600 g for 20 minutes at $4^{o}C$, followed by immediate separation of the supernatant.

The general scheme for radioimmunoassays used in this study is shown in table 2.2 and is explained below.

1. Blank. A tube with all assay reagents except antibody which alows non-specific binding of labelled hormone to be assessed.
2. A tube containing half the amount of added label which is added to other tubes and is useful for assessing whether greater sensitivity could have been achieved.
3. A tube that contains twice the amount of label added to the other assay tubes and is used to assess the sensitivity of the assay with respect to the amount of label added.
4. Tubes containing no unlabelled hormone (but which contain hormone label and antibody etc) which are run to assess any drift which may occur as a result of reagent deterioration or syringe fatigue.
5. A tube containing excess antibody which will bind all of the bindable hormone and provide a measure of the immunological integrity of the labelled hormone and the quality of the label.

Statistical Analysis

All the results of radioimmunoassay are given as means with the standard error of the mean. Statistical analysis was performed by one-way analysis of variance for each peptide separately using the Statgraphics programme available with the Compaq 386 PC. Individual differences between control and treament groups were subsequently determined by unpaired t-tests.

TABLE 2.1 Details of conjugate, antisera and labels used in radioimmunoassay

Peptide	Antigen	Conjugation	125-I label	RIA antibody dilution	Senitivity* (fmol/tube)
GAL	Porcine GAL	Unconjugated	Porcine GAL	1:48,000	2.0
GH-RH	Rat GH-RH/BSA	Carbo	Rat GH-RH	1:160,000	1.5
NT	NT/BSA	B.D.B.	Bovine NT	1:80,000	0.4
NPY	Porcine NPY/BSA	Glut	Porcine NPY	1:120,000	0.4
SP	Ovine SP/BSA	Glut	(Tyr-8) SP	1:8,000	0.3
SRIF	Ovine SRIF/BSA	Carbo	Ovine SRIF	1:80,000	0.4
VIP	Porcine VIP/BSA	Carbo	Porcine VIP	1:320,000	0.3

NPY = Neuropeptide Y; VIP = Vasoactive Intestinal Peptide; NT = Neurotensin; SP = Substance P; BSA = Bovine Serum Albumin; Glut = Glutaraldehyde; Carbo = Carbodiimide; B.D.B. = Bis Diazotized Benzidene; * = 95% Confidence Limit from zero.

TABLE 2.2 The standard recipe for radioimmunoassay

Blank	0.5 X Label	2 X Label	Zero	Standard	Sample
Buffer 700 ul	Buffer 650 ul	Buffer 500 ul	Buffer 600 ul	Buffer 500-600 ul	Buffer 600 ul
0.5M Acetic Acid 2-20 ul	0.5M Acetic Acid 2-20 ul	0.5M Acetic Acid 2-20 ul	0.5M Acetic Acid 2-20 ul	0.5M Acetic Acid 2-20 ul	Tissue Extracts 2-20 ul
Label 100 ul	Label 50 ul	Label 200 ul	Label 100 ul	Label 100 ul	Label 100 ul
	Antibody 100ul	Antibody 100 ul	Antibody 100 ul	Antibody 100 ul	Antibody 100 ul

CHAPTER 3

THYROID HORMONE MANIPULATIONS

INTRODUCTION

The secretion of thyroid hormones by the thyroid gland is regulated by the anterior pituitary hormone thyrotrophin (TSH) the synthesis and release of which is regulated in a negative feedback fashion by circulating levels of thyroid hormones (Wong CC et al 1980, Chin WW et al 1985, Mirell CJ et al 1987, Franklyn JA et al 1987). Thyroid hormone status has also been found to influence the production of growth hormone (GH) and prolactin (PRL). The effect of alterations in thyroid hormone secretion, especially hypothyroidism, on the anterior pituitary has been extensively studied. The secretion of GH and the accumulation of GH mRNA in cultured rat pituitary tumor cells has been shown to be stimulated by the addition of thyroid hormones to the culture medium (Dobner PR et al 1981, Evans RM et al 1982, Yaffe BM and Samuels HH 1984). The synthesis and secretion of GH is impaired in the hypothyroid state in both rats and humans (Peake GT et al 1973, Hervas F et al 1975, Coiro V et al 1979, Mirell CJ et al 1987). In rats hypothyroidism has also been associated with both decreased (Wong CC et al 1980) and increased prolactin production (Mirell CJ et al 1987).

Prior to this study thyroid hormone status had been shown to affect the anterior pituitary peptide content of NT and SP (Goedert M et al 1982, Aronin N et al 1984) suggesting that thyroid hormone status influences the synthesis of at least some minority pituitary peptides. However, the mechanism of these effects was not clear because there

were no reports of the effects of thyroid hormone status on NT or PPT mRNA levels. Furthermore, the effects of thyroid hormone manipulations on the content of GAL, NPY, and VIP and their respective mRNAs had not been reported. Results from experiments in this section of the study concerning the effects of thyroid hormone manipulations on NPY, NT, SP and VIP have been published (Jones PM et al 1989) using data derived from a different group of animals to those described in this chapter. Following publication of those results the manipulations were repeated in order to extend the original observations and data from the later series of experiments is given in this chapter.

ANIMALS

Male Wistar rats weighing 200-250 g, (supplied by Interfauna Ltd), were manipulated in the following ways:

1. Surgical thyroidectomy. Operations were performed by the supplier and experiments were timed to start five days after surgery. Animals received calcium lactate (0.1% w/v) in their drinking water in the immediate post-operative period to compensate for the removal of the parathyroid glands.

2. Surgical thyroidectomy with thyroxine (T4) replacment. Operations were performed by the supplier and experiments were timed to start five days after surgery from which time animals were injected subcutaneously daily with 5 ug T4. Animals also received calcium lactate (0.1%) in their drinking water to compensate for the removal of the parathyroid glands.

3. Chronic-thyroxine (T4) treatment. Animals were injected subcutaneously daily with 25 ug T4 (Sigma).

All groups were maintained for 14 days. Radioimmunoassay and Northern blot analysis were carried out as described in the materials and methods chapter.

RESULTS

TABLE 3.1 Thyroid hormone manipulations tissue weights and RNA yields

Anterior pituitary

		Tissue wet weight	Total RNA	
C	(a)	59 mg	320 ug	(n = 5)
TX	(b)	55 mg	302 ug	(n = 5)
R	(c)	60 mg	290 ug	(n = 5)
T4	(d)	58 mg	360 ug	(n = 5)

Hypothalamus

	Tissue wet weight	Total RNA	
C	318 mg	450 ug	(n = 5)
TX	206 mg	410 ug	(n = 5)
R	238 mg	350 ug	(n = 5)
T4	264 mg	375 ug	(n = 5)

(a) C = control group

(b) TX = thyroidectomised group

(c) R = thyroidectomised and T4 replaced group

(d) T4 = chronic T4 treated group

All values are for pooled groups of tissues

TABLE 3.2 Thyroid hormone manipulations anterior pituitary tissue peptide contents

Group	C	TX	R	T4
GAL	90 ± 6	36 + 2***	81 ± 7	82 ± 6
NPY	15 ± 4	335 ± 58***	587 ± 89***	12 ± 3
NT	162 ± 29	21 ± 9***	88 ± 21*	145 ± 24
SP	199 ± 32	581 ± 90***	214 ± 29	54 ± 13**
VIP	417 ± 77	1,386 ± 396***	320 ± 44	226 ± 24**

Mean values are femtomoles per gland (n = 9) determined by radioimmunoassay, given with standard error of the mean.

* Versus control, $p<0.05$

** Versus control, $p<0.01$

*** Versus control, $p<0.001$

TABLE 3.3 Thyroid hormone manipulations scanning densitometer analysis of Northern blots

A. Minority peptides

Group	C	TX	R	T4
GAL	100%	36%	58%	39%
NPY	100%	21,760%	970%	128%
NT	100%	38%	ND	ND
PPT-A	100%	1,744%	31%	2%
VIP	100%	680%	23%	8%

B. Anterior pituitary hormones

Group	C	TX	R	T4
GH	100%	18%	108%	97%
PRL	100%	32%	91%	105%
TSH-B	100%	725%	<DL	<DL
POMC	100%	102%	100%	98%

ND = experiment not done (due to exhaustion of material)

<DL = below detection limit

Values for treatment groups are expressed as percentages relative to controls.

TABLE 3. 4 Thyroid hormone manipulations hypothalamic tissue peptide contents

Central hypothalamus

Group	C	TX	R	T4
GAL	31 ± 2	34 ± 2	32 ± 1	31 ± 2
NPY	129 ± 7	122 ± 8	118 ± 13	132 ± 11
NT	107 ± 4	104 ± 6	111 ± 4	101 ± 6
SP	276 ± 8	284 ± 13	286 ± 13	279 ± 13
VIP	37 ± 2	33 ± 2	36 ± 2	35 ± 2

Lateral hypothalamus

Group	C	TX	R	T4
GAL	29 ± 1	30 ± 2	30 ± 2	29 ± 1
NPY	62 ± 3	70 ± 4	56 ± 6	62 ± 5
NT	113 ± 7	116 ± 6	124 ± 12	129 ± 13
SP	317 ± 18	328 ± 14	328 ± 19	365 ± 24
VIP	11 ± 1	11 ± 1	11 ± 1	12 ± 1

Mean values are picomoles/g wet weight tissue as determined by radioimmunoassay, given with standard errors of the mean (n = 9).

LEGEND TO PLATE 1 Representative Northern Blots From Thyroid Hormone Manipulations

C = control group

TX = thyroidectomised group

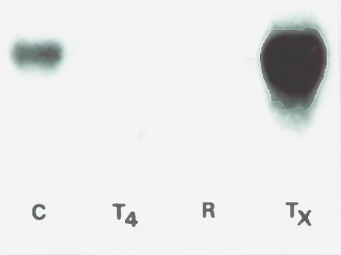
R = thyroidectomised and T4 replaced group

T4 = chronic T4 treated group

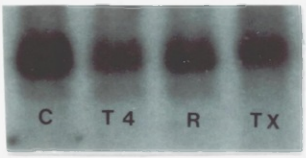
For the galanin, neuropeptide Y, neurotensin, preprotachykinin-A and vasoactive intestinal blots all lanes contained 40 ug of total RNA except for those marked 5 which contained 5 ug of total RNA. For the thyrotrophin-beta subunit blot all lanes contained 5 ug of total RNA. For the neurotensin and thyrotrophin-beta blots DNA probes were used and for the others RNA probes were used.

PLATE 1

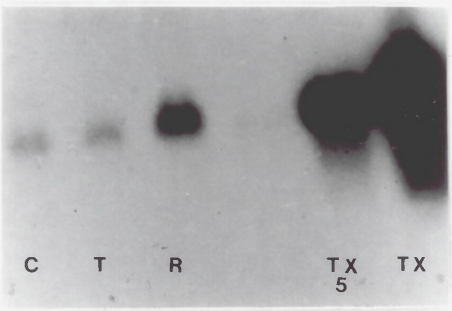
THYROID STIMULATING HORMONE-beta



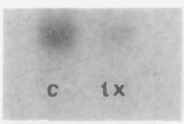
GALANIN



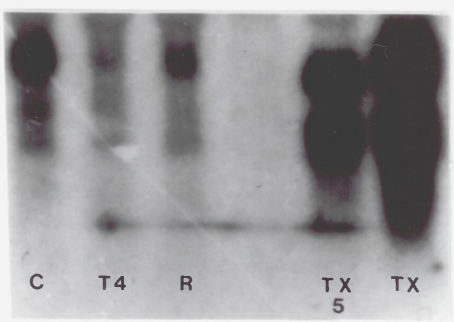
NEUROPEPTIDE Y



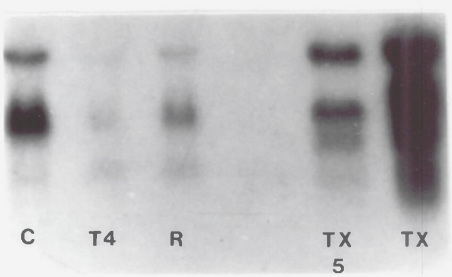
NEUROTENSIN



PREPROTACHYKININ-A



VASOACTIVE INTESTINAL PEPTIDE



Anterior Pituitary Hormone mRNA Levels

Analysis of total anterior pituitary RNA using a rat TSH-beta subunit specific cDNA fragment revealed that there were dramatic differences in levels of TSH-beta mRNA amongst the control and experimental groups. Both chronic-T4 treatment and T4 replacement in thyroidectomised rats decreased TSH-beta mRNA to undetectable levels, whilst thyroidectomy elicited a large increase in TSH-beta mRNA (>7-fold). Thyroidectomy decreased both GH and PRL mRNAs to approximately 20% and 30% respectively of control values whilst T4 treatment had no significant effect on levels of either mRNA. Proopiomelanocortin mRNA levels were unaffected by either thyroidectomy, thyroidectomy together with T4 replacement or chronic-T4 treatment.

Minority Peptide Concentrations

Thyroidectomy decreased pituitary GAL peptide content to less than 50% of control values (36 $\pm$ 2 vs control 90 $\pm$ 6 fmol/gland $p<0.001$). Neither chronic-T4 treatment or T4 replacement in thyroidectomised animals had any significant effect on pituitary GAL content.

In the anterior pituitaries of thyroidectomised animals the content of NPY was increased dramatically from 15 $\pm$ 4 fmol/gland in control animals to 335 $\pm$ 58 fmol/gland ($p<0.001$) whilst T4 treatment had no effect on NPY content. In the thyroidectomised and T4 replaced group NPY content was increased to 587 $\pm$ 89 fmol/gland from 15 $\pm$

4 fmol/gland in control animals ($p<0.001$).
Neurotensin showed a dramatic decrease in pituitary tissue peptide content from 162 ± 29 fmol/gland in control animals to 21 ± 9 fmol/gland ($p<0.001$) in thyroidectomised animals whilst T4 treatment produced no significant effect. In the thyroidectomised and T4 replaced group NT content was decreased from 162 ± 29 fmol/gland in control animals to 88 ± 21 fmol/gland ($p<0.05$)

Substance P showed a significant increase from 199 ± 32 fmol/gland in the pituitaries of control animals to 581 ± 90 fmol/gland in thyroidectomised rats ($p<0.001$). Chronic-T4 treatment produced a decrease in pituitary SP peptide content from 199 ± 32 fmol/gland in controls to 54 ± 13 fmol/gland ($p<0.01$) and thyroidectomy with T4 replacement had the effect of restoring SP peptide content to a level not significantly different from that of the control group (215 ± 30 fmol/gland vs control 199 ± 32 fmol/gland).

Vasoactive intestinal peptide showed a dramatic increase in anterior pituitary tissue peptide content from 417 ± 77 fmol/gland in controls to 1,386 ± 396 fmol/gland in thyroidectomised rat pituitaries ($p<0.001$). Chronic-T4 treatment produced a reduction in pituitary VIP content to approximately 50% of control values (226 ± 24 vs control 417 ± 77 fmol/gland, $p<0.01$), whilst in the T4 replaced group pituitary VIP levels were not significantly different from control values (321 ± 44 vs control 417 ± 77 fmol/gland).

In both central and lateral hypothalamic regions none of the 5 peptides showed any significant alteration following any of the treatments (Table 4).

Minority Peptide mRNA Levels

The NPY probe hybridised with a band of approximately 800 nucleotides, corresponding in length to the previously reported size of rat NPY mRNA (Larhammar D et al 1987). The PPT-A probe detected a band of approximately 1,200 nucleotides, the same size as previously reported for rat PPT-A mRNA (Krause JE et al 1987). The NT probe hybridised to a 1.0Kb band in the anterior pituitary, corresponding to the previously reported size of NT mRNA (Kislauskis E et al 1988). The VIP probe detected a band of approximately 1.7Kb in the hypothalamus and anterior pituitary, corresponding to the previously reported size of VIP mRNA (Linder S et al 1987) and also detected a second transcript of approximately 1.1Kb in the anterior pituitary which would appear to be unique to this tissue.

Northern blot analysis showed that GAL mRNA content was decreased in the pituitaries of thyroidectomised and chronically-T4 treated animals to approximately the same extent (36% and 39% of control levels respectively), and to a lesser extent in the thyroidectomised and T4 replaced group (58% of control).

Anterior pituitary NPY mRNA in thyroidectomised animals was increased approximately 200-fold relative to controls. Chronic-T4 treatment appeared to have no effect on NPY mRNA levels, whereas in the thyroidectomised and T4 replaced group NPY mRNA was increased approximately 10-fold over control levels.

Northern blot analysis of NT mRNA demonstrated a decrease to approximately 38% of control levels in thyroidectomised animals but shortage of RNA prevented assessment of the effects of thyroidectomy and T4 replacement or chronic-T4 treatment.

Thyroidectomy produced an increase of approximately 17-fold in pituitary PPT-A mRNA levels. In chronically-T4 treated animals PPT-A mRNA levels were depressed to such a level as to be almost undetectable (2% of control levels), whilst in the thyroidectomised and T4 replaced group PPT mRNA levels were approximately 31% of control levels.

Northern blot analysis indicated that thyroidectomy increased VIP mRNA levels approximately 7-fold, whilst, like SP, pituitary VIP mRNA levels were dramatically depressed in the chronically-T4 treated group (to approximately 8% of control levels) and to a lesser extent (23% of control levels) in the thyroidectomised and T4 replaced group.

No alteration was seen in hypothalamic mRNA for any of the five peptides examined following any of the treatments.

DISCUSSION

The aim of this section of the study was to alter the synthesis of TSH and observe the effects on pituitary hormones and minority peptides. Thyrotrophin-beta subunit mRNA showed classical negative feedback responses to the manipulations as has been previously demonstrated to occur following such treatments (Franklyn JA et al 1987, Mirell CJ et al 1987). Thyroidectomy produced its characteristic negative effects on GH and PRL mRNAs (Franklyn JA et al 1987, Samuels MH et al 1989) whilst POMC mRNA was unaffected as has been previously reported (Samuels MH et al 1989). The minority pituitary peptides were affected by thyroid hormone status with GAL, NPY, NT, SP and VIP in the anterior pituitary showing differential, tissue specific responses to alterations in thyroid hormone status. The effects of T4 replacement in thyroidectomised animals on TSH-beta, PPT-A and VIP mRNA suggested that the dose of T4 given was in excess of physiological requirements rendering the animals hyperthyroid.

The effects of thyroid hormone manipulations on pituitary GAL have not been reported. Thyroidectomy decreased both GAL peptide and mRNA to less than 50% of control values. Chronic T4 treatment had no affect on peptide content but decreased GAL mRNA to approximately 60% of control values. This would indicate that peptide turnover and synthesis were differentially affected by these manipulations. Galanin-immunoreactivity (GAL-IR) has been shown by immunocytochemistry to be present mainly in somatotrophs and occasionally in thyrotrophs in the male rat pituitary

(Steel JH et al 1989). This suggests that some functional link may exist between GAL and somatotroph and/or thyrotroph function. Data from physiological studies supports an involvement of GAL in GH regulation, whereas there are no reports of GAL having any effect on TSH secretion in any system. Injection of GAL both intravenously and into the third ventricle of male rats resulted in elevated serum levels of GH (Murakami Y et al 1987, Melander T et al 1987, Ottlecz A et al 1988). Incubation of GAL with cultured anterior pituitary cells was reported by different groups to have either no effect on GH release (Ottlecz A et al 1986) or to increase the release of GH (Gabriel SM et al 1988). When GAL antiserum was injected into the third ventricle of male rats a decrease in serum GH levels was observed (Ottlecz A et al 1988). In man the response of serum GH to GHRH was increased more than 3-fold when GHRH was injected in combination with GAL (Davis TM et al 1987). If GAL is synthesised by somatotrophs then the decrease in locally produced GAL seen in the thyroidectomised state may result from coordinate regulation of a minority peptide colocalised with a pituitary hormone. This was suggested to occur for another minority pituitary peptide, neuromedin B, which was found to be co-localised with TSH, and the immunoreactive content of neuromedin B was shown to change in parallel with TSH content following thyroidectomy (Steel J et al 1988).

The response of NPY to these manipulations was somewhat unusual, with dramatic increases in peptide and mRNA content observed in both the thyroidectomised and thyroidectomised and T4 replaced groups. Neuropeptide Y peptide and mRNA content did not alter significantly in the chronically-T4 treated group, suggesting that unlike SP and VIP (see below) hyperthyroidism had no effect on pituitary NPY synthesis. An unusual aspect of these results is the high levels of NPY peptide and mRNA seen in the thyroidectomised and T4 replaced group. This might indicate that pituitary NPY regulation is dependent on some other factor produced by the thyroid gland such as calcitonin (Foster GV et al 1964). The tissue peptide content of NPY was not significantly different in the thyroidectomised and T4 replaced group in comparison to the thyroidectomised group whereas NPY mRNA was approximately 20-fold more abundant in the latter group than in the former implying that peptide synthesis and turnover were different in the two groups. A possible explanation for the response of NPY to thyroidectomy and its similarity to that of TSH is suggested from immunocytochemical data. Neuropeptide Y immunoreactive cells were shown to be present in normal rat anterior pituitaries and increased in number in hypothyroid rats (Jones PM et al 1989). In both groups of animals these cells were identified as a subset of thyrotrophs indicating a possible link with TSH secretion. However, there are at present no reports of any effect of NPY on TSH release which might indicate that NPY is synthesised by thyrotrophs but affects other pituitary cell types.

Thyroidectomy had a negative effect on NT, with peptide content being reduced to less than 20% and mRNA being reduced to approximately 40% of control values. A decrease in NT tissue peptide content following thyroidectomy has been reported previously (Goedert M et al 1982, Goedert M et al 1984) and confirms the results of the present study. In the thyroidectomised and T4 replaced group NT content was decreased relative to the control group. The significance of this is uncertain as these animals would appear to have been hyperthyroid, as judged by the effects on TSH-beta, PPT-A and VIP mRNAs, but in the chronic T4 treatment group NT content was not significantly different to the control group. Neurotensin immunoreactivity was demonstrated in anterior pituitary cells (Goedert M et al 1982) but at present nothing is known about the colocalisation of NT with anterior pituitary hormones. There is evidence for a possible involvement of NT with the pituitary hormones which were affected by thyroidectomy ie TSH, GH and PRL. Injection of NT into the lateral ventricle of male rats decreased serum levels of GH and TSH (Aronin N et al 1986) whilst intravenous injection of NT into male rats increased serum GH and TSH (Aronin N et al 1986). Microinjection of NT into the third ventricle of ovariectomised female rats increased serum GH levels without affecting TSH release, whilst intravenous injection of NT into ovariectomised female rats had no effect on GH but elevated plasma TSH (Aronin N et al 1986). The incubation of NT with hemipituitaries from ovariectomised female rats had no effect on GH release, but enhanced the release of TSH

(Aronin N et al 1986). When NT was added to primary cultures of anterior pituitary cells from intact female rats, basal TSH secretion was significantly reduced and the stimulation of TSH secretion by TRH was abolished (Aronin N et al 1986). Neurotensin has also been shown to stimulate the secretion of PRL both in vivo and in vitro (see chapter 5 for details). The decrease in NT observed in thyroidectomised rats might indicate that NT is synthesised in either somatotrophs or lactotrophs and is regulated coordinately with the hormone produced by either cell type. Colocalisation data will aid in indicating with which pituitary hormones NT may be involved.

The effects of thyroid hormone manipulations on SP peptide content have previously been reported (Aronin N et al 1984) and confirm the results of the present study. After completion of this section of the study a report appeared about the effects of thyroid hormone status on PPT-A mRNA in the rat pituitary (Jonassen JA et al 1987) which also agrees with the findings of the present study. Substance P-like immunoreactivity (SP-IR) has been demonstrated in the rodent pituitary, but there is disagreement about colocalisation with pituitary hormones. Morel and co-workers found that in the rat, SP-IR was present in the lactotroph and gonadotroph (Morel G et al 1982b) whilst DePalatis and colleagues found SP-IR present in a sub-population of thyrotrophs in the guinea pig anterior pituitary (DePalatis LR et al 1982). If SP was present in thyrotrophs then it might be co-regulated with TSH in response to the appropriate stimuli. However, there is

only a small amount of evidence for a link between SP and thyrotroph function. Intraventricular injection of SP into male or ovariectomised female rats had no effect on TSH release (Aronin N et al 1986, Arisawa M et al 1989) but stimulated TSH release in ovariectomised female rats primed with oestrogen (Arisawa M et al 1989). Incubation of hemipituitaries from either ovariectomised female or normal male rats with SP failed to produce any effect on TSH release (Aronin N et al 1986, Arisawa M et al 1989). Thyroidectomy has been reported to have no effect on gonadotrophin synthesis (Samuels MH et al 1989) making it unlikely that the increase in SP seen in this state was related to any alteration in gonadotroph function. A stimulatory effect of SP on the release of PRL has been demonstrated both in vivo and in vitro (see chapter five for details). This might indicate that the response of SP to thyroidectomy is a feedback response to the decrease in PRL synthesis seen in this state.

The response of VIP to these manipulations was similar to that of SP. Thyroidectomy produced an increase and hyperthyroidism a decrease in VIP peptide and mRNA levels. A report of the effects of thyroidectomy on anterior pituitary VIP content has been published (Lam KSL et al 1989) confirming the results of the present study. The presence of VIP-immunoreactivity (VIP-IR) has been demonstrated in lactotrophs of normal male and female rats by immunocytochemical localisation (Morel G et al 1982c) and also shown to be present in the lactotrophs of oestrogen treated female rats (Steel JH et al 1989).

However, in their report Lam and co-workers found that in hypothyroid rat pituitaries VIP-IR was present in a population of cells which were neither lactotrophs nor thyrotrophs and have yet to be identified (Lam KSL et al 1989). Despite a variety of data indicating that VIP is synthesised by lactotrophs (see chapter 5 for details) Lam and colleagues felt that their results cast doubt on the colocalisation of VIP with PRL not only from the results of their localisation studies but also from the opposite responses of VIP and PRL to thyroidectomy. There may be an explanation for the interesting and unexpected findings of Lam and co-workers. Their results may reflect plasticity of VIP expression, with VIP synthesis being inducible in cell types other than lactotrophs under certain conditions. Alternatively, VIP may be normally expressed at low levels in cell types other than the lactotroph and the synthesis of VIP could be stimulated in these cells in the hypothyroid state. It should be noted that both groups were unable to localise VIP in any cell type in normal, untreated rats.

CHAPTER 4

ADRENAL HORMONE MANIPULATIONS

INTRODUCTION

Secretion of the glucocorticoid hormone, corticosterone from the adrenal gland is controlled by the anterior pituitary hormone, adrenocorticotrophin (ACTH). In the anterior pituitary the secretion of ACTH and transcription of the proopiomelanocortin (POMC) gene are regulated by levels of serum corticosterone in a negative feedback fashion (Eberwine JH and Roberts JL 1984, Birnberg NC et al 1983, Autelitano DJ et al 1987). The secretion of GH and the gonadotrophic hormones have also been reported to be influenced by glucocorticoids. Administration of the artificial glucocorticoid, dexamethasone, increased GH secretion in adrenalectomised rats (Beinfeld MC and Packman PM 1980) and enhanced the GH releasing effect of GHRH in both intact and adrenalectomised rats (Wehrenberg WB et al 1983). Adrenalectomy decreased GH mRNA in the rat pituitary, an effect which was reversed by dexamethasone treatment (Martinoli MG and Pelletier G 1989). Dexamethasone was shown to increase GH-RH stimulated GH secretion and mRNA accumulation in primary cultures of rat anterior pituitary cells (Vale W et al 1983, Fukata J and Martin JB 1986). Injection of dexamethasone into female rats was reported to suppress LH secretion (Euker JS et al 1975) and prevent ovulation (Baldwin DM and Sawyer CH 1974) whilst cultured rat anterior pituitary cells showed an increase in secretion of FSH in response to LHRH in the presence of dexamethasone (Suter DE and Schwartz NB 1985).

Relatively little work has been published on the influence of adrenal hormone status on pituitary minority peptides in comparison to the work published on the effects of alterations in thyroid and sex hormone status. In the rat anterior pituitary, NT and SP peptide levels were reported to be unaltered by adrenalectomy (Goedert M et al 1982, Coslovsky R et al 1984), although the effects of hypercortilism on either NT or SP are unreported. A doubling in rat anterior pituitary VIP content has been reported to occur 28 days after bilateral adrenalectomy (Rotsztejn WH et al 1980) although the effects of adrenalectomy on VIP mRNA and the effects of hypercortilism on either VIP peptide or mRNA content are unreported.

ANIMALS

Male Wistar rats, supplied by Charles Rivers Breeding Laboratories, weighing between 200g and 250g each were manipulated in the following ways:

1. Surgical bilateral adrenalectomy. Operations were performed by the supplier and the animals were timed from 5 days after surgery and were maintained on 0.9% saline to compensate for the effects of adrenalectomy on salt balance.

2. Surgical bilateral adrenalectomy together with surgical bilateral castration. Operations were performed by the supplier to produce animals lacking in both adrenal and gonadal steroid hormones. Animals were timed from 5 days post surgery and maintained on 0.9% N saline.

3. Chronic dexamethasone treatment. Animals received daily injections of 2 mg dexamethasone (Merck, Sharpe and Dome) injected sub-cutaneously in sterile saline.

4. Controls were sham adrenalectomised.

All treatment groups were maintained for 28 days. Animals were sacrificed and tissues processed as described in materials and methods.

RESULTS

TABLE 4.1 Adrenal hormone manipulations tissue weights and RNA yields

Anterior pituitaries

	Wet weight	Total RNA	
C (a)	61 mg	450 ug	(n = 5)
AX (b)	69 mg	415 ug	(n = 5)
AC (c)	58 mg	320 ug	(n = 5)
DX (d)	47 mg	290 ug	(n = 5)

Hypothalamus

	Wet weight	Total RNA	
C	234 mg	394 ug	(n = 5)
AX	274 mg	430 ug	(n = 5)
AC	200 mg	470 ug	(n = 5)
DX	201 mg	340 ug	(n = 5)

(a) C = control group

(b) AX = adrenalectomised group

(c) AC = adrenalectomised and castrated group

(d) DX = high dose dexamethasone treated group

All values are for pooled groups of tissues

TABLE 4.2 Adrenal hormone manipulations
anterior pituitary tissue peptide contents

Group	C	AX	AC	DX
GAL	84 ± 8	111 ± 7*	43 ± 6**	53 ± 4***
NPY	17 ± 3	16 ± 3	7 ± 3**	18 ± 3
NT	299 ± 24	277 ± 41	238 ± 32	119 ± 15***
SP	337 ± 27	177 ± 21***	157 ± 35***	37 ± 6***
VIP	112 ± 13	335 ± 35***	82 ± 7*	27 ± 4***

Mean values are femtomoles per gland (n = 9) as determined by radioimmunoassay, given with standard error of the mean.

* Versus control, $p<0.05$

** Versus control, $p<0.01$

*** Versus control, $p<0.001$

TABLE 4.3 Adrenal hormone manipulations scanning densitometer analysis of Northern blots

A. Minority pituitary peptides

Group	C	AX	AC	DX
GAL	100%	110%	115%	3,730%
NPY	100%	88%	118%	121%
NT	100%	105%	91%	71%
PPT-A	100%	53%	68%	3%
VIP	100%	230%	72%	11%

B. Anterior pituitary hormones

Group	C	AX	AC	DX
POMC	100%	260%	220%	21%
GH	100%	58%	65%	108%
LH-beta	100%	103%	1,300%	210%

Values for treatment groups are expressed as percentages relative to controls.

TABLE 4.4 Adrenal hormone manipulations hypothalamic tissue peptide contents

A) Central Hypothalamus

Group	C	AX	AC	DX
GAL	16 ± 1	15 ± 1	16 ± 1	19 ± 1
NPY	83 ± 5	72 ± 7	57 ± 9*	104 ± 7*
NT	105 ± 15	108 ± 19	91 ± 17	123 ± 10
SP	160 ± 8	160 ± 11	139 ± 7	156 ± 9
VIP	21 ± 1	17 ± 0	17 ± 1	21 ± 1

B) Lateral Hypothalamus

Group	C	AX	AC	DX
GAL	19 ± 1	18 ± 1	17 ± 1	22 ± 1
NPY	45 ± 3	34 ± 6	30 ± 5*	60 ± 5*
NT	122 ± 6	115 ± 8	109 ± 5	139 ± 8
SP	183 ± 11	169 ± 10	171 ± 9	199 ± 15
VIP	10 ± 0	9 ± 0	10 ± 1	10 ± 1

Mean values are picomoles/g wet weight tissue (n = 9) as determined by radioimmunoassay, given with standard errors.

* Versus control, $p<0.05$

LEGEND TO PLATE 2 Representative Northern blots from adrenal hormone manipulations

C = control group

AX = adrenalectomised group

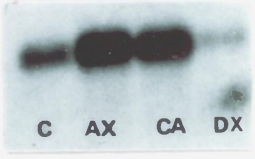
AC = adrenalectomised and castrated group

DX = high dose dexamethasone treated group

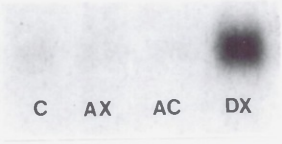
For the Northern blot hybridised with the proopiomelanocortin probe, 5 ug of total RNA was loaded in each lane and for all other blots 50 ug of total RNA was loaded into each lane. The galanin, neurotensin and proopiomelanocortin hybridisations were carried out using DNA probes and the others using RNA probes.

PLATE 2

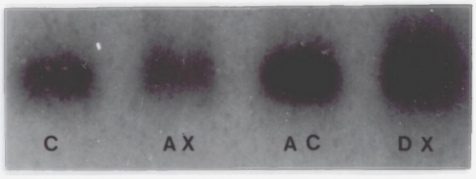
PROOPIOMELANOCORTIN



GALANIN

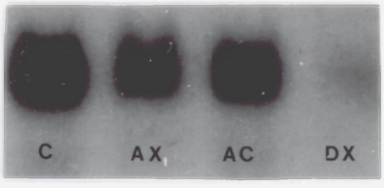


NEUROPEPTIDE Y

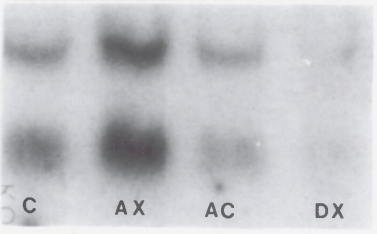


NEUROTENSIN

PREPROTACHYKININ-A



VASOACTIVE
INTESTINAL
PEPTIDE



Anterior Pituitary Hormone mRNA Levels

Analysis of total rat anterior pituitary RNA using a rat POMC cDNA fragment demonstrated that there were differences in the amount of POMC mRNA between the control and treated groups. In both the adrenalectomised and combined adrenalectomised and castrated groups of animals the amount of POMC mRNA was significantly elevated above controls (>2.0-fold in each case), whilst in the high-dose dexamethasone treated group POMC mRNA was reduced to approximately 20% of control levels. Levels of LH-beta subunit mRNA were unaffected by adrenalectomy but were significantly elevated in the adrenalectomised and castrated group to approximately 13-fold the amount in the control group, and in the high-dose dexamethasone treated group LH-beta mRNA was approximately twice the level of the control group. The amount of GH mRNA was decreased in both the adrenalectomised and combined adrenalectomised and castrated groups of animals but was unaffected by high-dose dexamethasone treatment.

Minority Peptide Concentrations

In the adrenalectomised group anterior pituitary GAL content was increased to approximately 150% of control levels (111 $\pm$ 7 vs control 84 $\pm$ 8 fmol/gland, $p<0.05$), whilst in the adrenalectomised and castrated group GAL content was decreased to approximately 50% of control levels (43 $\pm$ 6 vs control 84 $\pm$ 8 fmol/gland, $p<0.001$) and in the high-dose dexamethasone treated group GAL content was also decreased (53 $\pm$ 4 vs control 84 $\pm$ 8, $p<0.001$).

Neurotensin pituitary content was decreased by chronic dexamethasone treatment from 299 ± 24 fmol/gland in controls to 119 ± 15 fmol gland ($p<0.001$) but was unaffected by any of the other treatments.

Neuropeptide Y pituitary content was decreased in the adrenalectomised and castrated group (7 ± 3 vs control 17 ± 3 fmol/gland, $p<0.01$) but was unaffected by any of the other treatments.

Substance P showed significant reductions in anterior pituitary tissue peptide content in both the adrenalectomised (177 ± 21 fmol/gland vs control 337 ± 27 fmol/gland, $p<0.001$) and combined adrenalectomised and castrated groups (157 ± 21 fmol/gland vs control 337 ± 27 fmol/gland, $p<0.001$) and an even greater reduction, to approximately 10% of control levels, in the dexamethasone treated group (37 ± 6 fmol/gland vs control 337 ± 27 fmol/gland, $p<0.001$).

Anterior pituitary VIP content increased to approximately 300% of control levels following adrenalectomy (335 ± 35 fmol/gland vs control 112 ± 13 fmol/gland, $p<0.001$) whereas in the adrenalectomised and castrated group a small decrease was seen (82 ± 7 fmol/gland vs control 112 ± 13 fmol/gland, $p<0.05$) and a significant reduction to approximately 25% of control levels was observed in the dexamethasone treated animals (27 ± 4 fmol/gland vs control 112 ± 13 fmol/gland, $p<0.001$).

Galanin, NPY, SP and VIP hypothalamic peptide contents

were unaffected by any of the four treatments. The content of NPY in the central and lateral hypothalamic regions was decreased in the adrenalectomised and castrated group (57 ± 9 vs control 83 ± 5 pmol/g wet weight and 30 ± 5 vs control 45 ± 3 pmol/g wet weight, $p<0.05$ respectively). Central and lateral hypothalamic contents were increased in the dexamethasone treated group (104 ± 7 vs control 83 ± 5 pmol/g wet weight and 60 ± 5 vs control 45 ± 3 pmol/g wet weight, $p<0.05$ respectively).

Minority peptide mRNA levels

Anterior pituitary galanin mRNA was unaffected in both the adrenalectomised and adrenalectomised and castrated groups of animals but in the high-dose dexamethasone treated group was increased to nearly 40 times control levels.

Anterior pituitary NPY mRNA was not affected by adrenalectomy but was elevated slightly in both the adrenalectomised and castrated and high-dose dexamethasone treated groups of animals (118% and 121% of control levels respectively).

Levels of NT mRNA were unaltered in either the adrenalectomised or adrenalectomised and castrated groups of animals but in the high-dose dexamethasone treated group a decrease to approximately 70% of control values was observed.

Preprotachykinin-A mRNA showed significant reductions compared to the control group in all three manipulations with adrenalectomy producing a decrease to approximately 50%, adrenalectomy and castration a decrease to approximately 70% and chronic dexamethasone treatment a decrease to approximately 3% of control values.

Levels of VIP mRNA also showed significant differences between the treatment groups and controls with an increase observed in the adrenalectomised group to approximately 230% of control values, whereas in the adrenalectomised and castrated group there was a decrease to approximately 70% of control levels and in the high-dose dexamethasone treated group a decrease to approximately 11% of control levels was observed.

No alterations in hypothalamic mRNA levels were observed for any peptide following any of the treatments.

DISCUSSION

The effects of the manipulations on POMC and LH-beta mRNA levels demonstrated the effectiveness of the treatments in altering the synthesis of these hormones. The manipulations produced their characteristic effects on POMC and LH-beta mRNA levels with both peptides showing classical negative feedback effects in response to changes in hormonal status. As for the minority pituitary peptides, some unusual and unexpected results were observed, with NPY and NT showing very little response to the manipulations.

Galanin mRNA was unaffected in either the adrenalectomised or combined adrenalectomised and castrated groups, whilst GAL peptide content showed differential responses to the two treatments, being increased in the former and decreased in the latter group. Such differential responses of peptide and mRNA indicates that alterations in adrenal hormone status differentially affect GAL synthesis and peptide turnover. This occured dramatically in the chronic dexamethasone treated group where GAL peptide content was again decreased whilst a 37-fold increase in GAL mRNA content was seen. The changes in GAL peptide content following these manipulations and the inducibility of GAL mRNA by dexamethasone were unexpected as there are no reports of any effect of GAL on the pituitary-adrenal axis and this will require further investigation.

The only manipulation which affected the anterior pituitary content of NPY was combined adrenalectomy and castration where a decrease in peptide content was observed without any detectable alteration in mRNA level. The lack of alteration in mRNA might imply that this manipulation had affected peptide storage and/or release without affecting peptide synthesis. No alteration in NPY content or mRNA was observed after adrenalectomy or chronic dexamethasone treatment which might indicate a general lack of involvement of locally produced NPY in the regulation of ACTH secretion. This would be in accord with the reported failure of NPY to produce any effect on ACTH secretion following systemic administration in rats or incubation with primary cultures of rat anterior pituitary cells (Kerkerian L et al 1985). The central and lateral hypothalamic contents of NPY were decreased in the combined adrenalectomised and castrated group and increased in the dexamethasone treated group. No alteration in hypothalamic NPY mRNA was observed in any of the treatment groups indicating that although no detectable alterations in NPY synthesis were observed, hypothalamic NPY may be responsive to alterations in adrenal hormone status. This is supported by experimental studies indicating that hypothalamic NPY may be involved in regulation of the pituitary-adrenal axis (Liposits Zs et al 1988, Wahlestedt C et al 1987, Haas DA and George SR 1987).

The lack of effect of adrenalectomy on NT peptide and mRNA content is in aggreement with the earlier results of Goedert et al who demonstrated that NT peptide content in the rat anterior pituitary was unaffected by adrenalectomy (Goedert M et al 1984). However the effects of high dose dexamethasone treament were not reported and were found in this study to produce a decrease in both NT content and NT mRNA indicating that a decrease in NT synthesis had occurred. The incubation of NT with rat anterior pituitary fragments was reported to have no effect on basal or CRF stimulated ACTH release (Aronin N et al 1986). Taken together with the unresponsiveness of NT to adrenalectomy this might indicate that NT synthesised in the anterior pituitary does not function in regulating corticotroph function and that the decrease in NT seen in the dexamethasone treated group was a non specific effect of this treatment.

Both adrenalectomy and adrenalectomy together with castration produced significant decreases in pituitary SP peptide content which occurred co-ordinately with decreases in PPT-A mRNA. High-dose dexamethasone treatment produced even greater decreases in both peptide and mRNA with both being reduced to 10% or less of control levels. In contrast to the findings of the present study, Coslovsky and co-workers reported finding no change in the anterior pituitary peptide content of SP in adrenalectomised male rats (Coslovsky R et al 1984). The discrepancy between the findings of that study and the

present may result from differences between the strains of animals used in the two studies and also in the duration of the experiments. Wistar rats were used in this study and sacrificed after 28 days, whilst Coslovsky and co-workers used Sprague-Dawley rats which were sacrificed after 14 days. There are no reports of the effects of high-dose dexamethasone treatment on pituitary SP. The negative response of SP to both adrenalectomy and high dose dexamethasone treatment is unusual and has not so far been reported for any pituitary hormone in response to endocrine manipulations. There is evidence that SP may affect the pituitary-adrenal axis directly at the level of the anterior pituitary. Substance P was shown to significantly inhibit the stimulation of ACTH release induced by both CRF and arginine vasopressin from cultured rat anterior pituitary quarters (Aronin N et al 1986) and to stimulate the release of B-endorphin and B-lipotropin from dispersed rat anterior pituitary cells (Aronin N et al 1986). Together with the results of the present study this suggests that locally synthesised SP may affect corticotroph function and further investigation of the effects of SP on the secretion of POMC derived peptides is indicated.

In its responses to adrenalectomy and dexamethasone treatment VIP appeared to be showing a negative feedback type of response similar to that of POMC. However, VIP peptide and mRNA were decreased in the adrenalectomised and castrated group. Pituitary VIP content has previously

been reported to be increased to approximately 225% of control levels after four weeks of bilateral adrenalectomy, confirming the results of the present study (Rotsztejn WH et al 1980a). However, the effects of treament with high doses of dexamethasone were not reported. In respect to the pituitary-adrenal axis there is evidence to suggest that VIP may directly influence ACTH secretion from the anterior pituitary. Incubation of VIP with cultured human pituitary adenomas (White MC et al 1982, Oliva D et al 1982) and the mouse pituitary tumour derived AtT-20 cell line has been shown to stimulate the release of adrenocorticotropin (ACTH) (Westendorf JM et al 1983, Westendorf JM and Schonbrunn A 1985, Reisine TD et al 1982). Glucocorticoids have also been shown to modify the effects of VIP on the release of other anterior pituitary hormones. Addition of dexamethasone to cultured rat anterior pituitary cells was shown to modify the responsiveness of lactotrophs to VIP and to inhibit VIP induced PRL release (Rotszteyn WH et al 1980b, Rotszteyn WH et al 1981). Vasoactive intestinal peptide was also shown to produce a potent stimulatory effect on GH secretion from reaggregates of rat anterior pituitary cells only when they were cultured in the presence of dexamethasone (Denef C et al 1985). One of the most striking aspects of these results is the decrease in pituitary VIP in the combined adrenalectomised and castrated group whereas VIP was increased in the adrenalectomised group. The lack of effect of castration on pituitary VIP (see chapter 6) indicates that the decrease in VIP seen in the combined adrenalectomised and

castrated group is an effect manifest only in the absence of adrenal and gonadal factors. A possible interaction of sex hormones with glucocorticoids at the level of the anterior pituitary is supported by evidence of a link between corticotroph function and gonadotroph function. Immunocytochemical staining identified a sub-population of anterior pituitary cells containing both ACTH and FSH or LH. These cells were more abundant in the neonatal period than in adult life and were postulated to function in adrenal-gonadal maturation or to represent a form of stem cell (Childs GV et al 1982a). Acute experiments demonstrated adrenalectomy to have a delaying effect on the rise in serum gonadotrophins following castration in male rats (Lorenzen JR et al 1980) and also to inhibit the hypertrophy of gonadotrophs which occurs immediately after castration (Childs GV et al 1983).

CHAPTER 5

FEMALE SEX HORMONE MANIPULATIONS

INTRODUCTION

In female rats, the release of the anterior pituitary gonadotrophic hormones, follicle stimulating hormone (FSH) and luteinising hormone (LH), are regulated in a negative feedback fashion by the secretion of ovarian steroid hormones (Counis R et al 1983, Gharib SD et al 1986, Gharib SD et al 1987) and in a positive way by LHRH from the hypothalamus. The ovarian steroid hormone, oestrogen, is also known to influence the secretion of PRL and has been reported to affect GH secretion. Oestrogen has been shown to stimulate the synthesis of PRL (Maurer AR and Gorski J 1977) and the accumulation of PRL mRNA (Seo H et al 1979, Yamamoto N et al 1986). Gonadectomy of 30 day old female rats was found to result in a significant increase in longitudinal bone growth and weight gain (Jansson J-O et al 1983) whilst replacement with oestrogen reversed these effects. Oestrogen has also been reported to stimulate basal and GHRH induced release of GH from cultured rat anterior pituitary cells (Simard J et al 1986).

Alterations in female sex hormone status have previously been shown to produce changes in peptide levels of SP (Coslovsky R et al 1984, DePalatis LR et al 1985) and VIP (Maletti M et al 1982) but the effects of these manipulations on SP and VIP mRNA levels have not been documented. The effects of manipulations of female gonadal status on pituitary NPY and NT have not been documented. The rat GAL mRNA sequence was originally

obtained by random screening and sequencing of enriched sequences from an oestrogen induced pituitary tumor cDNA library (Vrontakis ME et al 1987). This suggested that GAL was an oestrogen inducible minority pituitary peptide making it an interesting peptide to study.

ANIMALS

Female Wistar rats weighing between 180 - 200 g (supplied by Interfauna Ltd.) were manipulated in 2 groups, each with its own controls in the following ways:

Group A:

1. Surgical ovariectomy. Operations were performed by the supplier and experiments were timed from 5 days post-surgery.

2. Surgical ovariectomy with oestrogen replacement. Operations were performed by the supplier and oestrogen replacement began 5 days after surgery when each animal was injected s.c. with 5 ug 17beta oestradiol (Sigma) in 100 ul of safflower oil as a physiological replacement dose.

3. Chronic oestrogen treament. Intact female rats received 2 depot injections of 2 mg 17b oestradiol in oil on days 1 and 14 of treatment.

4. Controls were sham ovariectomised.

All treatment groups were maintained for 28 days. Animals were sacrificed during the period of diestrus which was determined by daily vaginal smears through 2 consective cycles. Sacrifice and tissue processing was carried out as described in materials and methods.

Group B:

1. Medical castration. Intact female rats were injected subcutaneously with the LHRH superagonist D-Ser (BU^t), $Azgly^{10}$-LHRH (Zoladex depot 3.6 mg ICI Pharmaceuticals Ltd) to remove the anterior pituitary from hypothalamic LHRH regulation (see page 142).

2. Combined surgical and medical castration. Operations were performed by the supplier and timed from five days post surgery at which time the animals were given a subcutaneous depot injection of 3.6 mg of superagonist D-Ser (BU^t), $Azgly^{10}$-LHRH .

3. Oestrogen antagonist treatment. Intact female rats were given daily s.c. injections of 50 ug of the anti-oestrogen drug tamoxifen (Aldrich Chemical Co Ltd).

4. Controls were sham ovariectomised and given daily subcutaneous injections of saline.

All treatment groups were maintained for 28 days. Animals were sacrificed during the period of diestrus which was determined by daily vaginal smears through 2 consective cycles. Sacrifice and tissue processing was carried out as described in materials and methods.

RESULTS

TABLE 5.1 Female sex hormone manipulations tissue weights and RNA yields - Group A

Anterior pituitary

		Wet weight	Total RNA	
C	(a)	62 mg	500 ug	(n = 6)
O	(b)	70 mg	355 ug	(n = 6)
R	(c)	134 mg	1,300 ug	(n = 6)
E2	(d)	80 mg	780 ug	(n = 4)

Hypothalamus

	Wet weight	Total RNA	
C	228 mg	290 ug	(n = 6)
O	200 mg	275 ug	(n = 6)
R	220 mg	315 ug	(n = 6)
E2	225 mg	330 ug	(n = 6)

(a) C = controls

(b) O = ovariectomised

(c) R = ovariectomised with oestrogen replacement

(d) E2 = high dose oestrogen treatment

All values are for pooled groups of tissues

TABLE 5.2 Female sex hormone manipulations tissue weights and RNA yields - Group B

Anterior Pituitary

		Wet weight	Total RNA	
C	(e)	70 mg	448 ug	(n = 6)
A	(f)	49 mg	275 ug	(n = 5)
OA	(g)	51 mg	225 ug	(n = 5)
T	(T)	46 mg	260 ug	(n = 5)

Hypothalamus

	Wet weight	Total RNA	
C	250 mg	800 ug	(n = 6)
A	252 mg	350 ug	(n = 6)
OA	235 mg	740 ug	(n = 6)
T	217 mg	330 ug	(n = 6)

(e) C = controls

(f) A = LHRH super-agonist treatment

(g) OA = ovariectomised and LHRH super-agonist treatment

(h) T = tamoxifen treatment

All values are for pooled groups of tissues

TABLE 5.3 Female sex hormone manipulations anterior pituitary tissue peptide contents

Group A

	C	O	R	E2
GAL	223 ± 14	33 ± 3***	368 ± 14**	373 ± 14**
NPY	20 ± 2	35 ± 4**	21 ± 3	16 ± 2
NT	62 ± 7	85 ± 8*	8 ± 3***	5 ± 2***
SP	29 ± 2	130 ± 25***	27 ± 5	18 ± 3**
VIP	286 ± 26	201 ± 19*	6422 ± 458***	9962 ± 776***

Group B

	C	A	OA	T
GAL	233 ± 6	39 ± 5***	28 ± 6***	202 ± 7
NPY	24 ± 7	19 ± 2	19 ± 3	18 ± 7
NT	66 ± 3	64 ± 5	75 ± 5	59 ± 7
SP	31 ± 6	78 ± 24*	52 ± 12*	35 ± 8
VIP	240 ± 20	204 ± 19	228 ± 27	10 ± 2***

Values are femtomoles/gland (n = 9) as determined by radioimmunoassay. Mean values are given with standard errors of the mean.

* Versus control, $p<0.05$

** Versus control, $p<0.01$

*** Versus control, $p<0.001$

TABLE 5.4 Female sex hormone manipulations scanning densitometer analysis of Northern blots - anterior pituitary hormones

Treatment Group A

	C	O	R	E2
LH-b	100%	1,500%	14%	2%
PRL	100%	50%	187%	173%
GH	100%	176%	84%	51%

Treatment Group B

	C	A	OA	T
LH-b	100%	36%	44%	70%
PRL	100%	100%	103%	105%
GH	100%	104%	110%	104%

Values for treatment groups are expressed as percentages relative to controls.

TABLE 5.5 Female sex hormone manipulations scanning densitometer analysis of Northern blots - minority pituitary peptides

Treatment Group A

	C	O	R	E2
GAL	100%	<DL	475%	700%
NPY	100%	230%	104%	112%
NT	100%	257%	<DL	<DL
PPT-A	100%	300%	35%	25%
VIP	100%	<DL	3,800%	7,700%

Treatment Group B

	C	A	OA	T
GAL	100%	7%	9%	102%
NPY	100%	86%	190%	120%
NT	100%	330%	870%	158%
PPT-A	100%	318%	540%	180%
VIP	ND	ND	ND	ND

Values for treatment groups are expressed as percentages relative to controls.

<DL = Below detection limit

ND = Not done

TABLE 5.6 Female sex hormone manipulations hypothalamic tissue peptide contents - Group A

Central Hypothalamus

	C	O	R	E2
GAL	34 ± 3	30 ± 2	35 ± 2	37 ± 3
NPY	107 ± 12	93 ± 9	98 ± 6	115 ± 9
NT	129 ± 10	115 ± 6	147 ± 12	152 ± 9
SP	141 ± 7	130 ± 11	136 ± 13	158 ± 8
VIP	35 ± 2	33 ± 1	31 ± 1	35 ± 1

Lateral Hypothalamus

	C	O	R	E2
GAL	33 ± 3	32 ± 3	34 ± 2	40 ± 4
NPY	43 ± 7	38 ± 6	49 ± 5	35 ± 7
NT	144 ± 14	121 ± 10	154 ± 14	140 ± 11
SP	164 ± 14	194 ± 12	192 ± 19	186 ± 14
VIP	18 ± 0	20 ± 1	19 ± 1	17 ± 1

Values are picomoles/g wet weight (n = 9) as determined by radioimmunoassay. Mean values are given with standard errors of the mean.

TABLE 5.7 Female sex hormone manipulations hypothalamic tissue peptide contents - Group B

Central Hypothalamus

	C	A	OA	T
GAL	19 ± 1	18 ± 1	17 ± 1	18 ± 1
NPY	107 ± 13	103 ± 17	90 ± 10	103 ± 13
NT	142 ± 11	108 ± 5	111 ± 9	113 ± 7
SP	138 ± 12	92 ± 7	94 ± 15	101 ± 12
VIP	36 ± 1	35 ± 1	37 ± 2	35 ± 2

Lateral Hypothalamus

	C	A	OA	T
GAL	18 ± 1	18 ± 0	18 ± 1	18 ± 1
NPY	33 ± 5	28 ± 4	27 ± 6	35 ± 3
NT	141 ± 12	108 ± 5	105 ± 10	116 ± 11
SP	145 ± 16	114 ± 14	111 ± 20	124 ± 18
VIP	12 ± 1	11 ± 1	11 ± 0	13 ± 1

Values are means in picomoles/g wet weight (n = 9) as determined by radioimmunoassay, given with standard errors of the mean.

LEGEND TO PLATE 3 Representative Northern blots from female sex hormone manipulations - Group A

C = controls

O = ovariectomised

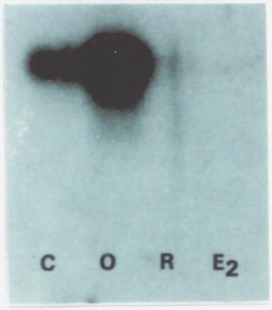
R = ovariectomised with oestrogen replacement

E2 = high dose oestrogen treatment

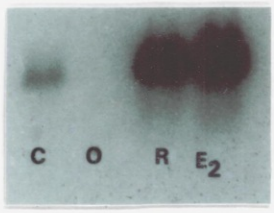
For the luteinising hormone beta subunit Northern blot 5 ug of total RNA was loaded in each lane, whilst for all the other blots 50 ug of total RNA was loaded in each lane. All hybridisations were carried out using DNA probes.

PLATE 3

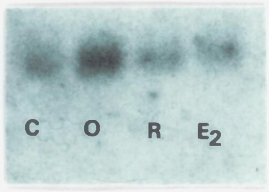
LUTEINISING HORMONE-Beta



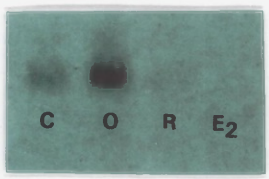
GALANIN



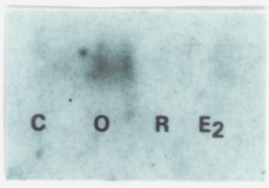
NEUROPEPTIDE Y



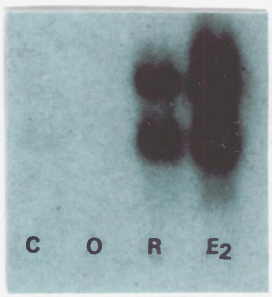
NEUROTENSIN



PREPROTACHYKININ-A



VASOACTIVE INTESTINAL PEPTIDE



LEGEND TO PLATE 4 Representative Northern blots from female sex hormone manipulations - Group B

C = controls

A = LHRH super-agonist treated

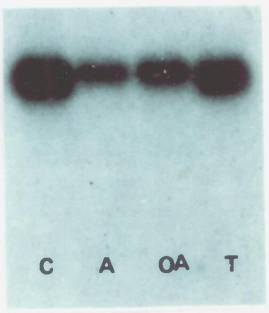
OA = ovariectomised and LHRH super-agonist treated

T = tamoxifen treatment

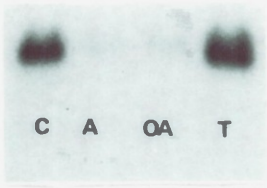
For the luteinising hormone beta subunit Northern blot 5 ug of total RNA was loaded onto each lane, whilst for all the other blots 50 ug of total RNA was loaded into each lane. All hybridisations were carried out using DNA probes.

PLATE 4

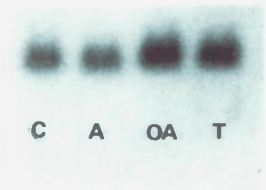
LUTEINISING HORMONE-Beta



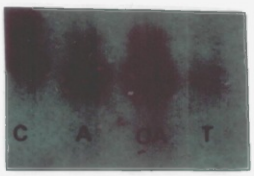
GALANIN



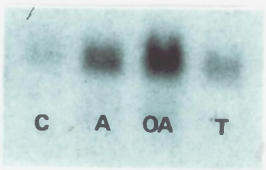
NEUROPEPTIDE Y



NEUROTENSIN



PREPROTACHYKININ-A



Anterior Pituitary Hormone mRNA Levels

Analysis of total pituitary RNA with a rat LH-beta cDNA probe demonstrated that there were dramatic differences in levels of LH-beta mRNA among the different groups. In the ovariectomised group LH-beta mRNA was increased approximately 15-fold relative to the control group. Treatment with high doses of oestrogen resulted in a decrease in LH-beta mRNA to approximately 2% of control values. In both groups of animals that received LHRH superagonist treatment, LH-beta mRNA was decreased to approximately 40% of control levels, whilst tamoxifen treatment produced a decrease in LH-beta mRNA to approximately 70% of control values. Prolactin mRNA showed a decrease in the ovariectomised group to approximately 50% of control values, and was increased in both the ovariectomised and oestrogen replaced and chronic oestrogen treated groups to approximately 170 - 190% of control values. In the 2 groups which received LHRH superagonist treatment and in the tamoxifen treated group PRL mRNA was unaltered. Growth hormone mRNA was increased to approximately 176% of control values by ovariectomy and was decreased by chronic oestrogen treatment to approximately 50% of control values but was unaffected in the groups which received LHRH superagonist treatment and in the tamoxifen treated group.

Minority Peptide Concentrations

Anterior pituitary GAL content showed significant alterations in nearly all groups. Ovariectomy produced a

significant decrease in GAL content (33 ± 3 fmol/gland vs control 223 ± 14 fmol/gland, $p<0.001$) whilst both ovariectomy with oestrogen replacement and chronic oestrogen treatment produced significant increases in pituitary GAL content (368 ± 14 fmol/gland and 373 ± 14 fmol/gland respectively vs control 223 ± 14 fmol/gland, $p<0.01$ for each). Tamoxifen treatment had no significant effect on GAL content whilst both groups of animals which received the LHRH superagonist treatment had significantly decreased GAL contents (39 ± 5 and 28 ± 6 fmol/gland vs control 233 ± 6 fmol/gland, $p<0.001$ for each).

Anterior pituitary NT content in group A showed some significant alterations following manipulations. Ovariectomy increased NT content (85 ± 8 vs control 62 ± 7 fmol/gland, $p<0.05$) whilst both ovariectomy with oestrogen replacement and chronic oestrogen treatment significantly lowered NT content (8 ± 3 and 5 ± 2 fmol/gland respectively vs control 62 ± 7 fmol/gland, $p<0.001$ for each). In treatment group B no significant alteration in NT content were observed.

The anterior pituitary content of NPY was significantly altered in only one group of animals out of both treatment groups. In group A, surgical ovariectomy increased NPY content (35 ± 4 fmol/gland vs control 20 ± 2.0 fmol/gland, $p<0.01$).

Anterior pituitary SP content in group A was increased by ovariectomy (130 ± 25 vs control 29 ± 2 fmol/gland, $p<0.001$), unaltered by ovariectomy and oestrogen replacement and decreased by chronic oestrogen treatment (18 ± 3 vs control 29 ± 2 fmol/gland, $p<0.01$). Substance P content in group B was increased in both groups which had been treated with the LHRH super agonist (31 ± 10 and 34 ± 8 vs control 18 ± 3 fmol/gland, $p<0.05$) whilst tamoxifen treatment had no significant effect on SP content.

Anterior pituitary VIP content in group A was decreased by surgical ovariectomy (201 ± 19 vs control 286 ± 26 fmol/gland, $p<0.05$) and increased by both ovariectomy and oestrogen replacement and chronic oestrogen treatment (6,422 ± 458 and 9,662 ± 776 fmol/gland respectively vs control 286 ± 26 fmol/gland, $p<0.001$). In group B no alteration in VIP content was observed in either of the two groups which received the LHRH superagonist treatment whilst tamoxifen treatment decreased VIP content dramatically (10 ± 2 fmol/gland vs control 240 ± 20 fmol/gland, $p<0.001$).

No significant alteration was observed in the hypothalamic content of any peptide in any of the treatment groups.

Minority peptide mRNA levels

Alterations in GAL mRNA were observed in nearly all groups. In group A, surgical ovariectomy decreased GAL

mRNA so much as to render it virtually undetectable whilst ovariectomy with oestrogen replacement and chronic oestrogen treatment increased GAL mRNA (to approximately 475% and 700% of control values respectively). In treatment group B, GAL mRNA levels were unaffected by tamoxifen treatment but were decreased to roughly the same extent (approximately 10% of control levels) in the two groups which received LHRH superagonist treatment.

Neurotensin mRNA showed alterations in most groups. Neurotensin mRNA in group A was increased to approximately 260% of control values by ovariectomy and decreased to undetectable levels by both ovariectomy with oestrogen replacement and chronic oestrogen treatment. In treatment group B, medical castration increased NT mRNA to approximately 330% of control values and combined medical and surgical castration group further increased NT mRNA to approximately 870% of control values whilst tamoxifen treatment elevated NT mRNA to approximately 158% of control levels.

Neuropeptide Y mRNA showed few responses to the manipulations. In group A, an increase in NPY mRNA to approximately 230% of control levels was observed in the surgically ovariectomised group and in group B an increase to approximately 190% of control values observed in the ovariectomised and LHRH superagonist treated group.

Levels of anterior pituitary PPT-A mRNA in group A were increased by surgical ovariectomy (to approximately 300% of control values) and decreased by both ovariectomy with oestrogen replacement and chronic oestrogen treatment (to 35% and 25% of control levels respectively). In group B, medical castration increased PPT-A mRNA and combined medical and surgical castration increased it further (to approximately 320% and 640% of control levels respectively). Tamoxifen treatment produced an increase in PPT-A mRNA levels to approximately 180% of control values.

Anterior pituitary VIP mRNA in group A was decreased to an undetectable level by ovariectomy and increased dramatically by ovariectomy with oestrogen replacement (to approximately 3,800% of control values) and increased further (to 7,700% of control levels) by chronic oestrogen treatment. The yields of RNA in group B were too low to allow probing for all five peptides and VIP was ommitted.

No alterations in hypothalamic mRNA were observed for any peptide following any of the treatments.

DISCUSSION

This series of manipulations successfully produced alterations in the synthesis of the gonadotrophic hormone LH. Luteininsing hormone-beta mRNA showed negative feedback responses to the alterations in endocrine status, being increased by surgical ovariectomy and decreased by oestrogen treatment. Treatment with the LHRH superagonist disconnects the anterior pituitary from hypothalamic LHRH regulation. Gonadotrophs become desensitised to the effects of hypothalamic LHRH following continuous treatment with LHRH or an analogue, as in the present study, as a result of receptor downregulation (Clayton RN 1982, Lalloz MRA et al 1988). The decrease in LH-beta mRNA seen in both groups of animals treated with the analogue demonstrated the effectiveness of this treament. Tamoxifen is a long acting estrogen antagonist which also shows agonist properties and produces species, organ and cell type specific effects, the mechanisms of which are not fully understood (Furr BJA and Jordan VC 1984).

In group A GAL showed a positive correlation with oestrogen status, being decreased in ovariectomised animals and increased in those treated with oestrogen. The effects of alterations in female sex hormone status on GAL mRNA have been reported by Kaplan and co-workers which confirm the results of the present study (Kaplan LM et al 1988a). In group B treatment with the LHRH super agonist reduced GAL peptide and mRNA in both intact and ovariectomised animals to roughly the same level indicating that pituitary GAL synthesis may be dependent

upon hypothalamic LHRH. Immunocytochemical staining revealed that in normal female rats galanin-immunoreactivity (GAL-IR) was present mainly in lactotrophs, with some minor staining in somatotrophs and thyrotrophs (Steel JH et al 1989). Oestrogen treatment of female rats increased the frequency of cells showing GAL-IR with most positive cells observed to be lactotrophs, whilst in ovariectomised rats GAL-IR was present in somatotrophs and a few thyrotrophs but not in lactotrophs (Steel JH et al 1989). This suggests that GAL may be involved in regulating PRL secretion, and there is experimental evidence to support this. Infusion of GAL into the third ventricle of male rats resulted in elevated serum levels of PRL (Melander T et al 1987, Ottlecz A et al 1988) whereas intravenous injection of GAL had no effect on serum PRL levels (Ottlecz A et al 1988). The immunocytochemical data taken together with the results of the present study supports a role for GAL in regulating PRL secretion.

In group A, surgical ovariectomy was the only manipulation to produce any significant effect on pituitary NPY with an approximate doubling in peptide and mRNA content seen in this group. In group B, no manipulation produced any alteration in NPY peptide content, whilst combined surgical and medical castration increased NPY mRNA to approximately 190% of control values. The general lack of response of pituitary NPY to these manipulations was somewhat unexpected as there is extensive evidence to suggest that NPY affects gonadotroph function. Central

administration of NPY has been shown to affect circulating levels of LH in female rats (Kalra SP and Crowley WR 1984, McDonald JK et al 1985). The incubation of NPY with cultured hemipituitaries from ovariectomised rats stimulated the release of LH and enhanced the LH releasing effect of LHRH (Crowley WR et al 1987). The results of this section of the study together with the detection of NPY in a sub-set of thyrotrophs by immunocytochemistry (discussed in chapter 3) indicate that NPY synthesised in the anterior pituitary may not function in regulating gonadotrophin secretion.

The effects of alterations in female sex hormone status on pituitary NT have not been reported. In group A ovariectomy resulted in an increase in NT content and mRNA whilst oestrogen replacement and chronic oestrogen treatment produced decreases in both peptide and mRNA content. In group B, no alterations in NT peptide content were observed whilst medical castration increased NT mRNA and combined medical and surgical castration produced a further increase in NT mRNA indicating that NT synthesis and peptide turnover were differentially affected by these treatments. At present nothing is known about the colocalisation of NT with pituitary hormones, but experimental data suggests that NT may be involved in regulating the secretion of PRL. Microinjection of NT into the lateral ventricle or third ventricle of male and ovariectomised female rats decreased PRL secretion (Aronin N et al 1986). Systemic administration of NT increased serum PRL levels in male rats (Aronin N et al 1986) and in

ovariectomised rats (Aronin N et al 1986). Following the addition of NT to cultured hemipituitaries from male and ovariectomised female rats (Aronin N et al 1986) and dispersed pituitary cells from both male and intact female rats (Memo M et al 1986) PRL release was stimulated. However, a decrease in PRL secretion from cultured and perifused rat pituitary cells following the addition of NT has been reported (Aronin N et al 1986). Injection of NT antiserum into the third ventricle of untreated ovariectomised rats and ovariectomised rats pretreated with oestrogen and progesterone elevated plasma PRL in both groups and also in untreated male rats (Vijayan E et al 1988). Intravenous injection of the same antiserum decreased serum PRL levels in the same two groups of female rats, but had no effect on PRL levels in males (Vijayan E et al 1988). There is also a small amount of evidence to suggest that NT may affect gonadotrophin secretion. Injection of NT into the third ventricle of ovariectomised rats decreased serum LH levels (Aronin N et al 1986), whilst microinjection into the medial preoptic area of intact or ovariectomised rats increased serum LH levels (Ferris CF et al 1984). A role for NT in the regulation of PRL and possibly gonadotrophin secretion is indicated by the above data and without information on the colocalisation of NT and further investigation of the effects of NT on the release of these hormones it is uncertain with which of these hormones NT may be involved.

In group A, ovariectomy increased and oestrogen treatment decreased SP and PPT-A mRNA. In group B, both medical

castration and combined surgical and medical castration increased SP and PPT-A mRNA. Peptide levels of SP in the female rat anterior pituitary have previously been shown to increase after ovariectomy and decrease following oestrogen treatment (Coslovsky R et al 1984, DePalatis LR et al 1985) confirming the results of the present study. Substance P immunoreactivity (SP-IR) was reported to be present in lactotrophs and gonadotrophs in the rat pituitary (Morel G et al 1982b) and experimental data support a role for SP in regulating the function of both cell types. Injection of SP into the third ventricle of ovariectomised rats elevated plasma LH levels (Aronin N et al 1986) whilst intravenous injection of SP into ovariectomised rats decreased serum LH levels (Aronin N et al 1986). Incubation of SP with hemipituitaries from ovariectomised rats had no effect on LH release (Aronin N et al 1986). Intravenous injection of an SP antiserum resulted in an increase in serum LH and FSH levels (Aronin N et al 1986). No effect on PRL secretion was seen when SP was injected into the lateral ventricle of male rats (Aronin N et al 1986) but intravenous administration of SP increased serum PRL levels in male rats (Aronin N et al 1986) and in ovariectomised female rats (Aronin N et al 1986). Addition of SP to cultured hemipituitaries from ovariectomised rats increased PRL release (Aronin N et al 1986). Intravenous injection of SP antiserum into hyperprolactinaemic male and female rats reduced circulating PRL levels but had no effect on the proestrous surge of PRL (Debeljuk L et al 1987, Debeljuk L et al 1988). The above data taken with the results of the

present study supports a role for SP in modulating the functions of either lactotrophs or gonadotrophs or both cell types.

Vasoactive intestinal peptide mRNA and peptide content in treatment group A was decreased by ovariectomy and increased by oestrogen treatment. In group B neither medical castration nor combined medical and surgical castration had any effect on VIP peptide content. Tamoxifen treatment decreased VIP content, indicating that this treatment may have an effect similar to that of ovariectomy. The presence of VIP was reported in rat lactotrophs by immunocytochemical localisation (Morel G et al 1982c) and confirmed by Steel and colleagues who were unable to identify the colocalising cell type in normal male or female rat pituitaries, but demonstrated that after oestrogen treatment VIP-immunoreactive cells were clearly identifiable as lactotrophs in female rats (Steel JH et al 1989). A relationship between pituitary VIP and PRL secretion has been suggested by a variety of evidence. Chronic treatment of rats with oestrogen increased pituitary VIP content (Maletti M et al 1982, Prysor-Jones RA et al 1988). Mutant strains of mice with pituitaries deficient in PRL producing cells have a significantly reduced anterior pituitary VIP content (Noguchi Y et al 1988). Addition of VIP antiserum to cultured rat anterior pituitary cells decreased PRL release (Hagen TC et al 1986). The reverse haemolytic plaque assay system demonstrated the release of VIP from lactotrophs and

showed that VIP antiserum or a VIP antagonist could reversibly block PRL secretion from these cells (Nagy G et al 1988). Both intraventricular and intravenous injection of VIP increased PRL secretion in rats (Kato Y et al 1978). Incubation of rat pituitary tumour cells with VIP produced an increase in PRL secretion and PRL mRNA levels (Carrillo AJ et al 1985). Intravenous injection of VIP antiserum blocked stress-induced PRL release, and reduced suckling-induced PRL release (Abe H et al 1985). This data, together with the results of the present study supports a role for VIP in the regulation of PRL release.

CHAPTER 6

MALE SEX HORMONE MANIPULATIONS

INTRODUCTION

In the male rat the synthesis and secretion of the gonadotrophic hormones LH and FSH are negatively regulated by circulating levels of the gonadal steroid hormone testosterone (Gharib SD et al 1986, Steiner RA et al 1982, Gharib SD et al 1987). The secretion of GH has also been reported to be affected by testosterone and the pattern of GH release is different in male and female rats. In male rats GH is secreted in regular episodic bursts which occur at 3- to 4-hour intervals, with low or undetectable levels between secretory peaks (Eden S 1979, Tannenbaum GS and Martin JB 1976). In females, the peaks occur less regularly, and levels between peaks are elevated compared to those in males (Eden S 1979). Orchidectomy of neonatal male rats resulted in a decrease in body weight gain and longitudinal bone growth, an effect reversed by testosterone treatment (Jansson J-O et al 1985). The growth stimulating properties of testosterone appear to operate via the anterior pituitary from the demonstration that testosterone treatment caused a dose-dependent increase in longitudinal bone growth and weight increase in gonadectomised male rats, but had no effect on male rats that had been hypophysectomised as well as gonadectomised and treated with GH (Jansson J-O et al 1983).

Previous reports have shown that minority pituitary peptides do not appear to be dramatically influenced by male sex hormone status. Manipulations of male sex

hormone status were reported to have no effect on rat anterior pituitary GAL mRNA (Kaplan LM et al 1988). In the rat anterior pituitary NT content was reported to be unaffected by castration (Goedert M et al 1982) and the content of SP was reported by different groups to be either unaltered or decreased by castration (Coslovsky R et al 1984, DePalatis LR et al 1985). The effects of these manipulations on NPY and VIP have not been reported.

ANIMALS

Rats were male Wistars weighing between 200 - 230 g supplied by Interfauna Ltd. The following manipulations were carried out;

1. Surgical castration. Operations were performed by the supplier and experiments were timed from 5 days post-surgery.

2. Surgical castration with testosterone replacement. Operations were performed by the supplier and testosterone replacement began 5 days after surgery. Each animal received daily subcutaneous injections of 500 ug of testosterone propionate (Sigma).

3. Chronic testosterone treatment. Intact animals received 100 mg of testosterone propionate administered in 4 doses over 28 days injected subcutaneously in depot.

4. Controls were sham operated.

All treatment groups were maintained for 28 days. Animals were sacrificed and tissues processed as described in materials and methods.

RESULTS

TABLE 6.1 Male sex hormone manipulations tissue weights and RNA yields

Anterior pituitary

	Wet weight	Total RNA	
C (a)	50 mg	310 ug	(n = 5)
CX (b)	62 mg	325 ug	(n = 5)
R (c)	43 mg	280 ug	(n = 5)
T (d)	42 mg	290 ug	(n = 5)

Hypothalamus

	Wet weight	Total RNA	
C	191 mg	315 ug	(n = 5)
CX	227 mg	333 ug	(n = 5)
R	219 mg	380 ug	(n = 5)
T	189 mg	295 ug	(n = 5)

(a) C = controls

(b) CX = castrated

(c) R = castrated and testosterone replaced

(d) T = chronic testosterone treated

All values are for pooled groups of tissues

TABLE 6.2 Male sex hormone manipulations anterior pituitary tissue peptide contents

Group	C	CX	R	T
GAL	80 ± 7	85 ± 6	84 ± 8	84 ± 6
NPY	12 ± 3	15 ± 2	10 ± 3	13 ± 2
NT	188 ± 40	230 ± 32	142 ± 35	167 ± 32
SP	175 ± 18	158 ± 22	204 ± 11	158 ± 23
VIP	233 ± 27	208 ± 36	169 ± 24	55 ± 3*

Mean values are femtomoles per gland (n = 9) given with standard error of the mean.

* Versus control, $p<0.001$

TABLE 6. 3 Male sex hormone manipulations scanning densitometer analysis of Northern blots

i) minority pituitary peptides

Group	C	CX	R	T
GAL	100%	104%	98%	97%
NPY	100%	112%	96%	118%
NT	100%	101%	103%	97%
PPT-A	100%	48%	108%	178%
VIP	100%	103%	54%	48%

ii) anterior pituitary hormones

Group	C	CX	R	T
LH-b	100%	1,100%	14%	<DL
GH	100%	111%	104%	96%

<DL = Below detection limit

Values for treatment groups are expressed as percentages relative to controls.

TABLE 6.4 Male sex hormone manipulations hypothalamic tissue peptide contents

A) Central hypothalamus

Group	C	CX	R	T
GAL	13 ± 1	13 ± 1	12 ± 0	13 ± 1
NPY	121 ± 6	110 ± 8	115 ± 7	126 ± 5
NT	104 ± 7	108 ± 5	109 ± 9	108 ± 6
SP	141 ± 10	145 ± 6	140 ± 5	142 ± 6
VIP	24 ± 1	19 ± 1	21 ± 1	20 ± 1

B) Lateral hypothalamus

Group	C	CX	R	T
GAL	11 ± 1	13 ± 1	12 ± 1	12 ± 1
NPY	63 ± 3	65 ± 9	66 ± 5	67 ± 2
NT	142 ± 9	134 ± 7	131 ± 5	149 ± 7
SP	180 ± 13	199 ± 10	167 ± 8	184 ± 11
VIP	17 ± 1	17 ± 1	16 ± 1	15 ± 1

Mean values are picomoles per g of tissue (wet weight, n = 9). The standard error of the mean is given with each value.

LEGEND TO PLATE 5 Representative Northern blots from male sex hormone manipulations

C = controls

CX = castrated

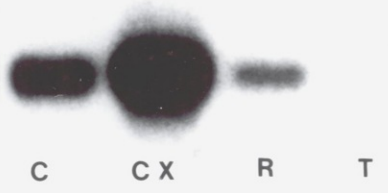
R = castrated and testosterone replaced

T = chronic testosterone treated

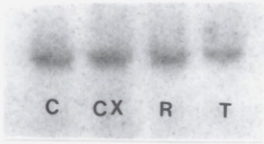
For the luteinising hormone-beta subunit Northern blot all lanes were loaded with 5 ug of total RNA and all other blots were loaded with 50 ug of total RNA in each lane. The luteinising hormone-beta subunit, galanin, neurotensin aand neuropeptide Y Northern blots were carried out using DNA probes and the preprotachykinin-A and vasoactive intestinal peptide blots were carried out with RNA probes.

PLATE 5

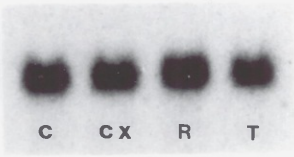
LUTEINISING HORMONE-Beta



GALANIN



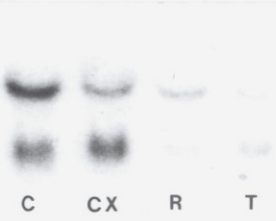
NEUROPEPTIDE Y



NEUROTENSIN

PREPROTACHYKININ-A

VASOACTIVE INTESTINAL PEPTIDE



Anterior Pituitary Hormone mRNA Levels

Analysis of total anterior pituitary RNA with an LH-beta specific cDNA probe demonstrated that there were dramatic differences in the content of LH-beta mRNA between the different groups. In the castrated group LH-beta mRNA was increased approximately 11-fold relative to controls. In the castrated and testosterone replaced group LH-beta mRNA was decreased to approximately 14% of control values whilst in the chronic testosterone treated group LH-beta mRNA was decreased to an undetectable level. Growth hormone mRNA showed no alteration following any of the treatments.

Minority Peptide Concentrations

The content of GAL in the anterior pituitary was not significantly affected by either castration, castration with testosterone replacement or chronic testosterone treatment.

The anterior pituitary content of NPY showed no significant alteration in response to any manipulation.

The anterior pituitary content of NT similarly showed no response to any manipulation.

Substance P pituitary content was also not significantly affected by any manipulation.

The pituitary content of VIP was unaffected by both castration and castration together with testosterone replacement. In the chronic testosterone treated group VIP content showed a significant decrease to 54.8 $\pm$ 2.6 fmol/gland from 233.1 $\pm$ 26.9 fmol/gland in control animals ($p<0.001$).

No significant alteration was seen in the hypothalamic content of any peptide in any treatment group.

Minority Peptide mRNA Levels

No alteration in GAL, NPY, or NT mRNA relative to controls was observed in any of the treatment groups.

Substance P showed a decrease to approximately 48% of control levels in the castrated group, showed no alteration in the castrated and testosterone replaced group and was increased in the chronic testosterone treated group to approximately 178% of control levels.

The level of VIP mRNA was unaffected by castration but was decreased in the castrated and testosterone replaced group to approximately 54% of control levels and was also decreased in the chronic testosterone treated group to approximately 48% of control levels.

No alteration in hypothalamic mRNA levels were observed for any peptide following any of the manipulations.

DISCUSSION

The manipulations successfully produced alterations in gonadotrophin synthesis as judged by the effects on LH-beta mRNA. Luteinising hormone-beta mRNA showed negative feedback responses to the alterations in endocrine status, being increased by castration and decreased by testosterone treatment. The minority pituitary peptides were on the whole largely unresponsive to these manipulations.

Galanin mRNA and peptide content was unaltered in any treatment group. The effects of sex hormone manipulations on GAL mRNA in male rats have been reported by Kaplan and co-workers (Kaplan LM et al 1988a) who found that castration had no effect on pituitary GAL mRNA, whilst treatment with testosterone resulted in a 2-fold increase in GAL mRNA. This increase was attributed by the authors to tissue aromatisation of testosterone to 17beta-oestradiol and was not regarded as a direct effect of testosterone treatment (Kaplan LM et al 1988a). The results of the present study confirm those of Kaplan and co-workers and indicate a lack of involvement of pituitary GAL in the regulation of gonadotrophin secretion in male rats.

Neuropeptide Y was unaffected in terms of either mRNA or peptide content by any treatment. This lack of effect may be regarded as somewhat unexpected from the results of experimental physiological studies, which suggested a role for NPY in the regulation of gonadotrophin secretion. In

female rats NPY has been shown to affect LH secretion (see chapter 5) and in male rats central administration of NPY was shown to alter circulating levels of LH (Kerkerian L et al 1985). In female rats pituitary NPY showed little response to alterations in sex hormone status (see chapter 5) and the lack of response in male rats further suggests that pituitary NPY is not involved in gonadotrophin secretion.

The effects of male castration on NT were previously reported by Goedert and co-workers who found no alteration in pituitary NT content in castrated male rats (Goedert M et al 1982) confirming the results of the present study. The effects of chronic testosterone treatment were not reported and were found in the present study to have no effect on pituitary NT.

Substance P peptide content was not significantly affected by any manipulation. The pituitary peptide content of SP in male rats sacrificed 6 weeks after castration was reported to decrease to approximately 60% of control values (DePalatis LR et al 1985). However, in another report, animals sacrificed at both 14 days and 45 days after castration did not have pituitary SP contents which were significantly different from controls (Coslovsky R et al 1984). Reasons for the discrepancies in the results of these two groups are unclear. Both groups used animals of the Sprague-Dawley strain and the time periods of the experiments (42 days in the former and 45 days in the latter) are similar. In the present study SP peptide

content in the castrated and chronic testosterone treated groups was not significantly different from the control group whilst PPT-A mRNA was observed to decrease and increase in these groups respectively. This suggests that alterations in the synthesis of SP may have occurred which did not affect peptide turnover sufficiently enough to produce detectable changes in content.

The effects of alterations in male sex hormone status on anterior pituitary VIP have not previously been reported. The response of VIP to the manipulations was somewhat unusual, with no alteration in VIP peptide or mRNA content observed in the castrated group whilst the castrated and replaced group showed no alteration in peptide content but a decrease in VIP mRNA. The chronic testosterone treated group showed a significant decrease in VIP peptide content together with a decrease in VIP mRNA indicating that tesosterone exerts a negative effect on anterior pituitary VIP in the male rat. However, the lack of effect of castration on VIP peptide and mRNA indicates that the effects of chronic testosterone treatment may be indirect.

CHAPTER 7

THE INFLUENCE OF ENDOCRINE STATUS ON GROWTH HORMONE

INTRODUCTION

This chapter describes the effects of the alterations in endocrine status carried out in this study on the hypothalamic regulation of growth hormone (GH) secretion. The secretion of GH is regulated by two hypothalamic hormones growth hormone-releasing hormone (GHRH) and somatostatin (SRIF) as well as by peripheral hormones such as glucocorticoid, thyroid and sex steroid hormones. In both humans and rats hypothyroidism produces lowered pituitary and serum GH levels (Peake GT et al 1973, Hervas F et al 1975, Coiro V et al 1979) and results in an abnormal response to GHRH (Williams T et al 1985, Root JL et al 1985). Hyperthyroidism is also known to impair GH secretion and has been reported to reduce the rise in plasma GH levels in response to exogenous GHRH, without affecting basal plasma GH levels (Wakabayashi I et al 1985). The effects of altered thyroid hormone status, especially hypothyroidism, have been extensively studied and the effects on GH release and gene expression are well characterised. However, little is known about the effects of thyroid hormone status on hypothalamic regulation of GH synthesis. Glucocorticoids have been reported to affect the synthesis and secretion of GH. Systemic adiministration of dexamethasone in rats was reported to increase GH secretion (Beinfeld MC and Packman PM 1980) and enhance the GH releasing effect of GHRH (Wehrenberg WB et al 1983). Glucocorticoids were shown to enhance the stimulatory effect of GHRH on GH secretion and GH mRNA accumulation in primary cultures of rat pituitary cells

(Vale W et al 1983). There are no reports of the effects of alterations in adrenal hormone status on hypothalamic GH regulation. Gonadal steroid hormones have been reported to affect GH secretion, although their effects have not been as well characterised as those of glucocorticoid and thyroid hormones. Some groups have reported that oestrogen stimulates the release of GH from cultured rat anterior pituitary cells (Simard J et al 1986, Wehrenberg WB et al 1985) whilst others found it to have no effect on basal or GHRH stimulated GH release from such cultures (Webb CB et al 1983). Castration of neonatal male rats was reported to decrease body weight gain and longitudinal bone growth, effects which could be reversed by testosterone replacement (Jansson J-O et al 1985).

RESULTS

The results given here are for animals manipulated as described in chapters 3 - 6.

TABLE 7.1 Hypothalamic and pituitary mRNA levels

A) Thyroid hormone manipulations

	C	TX	T4
SRIF	100%	109% ± 7%	103% ± 4%
GHRH	100%	540% ± 80%	48% ± 5%
GH	100%	18% ± 9%	97% ± 8%

B) Adrenal hormone manipulations

	C	AX	AC	DX
SRIF	100%	101%	95%	97%
GHRH	100%	112%	104%	95%
GH	100%	58%	65%	108%

Means and standard errors were calculated for the hypothalamic and pituitary mRNAs in hypo- and hyperthyroid animals as these manipulations were repeated several times. It should be noted that these values include but do not solely represent data derived from the animals described in chapter 3.

C) Female sex hormone manipulations - group A

	C	O	R	E2
SRIF	100%	102%	104%	100%
GHRH	100%	104%	101%	98%
GH	100%	176%	84%	51%

C) Female sex hormone manipulations - group B

	C	A	OA	T
SRIF	100%	111%	107%	94%
GHRH	100%	108%	98%	102%
GH	100%	104%	110%	104%

D) Male sex hormone manipulations

	C	CX	R	T
SRIF	100%	109%	107%	92%
GHRH	100%	88%	102%	104%
GH	100%	111%	104%	96%

Values for treatment groups are expressed as percentages relative to controls.

TABLE 7.2 Hypothalamic GHRH and SRIF content in thyroid manipulations

	lateral hypothalamus		central hypothalamus	
	GHRH	SRIF	GHRH	SRIF
Con	14 ± 1.2	2133 ± 71	77.3 ± 5.8	3603 ± 167
TX	11 ± 2.1	2176 ± 155	36.6 ± 2.3*	3534 ± 160
T4	12 ± 1.3	2440 ± 352	54.0 ± 4.3**	3094 ± 159

Mean values are picomoles/g wet weight tissue as determined by radioimmunoassay (n = 12).

* $p<0.01$

** $p<0.05$)

LEGEND TO PLATE 6 Representative Northern blots of the effects of manipulations on growth hormone mRNA

THYROID HORMONE MANIPULATIONS

C = control
TX = thyroidectomised
T4 = chronic thyroxine treated

ADRENAL HORMONE MANIPULATIONS

C = control
AX = adrenalectomised
AC = adrenalectomised and castrated
DX = dexamethasone treated

FEMALE SEX HORMONE MANIPULATIONS (group A only is shown)

C = control
O = ovariectomised
R = ovariectomised and oestrogen replaced
E2 = chronic oestrogen treated

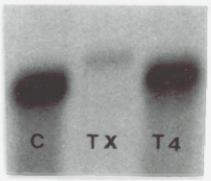
MALE SEX HORMONE MANIPULATIONS

C = control
CX = castrated
R = castrated and testosterone replaced
T+ = chronic testosterone treated

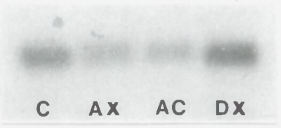
For each Northern blot all lanes contained 5 ug of total RNA and all hybridisations were carried out using DNA probes.

PLATE 6

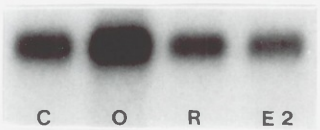
THYROID
HORMONE
MANIPULATIONS



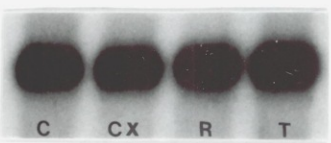
ADRENAL
HORMONE
MANIPULATIONS



FEMALE
SEX HORMONE
MANIPULATIONS



MALE
SEX HORMONE
MANIPULATIONS



LEGEND TO PLATE 7 Representative Northern blots of the effects of thyroid hormone manipulations on hypothalamic growth hormone-releasing hormone mRNA and somatostatin mRNA and on anterior pituitary growth hormone mRNA following RNase H digestion

C = control

T4 = chronic thyroxine treated

TX = thyroidectomised

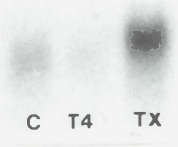
C+ = control pituitary RNA hybridised with oligo(dT) and digested with RNase H

TX+ = thyroidectomised pituitary RNA hybrised with oligo(dT) and digested with RNase H

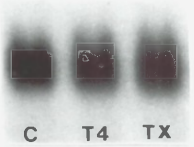
The Northern blot for growth hormone releasing hormone contained 50 ug of total RNA in each lane, the somatostatin blot contained 20 ug of total RNA in each lane and the blot for growth hormone contained 5 ug in each lane. All hybridisations were carried out using DNA probes.

PLATE 7

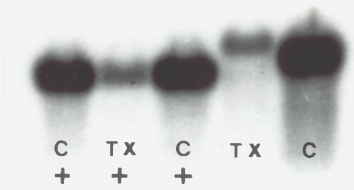
GROWTH HORMONE
RELEASING HORMONE



SOMATOSTATIN



GROWTH HORMONE



Anterior Pituitary Hormone mRNA Levels

In the anterior pituitaries of the hypothyroid group GH mRNA levels were decreased to approximately 20% of control levels. In addition to the decrease in amount of GH mRNA observed in the hypothyroid state a change in GH mRNA structure was also observed with the mature GH mRNA increasing in size by approximately 100 - 150 nucleotides. In the hyperthyroid group GH mRNA levels were unaffected relative to control levels and there was no observable alteration in GH mRNA structure. Following RNAse H digestion of oligo-dT hybridised total anterior pituitary RNA and analysis of the resulting de-adenylated products, a GH mRNA of equal length was observed in RNA from both control and thyroidectomised pituitaries. Estimation of the length of GH mRNAs by comparison with the positions of 28S and 18S ribosomal RNAs showed that in untreated control pituitaries the GH mRNA was approximately 900 nucleotides in length and in thyroidectomised pituitaries was approximately 1,000 nucleotides in length. Following RNAse H digestion the GH mRNA in RNA from both control and thyroidectomized pituitaries was approximately 780 nucleotides in length.

In the adrenal hormone manipulated animals GH mRNA was decreased to approximately 60% of control values in both the adrenalectomised and adrenalectomised and castrated groups and was unaffected by chronic dexamethasone treatment.

In the female sex hormone manipulation group A, GH mRNA was increased to approximately 175% of control values by ovariectomy and decreased by chronic oestrogen treatment to approximately 50% of control values. No alteration in GH mRNA was observed in the female sex hormone manipulation group B or in the male sex hormone manipulated group.

Hypothalamic mRNA levels

Northern blot analysis of total hypothalamic RNA demonstrated that SRIF mRNA showed no significant alteration in any treatment group of the four manipulation series. Levels of GHRH mRNA were altered in the thyroid hormone manipulated animals with no significant alteration observed in any group in the other three series of endocrine manipulations. Growth hormone-releasing hormone mRNA was altered in both hypo and hyperthyroid animals although in differential fashion with an increase to 5.4 $\pm$ 0.8 (n = 5) times control levels being seen in the thyroidectomised group and a decrease to 0.48 $\pm$ 0.05 (n = 4) of control levels being seen in the T4 treated group.

Hypothalamic Peptide Contents

Neither central or lateral hypothalamic regions showed significant differences in SRIF content in animals from either the hypothyroid group or the hyperthyroid group.

Both thyroidectomy and T4 treatment produced significant decreases in central hypothalamic GHRH content with the

thyroidectomised group showing the greatest decrease to approximately 50% of control levels (36.6 $\pm$ 2.3 vs 77.3 $\pm$ 5.8 pmol/g, $P<0.01$) and the T4 treated group showing a decrease to approximately 70% of control levels (54.0 $\pm$ 4.3 vs 77.3 $\pm$ 5.8 pmol/g, $P<0.05$). No significant difference was observed in GHRH content of the lateral hypothalamic region.

DISCUSSION

The results of this section of the study demonstrate that the methods employed in this study were capable of producing and detecting alterations in a hypothalamic peptide known to be involved in regulating pituitary function. Growth hormone-releasing hormone mRNA was unresponsive to alterations in adrenal or gonadal status but was altered following manipulations of thyroid hormone status, being increased by thyroidectomy and decreased by T4 treatment. Somatostatin mRNA showed no alteration in any group in any of the manipulation series. Following observation of the alterations in GHRH mRNA in the thyroidectomised and T4 treated groups the peptide content of GHRH and SRIF was measured in these groups. Alterations in hypothalamic GHRH peptide content were observed in both the thyroidectomised and T4 treated animals whilst SRIF content was unaltered in either group. In the hypothalami of both hypothyroid and hyperthyroid rats decreases in GHRH peptide content to approximately 50% and 70% respectively of control values were observed. The depletion in GHRH peptide content was of similar magnitude to that described in a previous report of the effects of thyroidectomy on hypothalamic GHRH peptide content (Katakami H et al 1986). The significance of this phenomenon is uncertain and might reflect some posttranscriptional derangement in GHRH synthesis, possibly through an alteration in GHRH processing resulting in an altered molecular form of the peptide or by inhibition of translation of GHRH mRNA. An alternative explanation might be hypothalamic feedback regulation of

GH secretion in reponse to the abnormally low levels of circulating GH. Thyroid hormone has been shown to be directly involved in GH gene transcription (Koenig RJ et al 1987, Glass CK et al 1987, Forman BM et al 1988) suggesting that the presence of thyroid hormone is a necessary prerequisite for normal GH synthesis. The abolition of pulsatile GH secretion (Martin D et al 1985) and the blunting of the GH releasing effect of GHRH in humans (Williams T et al 1985) and rats (Root JL et al 1985, Dieguez C et al 1985) supports this theory and suggests that in the hypothyroid state GH production is effectively disconnected from hypothalamic control (Martin D et al 1985). In this case the increase in mRNA and decrease in peptide content might reflect an increased rate of GHRH synthesis and release similar to the feedback effects of thyroidectomy on TSH production which have been shown to produce an increase in TSH beta subunit mRNA levels (Franklyn JA et al 1987) together with a decrease in TSH peptide content (Spira O et al 1979). It is interesting to note that a similar phenomenon to the increase in GHRH mRNA paralleled by a decrease in GHRH tissue peptide content was reported to occcur in rats as a response to hypophysectomy (Chomczynski P et al 1988). The similarity in the effects on GHRH following thyroidectomy and hypophysectomy may reflect a common increase in GHRH synthesis and secretion as a feedback response to the decrease in GH synthesis which resulted from both treatments. Confirmation of this would have required measurement of GHRH in the portal circulation which was beyond the scope of this study.

In the hyperthyroid group GHRH showed significant decreases in both peptide and mRNA levels. The significance of this observation is less easy to interpret as the effects of thyroid hormone excess on GH production have been relatively poorly studied in comparison to the effects of thyroid hormone deprivation. A possible explanation may be that this reflects a decrease in GHRH synthesis and secretion. It is of interest to note that rats treated with high doses of T4 during the neonatal period have been reported to develop a permanent derangement of the hypothalamo-hypophyseal-thyroid axis which results in decreased body weight, slight hypothyroidism and significantly lowered levels of pituitary GH (Pascual-Leone AM et al 1983, Bakke JL et al 1974). The alteration in pituitary GH content seen in this state was shown not to result from the mild hypothyroidism and was suggested to result from some other derangement of GH secretion produced by thyroid hormone treatment (Pascual-Leone AM et al 1983). It is possible that this may have a similar basis to the impairment of GH secretion following T4 treatment observed in this study and further work will be necessary to investigate this possibility. Further work will also be necessary to determine whether the effects on GHRH observed in hyperthyroid rats are of a transient or permanent nature. As SRIF showed no alterations in peptide or mRNA levels in the T4 treated animals this would suggest that hyperthyroidism influences the GH axis mainly through a modulation of GHRH production.

Growth hormone mRNA was altered by thyroid hormone, adrenal hormone and female sex hormone manipulations, but not by male sex hormone manipulations. The negative effects of thyroidectomy and adrenalectomy on GH mRNA have been previously reported and confirm the results of this study (Franklyn JA et al 1987, Martinoli MG and Pelletier G 1989). The effects of alterations in female sex hormone status on GH mRNA in vivo have not been previously reported. The results showed that GH mRNA appeared to be negatively regulated by oestrogen status, being increased by ovariectomy and decreased by oestrogen treatment. In addition to decreasing in amount in hypothyroid rats the mature GH mRNA transcript was observed to increase in size by approximately 100 nucleotides. This was an unexpected result which has not to the best of our knowledge been previously reported and was found to be experimentally reproducible by the repeated observation of this phenomenon in five groups of thyroidectomized animals on separate occasions. The results of RNase H digestion demonstrated that an increase in size of the GH mRNA poly(A) tail entirely accounted for the increase in the size of this mRNA observed in hypothyroid rat pituitary.

The significance of the enlarged GH poly(A) tract is uncertain. Alterations in poly(A) tail length have been associated with alterations in mRNA stability (Zeevi M et al 1982, Paek I and Axel R 1987) and proposed to affect the translational efficiency of RNA (Jacobson A and Favreau M 1983, Palatnik CM et al 1984, Rosenthal ET et al 1983). Alterations in the length of the poly(A) tails of

certain specific maternal mRNAs have been shown to occur following fertilisation in Spisula and Xenopus oocytes (Rosenthal ET et al 1983, Colot HV and Rosbash M 1982). Also, specific mRNAs in cultured cell lines have been shown to increase poly(A) tail length in response to hormonal or physiological stimuli (Paek I and Axel 1987), Muschel R et al 1986) whilst in-vivo certain RNAs have been demonstrated to alter the length of their polyadenylated tails in response to physiological stimuli and circadian rhythms (Carrazana EJ et al 1988, Mercer JFB and Wake SA 1985, Robinson BG et al 1988). Whether this increase in poly(A) tail length reflects alterations in the stability or translational efficiency of this mRNA remains to be determined. Similarly, the precise cause of the increase also remains to be determined as it may be a direct result of thyroidectomy or result from alterations in the secretion of other pituitary hormones or from some derangement of physiological or metabolic state.

These results suggest that hypothalamic regulation of GH is unaffected by alterations in adrenal or sex hormone status but is affected by altered thyroid status. Alterations in thyroid hormone status may also affect growth hormone synthesis at the pre-translational level.

CHAPTER 8

CONCLUSION

Summary of Results

One of the most important contributions of the study has been the demonstration of the presence of mRNAs encoding NPY, NT and VIP in the rat anterior pituitary gland. At the outset of this study there was relatively little evidence for minority peptide synthesis in the anterior pituitary gland. The interest in and importance of this is illustrated by reports published, during the period of the study, of the presence in the anterior pituitary of mRNAs encoding various peptides such as galanin, inhibin, insulin-like growth factor I, preprotachykinin-A, somatostatin and vasopressin (see introduction). The results of this study together with the results from these other groups strongly suggests that the anterior pituitary synthesises a variety of biologically active peptides which may be of importance in regulating anterior pituitary function. Furthermore, the study has shown that peptide and mRNA concentrations of GAL, NPY, NT, SP and VIP all vary with changes in endocrine status, supporting the hypothesis that these peptides have physiological functions in the anterior pituitary. The study has also shown that the influence of alterations in endocrine status on GAL, NT, SP and VIP is greater than was previously known.

In common with most work in a new field this study is primarily descriptive in nature. However, the results of the study in some instances supported roles for these peptides in the regulation of pituitary hormone secretion previously suggested by immunocytochemical and/or

experimental data and in other instances indicated further avenues of investigation. This has been discussed in the relevant results chapters and will only be touched upon briefly here.

The results of this study support a link between GAL and PRL secretion suggested by the experimental effects of GAL on pituitary hormone secretion and the results of immunocytochemistry (see chapter 5). Thyroidectomy had a negative effect on GAL synthesis which might be connected with the decrease in PRL synthesis seen in this state or may indicate that GAL has some other function in male rats. It is unlikely that the response of GAL to the adrenal hormone manipulations can be explained in terms of PRL regulation (chapter 4) and there is consequently a need to investigate the effects of GAL on the secretion of POMC derived peptides.

Most of the available experimental data regarding NPY point to a role in the regulation of gonadotroph function (chapters 5 and 6). However, manipulation of male and female rat sex hormone status had little or no effect on pituitary NPY whilst thyroidectomy produced a dramatic increase in both NPY peptide and mRNA content (chapters 3, 5 and 6). This might indicate that pituitary NPY is not involved in the regulation of gonadotrophin secretion but has some other local function. Further investigations into the effects of NPY on the secretion of anterior pituitary hormones is indicated, particularly those which are affected by thyroidectomy ie TSH, GH and PRL.

In those manipulations which affected PRL synthesis, namely thyroidectomy and the group A female sex hormone manipulations (chapters 3 and 5), NT synthesis was also altered, supporting a role for NT in the regulation of PRL production, previously suggested by experimental evidence (chapter 5). The effects of the female sex hormone manipulations on NT might also imply that NT affects gonadotroph function and further investigation of this as well as immunocytochemical localisation data is required.

Substance P was significantly affected by the thyroid, adrenal and female sex hormone manipulations (chapters 3, 4 and 5). This might indicate involvement in thyrotroph, corticotroph, gonadotroph and lactotroph function. There is experimental evidence that SP may affect the function of all these cell types (chapters 3, 4 and 5) and the effects of SP on hormone release require further investigation.

The response of VIP to the group A female sex hormone manipulations supported a role for VIP in the regulation of lactotroph function (chapter 5). The effects of thyroid hormone and adrenal hormone manipulations on VIP suggested that VIP may affect the release of other pituitary hormones (chapters 3 and 4).

The results of the study demonstrated that in the hypothalamus, GAL, NPY, NT, SP and VIP (with the exception of NPY in the adrenalectomised group) did not appear to alter in response to the alterations in endocrine status.

This might indicate, that, at the hypothalamic level, these peptides are not involved in regulating the responses of the anterior pituitary to alterations in endocrine status. Another possibility is that in the hypothalamus such peptides may function by modulating the secretion of hypophyseotropic hormones. It is possible that the techniques employed in this study may have been too gross to allow detection of alterations in hypothalamic peptides. Immunoreactive NPY content was reported to be decreased in the median eminence, arcuate and ventromedial nuclei in the hypothalami of male rats fourteen days after castration, but was unaltered in the medial preoptic area, dorsomedial and paraventricular nuclei (Sahu A et al 1987, Sahu A et al 1989). Neurotensin mRNA was reported to increase threefold in the medial preoptic region in female rats following chronic oestrogen treatment for 14 days (Alexander M et al 1989). In the present study no change was seen in hypothalamic NPY peptide content or mRNA in the male sex hormone manipulations or in NT peptide or mRNA in the female sex hormone manipulations. It is possible that changes in these peptides and their mRNAs may have occured in discrete regions of the hypothalamus (such as those looked in the reports of Sahu et al and Alexander et al, mentioned above), which might have been detected had peptide and mRNA contents been measured in these areas. However, such detailed dissection and measurements were not feasible in a study of this nature.

The second part of this study was concerned with examining the influence of alterations in endocrine status on the hypothalamic regulation of GH secretion. This demonstrated that hypothalamic peptides could alter in response to these endocrine manipulations and that such changes in peptide and mRNA content were detectable by the methods employed here. In the thyroid hormone manipulated group alterations were seen in the hypophyseotropic hormone GHRH, which is involved in regulating GH secretion. The results indicated that thyroidectomy increased GHRH synthesis and secretion, which would accord with the function of thyroid hormone as a transcriptional regulator of GH synthesis and agree with earlier suggestions that thyroidectomy effectively disconnects the somatotroph from normal hypothalamic regulation. Hyperthyroidism appeared to decrease GHRH synthesis and storage, and presumably altered its release which may account for and/or contribute to the defect in GH secretion reported to occur in hyperthyroid animals.

Further Research

Apart from further research into the effects of these peptides on hormone secretion as mentioned above, there are other questions about these peptides in the anterior pituitary which remain to be answered. With the exception of VIP there is little evidence that these peptides produce local effects on anterior pituitary function. Evidence of local actions comes from blocking the actions of minority peptides released from cultured anterior pituitary cells by immunoneutralisation with peptide-specific antisera or antagonists. This was done for VIP released from rat anterior pituitary cells in both monolayer cultures and the reverse haemolytic plaque assay, demonstrating that VIP stimulated the secretion of PRL (Hagen TC et al 1986, Nagy G et al 1988). Experiments of this type now need to be carried out on GAL, NPY, NT and SP, not only to determine whether these peptides have local functions but also to shed light upon the nature of such functions.

As part of the investigation into the nature of locally acting systems in the anterior pituitary it is important to determine which cell types synthesise which minority peptides. As mentioned in the introduction, immunocytochemical co-localisation cannot rule out the possibility that a peptide may be present in a cell type as the result of uptake of exogenously produced peptide, and this area requires more detailed investigation. The reverse haemolytic plaque assay is a good way of investigating which cell types secrete minority peptides

but possibly the best method would be the combined use of in situ hybridisation together with immunocytochemical localisation. This should provide a better picture of the situation within the tissue in vivo and might possibly provide information about the site(s) of peptide synthesis and peptide action. Certain minority pituitary peptides such as SP and GAL have been found by immunocytochemistry to be present in more than one cell type and it may be that these peptides are synthesised in one cell type and taken up by others which are targets for their actions. Also, the use of these techniques may provide useful information on the spatial relationships of the cells, which in a tissue with a highly organised structure such as the pituitary would be of interest.

The identity of the cell types within the anterior pituitary that are the targets for the actions of these peptides is probably the issue of greatest importance as this will aid in the elucidation of their functions. Immunoneutralisation experiments, such as those mentioned above will shed light on this issue, and another useful approach might be the combined use of the techniques of immunocytochemistry and radioreceptor labelling. Labelled minority peptides could be added to cultured anterior pituitary cells and the cells to which they had bound identified by immunocytochemistry. Such an approach would also allow investigation of the effects of alterations in endocrine status on the expression of minority peptide receptors. Hormonal status has been reported to affect the numbers of receptor binding sites for certain

hypophyseotropic hormones and minority pituitary peptides. Receptors for SP and angiotensin II in the female rat pituitary have been shown to be decreased following oestrogen treatment (Kerdelhue B et al 1985, Chen F-C M and Printz MP 1983). Anterior pituitary receptors for GHRH were reduced by adrenalectomy and restored by ddexamethasone replacement (Seifert H et al 1985). Testosterone has been shown to decrease LHRH binding sites in cultured anterior pituitary cells (Giguere V et al 1981) and testosterone administration in male rats shown to decrease LHRH binding to LH secreting gonadotrophs (Tibolt RE and Childs GV 1985). Oestrogen treatment of rat anterior pituitary cells in a reverse haemolytic plaque assay increased the fraction of gonadotrophs secreting LH (Smith PF et al 1984). This led to the suggestion that the increase in LHRH receptors seen during the preovulatory surge in vivo (Clayton RN et al 1980) may have resulted from the induction of receptors upon a sub-set of cells which did not previously possess LHRH receptors (Smith PF et al 1984).

Apart from the questions outlined above there are a number of other areas relating to the anterior pituitary which deserve investigation.

The immunocytochemistry results of Lam and colleagues and Steel and co-workers for VIP raised the possibility of plasticity of minority peptide expression. It might be the case that minority peptides such as VIP are normally

expressed in more than one cell type and that alterations in endocrine status result in abnormally high levels of synthesis only in certain cells for reasons which are at present unclear. Alternatively their results may represent true plasticity of expression where the expression of a gene is induced in a cell where previously it was not expressed, and this interesting possibility should be investigated.

An area of interest which may be related to the question of plasticity of expression is the classification of anterior pituitary secretory cell types, their development and relationships with each other. As stated in the introduction, the anterior pituitary is made up of five secretory cell types. However, this is an oversimplification of the situation. Luteinising hormone and FSH are known to be colocalised in some cells (Herbert DC 1976, Childs GV et al 1977) and it is uncertain whether monohormonal gonadotrophs containing only LH or FSH are immature stem cells or whether they represent different cell types from the ones which contain both hormones. Adrenocorticotrophin was reported to be present in a subpopulation of rat gonadotrophs (Moriarty GC and Garner LL 1977) which were particularly abundant in the neo-natal period but persisted at low levels in the adult (Childs GV et al 1982). A cell type in the rat pituitary which synthesises both GH and PRL, known as the mammosomatotroph, has been detected by immunocytochemistry (Nikitovitch-Winer MB et al 1987) and the reverse haemolytic plaque assay (Frawley LS et al 1985).

Another area that may warrant investigation is the regulation of anterior pituitary cell division. Hypothalamic releasing hormones such as GHRH and LHRH have been reported to affect the division of rat anterior pituitary cells (Billestrup N et al 1986, Sakai T et al 1988) and 2 of the peptides looked at in this study have been reported to possess growth factor properties in certain systems. Substance P stimulated DNA synthesis in rat aorta smooth muscle cell and human skin fibroblast cultures (Nilsson J et al 1985) and VIP stimulated DNA synthesis in cultures of human pituitary adenomas (Prysor-Jones et al 1989). It is known that populations of anterior pituitary cell types alter dramatically in response to endocrine manipulations such as those carried out in this study. Thyroidectomy was shown to cause hyperplasia of thyrotrophs (Surks M and DeFesi CR 1977, DeFesi CR et al 1979), adrenalectomy stimulated the division of corticotrophs (Westlund KN et al 1985, Gertz BJ et al 1987) and castration caused hyperplasia of gonadotrophs in both male and female rats (Childs GV et al 1982, Romano MI et al 1984,). Administration of oestrogen stimulated the synthesis of DNA and the proliferation of lactotrophs in the rat anterior pituitary in vivo (Lloyd RV 1983, Burdman JA et al 1983). The changes in minority pituitary peptides observed after the manipulations carried out in this study and the alterations in anterior pituitary cell populations which occur after such treatments raises the possibility that these peptides could function as local regulators of cell division.

REFERENCES

Abe H, Engler D, Molitch ME, Bollinger-Gruber J, Reichlin S 1985 Vasoactive intestinal peptide is a mediator of prolactin release in the rat. Endocrinology 116: 1383-1390

Adler L 1914 Metamorphosenstudaien an Batrachierlarven. 1. Exstirpation endokriner Dreusen: A. Exstirpation der hypophyse. Arch Entwicklungsmech. Organ 39: 21-45

Aguilera G, Hyde CL, Catt KJ 1982 Angiotensin II receptors and prolactin release in pituitary lactotrophs. Endocrinology 111: 1045-1050

Ahren B, Alumets J, Ericson M, Fahrenkrug J, Fahrenkrug I, Hakanson R, Hedner P, Loren I, Melander A, Rerup C, Sundler F 1980 VIP occurs in intrathyroidal nerves and stimulates thyroid hormone secretion. Nature 287: 343-348

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD 1983 Chemical signalling between cells. Chapter 13 in: Molecular Biology of the Cell. Garland Publishing Inc

Alexander MJ, Dobner PR, Miller MA, Bullock BP, Dorsa DM, Leeman SE 1989 Estrogen induces neurotensin/neuromedin N messenger ribonucleic acid in a preoptic nucleus essential for the preovulatory surge of luteinising hormone in the rat. Endocrinology 125: 2111-2117

Allen JM, Yeats JC, Adrian TE, Bloom SR 1984 Radioimmunoassay of neuropeptide Y. Regul. Pept. 8: 61-66

Allen JM, Bloom SR 1986 Neuropeptide Y: a putative neurotransmitter. Neurochem. Int. 8: 1

Allen LG, Kalra PS, Crowley WR, Kalra SP 1985 Comparison of the effects of neuropeptide Y and adrenergic agents on LH release and food intake in male rats. Life Sci. 37: 617-623

Alwine JC, Kemp DJ, Parker BA, Reiser J, Renart J, Stark GR, Wahl GM 1979 Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Methods in Enzymology 68: 220-242

Ambrose BJB, Pless RC 1987 DNA sequencing: Chemical methods chapter. In: Berger SL, Kimmel AR (eds) Methods in Enzymology: Guide to Molecular Cloning Techniques. Academic Press, New York, vol 152, chapter 56

Antoni FA, Holmes MC, Makara GB, Karteszi M, Laszlo FA 1984 Evidence that the effects of arginine-8-vasopressin (AVP) on pituitary corticotropin (ACTH) release are mediated by a novel type of receptor. Peptides 5: 519-522

Antoni FA 1986 Oxytocin receptors in rat adenohypophysis: evidence from radioligand binding studies. Endocrinology 119: 2393-2395

Arisawa M, Snyder GD, McCann SM 1989 Effect of substance P on thyrotropin secretion from the pituitary gland in the rat. Peptides 10: 763-766

Arnaout MA, Garthwaite TL, Martinson DR, Hagen TC 1986 Vasoactive intestinal polypeptide is synthesised in anterior pituitary tissue. Endocrinology 119: 2052-2056

Aronin N, Morency K, Leeman SE, Braverman LE, Coslovsky R 1984 Regulation by thyroid hormone of the concentration of substance P in the rat anterior pituitary. Endocrinology 114: 2138-2142

Aronin N, Coslovsky R, Leeman SE 1986 Substance P and neurotensin: their roles in the regulation of anterior pituitary function. Ann. Rev. Physiol. 48: 537-549

Aronin N, Coslovsky R, Chase K 1988 Hypothyroidism increases substance P concentrations in the heterotropic anterior pituitary. Endocrinology 122: 2911-2914

Aschner B 1912 Ueber die funktion der hypophyse. Pfleuger's Arch Ges Physiol 146: 1-146

Ascoli G, Legnani T 1912 Die folgen d Exstirpation d Hypophyse. Muenchn med Wochenschr 59: 518-521

Autelitano DJ, Clements JA, Nikolaidis I, Canny BJ, Funder JW 1987 Concomitant dopaminergic and glucocorticoid control of pituitary proopiomelanocortin messenger ribonucleic acid and b-endorphin levels. Endocrinology 121: 1689-1696

Baes M, Allaerts W, Denef C 1987 Evidence for functional communication between folliculo-stellate cells and hormone-secreting cells in perifused anterior pituitary cell aggregates. Endocrinology 120: 685-691

Bakke JL, Lawrence N, Wilber JF 1974 The late effects of neonatal hyperthyroidism upon the hypothalamic-pituitary-thyroid axis in the rat. Endocrinology 95: 406-410

Baldwin DM, Sawyer CH 1974 Effects of dexamethasone on LH release and ovulation in the cyclic rat. Endocrinology 84: 308-312

Bauer FE, Adrian TE, Christofides ND, Ferri G-L, Yanaihara N, Polak JM, Bloom SR 1986 Distribution and molecular heterogeneity of galanin in human, pig, guinea pig, and rat gastrointestinal tracts. Gastroenterology 91: 877-883

Beinfeld MC, Packman PM 1980 Corticosterone increases the synthesis of a soluble protein in pituitary glands from adrenalectomised male rats which precipitates with ani-rat growth antiserum. FEBS. Lett. 118: 85-88

Besson J, Rotsztejn W, Laburthe M, Epelbaum J, Beaudet A, Kordon C, Rosselin G 1979 Vasoactive intestinal peptide (VIP). Brain distribution, subcellular localisation and effect of deafferentation of the hypothalamus in male rats. Brain Research 165: 79-85

Bilestrup N, Swanson LW, Vale W 1986 Growth hormone-releasing factor stimulates proliferation of somatotrophs in vitro.
Proc. Natl. Acad. Sci. USA 83: 6854-6857

Binoux M, Hossenlopp P, Lassarre C, Hardouin N 1981 Production of insulin-like growth factors and their carrier gy rat pituitary gland and brain explants in culture. FEBS. Lett. 124: 178-184

Birnberg NC, Lissitzky J-C, Hinman M, Herbert E 1983 Glucocorticoids regulate proopiomelanocortin gene expression in vivo at the levels of transcription and secretion. Proc. Natl. Acad. Sci. USA 80: 6982-6986

Blackburn AM, Bloom SR 1979 A radioimmunoassay for neurotensin in human plasma. J. Endocrinol. 83: 175-181

Boockfor FR, Frawley LS 1987 Functional variations among prolactin cells from different pituitary regions.
Endocrinology 120: 874-879

Brazeau P, Vale W, Burgus R et al 1973 Hypothalamic polypeptide that inhibits immunoreactive pituitary growth hormone. Science 179: 77-79

Brown M, Vale W 1976 Effects of neurotensin and substance P on plasma insulin, glucagon and glucose levels. Endocrinology 98: 819-822

Bryant MG, Bloom SR, Polak JM, Albuquerque RH, Modlin I, Pearse AGE 1976 Possible dual role for vasoactive intestinal peptide as gastrointestinal hormone and neurotransmitter substance. Lancet 1: 991-995

Burdman JA, Calabrese MT, MacLeod RM 1983 Hyperprolactinaemia and DNA synthesis in the pituitary gland of the rat. J. Endocrinol. 97: 65-74

Bury RW, Mashford ML 1976 Biological activity of C-terminal partial sequences of substance P.
Journal of Medical Chemistry 19: 854-858

Carraway R, Leeman SE 1973 The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami.
J. Biol. Chem. 248: 6854-6861

Carraway R, Leeman SE 1975 The amino acid sequence of a hypothalamic peptide neurotensin.
J. Biol. Chem. 250: 1907-1911

Carrazana EJ, Pasieka KB, Majzoub JA 1988 The vasopressin mRNA poly (A) tract is unusually long and increases during stimulation of vasopressin gene expression in vivo. Mol. Cell. Biol. 8: 2267-2274

Carrillo AJ, Pool TB, Sharp ZD 1985 Vasoactive intestinal peptide increases prolactin messenger ribonucleic acid content in GH3 cells.
Endocrinology 116: 202-206

Chang MM, Leeman SE 1970 Isolation of a sialogogic peptide from bovine hypothalamic tissue and its characterisation as substance P.
J. Biol. Chem. 245: 4784-4790

Chateau M, Marchetti J, Burlet A, Boulange M 1979 Evidence of vasopressin in adenohypophysis: research into its role in corticotrope activity.
Neuroendocrinology 28: 25-35

Chen F-CM, Printz MP 1983 Chronic estrogen treatment reduces angiotensin II receptors in the anterior pituitary. Endocrinology 113: 1503-1510

Cheung CY 1983 Prolactin suppresses luteinising hormone secretion and pituitary responsiveness to luteinising hormone-releasing hormone by a direct action at the anterior pituitary. Endocrinology 113: 632-638

Chihara K, Arimura A, Coy DH, Schally AV 1978 Studies on the interaction of endorphins, substance P, and endogenous somatostatin in growth hormone and prolactin release in rats. Endocrinology 102: 281-290

Chihara K, Arimura A, Schally AV 1979 Immunoreactive somatostatin in rat hypophysial portal blood.
Endocrinology 104: 1434-1441

Childs (Moriarty) GV, Cole DE et al 1978 Endogenous thyrotropin-releasing hormone in the anterior pituitary: sites of activity as identified by immunocytochemical staining. J. Histochem. Cytochem. 26: 901-908

Childs (Moriarty) GV, Ellison DG, Lorenzen JR, Collins TJ, Schwartz NB 1982 Immunohistochemical studies of gonadotropin storage in developing castration cells.
Endocrinology 111: 1318-1328

Childs (Moriarty) GV, Ellison DG, Ramaley JA 1982a Storage of anterior lobe adrenocorticotropin in corticotropes and a subpopulation of gonadotropes during the stress-nonresponsive period in the neonatal male rat.
Endocrinology 110: 1676-1692

Childs GV, Ellison DG, Lorenzen JR, Collins TJ, Schwartz NB 1983 Retarded development of castration cells after adrenalectomy or sham adrenalectomy. Endocrinology 113: 166-177

Chin WW, Shupnik MA, Ross DS, Habener JF, Ridgway EC 1985 Regulation of the alpha and thyrotropin beta-subunit messenger ribonucleic acids by thyroid hormones. Endocrinology 116: 873-878

Ching M 1982 Correlative surges of LHRH, LH and FSH in pituitary stalk plasma and systemic of rat during proestrus. Neuroendocrinology 34: 279-285

Chomcszynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium-thiocyanate-phenol-chlorophorm extraction. Anal. Biochem. 162: 156-159

Chomczynski P, Downs TR, Frohman LA 1988 Feedback regulation of growth hormone (GH)-releasing hormone gene expression by GH in rat hypothalamus. Molecular Endocrinology 2: 236-241

Chronwall BM, DiMaggio DA, Massari VJ, Pickel VM, Ruggiero DA, O' Donohue TL 1985 The anatomy of neuropeptide-Y-containing neurons in rat brain. Neuroscience 15: 1159-1181

Clark JH, Markaverich BM 1982 The agonistic-antagonistic properties of clomiphene: a review. Pharmacol. Ther. 15: 467-519

Clayton RN 1982 Gonadotrophin-releasing hormone modulation of its own pituitary receptors: evidence for biphasic regulation. Endocrinology 111: 152-161

Clayton RN, Solano AR, Garcia-Vela A, Dufau ML, Catt KJ 1980 Regulation of pituitary receptors for gonadotropin-releasing hormone during the rat estrous cycle. Endocrinology 107: 699-706

Coiro V, Braverman LE, Christianson D, Fang S-L, Goodman HM 1979 Effect of hypothyroidism and thyroxine replacement on growth hormone in the rat. Endocrinology 105: 641-646

Colot HV, Rosbash M 1982 Behaviour of individual maternal pA+ RNAs during embryogenesis of Xenopus laevis. Dev. Biol. 94: 79-86

Coslovsky R, Evans RW, Leeman SE, Braverman LE, Aronin N 1984 The effects of gonadal steroids on the content of substance P in the rat anterior pituitary. Endocrinology 115: 2285-2289

Counis R, Corbani M, Jutisz M 1983 Estradiol regulates mRNAs encoding precursors to rat lutropin (LH) and follitropin (FSH) subunits.
Biochem. Biophys. Res. Com. 114: 65-72

Crowe J, Cushing HW, Homans J 1910 Experimental hypophysectomy. Bull. John Hopkins Hosp. 21: 127-169

Crowley WR, Hassid A, Kalra SP 1987 Neuropeptide Y enhances the release of luteinizing hormone (LH) induced by LH -releasing hormone. Endocrinology 120: 941-945

Cushing HW 1910 The functions of the pituitary body. Am. J. Med. Sci. 139: 473-484

Dandy WE 1913 The nerve supply to the pituitary body. Am. J. Anat. 15: 333-343

Daughaday WH 1985 The anterior pituitary. Chapter 18 in: Williams Textbook of Endocrinology, Wilson JD and Foster DW eds. WB Saunders Co

Davis TM, Burrin JM, Bloom SR 1987 Growth hormone (GH) release in response to GH-releasing hormone in man is 3-fold enhanced by galanin.
J. Clin. Endocrinol. Metab. 65: 1248-1252

Debeljuk L, Lasaga M, Horvath J, Duvilanski B, Seilicovich A, del Carmen Diaz M 1987 Effect of an anti-substance P serum on prolactin and gonadotropins in hyperprolactinemic rats. Regul. Pept. 19: 91-98

Debeljuk L, Lasaga M, Afione S, Duvilanski B, del Carmen Diaz M 1988 Effect of passive immunization against substance P in rats with hyperprolactinemia.
Peptides 9: 933-936

DeFesi CR, Astier HS, Surks MI 1979 Kinetics of thyrotrophs and somatotrophs during development of hypothyroidism and L-triiodothyronine treatment of hypothyroid rats. Endocrinology 104: 1172-1180

Deftos LJ, Burton BD, Catherwod BD, Bone HG, Parthermore JG, Guillemin R, Watkins WB, Moore RY 1978 Demonstration by immunoperoxidase histochemistry of calcitonin in the anterior lobe of the anterior pituitary.
J. Clin. Endocrinol. Metab. 47: 475-480

Del Sal G, Manfioletti G, Schneider C 1988 A one-tube plasmid DNA mini-preparation suitable for sequencing.
Nuc. Acids Res. 16: 9878

Denef C, Andries M 1983 Evidence for paracrine interaction between gonadotrophs and lactotrophs in pituitary cell aggregates. Endocrinology 112: 813-822

Denef C, Schramme C, Baes M 1985 Stimulation of growth hormone release by vasoactive intestinal peptide and peptide PHI in rat anterior pituitary reaggregates. Neuroendocrinology 40: 88-91

DePalatis LR, Fiorindo RP, Ho RH 1982 Substance P immunoreactivity in the anterior gland of the guinea pig Endocrinology 110: 282-284

DePalatis LR, Khorram O, McCann SM 1985 Age-, Sex-, and gonadal steroid-related changes in immunoreactive substance P in the rat anterior pituitary gland. Endocrinology 117: 1368-1373

Dobner PR, Kawasaki ES, Yu L-Y, Bancroft FC 1981 Thyroid or glucocorticoid hormone induces pre-growth-hormone mRNA and its probable nuclear precursor in rat pituitary cells. Proc. Natl. Acad. Sci. USA 78: 2230-2234

Domin J, Ghatei MA, Chohan P, Bloom SR 1987 Neuromedin U - a study of its distribution in the rat. Peptides 8: 779-784

Domin J, Steel JH, Adolphus N, Burrin JM, Leonhardt U, Polak JM, Bloom SR 1989 The anterior pituitary content of neuromedin U-like immunoreactivity is altered by thyrotrophin-releasing hormone and thyroid status in the rat. J. Endocrinol. 122: 471-476

Duello TM, Nett TM, Farquhar MG 1983 Fate of a gonadotropin-releasing hormone internalised by rat pituitary gonadotrophs. Endocrinology 112: 1-10

Eberwine JH, Roberts JL 1984 Glucocorticoid regulation of pro-opiomelanocortin gene transcription in the rat pituitary. J. Biol. Chem. 259: 2166-2170

Eden S 1979 Age- and sex-related differences in episodic growth hormone secretion in the rat. Endocrinology 105: 555-560

Edvinsson L, Emson P, McCulloch J, Tatemoto K, Uddman R 1983 Neuropeptide Y: cerebrovascular innervation and vasomotor effects in the cat. Neurosci. Lett. 43: 79-84

Edvinsson L, Ekblad E, Hakanson R, Wahlestedt C 1985 Neuropeptide Y potentiates the effects of various vasoconstrictor agents on rabbit blodd vessels. British Journal of Pharmacology 83: 519-525

Emson PC, Fahrenkrug J, Schaffalitzky de Muckadell OB, Jessell TM, Iversen LL 1978 Vasoactive intestinal polypeptide (VIP): vesicular localisation and potassium evoked release from rat hypothalamus. Brain Research 143: 174-178

Emson PC, Goedert M, Horsfield P, Rioux F, St Pierre S 1982 The regional distribution and chromatographic characterisation of neurotensin-like immunoreactivity in the rat central nervous system. J. Neurochem. 38: 992-999

Euker JS, Meites J, Riegle GD 1975 Effects of acute stress on serum LH and prolactin in intact, castrate and dexamethasone treated male rats. Endocrinology 96: 85-89

Evans HM, Long JA 1921 The effect of the anterior lobe administered intraperitoneally upon growth, maturity and oestrus cycles of the rat. Anat. Rec. 21: 62-62

Evans RM, Birnberg NC, Rosenfeld MG 1982 Glucocorticoid and thyroid hormones transcriptionally regulate growth hormone gene expression.
Proc. Natl. Acad. Sci. USA 79: 7659-7663

Fagin JA, Brown A, Melmed S 1988 Regulation of pituitary insulin-like growth factor-I messenger ribonucleic acid levels in rats bearing somatomammotropic tumors: implications for growth hormone regulation.
Endocrinology 122: 2204-2210

Feinberg AP, Vogelstein B 1983 A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6-9

Ferrara N, Schweigerer L, Neufeld G, Mitchell R, Gosparadowicz D 1987 Pituitary follicular cells produce basic fibroblast growth factor.
Proc. Natl. Acad. Sci. USA 84: 5773-5777

Ferris CF, Pan JX, Singer EA, Boyd ND, Carraway RE, Leeman SE 1984 Stimulation of luteinising hormone release after stereotaxic microinjection of neurotensin into the medial preoptic area of rats. Neuroendocrinology 38: 145-151

Feyrter F 1938 Uber die peripheren endokrinen (parakrinen) Drusen des Menschen. Leipzig : Ambrosius Barth

Fink G 1976 The development of the releasing factor concept. Clinical Endocrinology 5: 245s-260s

Fink G 1979 Feedback actions of target hormones on hypothalamus and pituitary with special reference to gonadal steroids. Ann. Rev. Physiol. 41: 571-585

Flynn JJ, Margules DL, Cooper CW 1981 Presence of immunoreactive calcitonin in the hypothalamus and the pituitary lobes of rat.
Brain Research Bulletin 61: 547-549

Forman BM, Yang C, Stanley F, Casanova J, Samuels HH 1988 C-erbA protooncogenes mediate thyroid hormone-dependant and independant regulation of the rat growth hormone and prolactin genes. Molecular Endocrinology 2: 902-911

Foster GV, Baghdiantz A, Kumar MA, Slack E, Soliman HA, MacIntyre I 1964 Thyroid origin of calcitonin. Nature 202: 1303-1305

Franklyn JA, Wood DF, Balfour NJ, Ramsden DB, Docherty K, Chin WW, Sheppard MC 1987 Effect of hypothyroidism and thyroid hormone replacement in vivo on pituitary cytoplasmic concentrations of thyrotropin-beta and alpha-subunit messenger ribonucleic acids. Endocrinology 120: 2279-2288

Franchimont P 1986 Foreword. Clinics in Endocrinology and Metabolism. 15: ix-xiii

Frawley LS, Bookfor FR, Hoeffler JP 1985 Identification by plaque assay of a pituitary cell type that secretes both growth hormone and prolactin. Endocrinology 116: 734-739

Friedgood HB 1936 Studies on the sympathetic nervous control of the anterior hypophysis with special reference to a neuro-humoral mechanism. Chapter 10 pages 635-698 Textbook of Endocrinology Williams RH (ed)

Furr BJA, Jordan VC 1984 The pharmacology and clinical uses of tamoxifen. Pharm. Ther. 25: 127-205

Furutani Y, Morimoto Y, Shibahara S, Noda M, Takahashi H, Hirose T, Asai M, Inayama S, Hayashida H, Miyata T, Numa S 1983 Cloning and sequence analysis of cDNA for ovine corticotropin releasing factor precursor. Nature 301: 537-540

Gabriel SM, Milbury CM, Nathanson JA, Martin JB 1988 Galanin stimulates rat pituitary growth hormone secretion in vitro. Life Sci. 42: 1981-1986

Gardner DG, Deschepper CF, Ganong WF, Hane S, Fiddes, Baxter JD, Lewicki J 1986 Extra-atrial expression of the gene for atrial natriuretic factor. Proc. Natl. Acad. Sci. USA 83: 6697-6701

Gertz BJ, Contreras LN, McComb DJ, Kovacs K, Tyrrell JB, Dallman MF 1987 Chronic administration of corticotropin-releasing factor increases pituitary corticotroph number. Endocrinology 120: 381-388

Gharib SD, Bowers SM, Need LR, Chin WW 1986 Regulation of rat luteinising hormone subunit messenger ribonucleic acids by gonadal steroid hormones. J. Clin. Invest. 77: 582-589

Gharib SD, Wierman ME, Badger TM, Chin WW 1987 Sex steroid hormone regulation of follicle-stimulating hormone subunit messenger ribonucleic acid (mRNA) levels in the rat. J. Clin. Invest. 80: 294-299

Giguere V, Lefebvre F-A, Labrie F 1981 Androgens decrease LHRH binding sites in rat anterior pituitary cells in culture. Endocrinology 108: 350-352

Glass CK, Franco R, Weinberger C, Albert VR, Evans RM, Rosenfeld MG 1987 A c -erb-A binding site in rat growth hormone gene mediates trans-activation by thyroid hormone. Nature 329: 738-741

Glembotski CC, Wildey GM, Gibson TR 1985 Molecular forms of immunoreactive atrial natriuretic peptide in the rat hypothalamus and atrium.
Biochem. Biophys. Res. Comm. 129: 671-678

Goedert M, Lightman SL, Nagy JI, Marley PD, Emson PC 1982 Neurotensin in the rat anterior pituitary gland.
Nature 298: 163-165

Goedert M, Lightman SL, Emson PC 1984 Neurotensin in the rat anterior pituitary gland: effects of endocrinological manipulations. Brain Research 299: 160-163

Goltzman D, Mitchell J 1985 Interaction of calcitonin and calcitonin gene-related peptide at receptor sites in target tissues. Science 227: 1343-1345

Gozes I, Tsafriri A 1986 Detection of vasoactive intestinal peptide-encoding messenger ribonucleic acid in the rat ovaries. Endocrinology 119: 2606-2610

Green JD, Harris GW 1949 Observation of the hypophysio-portal vessels of the living rat.
J. Physiol. 108: 359-361

Gross DS 1983 Hormone production in the hypophysial pars tuberalis of intact and hypophysectomised rats.
Endocrinology 112: 733-744

Gross DS, Turgeon JL, Waring DW 1984 The ovine pars tuberalis: a naturally occurring source of partially purified gonadotropes which secrete luteinising hormone in vitro. Endocrinology 114: 2084-2091

Haas DA, George SR 1987 Neuropeptide Y administration acutely increases hypothalamic corticotropin-releasing factor immunoreactivity: lack of effect in other rat brain regions. Life Sci. 41: 2725-2731

Hagen TC, Arnaout MA, Scherzer WJ, Martinson DR, Garthwaite TL 1986 Antisera to vasoactive intesinal polypeptide inhibit basal prolactin release from dispersed anterior pituitary cells. Neuroendocrinology 43: 641-645

Harris GW 1948 Neural control of the pituitary gland.
Physiol. Rev. 28: 139-179

Hauger RL, Aguilera G, Baukal AJ, Catt KJ 1982 Characterisation of angiotensin II receptors in the anterior pituitary gland.
Mol. Cell. Endocrinol. 25: 20316-20321

Hellmann W, Suzuki F, Ohkubo H, Nakanishi S, Ludwig G, Ganten D 1988 Angiotensinogen gene expression in extrahepatic rat tissues: application of a solution hybridisation assay.
Naunyn-Schmeidberg's Arch. Pharmacol. 338: 327-331

Herbert DC 1976 Immunocytochemical evidence that luteinising hormone (LH) and follicle stimulating hormone are present in the same cell type in the rhesus monkey pituitary gland. Endocrinology 98: 1554-1557

Hervas F, Morreale de Escobar G, Escobar del Rey F 1975 Rapid effects of single small doses of L-thyroxine and triiodo-thyronine on growth hormone, as studied in the rat by radioimmunoassay. Endocrinology 97: 91-101

Hoeffler JP, Frawley LS 1987 Hypothalamic factors differentially affect the proportions of cells that secrete growth hormone or prolactin.
Endocrinology 120: 791-795

Hokfelt T, Kellerth J-O, Nilsson G, Pernow B 1975 Substance P: localisation in the central nervous system and in some primary sensory neurons. Science 190: 889-898

Hopkins CR, Gregory H 1977 Topographical localisation of the receptors for luteinising hormone-releasing hormone on the surface of dissociated cells.
J. Cell. Biol. 75: 528-540

Jacobson A, Favreau M 1983 Possible involvement of poly(A) in protein synthesis.
Nuc. Acids. Res. 11: 6353-6368

Jansson J-O, Eden S, Isaksson O 1983 Sites of action of testosterone and estradiol on longitudinal bone growth.
Am. J. Physiol. 2244: E135-E140

Jansson J-O, Ekberg S, Isaksson O, Mode A, Gustafsson J-A 1985 Imprinting of growth hormone secretion, body growth, and hepatic steroid metabolism by neonatal testosterone.
Endocrinology 117: 1881-1889

Jonassen JA, Mullikin-Kilpatrick D, McAdam A, Leeman SE 1987 Thyroid hormone status regulates preprotachykinin-A gene expression in male rat anterior pituitary.
Endocrinology 121: 1555-1561

Jones PM, Ghatei MA, Steel J, O' Halloran DJ, Gon G, Legon S, Burrin JM, Leonhardt U, Polak JM, Bloom SR 1989 Evidence for neuropeptide Y synthesis in the rat anterior pituitary and the influence of thyroid hormone status: comparison with vasoactive intestinal peptide, substance P, and neurotensin. Endocrinology 125: 334-341

Jones PM, Burrin JM, Ghatei MA, O' Halloran DJ, Legon S, Bloom SR 1990 The influence of thyroid hormone status on the hypothalamo-hypophyseal growth hormone axis. Endocrinology (in press).

Kalra SP, Crowley WR 1984 Norepinephrine-like effects of neuropeptide Y on LH release in the rat. Life Sci. 35: 1173-1176

Kaplan LM, Gabriel SM, Koenig JI, Sunday ME, Spindel ER, Martin JB, Chin WW 1988a Galanin is an estrogen-inducible, secretory product of the rat anterior pituitary. Proc. Natl. Acad. Sci. USA 85: 7408-7412

Kaplan LM, Spindel ER, Isselbacher KJ, Chin WW 1988b Tissue-specific expression of the rat galanin gene. Proc. Natl. Acad. Sci. USA 85: 1065-1069

Kasper S, Worsley IG, Rowe JM, Shiu RPC, Friesen HG 1982 Chondrocyte growth factor from the human pituitary gland. J. Biol. Chem. 257: 5226-5230

Katakami H, Downs TR, Frohman LA 1986 Decreased hypothalamic growth hormone-releasing hormone content and pituitary responsiveness in hypothyroidism. J. Clin. Invest. 77: 1704-1711

Kato Y, Chihara K, Ohgo S, Iwasaki Y, Abe H, Imura H 1976 Growth hormone and prolactin release by substance P in rats. Life Sci. 19: 441-446

Kato Y, Shimatsu A, Matsushita N, Ohta H, Imura H 1984 Role of vasoactive intestinal peptide (VIP) in regulating the pituitary function in man. Peptides 5: 389-394

Kato Y, Iwasaki Y, Iwasaki J, Abe H, Yanihara N, Imura H 1978 Prolactin release by vasoactive intestinal peptide in rats. Endocrinology 103: 554-558

Kerdelhue B, Tartar A, Lenoir V, El Abed A, Hublau P, Milllar RP 1985 Binding studies of substance P anterior pituitary binding sites: changes in substance P binding sites during the rat estrous cycle. Regul. Pept. 10: 133-143

Kerkerian L, Guy J, Lefevre G, Pelletier G 1985 Effects of neuropeptide Y (NPY) on the release of anterior pituitary hormones in the rat. Peptides 6: 1201-1204

Kislauskis E, Bullock B, McNeil S, Dobner PR 1988 The rat gene encoding neurotensin and neuromedin N.
J. Biol. Chem. 263: 4963-4968

Kobrin MS, Asa SL, Samsoondar J, Kudlow JE 1987
Alpha-transforming growth factor in the bovine anterior pituitary gland: secretion by dispersed cells and immunohistochemical localisation.
Endocrinology 121: 1412-1416
Koenig RJ, Brent GA, Warne RL, Larsen PR, Moore DM 1987
Thyroid hormone receptor binds to a site in the rat growth hormone promoter required for induction by thyroid hormone. Proc. Natl. Acad. Sci. USA 84: 5670-5674

Korchak DM, Nilaver G, Beinfeild MC 1984 The development of motilin-like immunoreactivity in the rat cerebellum and pituitary as defined by radioimmunoassay.
Neurosci. Lett. 48: 267-272

Kirkouli SE, Glenn Stanley B, Leibowitz SF 1986 Galanin: stimulation of feeding induced by medial hypothalamic injection of this novel peptide.
Eur. J. Pharmacol. 122: 159-160

Krause JE, Chirgwin JM, Carter MS, Xu ZS, Hershey AD 1987
Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A.
Proc. Natl. Acad. Sci. USA 84: 881-884

Krug MS, Berger SL 1987 First-strand cDNA synthsesis primed with oligo(dT). In: Methods in Enzymology Volume 152, Guide to Molecular Cloning Techniques. Berger SL and Kimmel AR Eds.

Kudlow JE, Kobrin MS 1984 Secretion of epidermal growth factor-like mitogens by cultured cells from bovine anterior pituitary glands. Endocrinology 115: 911-917

Labrie F, DeLean A, Lagrace L 1978 Interactions of TRH, LHRH and somatostatin in the anterior pituitary gland.
Receptors and hormone action Vol 3
Bimbanmer L, O' Malley BW (eds)
London, Academic press

Lalloz MRA, Detta A, Clayton RN 1988
Gonadotropin-releasing hormone desensitisation preferentially inhibits expression of the luteinizing hormone beta-subunit gene in vivo.
Endocrinology 122: 1689-1694

Lam KSL, Lechan RM, Minamitani N, Segerson TP and Reichlin S 1989 Vasoactive intestinal peptide in the anterior pituitary is increased in hypothyroidism.
Endocrinology 124: 1077-1084

Larhammar D, Ericsson A, Persson H 1987 Structure and expression of the rat NPY gene.
Proc. Natl. Acad. Sci. USA 84: 2068-2071

Larsson LI, Fahrenkrug J, Schaffaklitgsky de Muckadell OB 1977 Occurrence of nerves containing vasoactive intestinal polypeptide immunoreactivity in the male genital tract. Life Sci. 21: 503-508

Leeman SE, Hammerschlag R 1967 Stimulation of salivary secretion by a factor extracted from hypothalamic tissue. Endocrinology 81: 803-807

Leitman DC, Murad F 1987 Atrial natriuretic factor receptor heterogeneity and stimulation of particulate guanylate cyclase and cyclic GMP accumulation. Endocrinol. Metab. Clin. North America 16: 79-105

Li CH 1972 Hormones of the adenohypophysis. Proceedings of the American Philososphical Society 116: 365-382

Linder S, Barkhem T, Norberg A, Persson H, Schalling M, Hokfelt T, Magnusson G 1987 Structure and expression of the gene encoding the vasoactive intestinal peptide precursor. Proc. Natl. Acad. Sci. USA 84: 605-611

Liposits Zs, Sievers L, Paull WK 1988 Neuropeptide-Y and ACTH-immunoreactive innervation of corticotropin releasing factor (CRF)-synthesising neurons in the hypothalamus of the rat. Histochemistry 88: 227-234

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

LLoyd RV 1983 Estrogen-induced hyperplasia and neoplasia in the rat anterior pituitary gland. Am. J. Path. 113: 198-206

Loh YP, Castro MG, Zeng FJ, Patel-Vaidya 1988 Presence of pro-vasopressin mRNA, neurophysin and arginine vasopressin in mouse anterior pituitary cells and the AtT-20 corticotrophic tumour cell line. J. Mol. Endocrinol 1: 39-48

Lorenzen JR, Schlepphorst C, Schwartz NB 1980 The interaction of castration and adrenalectomy on pituitary responses to loss of target gland negative feedback in the male rat. Endocrinology 106: 592-599

Lundberg JM, Terenius L, Hokfelt T, Tatemoto K, 1984 Comparative immunohistochemical and biochemical analysis of pancreatic polypeptide-like peptides with special reference to presence of Neuropeptide Y in central and peripheral neurons. J. Neurosci. 4: 2376-2386

Maletti M, Rostene (Rotszstejn) WH, Carr L, Scherrer H, Rotten D, Kordon C, Rosselin G 1982 Interaction between estradiol and prolactin on vasocative intestinal peptide concentrations in the hypothalamus and in the anterior pituitary of the female rat. Neurosci. Lett. 32: 307-309

Maniatis T, Fritsch EF, Sambrook J 1982 Molecular cloning: A laboratory manual. Cold Spring Harbour Laboratories, Cold Spring Harbour, New York.

Martin D, Epelbaum J, Bluet-Pajot M-T, Prelot M, Kordon C Durand D 1985 Thyroidectomy abolishes pulsatile growth hormone secretion without affecting hypothalamic somatostatin. Neuroendocrinology 41: 476-481

Martinoli MG, Pelletier G 1989 Thyroid and glucocorticoid hormone regulation of rat pituitary growth hormone messenger ribonucleic acid as revealed by in situ hybridisation. Endocrinology 125: 1246-1252

Matsumura M, Yamanoi A, Yamamoto S, Mori H, Saito S 1985 In vivo and in vitro effects of cholecystokinin octapeptide on the release of prolactin in rats. Horm. Metab. Res. 17: 293-297

Matsuo H, Baba Y, Nair RMG et al 1971 Structure of the porcine LH and FSH-releasing hormone. I. The proposed amino acid sequence. Biochem. Biophys. Res. Comm. 43: 1334-1339

Maurer AR, Gorski J 1977 Effects of estradiol-17b and pimozide on prolactin synthesis in male and female rats. Endocrinology 101: 76-84

McCann SM, Porter JC 1969 Hypothalamic stimulating and inhibiting hormones. Physiol. Rev. 49: 240-279

McDonald JK, Lumpkin MD, Samson WK, McCann S 1985 Neuropeptide Y affects secretion of luteinising hormone and growth hormone in ovariectomized rats. Proc. Natl. Acad. Sci. USA 82: 561-564

McGregor GP, Bloom SR 1983 Radioimmunoassay of substance P and its stability in tissue. Life Sci. 32: 655-659

McKenzie JC, Tanaka I, Misono KS, Ingami T 1985 Immunocytochemical localisation of atrial natriuretic factor in the kidney, adrenal medulla, pituitary and atrium of rat. J. Histochem. Cytochem. 33: 828-832

Melander T, Fuxe K, Harfstrand A, Eneroth P, Hokfelt T 1987 Effects of intraventricular injections of galanin on neuroendocrine functions in the male rat. Possible involvement of hypothalamic catecholamine neuronal systems. Acta. Physiol. Scand. 131: 25-32

Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR 1984 Efficient in vitro synthesis of biologically active RNA and RNA hybridisation probes from plasmids containing a bacteriophage SP6 promoter. Nuc. Acids Res. 12: 7053-7056

Memo M, Castelletti L, Valerio A, Missale C, Spano PF 1986 Identification of neurotensin receptors associated with calcium channels and prolactin release in rat pituitary. J. Neurochem. 47: 1682-1688

Mercer JFB, Wake SA 1985 An analysis of the rate of metallothionein mRNA poly(A) shortening using RNA blot hybridisation. Nuc. Acids Res. 13: 7929-7943

Meunier H, Rivier C, Evans RM, Vale W 1988 Gonadal and extragonadal expression of inhibin alpha, beta A and beta B subunits in various tissues predicts diverse functions. Proc. Natl. Acad. Sci. USA 85: 247-251

Mezey E, Palkovits M, de Kloet ER 1978 Evidence for pituitary-brain transport of a behaviorally potent ACTH analog. Life Sci. 22: 831-838

Mierendorf RC, Pfeffer D 1987 Direct sequencing of denatured plasmid DNA. In: Berger SL, Kimmel AR (eds) Methods in Enzymology: Guide to Molecular Cloning Techniques. Academic Press, New York, vol 152, chapter 58

Mirell CJ, Yanagisawa M, Lau R, Pekary AE, Chin WW, Hershman JM 1987 Influence of thyroidal status on pituitary content of thyrotropin beta- and alpha-subunit, growth hormone, and prolactin messenger nucleic acids. Molecular Endocrinology 1: 408-412

Mitchell SJ, Bloom SR 1978 Measurement of fasting and postprandial plasma VIP in man. Gut 19: 1043-1047

Morel G, Hemming F, Tonon MC, Vaudry H, Dubois MP, Coy D, Dubois PM 1982a Ultrastructural evidence for corticotropin-releasing factor (CRF)-like immunoreactivity in the rat pituitary gland. Biol. Cell. 44: 89-92

Morel G, Chayvialle JA, Kerdelhue B, Dubois PM 1982b Ultrastructural evidence for endogenous substance P like immunoreactivity in the rat pituitary gland. Neuroendocrinology 35: 86-92

Morel G, Besson J, Rosselin G, Dubois PM 1982c Ultrastructural evidence for endogenous vasoactive intestinal peptide-like immunoreactivity in the pituitary gland. Neuroendocrinology 34: 85-89

Morel G, Mesguich P, Dubois MP, Dubois PM 1983 ULtrastructural evidence for enogenous somatostatin-like immunoreactivity in the pituitary gland. Neuroendocrinology 36: 291-299

Morel G, Mesguich P, Dubois MP, Dubois PM 1984 Ultrastructural evidence for endogenous growth hormone-releasing factor (GRF)-like immunoreactivity in the the monkey pituitary gland. Neuroendocrinology 38: 123-133

Morel G, Gourdji D, Grouselle D, Brunet N, Tixier-Vidal A, Dubois PM 1985 Immunocytochemical evidence for in vivo internalisation of thyroliberin into rat pituitary target cells. Neuroendocrinology 41: 312-320

Morel G, Chabot JG, Dubois PM 1988 Ultrastructural evidence for oxytocin in the rat anterior pituitary gland. Acta Endocrinologica 117: 307-314

Morel G, Chabot J-G, Gossard F, Heisler S 1989 Is atrial natriuretic peptide synthesised and internalised by gonadotrophs ? Endocrinology 124: 1703-1710

Meunier H, Rivier C, Evans RM, Vale W 1988 Gonadal and extragonadal expression of inhibin alpha, betaA and betaB subunits in various tissues predicts diverse functions. Proc. Natl. Acad. Sci. USA 85: 247-251

Muraki K, Okahata H, Nishi Y, Usui T, Yamada H, Fujita S, Miyachi Y, Yanaihara N, Yajima H 1985 Distribution of neurotensin-like immunoreactivity in the hypothalamus, pituitary gland, and gastro-intestinal tract of rats. Acta Endocrinologica 110: 1-5

Murakami Y, Kato Y, Koshiyama H, Inoue T, Yanaihara N, Imura H 1987 Galanin stimulates growth hormone (GH) secretion via GH-releasing factor (GRF) in conscious rats. Eur. J. Pharmacol. 136: 415-418

Muschel R, Khoury G, Reid LM 1986 Regulation of insulin mRNA abundance and adenylation: dependance on hormones and matrix substrata. Mol. Cell. Biol. 6: 337-341

Nagata M, Mizunaga A, Ema S, Yoshimura F 1980 Various types of the pituitary folliculo-stellate cells involving the Siperstein's corticotroph in the normal rats. Endocronologica Japonica 27: 13-22

Nagy G, Mulchahey JJ, Neill JD 1988 Autocrine control of prolactin secretion by vasoactive intestinal peptide. Endocrinology 122: 364-366

Nair RMG, Barett JF, Bowers CY, Schally AV 1970 Structure of porcine thyrotropin releasing hormone. Biochemistry 9: 1103-1106

Nakane PK 1970 Classification of pituitary cell types by with immunoenzyme histochemistry. J. Histochem. Cytochem. 18: 9-20

Naruse M, Naruse K, McKenzie JC, Schelling P, Ingami T 1985 Regional distribution of renin and angiotensinogen in the brain of normotensive (WKY) and spontaneously hypertensive (SHR) rats. Brain Research 333: 147-150

Nikitovitch-Winer MB, Atkin J, Maley BE 1987 Colocalisation of prolactin and growth hormone within specific adenohypophyseal cells in male, female, and lactating female rats. Endocrinology 121: 625-630

Nilsson J, von Euler AM, Dalsgaard C-J 1985 Stimulation of connective tissue cell growth by substance P and substance K. Nature 315: 61-63

Nogami H, Yoshimura F 1982 Fine structural criteria of prolactin cells identified immunohistochemically in the male rat. Anatomical records 202: 261-274

Noguchi T, Sugisaki T, Kanamatsu T, Satoh I, Nishikawa N 1988 Reduced vasoactive intestinal polypeptide immunoreactivity in the pituitaries of hormone-deficient mutant mice. J. Endocrinol 118: 179-185

Oliva D, Nicosia S, Spada A, Giannattasio G 1982 VIP stimulates ACTH release and adenylate cyclase in human ACTH-secreting pituitary adenomas. Eur. J. Pharmacol. 83: 101-105

Oliver C, Mical RS, Porter JC 1977 Hypothalamic-pituitary vasculature: Evidence for retrograde blood flow in the pituitary stalk. Endocrinology 101: 598-604

Ottlecz A, Samson WK, McCann SM 1986 Galanin: evidence for a hypothalamic site of action to release growth hormone. Peptides 7: 51-53

Ottlecz A, Snyder GD, McCann SM 1988 Regulatory role of galanin in control of hypothalamic-anterior pituitary function. Proc. Natl. Acad. Sci. USA 85: 9861-9865

Ottesen B, Anderson AN, Gerstenbeurg T, Ulrichsen H, Manthorpe T, Fahrenkrug J 1981 VIP stimulates prolatin release in women. Lancet ii: 696

O' Donohue TL, Beinfeld MC, Chey WY, Chang TM, Nilaver G, Zimmerman EA, Yajima, H, Adachi H, Poth M, McDevitt RP, Jacobowitz DM 1981a Identification, charaacterisation and distribution of motilin immunoreactivity in the rat central nervous system. Peptides 2: 467-477

O' Donohue TL, Charlton CG, Miller RL, Boden G, Jacobowitz DM 1981b Identification, characterisation, and distribution of secretin immunoreactivity in rat and pig brain. Proc. Natl. Acad. Sci. USA 78: 5221-5224

O' Shaughnessy DJ 1982 Somatostatin In: Bloom SR and Long RG (eds.) Radioimmunoassay of gut regulatory peptides; chapter 18. WB Saunders Co Ltd, Eastborne, Sussex, UK

Paek I and Axel R 1987 Glucocoricoids enhance stability of human growth hormone mRNA. Mol. Cell. Biol. 7: 1496-1501

Pagesy P, Li JY, Rentier-Delrue F, Le Bouc Y, Martial JA, Peillon F 1989 Evidence of pre-prosomatostatin mRNA in human normal and tumoral anterior pituitary gland. Molecular Endocrinology 3: 1289-1294

Palatnik CM, Wilkins C, Jacobson A 1984 Translational control during early dictyostelium development: possible involvement of poly(A) sequences. Cell 36: 1017-1025

Pascual-Leone AM, Besa E, Hervas F, Escriva F, Alvarez C 1983 Effects of L-thyroxine treatment on pituitary GH content of adult rats with neonatal thyrotoxicosis. Acta Endocronologica 104: 340-344

Peake GT, Birge CA, Daughaday WH 1973 Alterations of radioimmunoassayable growth hormone and prolactin during hypothyroidism. Endocrinology 92: 487-493

Pearse AGE, Polak JM 1975 Immunocytochemical localisation of substance P in mammalian intestine. Histochemistry 43: 97-108

Pernow B 1983 Substance P Pharmacological Reviews 35: 85-141

Plotsky PM, Vale W 1984 Hemorrhage-induced secretion of corticotropin-releasing factor-like immunoreactivity into the rat hypophyseal portal circulation and its inhibition by glucocorticoids. Endocrinology 114: 164-196

Plotsky PM, Vale W 1985 Patterns of growth hormone-releasing factor and somatostatin secretion into the hypophysial-portal circulation of the rat. Science 230: 461-463

Poppa GT, Fielding U 1930 A portal circulation from the pituitary to the hypothalamic region. Journal of Anatomy 65: 88-91

Prysor-Jones RA, Silverlight J, Kennedy SJ, Jenkins JS 1988 Vasoactive intestinal peptide and the stimulation of lactotroph growth by oestradiol in rats. J. Endocrinol. 116: 259-265

Prysor-Jones RA, Silverlight J, Jenkins JS 1989 Oestradiol, vasoactive intestinal peptide and fibroblast growth factor in the growth of human pituitary tumour cells in vitro. J. Endocrinol. 120: 171-177

Powell CT, Ney C, Agarwal K 1985 A gastrin gene is expressed in both porcine pituitary and mucosal tissues. Nuc. Acids Res. 13: 7299-7305

Ramasharma K, Li CH 1986 Human seminal alpha-inhibins: detection in human pituitary, hypothalamus, and serum by immunoreactivity. Proc. Natl. Acad. Sci. USA 83: 3484-3486

Rehfeld JF 1978a Immunocytochemical studies on cholecystokinin. II. Distribution and molecular molecular heterogeneity in the central nervous system of man and hog. J. Biol. Chem. 253: 4022-4030.

Rehfeld JF 1978b Localisation of gastrins to neuro- and adenohypophysis. Nature 271: 771-773

Rehfeld JF 1987 Preprocholecystokinin processing in the normal human anterior pituitary.
Proc. Natl. Acad. Sci. USA 84: 3019-3023

Reichlin S 1988 Neuroendocrine significance of vasoactive intestinal polypeptide. Annals of the New York Academy of Sciences 527: 431-449

Reisine TD, Heisler S, Hook VYH, Axelrod J 1982 Multireceptor-induced release of adrenocorticotropin from anterior pituitary tumor cells.
Biochem. Biophys. Res. Comm. 108: 1251-1257

Rettori V, Milenkovic L, Fahim AM, Polak J, Bloom SR, McCann SM 1989 Role of neuromedin B in the control of the release of thyrotropin in the rat.
Proc. Natl. Acad. Sci. USA 86: 4789-4792

Rivier J, Spiess J, Thorner M, Vale W 1982 Characterisation of a growth hormone releasing factor from a human pancreatic islet tumor. Nature 300: 276-278

Robberecht P, Coy DH, De Neef P, Camus JC, Cauvin A, Waelbroeck M, Christophe J 1986 Interaction of vasoactive intestinal peptide (VIP) and N-terminally modified VIP analogs with rat pancreatic, hepatic and pituitary membranes. Eur. J. Biochem. 159: 45-49

Robinson BG, Frim DM, Schwartz WJ, Majzoub JA 1988 Vasopressin mRNA in the superchiasmatic nuclei: daily regulation of polyadenylate tail length.
Science 241: 342-344

Rodriguez-Sierra JF, Jacobowitz DM, Blake CA 1987 Effects of neuropeptide Y on LH, FSH and TSH release in male rats. Peptides 8: 539-542

Rockaeus A 1987 Galanin: a newly isolated biologically active neuropeptide.
Trends in Neurological Sciences 10: 158-164

Role LW, Leeman SE, Perlman RL 1981 Somatostatin and substance P inhibit catecholamine secretion from isolated cells of guinea pig medulla. Neuroscience 6: 1813-1820

Romano MI, Machiavelli GA, Perez RL, Carricarte V, Burdman JA 1984 Correlation between LH secretion in castrated rats with cellular proliferation and synthesis of DNA in the anterior pituitary gland. J. Endocrinol. 102: 13-18

Root JL, Duckett GE, Sweetland M, Strzelecki JA, Root AW 1985 Hypothyroidism blunts the growth hormone (GH)-releasing effect of human pancreatic GH releasing factor in the adult male rat in vivo and in vitro. Endocrinology 116: 1703-1706

Rosenthal ET, Tansey TR, Ruderman JV 1983 Sequence specific adenylations and deadenylations accompany changes in the translation of maternal messenger RNA after fertilisation of spisula oocytes. J. Mol. Biol. 166: 309-327

Rotsztejn WH, Besson J, Briaud B, Gagnant L, Rosselin G, Kordon C 1980a Effect of steroids on vasoactive intestinal peptide in discrete brain regions and peripheral tissues. Neuroendocrinology 31: 287-291

Rotsztejn WH, Benoist L, Besson J, Beraud G, Bluet-Pajot MT, Kordon C, Rosselin G, Duval J 1980b Effect of vasoactive intestinal peptide (VIP) on the release of adenohypophyseal hormones from purified cells obtained by unit gravity sedimentation. Neuroendocrinology 31: 282-286

Rotsztejn WH, Dussaillant M, Nobou F, Rosselin G 1981 Rapid glucocorticoid inhibition of vasoactive intestinal peptide-induced cyclic AMP accumulation and prolactin release in rat pituitary cells in culture. Proc. Natl. Acad. Sci. USA 78: 7584-7588

Sahu A, Crowley WR, Tatemoto K, Balasubramaniam A, Kalra SP 1987 Effects of neuropeptide Y, NPY analog (norleucine-4-NPY), galanin and neuropeptide K on LH release in ovariectomised (OVX), and OVX estrogen, progesterone-treated rats. Peptides 8: 921-926

Sahu A, Kalra SP, Crowley WR, Kalra PS 1989 Testosterone raises neuropeptide-Y concentration in selected hypothalamic sites and in vitro release from the medial basal hypothalamus of castrated male rats. Endocrinology 124: 410-414

Said SI 1984 Vasoactive intestinal polpeptide (VIP): current status. Peptides 15: 143-150

Sakai T, Inoue K, Hasegawa Y, Kurosumi K 1988 Effect of passive immunisation to gonadotropin-releasing hormone (GnRH) using GnRH antiserum on the mitotic activity of gonadotrophs in castrated male rats. Endocrinology 122: 2803-2808

Saiki R, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N 1985 Enymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1356

Saint-Andre JP, Rohmer V, Alhenc-Gelas F, Menard J, Bigorne J-C, Corvol P 1986 Presence of renin, angiotensinogen, and converting enzyme in human pituitary lactotroph cells and prolactin adenomas. J. Clin. Endocr. Metab. 63: 231-237

Samson WK, Said SI, McCann SM 1979 Radioimmunologic localisation of vasoactive intestinal peptide in hypothalamic and extrahypothalamic sites in the rat brain. Neurosci. Lett. 12: 265-269

Samson WK, Lumpkin MD, McCann SM 1984a Presence and possible action of secretin in the rat pituitary and hypothalamus. Life Sci 34: 155-163

Samson WK, Lumpkin MD, Nilaver G, McCann SM 1984b Motilin: a novel growth hormone releasing agent. Brain Research Bulletin 12: 57-62

Samson WK, Lumpkin MD, McCann SM 1986 Evidence for a physiological role for oxytocin in the control of prolactin. Endocrinology 119: 554-560

Samuels MH, Wierman ME, Wang C, Ridgway EC 1989 The effect of altered thyroid status on pituitary hormone messenger ribonucleic acid concentrations in the rat. Endocrinology 124: 2277-2282

Sanger F, Nicklen S, Coulson AR, 1977 DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5468

Sato S 1980 Postnatal development, sexual difference and sexual cyclic variation of prolactin cells in rats: special reference to the topographic affinity to a gonadotroph. Endocronologica Japonica 27: 573-583

Seethalakshmi L, Steinberger A, Steinberger E 1984 Pituitary binding of 3H labelled Sertoli cell factor in vitro: a potential radioreceptor assay for inhibin. Endocrinology 115: 1289-1294

Seifert H, Perrin M, Rivier J, Vale W 1985 Binding sites for growth hormone releasing factor on rat anterior pituitary cells. Nature 313: 487-489

Seifert H, Perrin M, Rivier J, Vale W 1985 Growth hormone-releasing factor binding sites in rat anterior pituitary membrane homogenates: modulation by glucocorticoids. Endocrinology 117: 424-426

Seo H, Martino E, Refetoff S, Vassart G 1979 The differential stimulatory effect of thyroid hormone and estrogen on growth hormone and prolactin is due to induction of specific mRNAs. Endocrinology 104: 1083-1090

Silvestre RA, Miralles P, Monge L, Moreno P, Villanueva ML, Marco J 1987a Effects of galanin on hormone secretion from the in situ perfused rat pancreas and on glucose production in rat hepatocytes in vitro. Endocrinology 121: 378-383

Silvestre RA, Miralles P, Monge L, Villanueva ML, Marco J 1987b Inhibitory effect of galanin on pancreatic polypeptide release by the perfused rat pancreas. Life Sci. 40: 1829-1833

Simard J, Hubert J-F, Hosseinzadeh T, Labrie F 1986 Stimulation of growth hormone release and synthesis by estrogens in rat anterior pituitary cells in culture. Endocrinology 119: 2004-2011

Shah GV, Epand RM, Orlowski RC 1988 Calcitonin inhibition of prolactin secretion in isolated rat pituitary cells. J. Endocrinol. 116: 279-286

Sheward WJ, Harmar AJ, Fraser HM, Fink G 1983 TRH in rat pituitary stalk blood and hypothalamus: studies with high performance liquid chromatography. Endocrinology 113: 1865-1869

Shimatsu A, Kato Y, Matsushita N, Katakami H, Yanaihara N, Imura H 1981 Immunoreactive vasoactive intestinal polypeptide in rat hypophyssial portal blood. Endocrinology 108: 395-398

Skofitsch G, Jacobowitz DM 1985 Quantitative distribution of calcitonin gene-related peptide in the rat central nervous system. Peptides 6: 1069-1073

Skofitsch G, Jacobowitz DM 1986 Quantitative distribution of galanin-like immunoreactivity in the rat central nevous system. Peptides 7: 609-615

Smith PE 1930 Hypophysectomy and replacement therapy in the rat. Am. J. Anat. 45: 205-272

Smith PE and Smith IP 1922 The repair and activation of the thyroid in the hypophysectomised tadpole by the parenteral administration of fresh anterior lobe of the bovine hypophysis. J. Med. Res. 43: 267-283

Smith PF, Frawley LS, Neill JD 1984 Detection of LH release from individual pituitary cells by the reverse hemolytic plaque assay: Estrogen increases the fraction of gonadotropes responding to GnRH. Endocrinology 115: 2484-2486

Smith S 1980 Role of prolactin in regulating gonadotropin secretion and gonad function in female rats. Federation Proceedings 39: 2571-2576

Southern EM 1975 Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Spira O, Birkenfeld A, Gross J, Gordon A 1979 TSH synthesis and release in the thyroidectomised rat. Acta Endocrinologica 92: 489-501

Sporn MB, Todaro GJ 1980 Autocrine secretion and malignant transformation of cells. New Eng. J. Med. 303: 878-880

Srikant CB, Patel YC 1981 Somatostatin receptors: identification and characterisation in rat membranes. Proc. Natl. Acad. Sci. USA 78: 3930-3934

Steele MK, Brownfield MS, Ganong WF 1982 Immunocytochemical localization of angiotensin immunoreactivity in gonadotrops and lactotrops of the rat anterior pituitary gland. Neuroendocrinology 35: 155-158

Steel JH, Van Noorden S, Ballesta J, Gibson SJ, Ghatei MA, Burrin J, Leonhart U, Domin J, Bloom SR, Polak JM 1988 Localization of 7B2, neuromedin B and neuromedin U in specific cell types of rat, mouse and human pituitary, in rat hypothalamus, and in 30 human pituitary and extra pituitary tumors. Endocrinology 122: 270-278

Steel JH, Gon G, O' Halloran DJ, Jones PM, Yanaihara N, Ishikawa H, Bloom SR, Polak JM 1989 Galanin and vasoactive intestinal polypeptide are colocalised with classical pituitary hormones and show plasticity of expression. Histochemistry 93: 183-189

Steiner RA, Bremner WJ, Clifton DK 1982 Regulation of luteinizing hormone pulse frequency and amplitude by testosterone in the adult male rat. Endocrinology 111: 2055-2061

Stricker P, Grueter F 1929 Action du lobe anterieur de l' hypophyse sur la montee laiteuse. C. R. Soc. Biol. Paris 99: 1978-1980

Surks M, DeFesi CR 1977 Determination of the cell number of each cell type in the anterior pituitary of euthyroid and hypothyroid rats. Endocrinology 101: 946-958

Suter DE, Schwartz NB 1985 Effects of glucocorticoids on secretion of luteinising hormone and follicle stimulating hormone by female rat pituitary cells in vitro. Endocrinology 117: 849-854

Sutton SW, Toyama TT, Otto S, Plotsky PM 1988 Evidence that neuropeptide Y (NPY) released into the hypophysial-portal circulation participates in priming gonadotropes to the effects of gonadotropin releasing hormone (GnRH). Endocrinology 123: 1208-1210

Suzuki Y, McMater D, Lederis K, Rorstad OP, 1984 Characterisation of the relaxant effects of vasoactive intestinal peptide (VIP) and PHI on isolated brain arteries. Brain Research 322: 9-16

Tannenbaum GS, Martin JB 1976 Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. Endocrinology 98: 562-568

Tatemoto K, Carlquist M, Mutt V 1982 Neuropeptide Y- a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296: 659-660

Tatemoto K, Rokaeus A, Fornvall H, McDonald TF, Mutt V 1983 Galanin - a novel biologically active peptide from porcine intestine. Feb. Lett. 164: 124-128

Tibolt RE, Childs GV 1985 Cytochemical and cytophysiological studies of gonadotropin-releasing hormone (GnRH) target cells in the male rat pituitary: differential effects of androgens and corticosterone on GnRH binding and gonadotropin release. Endocrinology 117: 396-404

Turkelson CM, Thomas CR, Arimura A, Chang D, Cang JK, Shimizu M 1982 In vitro potentiation of the activity of synthetic ovine corticotropin releasing factor by arginine vasopressin. Peptides 1: 111-115

Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J 1986 Purification and characterisation of an FSH releasing protein from porcine ovarian fluid. Nature 321: 776-779

Vale W, Vaughan J, Yamamoto G, Spiess J, Rivier J 1983 Effects of synthetic human pancreatic (tumor) GH releasing factor and somatostatin, triiodothyronine and dexamethasone on GH secretion in vitro. Endocrinology 112: 1553-1555

Vandesande F, Dierickx K 1975 Identification of the vasopressin producing and of the oxytocin producing neurons in the hypothalamic neurosecretory system of the rat. Cell. Tissue Res. 164: 153-162

Vijayan E, Samson WK, McCann SM 1978 Effects of intraventricular injection of gastrin on release of LH, prolactin, TSH and GH in conscious ovariectomised rats. Life Sci. 26: 321-327

Vijayan E, Samson WK, Said SI, McCann SM 1978 Vasoactive intestinal peptide: evidence for a hypothalamic site of action to release growth hormone, luteinising hormone and prolactin in conscious ovariectomised rats. Endocrinology 104: 53-57

Vijayan E, Carraway R, Leeman SE, McCann SM 1988 Use of antiserum to neurotensin reveals a physiological role for the peptide in rat prolactin release.
Proc. Natl. Acad. Sci. USA 85: 9866-9869

Vrontakis ME, Peden LM, Duckworth ML, Friesen HG 1987 Isolation and characterisation of a complementary DNA (Galanin) clone from estrogen-induced pituitary tumor messenger RNA. J. Biol. Chem. 35: 16755-16758

Wahlestedt C, Skagerberg G, Ekman R, Heilig M, Sundler F, Hakanson R 1987 Neuropeptide Y (NPY) in the area of the hypothalamic paraventricular nucleus activates the pituitary-adrenocortical axis in the rat.
Brain Research 417: 33-38

Wakabayashi I, Tonegawa Y, Ihara T, Hattori M, Shibasaki T, Ling N 1985 Plasma growth hormone response to human growth hormone-releasing factor in rats administered with chlorpromazine and antiserum against somatostatin.
Neuroendocrinology 41: 306-311

Watanabe YG, Daikoku S 1979 An immunohistochemical study on the cytogenesis of adenohypophysial cells in fetal rats. Developmental Biology 68: 557-567

Webb CB, Szabo M, Frohman LA 1983 Ectopic growth hormone-releasing factor and dibutyryl cyclic adenosine monophosphate-stimulated growth hormone release in vitro: effects of corticosterone and estradiol.
Endocrinology 113: 1191-1196

Wehrenberg WB, Baird A, Ying SY, Ling N 1985 The effects of testosterone and estrogen on the pituitary growth hormone response to growth hormone-releasing factor.
Biol. Reprod. 32: 369-371

Wehrenberg WB, Baird A, Ling N 1983 Potent interaction between glucocorticoids and growth hormone-releasing factor in vivo. Science 221: 556-561

Westendorf JM, Phillips MA, Schonbrunn A 1983 Vasoactive intestinal peptide stimulates hormone release from corticotrophic cells in culture.
Endocrinology 112: 550-557

Westendorf JM, Schonbrunn A 1985 Peptide specificity for stimulation of corticotropin secretion: activation of overlapping pathways by the vasoactive intestinal peptide family and corticotropin-releasing factor.
Endocrinology 116: 2528-2535

Westlund KN, Aguilera G, Childs GV 1985 Quantification of morphological changes in pituitary corticotropes produced by in vivo corticotropin-releasing factor stimulation and adrenalectomy. Endocrinology 116: 439-445

White MC, Adams EF, Loizou M, Mashiter K 1982 Vasoactive intestinal peptide stimulates adrenocorticotropin release from human corticotropinoma cells in culture: interaction with arginine vasopressin and hydrocortisone.
J. Clin. Endocrinol. Metab. 55: 967-972

Williams T, Maxon H, Thorner MO, Frohman LA 1985 Blunted growth hormone (GH) response to GH-releasing hormone in hypothyroidism resolves in the euthyroid state.
J. Clin. Endocrinol. Metab. 61: 454-456

Wong CC, Dohler K -D, von zur Muhlen 1980 Effects of tri-iodothyronine, thyroxine and isopropyl-di-iodothyronine on thyroid-stimulating hormone in serum and pituitary gland and on pituitary concentrations of prolactin, growth hormone, luteinising hormone and follicle stimulating hormone in hypothyroid rats. J. Endocrinol. 87: 255-263

Wynn PC, Aguilera G, Morell J, Catt KJ 1983 Properties and regulation of high-affinity pituitary receptors for corticotropin-releasing factor.
Biocem. Biophys. Res. Comm. 110: 602-608

Yamamoto N, Seo H, Suganama N, Matsui N, Nakane T, Kuwayama A, Kageyama N 1986 Effect of estrogen on prolactin mRNA in the rat pituitary.
Neuroendocrinology 42: 494-497

Yaffe BM, Samuels HH 1984 Hormonal regulation of the growth hormone gene: Relationship of the rate of transcription to the level of nuclear thyroid hormone-receptor complexes. J. Biol. Chem. 259: 6284-6291

Yoshimura F, Nogami H 1980 Immunohistochemical characterisation of pituitary stellate cells in rats.
Endocronologica Japonica 27: 43-51

Zeevi M, Nevins JR, Darnell JE 1982 Newly formed mRNA lacking polyadenylic acid enters the cytoplasm and the polyribosomes but has a shorter half-life in the absence of polyadenylic acid. Mol. Cell. Biol. 2: 517-525

Zondek B, Aschheim S 1927 Das hormon des hypophysenvorderlappens. Klin Wochenschr 6: 348-352

APPENDICES

A). Buffers, Solutions and Chemicals

1 M Tris pH 7.4, pH 7.6 and pH 8.0

121.1 g of Trizma base (Sigma) dissolved in 1 l of distilled water and titrated to required pH with concentrated HCl, filtered and autoclaved.

0.5 M EDTA pH 8.0

186.1 g of Ethylenediaminetetracetic acid - disodium salt (BDH) dissolved in 1 l of distilled water titrated to pH 8.0 with 10 n NaOH, filtered and autoclaved.

5 M NaCl

292.2 g of sodium chloride (BDH) dissolved in 1 l of distilled water, filtered and autoclaved.

1 M MgCl

203.3 g of magnesium chloride hexahydrate (BDH) dissolved in 1 l of distilled water, filtered and autoclaved.

2 M NaOAc pH 4.0 and pH 5.2

272.1 g of sodium acetate trihydrate (BDH) dissolved in 1 l of distilled water and adjusted to pH with glacial acetic acid, filtered and autoclaved.

20 % SDS

200 g of sodium dodecyl sulphate dissolved in 1 l of distilled water, used without further preparation.

20 X SSC

175.3 g of sodium chloride and 88.2 g of trisodium citrate (both BDH AnalaR grade) dissolved in 1 l of distilled water and brought to pH 7.0 by the addition of a few drops of 10 N NaOH and filtered, (= 3M Na Cl/0.3M Na citrate).

100 X TE

121.1 g of Trizma base and 3.7 g of ethylenediaminetetracetic acid dissolved in 1 l of distilled water (to give final concentrations of 1 M and 10 mM respectively) and adjusted to pH 7.0 with concentrated HCl, filtered and autoclaved.

TE

(a 1:100 dilution of 100 X TE in distilled water to give final concentrations of 10 mM Tris and 0.1 mM ethylenediaminetetracetic acid respectively)

0.5 M Sodium phosphate pH 6.6

Prepared by titrating 0.5 M disodium hydrogen orthophosphate with 0.5 M sodium dihydrogen orthophosphate until the desired pH was reached.

Luria-Bertani Medium (referred to as L broth or LB)

10g of Bacto-tryptone (Difco Laboratories, Detroit, Michigan, USA)
5g of Bacto-yeast extract (Difco Laboratories)
10g of Na Cl (BDH)
Dissolved in 1 l of water, adjusted to pH 7.5 with sodium hydroxide solution and autoclaved.

L-agar

15 g of Bacto-agar (Difco Laboratories, dissolved in 1 l of L broth and autoclaved to dissolve.

Phenol chrystalline solid, analar grade (BDH)

Prepared by dissolving crystals in distilled water until a saturated solution was obtained.

Formaldehyde solution (40% vol/vol BDH)

Chlorophorm (BDH)

Iso-propyl alcohol (propan-2-ol, BDH)

Formamide (BDH)

Denhart's solution

10 g each of Ficoll, polyvinylpirrolidone and bovine serum albumin dissolved in distiled water.

Sonicated, denatured herring sperm DNA (Sigma) 10 mg/ml in distilled water.

Dextran sulphate, analar grade (BDH)

Agarose (Sigma; Type II: medium EEO)

Kodak XAR5 diagnostic film (Eastman Kodak Company, Rochester, New York 14650, USA)

B). Agarose Gel Electrophoresis

i). Formaldehyde gels

Reagents

20 X MOPS solution:

0.4 M Morpholinopropanesulphonic acid (MOPS), pH7.0 (BDH). After dissolution the solution was titrated to pH 7.0 with 1N NaOH. For storage formaldehyde was added to a final concentration of 0.1% (vol/vol).

Method

One g of agarose was added to 87.5 ml of GDW in a 500 ml conical glass flask and boiled by heating in a microwave oven until all the agarose was observed to have dissolved. To this was then added 7.5 ml of 40% formaldehyde solution and 5 ml of 20 X MOPS in a fume hood. Whilst the agarose was being melted the gel casting plate was prepared. A strip of autoclave tape was placed around the edge of a 100 mm by 120 mm glass plate to completely enclose and seal it. The tape was sealed around the plate's edge and underside leaving a strip of tape approximately 10 mm high raised above the surface of the plate to form the gel mould. A bull dog clip was attached to each end of a gel comb to support the comb approximately 1 mm above the glass plate. The electrophoresis tank contained sufficient buffer (1 X MOPS) to completely cover the gel and samples were loaded by inserting the end off the tip into a well below the surface of the buffer and gently expelling the sample into the well. When the wells had been loaded the apparatus was connected to a power supply and the current turned on.

ii) Neutral Agarose Gels

Reagents

TBE 5 X stock solution

54 g of Tris base
27.5 g of boric acid
20 ml of 0.5 M EDTA
Dissolved in 1 l of distilled water to give final concentrations of 0.089 M, 0.089 M and 0.002 M respectively.

Method

Powdered agarose was added to 100 m 1 X TBE buffer and dissolved by heating in a microwave oven. Once all the crystals were observed to have dissolved 10 ul of a 10 mg/ml solution of ethidium bromide was added to the gel. A gel casting plate was prepared by taping around the edge of a 120 mm by 100 mm glass plate and fixing a comb in position over this in the same way as described for the preparation of formaldehyde gels. The molten agarose was poured into this and allowed to set at room temperature. Once set the gel was placed in an electrophoresis tank containing sufficient 1 X TBE buffer (plus 1 ug/ul ethidium bromide) to completely submerge the gel. After the wells were loaded the tank was connected to an electrophoresis power pack and the current was turned on. Initially the gel was run at 50 V until the dye was observed to have run out of the well and into the gel when the voltage was turned up to 100 V. The gel was run at this voltage until the marker dye was observed to have reached a desired stage at which point the DNA samples were visualised using a UV light source.

C. Publications

Jones PM, Burrin JM, Ghatei MA, O' Halloran DJ, Legon S, Bloom SR 1990 The influence of thyroid hormone status on the hypothalamo-hypophyseal growth hormone axis. Endocrinology (in press).

Jones PM, Ghatei MA, Steel J, O' Halloran DJ, Gon G, Legon S, Burrin JM, Leonhardt U, Polak JM, Bloom SR 1989 Evidence for neuropeptide Y synthesis in the rat anterior pituitary and the influence of thyroid hormone status: comparison with vasoactive intestinal peptide, substance P, and neurotensin. Endocrinology 125: 334-341

Steel JH, Gon G, O' Halloran DJ, Jones PM, Yanaihara N, Ishikawa H, Bloom SR, Polak JM 1989 Galanin and vasoactive intestinal polypeptide are colocalised with classical pituitary hormones and show plasticity of expression. Histochemistry 93: 183-189

Ferrier GJM, Pierson AM, Jones PM, Bloom SR, Girgis SI, Legon S 1989 Expression of the rat amylin (IAPP/DAP) gene. Journal of Molecular Endocrinology 3: R1-R4

Steel JH, Hamid Q, Van Noorden S, Jones PM, Denny P, Burrin J, Legon S, Bloom SR, Polak JM 1988 Combined use of in situ hybridisation and immunocytochemistry for the investigation of prolactin gene expression in immature, pubertal, pregnant, lactating and ovariectomised rats. Histochemistry 89: 75-80

Mulderry PK, Ghatei MA, Spokes RA, Jones PM, Pierson AM, Hamid Q, Kanse S, Amara SG, Burrin J, Legon S, Polak JM, Bloom SR 1988 Differential expression of alpha CGRP and beta CGRP by primary neurons of the rat. Neuroscience 25: 195-205

Gibson SJ, Polak JM, Giaid A, Hamid Q, Kar S, Jones PM, Denny P, Legon S, Amara S, Craig RK, Bloom SR, Penketh RJA, Rodek C, Ibrahim NBN, Dawson A 1988 Calcitonin gene related peptide messenger RNA is expressed in sensory neurones of the dorsal root ganglia and also in spinal motoneurones in man and rat. Neuroscience Letters 91: 283-288