THE CONTROL OF THE RELEASE OF SOME HYPOPHYSIAL PORTAL VESSEL PEPTIDES

by

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Dedicated

to My Parents

No amount of experimentation can ever prove me right; a single experiment can prove me wrong

Albert Einstein
(1879–1955)

Research is to see what everybody else has seen and to think what nobody else has thought.

Albert Szent-Györgyi
(1893–)

Statement in terms of Ph.D. regulation 2.4.15 of the post-graduate regulations of the University of Edinburgh, Scotland

I declare that this thesis was totally composed by myself, and that all the experimental work described herewith was performed by myself with the following exceptions:

i) quantification of pituitary LHRH binding sites and some radioimmunoassays to measure concentrations of plasma and pituitary luteinising hormone (LH) and plasma follicle stimulating hormone (FSH) for experiments in Chapter III were done by Dr. M.L. Aubert (Département de Pédriatrié et de Génétique, Université de Genève, Switzerland);

ii) transplantation of anterior pituitary glands under the kidney capsule, implantation of testosterone capsules and radioimmunoassays to measure plasma concentrations of LH, FSH and prolactin for experiments in Chapter IV were done by Dr. A.S. McNeilly (MRC Reproductive Biology Centre, Edinburgh, Scotland);

iii) the measurement of dopamine in plasma samples for experiments in Chapter IV was done with the assistance of Dr. V. Kapoor (Department of Medicine, Flinders Medical Centre, Adelaide, Australia);

iv) radioimmunoassays to determine plasma concentrations of cholecystokinin and gastrin (Chapter VI) were done by Professor G.J. Dockray (Department of Physiology, Liverpool University, England);

v) radioimmunoassays to determine plasma concentrations of vasoactive intestinal polypeptide (Chapter VII) were done by Dr. W.H. Rotsztejn (INSERM U55, Service de Diabétologie et d'Etudes Radioimmunologiques des Hormones Protéiques, Hôpital Saint-Antoine, Paris, France).

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ABSTRACT OF THESIS: The Control of the Release of Some Hypophysial Portal Vessel Peptides

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The aim of this thesis was to investigate hypothalamic-pituitary control by measuring the release of neuropeptides into pituitary stalk blood. The neuropeptides measured were luteinizing hormone-releasing hormone (LHRH) and the 'gut peptides' cholecystokinin (CCK), gastrin and vasoactive intestinal polypeptide (VIP).

Studies in castrated rats showed that, (1) despite marked increases in pituitary gonadotrophin secretion and the number of LHRH receptors in the anterior pituitary gland, the amount of LHRH in stalk blood was similar to that in control rats; (2) the release of LHRH into stalk blood induced by electrical stimulation of the median eminence (ME) was significantly lower than in control rats; (3) administration of oestradiol, 5α-dihydrotestosterone or testosterone (T), suppressed the post-castration rise in plasma luteinizing hormone (LH) but had no effect on LHRH released into stalk blood or the increased number of LHRH receptors in the anterior pituitary gland.

Experiments using intact and castrated rats made hyperprolactinaemic by implanting two anterior pituitary glands under the kidney capsule showed that, (1) the suppression of gonadotrophin release in intact and castrated hyperprolactinaemic rats was not accompanied by a decrease in LHRH release into stalk blood; (2) electrical stimulation of the ME was as effective in hyperprolactinaemic rats as in control rats in increasing LHRH release into stalk blood; (3) implantation of T capsules into castrated hyperprolactinaemic rats suppressed gonadotrophin but not LHRH secretion.

Catechol oestrogens stimulated the release of LH in pre-pubertal male and female rats but suppressed LH release induced by pregnant mare serum gonadotrophin.

CCK and VIP, but not gastrin, were released in significantly higher concentrations into stalk blood than into peripheral blood of adult male rats. Electrical stimulation of several areas of the brain known to contain CCK, gastrin or VIP did not alter the release of these peptides. Removal of the major peripheral source of CCK and gastrin (the gastric antrum) or VIP (the entire gut), significantly lowered CCK and gastrin concentrations but did not reduce VIP release into stalk blood. VIP release into stalk blood at various times of the oestrous cycle under Althesin, Ketalar, Sagatal or urethane anaesthesia showed no clear-cut changes. Therefore, it is unlikely that CCK and gastrin are physiological hypothalamic-pituitary regulatory factors. The physiological significance of the higher amounts of VIP in stalk blood compared with peripheral blood remains to be determined.

CONTENTS

CHAPTER I

Introduction

CHAPTER I

The involvement of the hypothalamus in the control of LH and FSH release by the anterior pituitary was first postulated over 30 years ago (Sawyer, Markee and Everett, 1950; Harris, 1955). Extensive research led to the isolation and synthesis of the hypothalamic peptide, luteinising hormone-releasing hormone (LHRH), which is released into hypophysial portal vessel blood on stimulation of higher brain centres. Since one of the main aims of this thesis was to investigate the control of the release of LHRH, the evidence supporting the hypothesis that LHRH is the major determinant of gonadotrophin release and that the hypothalamic dopaminergic system and gonadal steroids exert important modulatory influences is outlined below. The recent evidence suggesting that other peptides (e.g. cholecystokinin) may also modulate gonadotrophin secretion is also presented. In addition, the mechanisms controlling prolactin secretion and the effects of prolactin on gonadotrophin release are discussed with relevance to this thesis.

1.1 Neurohumoural Regulation of the Adenohypophysis

1.1.1 The Neurohumoural Hypothesis

The current concept of the main mechanism controlling the release of hormones from the adenohypophysis is that substances liberated into the hypothalamo-hypophysial portal circulation from nerve terminals in the median eminence modulate adenohypophysial secretions. This 'neurohumoural hypothesis' has been reviewed in detail by Harris (1955, 1972), Guillemin (1967), Everett (1969) and McCann and Porter (1969). The early observations that ovulation in some female mammals (e.g. rabbits) occurred only after mating (Heape, 1905; Barry, 1939) and that environmental factors played a role in reproductive functions (Marshall, 1936, 1942), first lead to

the hypothesis that the activity of the adenohypophysis was stimulated by sensory stimuli acting through reflex pathways in the central nervous system. This hypothesis was supported by demonstrations that ovulation and pseudopregnancy could be induced in the rabbit by applying a large electrical stimulus to the whole head (Marshall and Verney, 1936) or to localised areas of the hypothalamus (Harris, 1937; Haterius and Derbyshire, 1937). However, the demonstration by Popa and Fielding (1930, 1933) of the existence, in the human, of a portal vessel system between the hypothalamus and the adenohypophysis started serious investigations into the suggestion that stimuli from the hypothalamus or neurohypophysis modulated adenohypophysial secretions by 'humoural transmission', by way of the portal vasculature (Hinsey and Markee, 1933; Friedgood, 1936; Harris, 1937; Haterius, 1937). The initial controversy regarding the direction of the blood flow in the hypophysial portal vessels was resolved by studies in the living toad (Houssay, Biasotti and Sammartino, 1935), rat (Green and Harris, 1949) and mouse (Worthington, 1955) which clearly indicated that blood flowed from the hypothalamus towards the adenohypophysis, thus supporting the neurohumoural hypothesis. In addition, the unique arrangement of the hypophysial portal vessels which permitted the transport of substance between the hypothalamus and adenohypophysis, in the absence of direct innervation of the adenohypophysis, was confirmed to occur in up to 76 species studies of animals (Wislocki and King, 1936; Barrnett and Greep, 1951; Green, 1951; Török, 1954; Daniel and Pritchard, 1975).

Harris (1950) was the first to conclusively demonstrate that if the pituitary stalk of female rats was sectioned and vascular regeneration prevented by the placement of a plate between the cut ends of the stalk,

the uteri and ovaries became atrophic and a state of anoestrus was induced. If regeneration of the portal vessels was permitted, normal oestrous cycles and pseudopregnancy could be established. The functional importance of the portal vessels in the control of reproductive function was also confirmed in other mammals (Benoit and Assenmacher, 1953; Donovan and Harris, 1954; Fortier, Harris and McDonald, 1957; Daniel and Pritchard, 1958). Further support for the neurohumoural hypothesis came from experiments by Harris and Jacobsohn (1952) who transplanted pituitary tissue under the median eminence or temporal lobe of hypophysectomised rats. Transplants at both sites became well-vascularised but reproductive fertility only remained in those rats with transplants under the median eminence, which were vascularised by the hypophysial portal vessels as opposed to the systemic blood vessels, which vascularised the transplants under the temporal lobe. These experiments were extended and confirmed by Nikitovich-Winer and Everett (1958). The demonstration that crude extracts of the median eminence stimulated luteinising hormone (LH) release, determined by the ovarian ascorbic acid depletion test (McCann, Taleisnik and Friedman, 1960; Smith, 1961), and induced ovulation in the rabbit (Campbell, Feuer and Harris, 1960) provided the most direct evidence in support of the neurohumoural hypothesis for the control of adenohypophysial secretions.

1.1.2 The Hypophysial-Portal Vasculature

Detailed descriptions of the hypophysial-portal vasculature have been published by Daniel (1966), Porter, Ondo and Cramer (1974), Bergland and Page (1978, 1979) and Flerkó (1980). Apart from the rabbit (Harris, 1947), the adenohypophysis receives no direct arterial supply; its entire afferent vascular supply is provided by the

hypophysial portal vessels. The arterial blood supply of the median eminence, the pituitary and the pituitary stalk is derived from hypophysial arteries which arise from the circle of Willis or directly from the internal carotid arteries and form a network in the external zone of the median eminence called the primary capillary plexus. From this plexus, the so-called long portal vessels arise, which lie on the surface of the pituitary stalk. Another primary capillary bed lies in the neurohypophysis in rats, and derives its blood supply mainly from the inferior hypophysial arteries and comprise the so-called short portal vessels. When the pituitary stalk is cut, the so-called long portal vessels are severed (Daniel, 1966). The resulting infarct has been reported to destroy up to 78% of the adenohypophysis in the rat (Adams, Daniel and Pritchard, 1963).

There is little mixing of the blood carried to the different parts of the adenohypophysis by the long and short portal vessels. The outflow from the sinusoids of the adenohypophysis is through small collecting veins at the periphery of the lobe, which open into one of the adjacent veins. Bergland and Page (1978, 1979) have suggested that some venous blood from the adenohypophysis returns by way of the short portal vessels to the neurohypophysis, and from there to the systemic circulation, as postulated by Török (1954). In addition, there have been suggestions that there is retrograde blood flow in the portal vessels in the pituitary stalk (Ambach, Palkovits and Szentágothai, 1976; Sétáló, Vigh, Schally, Arimura and Flerkó, 1976; Oliver, Mical and Porter, 1977; Porter, Barnea, Cramer and Parker, 1978).

Portal vessel blood samples were first collected in the dog using a temporal approach to the vessels, followed by hypophysectomy (Porter and Jones, 1956). Subsequently, a parapharengeal approach to the

vessels was developed by Porter and Smith (1967) in the rat, and portal vessel blood samples collected from hypophysectomised rats after placement of a cannula over the cut stalk which was connected to a pump to withdraw blood. Worthington (1966) described a method involving a transpharyngeal approach to the portal vessels in the rat, in which portal vessel blood samples were collected from the cut end of the pituitary stalk. Worthington's technique avoided the contamination of portal vessel blood samples with blood from the infundibular artery which would be damaged in the process of hypophysectomy. However, the blood collected using Worthington's method is a mixture of portal vessel blood, small amounts of cerebrospinal fluid and pituitary sinosoidal blood. The portal vessel blood samples collected in the experiments described in this thesis were collected using a modification of Worthington's method (Fink and Jamieson, 1976), therefore the portal blood collected will be referred to as 'pituitary stalk blood' and all data assessed in terms of concentration (amount/volume) and content (amount/unit time).

1.1.3 Hypothalamo-Hypophysial Stimulating and Inhibiting Substances

Substances that modulate the release of adenohypophysial hormones reach the gland by way of the hypophysial portal vessels (Section 1.1.1). For a substance to be considered as a physiological stimulator or inhibitor of adenohypophysial hormones, it should therefore

i) be located within diffusion distances of the primary capillary plexus of the portal vessels (these substances would therefore be extractable from the hypothalamus, stalk-median eminence tissue);

ii) be identified, characterised and present in higher concentrations in portal vessel blood than in systemic blood;

iii) be capable of altering the rate of release of at least one hormone from adenohypophysial cells, under several experimental and environmental situations that alter the concentration of the substance(s) in portal vessel blood.

Thyrotrophin releasing hormone (TRH), characterised in 1969 (Burgus, Dunn, Ward, Vale, Amos and Guillemin, 1969; Bøler, Enzmann, Folkers, Bowers and Schally, 1969), was the first hypothalamic-pituitary peptide to be sequenced. The structures of the other hypothalamo-hypophysiotropic hormones to be characterised to date are LH-releasing hormone (LHRH: Matsuo, Baba, Nair, Arimura and Schally, 1971; Burgus, Butcher, Amoss, Ling, Monahan, Rivier, Fellows, Blackwell, Vale and Guillemin, 1972), somatostatin (Brazeau, Vale, Burgus, Ling, Butcher, Rivier and Guillemin, 1973), corticotrophin-releasing hormone (CRH: Vale, Speiss, Rivier and Rivier, 1961) and growth hormone-releasing hormone (GRH): Guillemin, Brazeau, Bohlen, Esch, Ling and Wehrenberg, 1982; Rivier, Spiess, Thorner and Vale, 1982). The evidence supporting a physiological role for the monoamine, dopamine (DA), as the major prolactin inhibiting factor is discussed in Section 1.4.2.

1.1.4 Luteinising Hormone-Releasing Hormone (LHRH) as a Neurohumoural Substance

The isolation and characterisation of LHRH in the porcine and ovine hypothalamus was reported (Burgus *et al.*, 1971; Matsuo *et al.*, 1971) almost a decade after the first demonstration of LH-releasing activity in hypothalamic extracts of rats and domestic animals

(Schally, Kastin and Arimura, 1972). The decapeptide LHRH, (pyro) Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, stimulates the secretion of both LH and follicle-stimulating hormone (FSH: Schally, Arimura, Kastin, Baba, Matsuo, Redding, Nair, Debeljuk and White, 1971; Koch, Chobsieng, Zor, Friedman and Lindner, 1973; McCann, 1974). Administration of anti-LHRH serum to intact or castrated rats lowered plasma concentrations of both LH and FSH (Koch *et al.*, 1973; Arimura, Debeljuk and Schally, 1974; Kerdelhue, Catin and Jutisz, 1975), indicating the key role of LHRH in maintaining secretion of both LH and FSH. However, there is evidence that FSH release is inhibited by a substance distinct from LHRH (Bowers, Currie, Johansson and Folkers, 1973; Shin and Kraicer, 1974; Chappel and Barraclough, 1976). Although an FSH-inhibiting hormone has not yet been isolated, a gonadal peptide named 'Inhibin' has been found to selectively inhibit FSH secretion at the level of the pituitary (Morris and Jackson, 1978; Franchimont, Verstraelen-Proyard, Hazee-Huelstein, Renard, Demoulin-Bourguignon and Hustin, 1979; Lumpkin, Negro-Vilar, Franchimont and McCann, 1981).

The highest concentration of radioimmunoassayable LHRH in the rat central nervous system is in the median eminence, with lower concentrations in the arcuate and ventromedial nuclei (Palkovits, Arimura, Brownstein, Schally and Saavedra, 1974; Brownstein, Palkovits, Saavedra and Kizer, 1976). Nerve terminals containing immunoreactive LHRH have been identified in the external layer of the median eminence surrounding the portal vessels (Pelletier, Labrie, Puviani, Arimura and Schally, 1974; Goldsmith and Ganong, 1975).

The concentration of LHRH in portal vessel blood has been found to be higher than in systemic blood, measured by bioassay (Fink,

Nallar and Worthington, 1967; Fink and Harris, 1970; Porter, Goldman and Wilber, 1970) and radioimmunoassay methods (Fink and Jamieson, 1976). LHRH release into portal vessel blood was shown to increase during the spontaneous LH surge on pro-oestrus in adult rats (Sarkar, Chiappa, Fink and Sherwood, 1976; Ching, 1982) and during the LH surge induced by pregnant mare serum gonadotrophin injections into immature female rats (Sarkar and Fink, 1979). The LHRH surges could only be demonstrated if anaesthesia was induced using the steroid anaesthetic, Althesin, which did not block the LH surge on pro-oestrus or ovulation when administered during the critical period (Section 1.2.3) on pro-oestrus (Sarkar *et al.*, 1976). Earlier attempts by Fink and Jamieson (1976), using urethane, and by Eskay, Mical and Porter (1977), using pentobarbitone, probably failed to demonstrate the LHRH surge because both urethane and pentobarbitone modulate LH release and ovulation (Section 1.2.3). The release of LHRH into portal vessel blood has also been modulated by experimental procedures that alter the steroid environment and by electrical stimulation of specific brain regions. Electrical stimulation of the medial preoptic area (mPOA) increased LHRH release into portal vessel blood (Fink and Jamieson, 1976). The amount of LHRH released by stimulation of the mPOA could be varied with different current strengths. In addition, the LHRH response was highest during the early afternoon on pro-oestrus and lowest on dioestrus. Stimulation of the median eminence and supra-chiasmatic nucleus resulted in greater and less release of LHRH, respectively, while stimulation of the amygdala, dorsal hippocampus or ventral hippocampus had no effect on the release of LHRH into portal vessel blood (Chiappa, Fink and Sherwood, 1977). Ovariectomy on diestrus produced a decrease in LHRH concentration in portal vessel blood on pro-oestrus that

could be restored or increased by oestrogen or testosterone administration (Sherwood, Chiappa and Fink, 1976; Chiappa *et al.*, 1977). The pulsatile release of LH in long-term ovariectomised rats (Gay and Sheth, 1972; Gallo, 1980) has been suggested to be induced by fluctuating LHRH concentrations in portal vessel blood (Sarkar and Fink, 1980). This concept is supported by the demonstration that intravenous injections of antiserum to LHRH to ovariectomised rats inhibited LH pulsatile release (Snabes and Kelch, 1979). The diurnal LH surge induced by oestrogen administered to long-term ovariectomised rats (Caligaris, Astrada and Taleisnik, 1971; Henderson, Baker and Fink, 1977) has also been correlated to the LHRH concentration in portal vessel blood (Sarkar and Fink, 1980). Oestrogen also potentiated LHRH release induced by mPOA stimulation (Sherwood *et al.*, 1976). Progesterone, on the other hand, decreased the LHRH response to ovariectomy (Sherwood *et al.*, 1976; Sarkar and Fink, 1979).

From the studies described it is evident that LHRH satisfies all the criteria required to classify a substance as a hypothalamo-hypophysial releasing factor as defined by the neurohumoural hypothesis (Section 1.1.1).

1.1.5 Neurohumoural Role for Peptides Found in the Brain and Gut

During the last decade several peptides, such as cholecystokinin (CCK: Dockray, 1976; Rehfeld, 1978b; Larsson and Rehfeld, 1979), neurotensin (Carraway and Leeman, 1973; Uhl and Snyder, 1976; Kobayashi, Brown and Vale, 1977), somatostatin (Schally, Dupont, Arimura, Redding, Nishi, Linthicum and Schlesinger, 1976; Kobayashi *et al.*, 1977; Krisch, 1979), Substance P (Pearse and Polak, 1975; Kanazawa and Jessel, 1976; Lungdahl, Hökfelt and Nilsson, 1978) and vasoactive intestinal polypeptide (VIP: Said and Mutt, 1970; Larsson,

Fahrenkrug, Schaffalitsky de Muckadell, Sundler, Håkanson and Rehfeld, 1976), have been demonstrated in gastrointestinal endocrine cells and in neurones in the peripheral and central nervous systems. The biological significance of this dual distribution of these peptides is not yet clear. One explanation put forward has been that endocrine and nerve-cells share a common embryological (neuroectodermal) origin and have similar histochemical properties (Pearse, 1976). Some of these properties have been summarised in the acronym APUD (Amine Precursor Uptake and Decarboxylation: Pearse, 1976). However, studies by Fontain and Le Douarin (1977) involving transplantation of quail tissues into chick embryos suggested that gut and pancreatic endocrine cells are derived from the endoderm rather than the ectoderm, as postulated by Pearse (1976). The possibility that changes in the pattern of gene expression in different tissues may account for the distribution of peptides in neural and endocrine cells has been reviewed by Dockray (1979). There is accumulating evidence which suggests that the same peptide functions both as a neurotransmitter (defined as a substance released by nerve endings that acts at synaptic junctions in the vicinity of its site of release) as well as a neurohormone (defined as a substance released by nerve endings and transported in the blood stream to its site of action). The evidence supporting a role for CCK and VIP as a neurotransmitter and a neurohormone, has been reviewed by Emson, Hunt, Rehfeld and Fahrenkrug (1979); Dockray (1982); Fahrenkrug and Emson (1982); Rosselin, Maletti, Besson and Rotsztejn (1982).

Both CCK and VIP have been reported to influence pituitary hormone release (Ruberg, Rotsztejn, Arancibia, Besson and Enjalbert, 1978; Vijayan, Samson and McCann, 1979a; Vijayan, Samson, Said and

McCann, 1979b; Morley, 1981). The presence of CCK and VIP in the hypothalamus and median eminence (Loren, Alumets, Håkanson and Sundler, 1979b; Roberts, Woodhams, Bryant, Crow, Bloom and Polak, 1980; Sims, Hoffman, Said and Zimmerman, 1980; Vanderhaeghen, Lotstra, de Mey and Gilles, 1980) and the cerebrospinal fluid (Fahrenkrug, Schaffalitsky de Muckdell and Fahrenkrug, 1977; Rehfeld and Kruse-Larsen, 1978), and the presence of VIP in the hypophysial-portal circulation (Said and Porter, 1979; Shimatsu, Kato, Matsushita, Katakami, Yanaihara and Imura, 1981) and in prolactin-secreting cells in the pituitary, which appear to have specific receptors for VIP (Nicosia, Spada, Borghi, Cartelazzi and Giannattasio, 1980; Morel, Besson, Rosselin and Dubois, 1982), is compatible with a role for CCK and VIP in the control of pituitary hormone release. However, the criteria for designating CCK and VIP as physiological hypothalamo-hypophysial regulating factors, as stated by the neurohumoural hypothesis (Section 1.1.1), has only partially been established in the case of VIP and still largely uninvestigated in the case of CCK. The evidence suggesting a role for VIP and CCK in modulating the secretion of adenohypophysial hormones is discussed in detail in Chapters VI and VII.

1.2 The Rat Reproductive Cycle

Adult female rats maintained on a schedule of 14 h light : 10 h darkness have cyclical gonadotrophin secretion patterns with ovulation occurring every 4 days. The absence of a similar phenomenon in the male rat has been attributed to sexual differentiation of the brain (Brown-Grant, 1973; McEwen, 1981).

1.2.1 Hormonal Changes During the Oestrous Cycle

The main hormonal changes over the four-day oestrous cycle of the rat can be summarised as follows:

i) The concentration of LH in peripheral plasma remains low until the afternoon (between 17.00 - 19.00 h) of pro-oestrus when a surge of LH secretion occurs and plasma LH concentrations may be up to fifty times those at dioestrus (Monroe, Rebar, Gay and Midgley, 1969; Brown-Grant, Exley and Naftolin, 1970; Piacsek, Schneider and Gay, 1971; Aiyer, Fink and Greig, 1974a; Blake, 1976).

ii) Plasma concentrations of FSH also increase with the rise in LH, but peak secretion does not occur until about 05.00 h of oestrus (Daane and Parlow, 1971; Butcher, Collins and Fugo, 1974; Fink and Aiyer, 1974).

iii) There is also a surge of PRL during the afternoon of pro-oestrus (Gay, Midgley and Niswender, 1970; Wuttke and Meites, 1970; Neill, Freeman and Tilson, 1971), which has been correlated with a decrease in the DA concentration in portal vessel blood (Ben-Jonathan, Oliver, Weiner, Mical and Porter, 1977; Plotsky, Gibbs and Neill, 1978).

iv) The concentration of LHRH in the hypophysial portal vessel blood increases rapidly during the afternoon of proestrus, reaching a peak at the same time as the peripheral plasma concentrations of LH (Sarkar *et al.*, 1976; Ching, 1982). A second smaller surge of LHRH which has been postulated to be related to peak FSH secretion, occurs between midnight of pro-oestrus and 02.00 h of oestrus (Sarkar *et al.*, 1976).

v) The gonadotrophin surges are preceded by an increase in plasma oestradiol-17β concentration which begins during the evening of dioestrus and reaches a peak during the morning and early afternoon of pro-oestrus (Ferin, Tempone, Zimmering and Van de Weil. 1969; Paicsek *et al.*, 1971; Brown-Grant *et al.*, 1972; Shaikh and Shaikh, 1975; Henderson *et al.*, 1977).

vi) An elevation in progesterone secretion begins before the LH surge but is markedly increased during and after the LH surge (Barraclough, Collu, Massa and Martini, 1971; Feder, Brown-Grant and Corker, 1971; Brown-Grant *et al.*, 1972).

An increase in pituitary responsiveness to LHRH on pro-oestrus (Section 1.3.2a; Aiyer, Chiappa and Fink, 1974b; Cooper, Fawcett and McCann, 1974; Gordon and Reichlin, 1974), together with a priming effect exerted by LHRH on the anterior pituitary (Section 1.2.2; Aiyer *et al.*, 1974a) ensures the culmination of all these hormonal changes in ovulation early on the morning of oestrus.

1.2.2 The Priming Effect of LHRH

The priming effect is the ability of LHRH to increase the responsiveness of gonadotrophs to subsequent exposures to itself (Aiyer *et al.*, 1974a, 1974b). The priming effect was demonstrated by the fact that the LH response to the second of two successive injections of LHRH (50 ng/rat, i.v.) and was maximal if the two injections were separated by 60 min (Aiyer *et al.*, 1974b). The effect was also elicited if endogenous LHRH release was increased by electrical stimulation of the mPOA, applied over two successive 15 min periods separated by 60 min (Fink, Chiappa and Aiyer, 1976). Since the content of LHRH in stalk blood during the two stimulations

periods was similar (Fink *et al.*, 1976), the priming effect could not be attributed to an increased secretion of LHRH induced by the second period of stimulation. Moreover, the priming effect was demonstrated in rats anaesthetised with pentobarbitone and infused with LHRH at a constant rate for 90 min or by the application of a constant stimulation to the mPOA (Fink *et al.*, 1976; Chiappa *et al.*, 1977). The priming effect was not mediated by adrenal or ovarian steroids since it can be demonstrated in adrenalectomised and acutely ovariectomised rats (Aiyer *et al.*, 1974b), although ovarian steroids (oestradiol and progesterone) determined the magnitude of the effect (Aiyer *et al.*, 1974b; Speight, Popkin, Watts and Fink, 1981; Padmanabhan, Leung and Convey, 1982). The priming effect was also elicited *in vitro* (Pickering and Fink, 1966, 1977) although the maximal effect occurred on pro-oestrus (Aiyer *et al.*, 1974b; Waring and Turgeon, 1980), while ovariectomy on the morning of dioestrus reduced the priming effect on the afternoon of pro-oestrus (Speight *et al.*, 1981) and the release of LHRH into stalk blood. Oestradiol (2.5 µg/rat, s.c.) administration immediately after ovariectomy only partially reversed the suppression of the priming effect produced after removal of the ovarian steroids (Aiyer *et al.*, 1974b). Studies done to determine the mechanism of the priming effect have been reviewed by Fink and Pickering (1980).

1.2.3 The Critical Period on Pro-oestrus

The administration of neural blocking agents, such as atropine, chlorpromazine, morphine or pentobarbital, in the early afternoon (14.00 - 16.00 h) on pro-oestrus blocked the pre-ovulatory LH surge and ovulation (Everett, Sawyer and Markee, 1949; Everett and Sawyer, 1950; Barraclough and Sawyer, 1955, 1957; Greig and Weisz, 1973), while stimulation

of the mPOA induced secretion of comparable amounts of LH, whichever of the drugs was used (Everett and Tyrey, 1982). The primary suppressive action of these drugs does not appear to be on the preoptico-infundibular tract (Section 1.3.3a), the availability or release of LHRH or the ability of the pituitary to release LH (Everett and Tyrey, 1982). However, the exact timing of the 'critical period' on pro-oestrus before which administration of the neural blocking drugs effectively inhibited ovulation was dependent on the lighting schedule of the animals (Everett, 1964, 1977). In rats maintained on a 14 h light : 10 h dark schedule, the critical period occurred 14 h after the midpoint of the dark period (Everett and Tejasen, 1967; Hoffman, 1970).

1.3 Control of Gonadotrophin Release

1.3.1 Role of the Hypothalamus

The neural signal that results in the release of LHRH into the hypophysial portal vessels and the pre-ovulatory LH surge is thought to be generated in the mPOA and suprachiasmatic nucleus regions of the rostral diencephalon, which contain LHRH cell bodies (Barraclough, 1966; Koves and Halasz, 1970). The importance of the neural connections between the mPOA and median eminence for ovulation has been demonstrated by lesioning studies and experiments involving deafferentiation of the medial basal hypothalamus (MBH) which included the paraventricular and arcuate nuclei (Barraclough and Gorski, 1961; Halasz and Pupp, 1965; Blake, Weiner, Gorski and Sawyer, 1972). The mPOA has also been postulated to initiate cyclic release of LH preceding ovulation in the rat, while the MBH appears responsible for tonic release of gonadotrophins during meto-oestrus and dioestrus, for follicular growth and maturation of the ovary (Barraclough and

Sawyer, 1961; Dorianski, Przekop and Polkowsta, 1980). Interruption of the amygdaloid complex and/or hippocampal afferents to the basal hypothalamus have no inhibitory effect on the oestrous cycle and ovulation (Velasco and Taleisnik, 1971; Brown-Grant and Raisman, 1972; Chiappa *et al.*, 1977). However, the LH surge induced by oestrogen in ovariectomised rats appears to involve the limbic system, particularly the amygdala, since lesions of neural pathways to the hypothalamus from the limbic system or bilateral lesions of the amygdala prevent the release of LH by oestrogen (Rabii, 1981).

1.3.2 The Role of Gonadal Steroids

The influence of gonadal steroids on the hypothalamo-hypophysial system has been extensively studied (Davidson, 1969; Goodman, 1978; Lipsett, 1979; McEwen and Parsons, 1982; Martini, 1982). Investigations into the mechanisms by which gonadotrophin secretion is regulated show that steroids exert both a stimulatory and inhibitory influence, depending on the duration, timing and level of exposure to the steroids. Oestrogen exerts an inhibitory influence on gonadotrophin secretion except on the morning and early afternoon of pro-oestrus during the cycle when oestrogen switches over to exerting a facilitatory effect.

(a) Positive feedback effects of steroids:

In rats, the spontaneous pre-ovulatory surge of LH on the afternoon of pro-oestrus depends on a significant increase in plasma oestradiol-17β during the evening of dioestrus and morning of pro-oestrus (Shirley, Wolinsky and Schwartz, 1968; Ferin *et al.*, 1969; Aiyer and Fink, 1974). Administration of antiserum to oestradiol-17β on dioestrus blocked the pre-ovulatory gonadotrophin surge and

ovulation (Ferin *et al.*, 1969; Neill *et al.*, 1971). The facilitatory effect of oestradiol-17β is enhanced by ovarian progesterone, secreted in response to LH on the afternoon of pro-oestrus (Everett, 1964; Rothchild, 1965; Brown-Grant and Naftolin, 1972; Mann and Barraclough, 1973; Aiyer and Fink, 1974). This enhancing effect of progesterone only occurred in oestrogen-primed rats (Everett, 1964; Aiyer and Fink, 1974; Brown-Grant, 1974); the reason for this has been suggested to be due to the fact that the concentration of progesterone receptors in the hypothalamus and pituitary is modulated by exposure to oestrogen (Maclusky and McEwen, 1978). Progesterone by itself has little or no effect on LHRH secretion (Sarkar and Fink, 1979) or gonadotrophin secretion (Everett, 1964; Brown-Grant and Naftolin, 1972; Aiyer and Fink, 1974).

There is substantial evidence to suggest that oestradiol and progesterone increase pituitary responsiveness by a direct action on gonadotrophs (Drouin, Lagacé and Labrie, 1976; Fink and Henderson, 1977; Hseuh, Erickson and Yen, 1978; Goodman and Knobil, 1981; Turgeon and Waring, 1981), in the absence of a significant increase in LH and LHRH release (Martin, Tyrey, Everett and Fellows, 1974; Sarkar *et al.*, 1976; Fink and Henderson, 1977; Lagacé, Massicotte and Labrie, 1980). The pituitary responsiveness to LHRH shows a gradual increase between 13.30 h of dioestrus to 15.00 h of pro-oestrus followed by a further abrupt increase between 15.00 h and 18.00 h on pro-oestrus (Aiyer *et al.*, 1974; Cooper *et al.*, 1974; Gordon and Reichlin, 1974). The initial increase in responsiveness depends on the rise in plasma oestradiol (Aiyer and Fink, 1974; Henderson *et al.*, 1977). Oestradiol exerts a biphasic effect on pituitary responsiveness to LHRH; it is inhibitory during the first few hours after administration

then 8-12 h later it exerts a facilitatory effect on the pituitary (Libertun, Orias and McCann, 1974; Henderson *et al*., 1977). The increase in progesterone which accompanies the LH surge, contributes to the later abrupt increase in pituitary responsiveness (Aiyer *et al*., 1974a; Fink and Henderson, 1977). Since administration of antiserum to progesterone does not block the LH surge (Ferin *et al*., 1969), it is postulated that progesterone does not initiate the increase in LH secretion.

A hypothalamic site of action for the positive feedback action of oestradiol, at the level of the preoptic area (POA) rather than the MBH (which appears to be the site for the negative feedback of progesterone), has been suggested by experiments in which oestradiol implants were placed in different regions of the brain (Goodman, 1978; Goodman and Knobil, 1981). In contrast, progesterone implants in the POA blocked oestradiol-induced LH (McLean, Change and Nikitivitch-Winer, 1975) and LHRH release (Sarkar *et al*., 1976). Moreover, progesterone decreased LHRH output in response to stimulation of the POA (Campbell, Schwartz and Firlit, 1977).

(b) Negative feedback effects of steroids:

The existence of a negative feedback relationship between gonadal steroids and pituitary gonadotroph secretion was first postulated by Moore and Price (1932). The negative feedback action of the gonadal steroids maintains plasma gonadotrophin at fairly constant low concentrations in the intact male rat and in the female rat, at all stages of the oestrous cycle except pro-oestrus (Gay *et al*., 1970). The importance of the negative feedback control of the pituitary by the gonadal steroids is demonstrated by the fact that removal of the gonadal steroids evokes a pronounced and sustained increase in circulating

concentrations of gonadotrophins (Gay and Midgley, 1969; Yamamoto, Diebel and Bogdanove, 1970; Tapper, Grieg and Brown-Grant, 1974). Administration of oestradiol, oestradiol and progesterone or testosterone to gonadectomised rats rapidly reverses the post-castration rise in plasma gonadotrophins (Gay and Bogdanove, 1969; Caligaris *et al.*, 1971; Kalra, Fawcett, Krulich and McCann, 1973; Negro-Vilar, Orias and McCann, 1973). Progesterone administration without oestradiol is ineffective in lowering the post-castration increase in gonadotrophin secretion (McCann, 1963; Schally, Bowers, Carter, Arimura, Redding and Saito, 1969), while administration of progesterone together with oestradiol results in an enhancement of the negative feedback action of oestradiol on LH secretion and a suppression of the positive feedback effect of oestradiol on LH secretion (Goodman, 1978).

The mechanism by which steroids exert the negative feedback in influence on gonadotrophin secretion appears to be by a modulation of pituitary responsiveness and hypothalamic LHRH secretion. Numerous studies have shown that androgens are capable of directly inhibiting the pituitary response to exogenous LHRH (Kingsley and Bogdanove, 1973; Cheung and Davidson, 1977; Labrie, Drouin, Ferland, Lagacé, Beaulieu, DeLean, Kelly, Caron Raymond, 1978), although intra-hypothalamic implants of testosterone suppressed plasma LH concentrations before any change in pituitary responsiveness was detected (Cheung and Davidson, 1977). While it has been demonstrated that LHRH neurones in the MBH respond to changes in plasma testosterone titres (Kalra, Kalra and Mitchell, 1977; Kalra and Kalra, 1980), there is no published study demonstrating a modulation of LHRH release by the androgens. In contrast, measurements of immunoreactive LHRH in portal vessel blood after oestradiol administration to ovariectomised

rats showed that the rise in LHRH secretion induced by ovariectomy could be reversed by oestradiol replacement (Sarkar and Fink, 1980). However, since ovariectomy increased (Cooper *et al.*, 1974; Henderson *et al.*, 1977) and oestradiol decreased (Libertun *et al.*, 1974; Debeljuk, Vilchez-Martinez, Arimura and Schally, 1974; Henderson *et al.*, 1977), the magnitude of the LH release in response to LHRH administration, a pituitary site of action for the negative feedback effect of oestradiol, has also been postulated. In addition, oestradiol, progesterone, oestradiol plus progesterone or testosterone inhibited the post-gonadectomy rise in LHRH receptors in the pituitary (Clayton and Catt, 1981; Fraser, Popkin, McNeilly and Sharpe, 1982b).

1.3.3 Role of Catecholamines in the Control of Gonadotrophin Secretion

Evidence of the involvement of brain catecholamines in the regulation of anterior pituitary secretion of gonadotrophins has been discussed in several reviews (Fink and Geffen, 1978; Weiner and Ganong, 1978; Barraclough and Wise, 1982). There is a vast amount of controversial literature on the numerous experiments carried out in this field. Most of the controversy is probably due to the complex experimental models used, the lack of knowledge of the specificity of the pharmacological drugs used and of the neuroanatomical pathways involved and the inadequacy of quantitative techniques. An overview of the control of gonadotrophin secretion by monoamines relevant to this thesis is given here.

(a) Neuroanatomy of the LHRH, dopamine (DA) and noradrenaline (NA) systems:

The LHRH system: The failure of early studies to demonstrate LHRH in perikarya (Baker, Dermody and Reel, 1974; King, Parsons, Erlansen and Williams, 1974; Gross, 1976) has been attributed to the concentration of LHRH in the perikarya being below the threshold of

the immunochemical techniques used, since subsequent studies in which drugs such as colchicine were used to raise the LHRH concentrations clearly demonstrated the existence of LHRH immunoreactivity in the rat central nervous system (Sétáló, Vigh, Schally, Arimura and Flerkó, 1976; Vigh, Sétáló, Schally, Arimura and Flerkó, 1978; Kawano and Daikoku, 1981). Conflicting conclusions have been reached concerning the location of LHRH in the perikarya, presumably due to differences in the LHRH antisera used. Some investigators favour the mPOA or the suprachiasmatic nucleus as the region where the majority of the LHRH cell bodies are localised (Barry, Dubois and Poulain, 1973; Sétáló *et al.*, 1976; Ibata, Watanabe, Kinoshita, Kubo, Sano, Sin, Hashimura and Imagawa, 1979), while others favour the arcuate nucleus (Zimmerman, Hsu, Ferin and Kozlowski, 1974; Naik, 1975). With the use of highly specific antisera to LHRH and refined methodology, such as the use of vibrotome sections of brains from normal untreated animals which enable immunohistochemical procedures to be carried out on unembedded tissue, the main regions which contain LHRH cell bodies have been demonstrated to be the septal and mPOA (Merchenthaler, Kovács, Lovács and Sétáló, 1980; Kawano and Daikoku, 1981; Witkin, Padan and Silverman, 1982). The presence of LHRH cell bodies in the MBH is still controversial (Kawano and Daikoku, 1981; Kelly and Ronnekliev, 1981; Shivers, Harlan, Morrell and Pfaff, 1981). Lesions which isolate the MBH reduced, but did not completely eliminate the LHRH in this region (Weiner, Pattou, Kerdelhué and Kordon, 1975; Brownstein, Arimura, Schally, Palkovits and Kizer, 1976; Taketani, Nozaki, Taga, Minaguchi, Kigawa, Sakamoto and Kobayashi, 1980). It has been suggested that these lesions failed to eliminate the ventral-most LHRH input to the MBH, although the presence of

neurones capable of synthesizing LHRH could explain why the MBH retains 20-30% of its LHRH content after total deafferentation (Taketani *et al.*, 1975; Weiner *et al.*, 1975; Brownstein *et al.*, 1976; Kordon, Patton, Herman and Palkovits, 1982).

A dense localisation of LHRH axons in the lateral perivascular region of the median eminence, surrounding the primary capillary plexus of the portal vessels, has been repeatedly reported (Baker *et al.*, 1974; King *et al.*, 1974; Naik, 1975; Sétáló, 1976). The major innervation of the median eminence originates from cell bodies in the septal and mPOA, passes through the MBH (retrochiasmatic area and anterior hypothalamus) and is joined by fibres from the bed nucleus of the stria terminalis. All these fibres merge at the level of the arcuate nucleus to terminate in the lateral layer of the median eminence (Sétáló *et al.*, 1978; Ibata *et al.*, 1979; Merchenthalar *et al.*, 1980; Kawano and Daikoku, 1981). The fibres from this preoptico-infundibular tract are suggested to release LHRH into portal vessel blood. Electrophysiological evidence has been presented for a role of the preoptico-infundibular pathway in the control of pituitary gonadotrophin release (Kawakama and Sakuma, 1976).

The organum vasculosum of the lamina terminalis (OVLT) is the other main region, apart from the median eminence, that receives a large number of LHRH axons (Brownstein, Palkovits, Tappaz, Saavedra and Kizer, 1976; Selmanoff, Wise and Barraclough, 1980). The LHRH cell bodies which project fibres to the OVLT are located mainly in the preoptic-septal region (Weiner *et al.*, 1975; Palkovits, Mezey, Ambach and Kivovics, 1978; Kawano and Daikoku, 1981; Witkin *et al.*, 1982). Since the venous drainage of the OVLT is directly into the systemic circulation, it seems unlikely that the LHRH terminals in the OVLT

contribute directly to the LHRH in the portal vessel blood (Palkovits *et al.*, 1978). However, it has been proposed that LHRH terminals in the OVLT release LHRH into the cerebrospinal fluid of the third ventricle, which is then taken up by tanycyctes of the median eminence and transported to pituitary portal capillaries (Ben-Jonathan, Mical and Porter, 1974; Scott, Dudley and Knigge, 1974; Goldgefter, 1976). This suggestion is supported by the demonstration that placement of radioactive materials of different molecular weights (Ondo, Eskay, Mical and Porter, 1973) or LHRH (Ben-Jonathan *et al.*, 1974), in the ventricular system rapidly results in the appearance of these substances in the hypophysial portal vessel blood.

The Dopamine (DA) system: The distribution of DA neurones in the rat brain has been studied primarily using histofluorescent techniques (Falck, Hillarp, Thieme and Torp, 1962; Dahlström and Fuxe, 1964; Ungerstedt, 1971; Lindvall and Björklund, 1974). Dopaminergic neurones in the hypothalamus are derived from two independent dopaminergic systems, the incertohypothalamic (ICH) and the tuberoinfundibular (TIF) systems. The ICH fibre system originates from the dorsal and caudal hypothalamic cell groups (A11, A13, A14) and project for short distances into the medial preoptic, suprachiasmatic, dorsomedial and anterior hypothalamic nuclei (Björklund, Lindvall and Nobin, 1975). The cell bodies of the TIF system are located in the arcuate nuclei and a portion of the periventicular nucleus just dorsal to the arcuate nucleus which terminate in the median eminence, pituitary stalk, pars nervosa and pars intermedia of the adenohypophysis (Björklund, Falck, Hromek, Owman and West, 1970; Smith and Fink, 1972; Björklund, Moore, Nobin and Stenevi, 1973). The axons of the TIF project ventrally to the median eminence and terminate in the peri-

capillary spaces around the primary capillary plexus that coalesces to form the hypophysial portal vessels (Fuxe and Hökfelt, 1966). That these TIF DA terminals secrete DA is suggested by the demonstration of significantly higher DA concentrations in the portal vessel blood than in the systemic circulation (Ben-Jonathan *et al.*, 1977).

The dorsolateral projection of the TIF DA axons to the median eminence terminates close to LHRH-containing axon terminals (Björklund *et al.*, 1973). By applying simultaneous histofluorescence and immunohistochemical techniques to rat median eminence tissue, it has been shown that LHRH and catecholamines do not co-exist in the same terminals (McNeill and Sladek, 1978; McNeill, Scott and Sladek, 1980). In addition, administration of 6-hydroxydopamine caused severe depletion of the DA content in the median eminence, without affecting the LHRH content (Kizer, Arimura, Schally and Brownstein, 1975). However, the close anatomical localisation of the DA- and LHRH-containing terminals does support the suggestion that DA modulates the secretion of LHRH into portal vessel blood, although axo-axonal contact between DA and LHRH contacts between DA- and LHRH-containing terminals have not yet been reported to be histologically visualised.

The Noradrenaline (NA) system: The major NA innervation of the hypothalamus is from the ventral NA bundle (Ungerstedt, 1971; Lindvall and Björklund, 1974; Kizer, Muth and Jacobowitz, 1976; Löftström, Johnsson and Fuxe, 1976), which forms one of the two major ascending NA pathways to the brain which is derived from the lower brainstem cell groups of the lateral tegmentum (Olsen and Fuxe, 1972). The ventral NA pathway innervates the whole of the hypothalamus including

the supraoptic, preoptic and arcuate nuclei, the internal layer of the median eminence and the retrochiasmatic area (Moore and Bloom, 1979; Palkovits, 1981). The other NA fibre system in the brain which originates in the locus coeruleus has been shown by autoradiographic tracing methods (Segal, Pickel and Bloom, 1974; Jones and Moore, 1977) to project only to the periventricular, paraventricular and supraoptic nuclei and the dorsal medial nucleus of the hypothalamus.

(b) Experimental evidence supporting a role of catecholamines in gonadotrophin secretion:

The experimental approaches used to elucidate the role of the catecholamines in gonadotrophin secretion have been reviewed by McCann and Moss (1975), Fink and Geffen (1978), Weiner and Ganong (1978), Sawyer (1979), Barraclough and Wise (1982). The contribution of central DA pathways in modulating the secretion of gonadotrophins in the cycling rat is still controversial. The existence of an inhibitory dopaminergic influence on LH secretion has been repeatedly claimed by Fuxe and his colleageus. For example, studies assessing changes in DA turnover showed that in the TIF DA terminals the turnover is decreased during the critical period of pro-oestrus (Fuxe, Hökfelt, Löfström *et al.*, 1976; Löfstrom, 1977), while the inhibitory feedback action of oestradiol on LH secretion induced a marked increase in DA turnover in the lateral perivascular region of the median eminence (Löfström, Eneroth, Gustaffson and Skett, 1977). In addition, ovulation was blocked by DA receptor agonists while blockade of the DA receptors by pimozide, antagonised this action of the DA agonists (Fuxe, Löfström, Agnati, Everitt, Hökfelt, Jonsson and Wiesel, 1975). On the other hand, a stimulatory role for DA in the control of LH secretion has been proposed by McCann and his colleagues, largely due to demonstrations

that intraventricular injections of DA agonists or infusions of DA stimulated LH secretion in oestrogen-primed female rats (Vijayan and McCann, 1978b). Support for both a stimulatory and an inhibitory influence of DA on LH secretion has accumulated, often resulting in further controversy. For example, *in vitro* studies showed that DA stimulated the release of LHRH from hypothalamic fragments (Rotsztejn, Charli, Pattou, Epelbaum and Kordon, 1976; Negro-Vilar and Ojeda, 1978), while lesions of DA fibres localised predominantly in the median eminence also results in a stimulation of the LHRH surge in prepubertal rats treated with pregnant mare serum gonadotrophin (PMSG: Sarkar, Smith and Fink, 1981). Studies with DA receptor agonists and antagonists have suggested that DA could either facilitate or inhibit LHRH and thus LH secretion (Sarkar and Fink, 1980). The existence of two or more pharmacologically distinct receptors with opposing effects may explain these findings.

The evidence favouring a stimulatory role of NA in LH secretion and ovulation in the rat (Rubinstein and Sawyer, 1970; Tima and Flerkó, 1974; Krieg and Sawyer, 1976) has been confirmed by a number of studies. These include the demonstration that the turnover of NA in the median eminence and mPOA is increased before the LH surge on pro-oestrus and in prepubertal rats treated with PMSG (Agnati, Fuxe, Löfström and Hökfelt, 1977; Löfström, 1977; Negro-Vilar, Chiocchio and Tremezzani, 1977; Rance, Wise, Selmanoff and Barraclough, 1981). In addition, castration increased the NA content of the median eminence (Chiocchio, Negro-Vilar and Tremezzani, 1976), while interference with the NA system reduced the post-castration increase in plasma LH concentrations (Drouva and Gallo, 1976; Gnodde and Schuiling, 1976). Furthermore, the steroid-induced suppression of the post-castration

rise in LH secretion was accompanied by a reduction in the turnover of NA in the median eminence (Löfstrom *et al.*, 1977). Acute injections of 6-hydroxydopamine, to lesion the ventral NA tract, blocked the spontaneous and steroid-induced surges of LH (Martinovic and McCann, 1977) although a permanent loss of oestrous cycles after lesions of the ascending NA systems did not occur (Nicholson, Greeley, Humm, Youngblood and Kizer, 1978; Clifton and Sawyer, 1979, 1980). However, α-adrenoceptor blockers (e.g. phenoxybenzamine) inhibited the PMSG-induced surges of LH and LHRH in the prepubertal rat (Sarkar and Fink, 1981; Sarkar, Smith and Fink, 1981). The facilitation of LHRH release into pituitary stalk blood by NA neurones is in agreement with studies that demonstrated that NA released LHRH from hypothalamic fragments *in vitro* (Negro-Vilar and Ojeda, 1978) and that catecholamines do not act directly on the pituitary gland to alter LH secretion (Schneider and McCann, 1970; Kamberi, Mical and Porter, 1970, 1971).

The coincidental localisation of DA cell bodies and oestrogen target areas in the periventricular and arcuate nucleus and the ability of catecholamine-containing neurones to concentrate tritiated oestrogen in the arcuate nucleus and median eminence (Grant and Stumpf, 1973), as well as immunofluorescence studies following pharmacological manipulations, have suggested that DA neurones are under feedback control by gonadal steroids. It was also suggested that an inhibition of LHRH release, by increased DA output, accounted for the inhibitory feedback action of gonadal steroids on gonadotrophin release. The release of LHRH from incubated medial basal hypothalamic fragments (Bennett, Edwardson, Holland, Jeffcoate and White, 1975) and the palisade layer of the median eminence (Rotsztejn *et al.*, 1976) was increased by DA,

provided the animals were pretreated with oestradiol (Rotsztejn, Charli, Pattou and Kordon, 1977). On the other hand, some studies suggest that the treatment of ovariectomised rats with gonadal steroids converts the action of DA to a stimulatory one with respect to LH release (Kawakami, Kimura, Manaka and Kawagoe, 1975; Vijayan and McCann, 1978b). In the male, the majority of the evidence favours a stimulatory role for oestrogens on DA turnover in the median eminence (Fuxe, Hökfelt and Nilsson, 1972; McEwen and Parsons, 1982) and release into portal vessel blood (Gudelsky, Nansel and Porter, 1981). A sex difference in the response of hypothalamic TIF DA systems during the cycle and in response to gonadectomy has also been found (Demarest, McKay, Riegle and Moore, 1981b; DePaolo, McCann and Negro-Vilar, 1982). The contribution of sex steroids to this sexual differentiation is, however, still under investigation.

1.4 Control of Prolactin Secretion

1.4.1 Hypothalamic Influence

In contrast to other pituitary hormones, the secretion of prolactin (PRL) in mammals is regulated by a predominantly inhibitory influence from the hypothalamus (MacLeod, 1976). This was suggested by studies showing that a dramatic increase in PRL release followed transplantation of the pituitary to sites such as the kidney capsule which are removed from the hypothalamic influence (Everett, 1954; Chen, Amenomori, Lu, Voogt and Meites, 1970) destruction of the hypothalamus or deafferentation of the medio-basal hypothalamus (Haun and Sawyer, 1960; Chen *et al.*, 1970; Bishop, Krulich, Fawcett and McCann, 1971; Blake, Scaramuzzi, Norman, Hilliard and Sawyer, 1973) or pituitary stalk section (Dienfenbach, Carmel, Frantz and Ferin, 1970; Kanematsu and

Sawyer, 1973). Moreover, pituitary cells maintained in culture released large amounts of PRL (MacLeod, 1976). Acid extracts of rat hypothalamus decreased the concentration of PRL in the medium, when the extracts were incubated with rat anterior pituitary gland, but not rat cerebral cortex; this suggested the existence of hypothalamic PRL inhibiting factors (PIF; Talwalker, Ratner and Meites, 1963). This view was supported by the fact that injection of hypothalamic extracts either systemically (Amenomorri and Meites, 1970) or into the hypophysial portal vessels (Kambieri, Mical and Porter, 1971) reduced plasma PRL concentrations but increased plasma LH and FSH concentrations.

1.4.2 Role of Biogenic Amines

A function of the hypothalamic catecholamines in regulating the secretion of PRL was first suggested by Kanematsu, Hillard and Sawyer (1963). They demonstrated that injections of reserpine, like electrolytic lesions of the hypothalamus, induced lactation and decreased pituitary PRL concentrations. Subsequent studies showed that administration of either monoamine oxidase (MAO) inhibitors that prevent catabolism of the catecholamines and so increased catecholamine concentrations, or L-dopa, the precursor of the catecholamines, inhibited the release of PRL and post-partum lactation in rats (Mizuno, Talwalker and Meites, 1964; Lu and Meites, 1972; Donoso, Banzan and Barcaglioni, 1974). Agents that reduced synthesis of catecholamines or blocked catecholamine receptors were found to increase PRL release (Meites and Clemens, 1972; Weiner and Ganong, 1978). The suppression of PRL release by infusion of a purified preparation of either procine PIF or catecholamines into hypophysial portal vessels of rats also supported a role of the catecholamines in regulating PRL release (Takahar, Arimura and Schally, 1974). Moreover, enzymatic digestion

of hypothalamic extracts with MAO or adsorption of catecholamines with alumina were found to abolish PIF activity (Shaar and Clemens, 1974). Intravenous administration of DA was later found to inhibit PRL secretion in a number of physiological situations including the oestrous cycle, sleep and stress as well as from PRL-secreting tumours (Weiner and Ganong, 1978). Since DA administered peripherally did not cross the blood-brain barrier, it was postulated that DA was acting at the level of the anterior pituitary or brain regions outside the blood-brain barrier such as the median eminence and other circumventricular organs. Evidence supporting the view that DA is a PIF includes,

i) the demonstration that TIF dopaminergic neurones terminate in the external layer of the median eminence, adjacent to the primary plexus of capillaries of the hypophysial portal circulation (Hökfelt, 1967; Fuxe, Hökfelt and Nilsson, 1969);

ii) the presence of DA in portal vessels supplying blood to the anterior pituitary of rats and monkeys at concentrations sufficient to inhibit pituitary PRL release (Ben-Jonathan, Oliver, Weiner, Mical and Porter, 1977; Gibbs and Neill, 1978; Neill, Frawley, Plotsky and Tindall, 1981);

iii) the suppression of PRL secretion from pituitary PRL cells and the pituitary gland by physiological concentrations of DA (MacLeod and Lehmeyer, 1974), or by DA agonists (Lamberts and MacLeod, 1978);

iv) the demonstration of specific DA receptors on the plasma membrane of pituitary cells (Caron, Beaulieu, Raymond, Gagne, Drouin,

Lefkowitz and Labrie, 1978; Calabro and MacLeod, 1978; Cronin, Roberts and Weiner, 1978);

v) the demonstration of an increase in DA turnover in the TIF DA neurones, but not in the nigrostriatal neurones, when circulating PRL concentrations are increased by injections of PRL (Gudelsky, Simpkins, Mueller, Meites and Moore, 1976; Hökfelt and Fuxe, 1972), pituitary transplants (Olsen, Fuxe and Hökfelt, 1972) or in lactating and pregnant rats (Fuxe *et al.*, 1969);

vi) that removal of pups from lactating mothers for 24 h, which reduced circulating PRL, resulted in a significant increase in DA concentrations in pituitary stalk blood (Ben-Jonathan, Neill, Arbogast, Peters and Hoefer, 1980), while a decrease in the DA content of the hypothalamus occurred within 5 min after the onset of suckling (Mena, Enjalbert, Carbonnel, Priam and Kordon, 1976; Chiocchio, Cannata, Cordero-Funes and Tramezzani, 1979);

vii) damage to TIF DA neurones was associated with development of PRL-secreting pituitary tumours in rats (Sarkar, Gottschall and Meites, 1982).

Several lines of evidence suggest that PIF activity in purified hypothalamic extracts devoid of catecholamines (Greibrokk, Currie, Johansson, Hansen, Folkers and Bowers, 1974; Enjalbert, Priam and Kordon, 1977; Schally, Redding, Arimura, Dupont and Linthicum, 1977), is due to γ-amino butyric acid (GABA), which may supplement the inhibitory influence of DA on PRL release (Schally *et al.*, 1977; Grandison and Guidotti, 1979; Dow, Fink, Grieve and Mitchell, 1982; Nicolleti, Canoninco, Rampello, Patti *et al.*, 1983). NA also inhibited PRL release from the anterior pituitary *in vitro* (MacLeod, 1969;

Birge, Jacobs, Hammer and Daughaday, 1970; Koch, Lu and Meites, 1970; Shaar and Clemens, 1974) and when injected into portal vessels (Takahara *et al.*, 1974). However, NA terminals in the median eminence are located predominantly in the internal zone, some distance from the primary portal capillaries (Swanson and Hartmen, 1975) and the concentration of NA in portal vessel blood was significantly lower than peripheral blood (Ben-Jonathan *et al.*, 1977). Although pharmacological studies suggest the NA may be involved in pulsatile release of PRL in oestrogen-induced and stress-induced release of PRL (Subramanian and Gala, 1976; Carr, Conway and Voogt, 1977; Langelier and Gala, 1977), it appears unlikely that NA-containing neurones are involved to any great extent in regulating unstimulated or suckling-induced release of PRL (Blake, Weiner, Gorski and Sawyer, 1972; Carr *et al.*, 1977; Weiner and Ganong, 1978).

A stimulatory role of serotonin (5HT) is generally accepted (Kambieri *et al.*, 1971; Lu and Meites, 1973; Caligaris and Taleisnik, 1974; Garthwaite and Hagen, 1979; Clemens and Shaar, 1980), involving activation of hypothalamic 5HT neurones originating from the midbrain raphe nucleus (Advis, Simpkins, Bennett and Meites, 1979; Van de Kar and Lorens, 1979). 5HT did not stimulate PRL release directly from the anterior pituitary (Birge *et al.*, 1970; Kambieri *et al.*, 1971). The mechanism of action of 5HT may involve stimulation of the release of vasoactive intestinal polypeptide (VIP), a putatitive PRF (Kato, Iwasaki, Iwasaki, Abe, Yanaihara and Imura, 1978; Vijayan, Samson, Said and McCann, 1979b; Shimatsu, Kato, Matushita, Katakami, Yanaihara and Imura, 1982), rather than inhibition of dopaminergic suppression of PRL release (Clemens, Rousch and Fuller, 1978; Lamberts and MacLeod, 1978) or suppression of DA release into portal vessel blood (Pilotte and Porter, 1981).

1.4.3 Prolactin Releasing Factors (PRF)

Evidence that PRL may be under stimulatory as well as inhibitory control was first presented by Nicoll, Yaron, Nutt and Daniels (1970), who found that hypothalamic extracts which initially inhibited PRL secretion from incubated pituitary glands, stimulated PRL release during prolonged periods of incubation.

(a) Thyrotrophin releasing hormone (TRH):

The tripeptide, pGlu-His-Pro-NH_2 (TRH) has been found to stimulate the release and synthesis of PRL in humans (Bowers, Friesen, Hwang, Guyda and Folkers, 1971; Jacobs, Snyder, Wilber, Utiger, Utiger and Daughadey, 1971; Tashijan, Barowsky and Jensen, 1971) and in rats (Mueller, Chen and Meites, 1973; Rivier and Vale, 1974). The minimum concentration of TRH that was required to increase plasma TSH concentration was also effective in stimulating PRL release (Noel Dimond, Wartofsky, Earll and Frantz, 1974). The demonstration that TRH stimulated PRL secretion by ectopic pituitary transplants in rats with bilateral median eminence lesions (Porteus and Malven, 1974) and by pituitary tumour cells *in vitro* (Tashijan *et al.*, 1971), as well as rat pituitary cell cultures (Yale, Blackwell, Grant and Guillemin, 1973), suggested that TRH acted directly on the pituitary gland and not through a hypothalamic releasing factor. In addition, TRH induced a rapid increase in the firing rates of PRL-secreting cells (Dufy, Vincent, Fleury, Du Pasquier, Gourdji and Tixier-Vidal, 1979). An increase in TRH concentration in portal vessel blood (Fink, Koch and Ben Aroya, 1983) and TSH concentrations in systemic blood at pro-oestrus (Brown-Grant, Dutton and ter Haar, 1977) has been associated with the pro-oestrous PRL surge. While TRH has been found to stimulate PRL release in the presence of DA and DA agonists (Hill-Samli

and MacLeod, 1974, 1975; Labrie, Beaulieu, Ferland, Raymond, Di Pauli, Caron, Veilleux, Denizeau, Euvrard, Raynaud and Boissier, 1979), the PRF activity of TRF was enhanced by a brief removal of DA from the perifusion system (Fagin and Neill, 1981). In lactating females, TRH injections do not release PRL when given before a brief suckling period but are highly effective if given after it (Grosvenor and Mena, 1980). It has been suggested, therefore, that although the small (~20%) decrease in the release of DA into stalk blood following mammary nerve stimulation (de Greef, Plotsky and Neill, 1981) cannot account for the 5- to 7-fold increase induced in PRL secretion, a brief decrease in hypothalamic DA secretion resulted in an increased responsiveness of the pituitary to the PRF(s) released by the hypothalamus, in response to suckling (Plotsky and Neill, 1982).

PRL and TSH secretions are, however, dissociated in a number of physiological conditions such as lactation (Gautvik, Tashijan, Kourides, Weintraub, Graeber, Maloof, Suzuki and Zukerman, 1974), when plasma PRL is elevated and plasma TSH concentrations are normal, and stress (Reichlin, 1966; Meites and Clemens, 1972; Harris, Christianson, Smith, Fang, Braverman and Vagenakis, 1978), when plasma PRL is elevated and plasma TSH concentrations may be lower than normal. The fact that extracts of the hypothalamus possess PRF activity distinct from TRH (Boyd, Spencer, Jackson and Reichlin, 1976; Szabo and Frohman, 1976; Grosvenor and Mena, 1980) supports the existence of additional PRF(s) distinct from TRH. This view is substantiated by pharmacological studies which show that administration of L-dopa or pilocarpine induced a marked fall in plasma PRL concentrations without affecting plasma TSH concentrations and oestrogen injections stimulated PRL release and decreased TSH secretion (Meites and Clemens, 1972;

Meites, 1977). The recent demonstration that in the ewe, TRH-immunisation suppresses TSH secretion without exerting any major influence on PRL secretion in a number of physiological situations (Fraser and McNeilly, 1982) also supports the view that TRH plays a minor role in the physiological control of PRL secretion.

(b) Oestrogens:

Oestrogens have also been found to stimulate PRL synthesis and release (Ratner, Talwalker and Meites, 1963; Chen and Meites, 1970; Ajika, Krulich, Fawcett and McCann, 1972; Frantz, Kleinberg and Noel, 1972; Neill, 1980). Oestrogens have been demonstrated to be essential for the surge of PRL on pro-oestrus, when a decrease of DA turnover in the hypothalamus has been reported to occur (Ahren, Fuxe, Hamberger and Hökfelt, 1971). Antiserum to oestrogens administered on dioestrus abolished the expected increase in plasma PRL on pro-oestrus (Neill *et al.*, 1971). This effect of the oestrogen antiserum could be prevented by concomitant injections of diethylstilbestrol (a synthetic oestrogen) with the antiserum. The presence of oestrogen receptors on lactotrophs (Keefer, Stumpf and Petrusz, 1976) and the demonstration that oestradiol-17β induced action potentials in pituitary cells (Dufy *et al.*, 1979) suggest that oestradiol could act on prolactotrophs to stimulate PRL release. Although the oestrogen-induced increase in plasma PRL concentrations does not appear to be due to the suppression of DA release into hypophysial portal vessel blood (Gudelsky *et al.*, 1981), oestrogen did reverse the inhibitory effect of DA on PRL secretion from cultured pituitary cells (Raymond, Beaulieu, Labrie and Bossier, 1978). Oestrogen treatment of ovariectomised rats either had no effect or decreased dopaminergic binding sites in the anterior pituitary (Di Paolo, Carmicheal, Labrie and Raymond,

1979; Heiman and Ben-Jonathan, 1982), while a significant increase occurred in the number of pituitary TRH binding sites in adult female rats treated with oestrogen (De Lean, Ferland, Drouin, Kelly and Labrie, 1977). These data suggest that the mechanism by which oestrogen stimulates PRL release may involve a facilitation of TRH-induced release of PRL and possibly a suppression of DA binding to lactotrophs, in addition to a direct effect of oestrogen on the anterior pituitary. An oestrogen-induced reduction in the capacity of the PRL cell to incorporate DA into PRL secretory granules (Gudelsky *et al.*, 1981) provides evidence for another site of action by which oestrogens may modulate PRL release. In primates, oestrogen reinforces the inhibitory effect of DA on PRL release and has little stimulatory effects on PRL release, in contrast to its potent PRL-releasing effects and antagonism of DA inhibition in rodents (Judd, Rigg and Yen, 1979).

(c) Other prolactin releasing factors:

The suggestion that some metabolites of oestrogens, the catechol-oestrogens, are more active than the oestrogens themselves in stimulating PRL secretions is discussed in Chapter V. A number of peptides found in the hypothalamus or pituitary also influence PRL release *in vivo* but not *in vitro* (Vale, Rivier and Brown, 1977; Neill, 1980).
Of these peptides that increase PRL secretion, neurotensin (NT) and vasoactive intestinal polypeptide (VIP) are the best candidates for physiological PRFs distinct from TRH (Carraway and Leeman, 1973; Enjalbert, Arancibia, Ruberg, Priam, Bluet-Pajot, Rotsztejn and Kordon, 1980; Vijayan and McCann, 1980; Enjalbert, Arancibia, Ruberg, Bluet-Pajot and Kordon, 1982). Both NT and VIP are found in cell bodies in the hypothalamus, in terminals of the median eminence and have been reported to be released from hypothalamic nerve terminals upon

depolarisation, by a calcium-dependent mechanism (Uhl and Snyder, 1976; Kobayashi, Brown and Vale, 1977). The PRL-releasing action of NT is additive to that of VIP and TRF (Ruberg, Rotsztejn, Arancibia, Besson and Enjalbert, 1978; Enjalbert, Arancibia, Priam, Bluet-Pajot and Kordon, 1982). The effects of NT or VIP on PRL cells do not involve either DA, opiate or GABA receptors since antagonists to these substances were ineffective in modulating PRL secretion by NT and VIP (Ruberg *et al.*, 1978; Enjalbert *et al.*, 1980). However, VIP-stimulated release of PRL is inhibited by L-dopa, the precursor of DA (Kato *et al.*, 1978) and VIP significantly diminished the effect of DA on PRL secretion from cultured pituitary cells (Kato *et al.*, 1978). NT-induced stimulation of PRL secretion was partially reversed by diphenhydramine (a histamine antagonist), suggesting an involvement of histamine pathways in the modulation of PRL release by NT (Carraway and Leeman, 1973; Vale *et al.*, 1977). VIP and NT immunoreactivity has been found in the anterior pituitary, although only VIP has been reported to be present in portal vessel blood at greater concentrations than in systemic blood (Said and Porter, 1979; Shimatsu *et al.*, 1981). In addition, serotonin, a potent stimulator of PRL release, has recently been reported to increase VIP release into portal vessel blood (Shimatsu *et al.*, 1982). Other details of the postulated mechanism of action of VIP are discussed in Chapter VII.

1.4.4 Hyperprolactinaemia and Hypogonadism

The existence of an inverse relationship between PRL and LH secretion together with an inhibition of ovarian cyclicity during lactation (Lu, Chen, Huang, Grandison, Marshall and Meites, 1976b; Smith, 1978c) and clinical abnormalities of the hypothalamo-hypophysial

axis (Besser and Edwards, 1972; Frantz, Kleinberg and Noel, 1972) has prompted investigations in to the physiological role of PRL in regulating the release of gonadotrophins. Hyperprolactinaemia in pathophysiological conditions in humans is frequently associated with hypogonadism (Bohnet, Dahlen, Wuttke and Schneider, 1976; Carter, Tyson, Tolis, Van Vliet, Faiman and Friesen, 1978), while ovulation and ovulatory surges of hormones are suppressed in nursing women (Delboye, Demaegd and Robyn, 1978; McNeilly, Howie and Houston, 1980). The galactorrhoea-amenorrhoea syndrome in non-nursing women represents another situation in which hyperprolactinaemia and decreased LH secretion occur, although this situation occurs in the absence of a suckling stimulus (Franks, Murray, Jequier, Steele, Nabarrow and Jacobs, 1975; Seki, Seki and Okumura, 1975; Kleinberg, Noel and Frantz, 1977). Treatments of pituitary tumours by surgery, irradiation or by DA agonists (e.g. Bromocriptine) that lower PRL concentrations, are found to effectively restore fertility and increase LH secretion (Von Werder, Eversmann, Rjosk and Fahlbusch, 1982).

A reduction in plasma gonadotrophin concentrations can be produced in experimental animals by inducing persistent hyperprolactinaemia, by placement of pituitary glands or pituitary tumours under the kidney capsule (Everett, 1954; Mena, Haiweg and Grosvenor, 1968; Grandison *et al.*, 1977). These animal models together with the lactating rat model have been studied to determine the mechanism(s) by which hyperprolactinaemia modulates gonadotrophin secretion (Smith, 1980), although in contrast to the animal models plasma PRL concentrations are not continuously elevated over the entire post-partum period (Lu *et al.*, 1976; Smith, 1978a). In the lactating rat, the suckling stimulus may be the primary factor responsible for reduced LH release

(Smith, 1978a), although high PRL concentrations in plasma contributed to the suppression of LH secretion during late lactation and after ovariectomy (Muralidhar, Maneckjee and Mougdal, 1977; Smith, 1978a). Certainly, the suckling stimulus facilitated the action of PRL in suppressing LH release (Muralidhar *et al.*, 1977; Smith, 1978a).

Several mechanisms have been proposed to explain how hyperprolactinaemia inhibits LH synthesis and release and delays the post-gonadectomy rise in plasma gonadotrophins. In general, they suggest that hypogonadism associated with pathological and experimental hyperprolactinaemia encompasses deficits over the entire hypothalamic-pituitary-gonadal axis. At the hypothalamic level, excess PRL increases the inhibitory influence of DA on LH release (Fuxe, Hökfelt and Nilsson, 1972; Hohn and Wuttke, 1978; Gudelsky and Porter, 1980; Moore, Demarest and Johnson, 1980) and suppresses the positive feedback effect of gonadal steroids on LH release (Glass, Shaw, Butt, Edwards and London, 1975; Smith, 1978b; Steger and Pelusa, 1978). The sensitivity of the hypothalamic-pituitary axis to negative feedback by gonadal steroids appears to be increased by hyperprolactinaemia (McNeilly, Sharpe, Davidson and Fraser, 1980b). On the other hand, the pituitary appears to be less sensitive to stimulation and priming by LHRH during hyperprolactinaemic conditions (Lu *et al.*, 1976a; Muralidhar *et al.*, 1977; Smith, 1978c, 1980; Winters and Loriaux, 1978; Greeley and Kizer, 1979; Wuchen and Cheung, 1981). The role of the adrenals and gonads in contributing to hypogonadism by hyperprolactinaemia is still not clearly established (Bartke, Smith, Micheal, Peron and Dalterio, 1977; McNeilly *et al.*, 1978; Greeley and Kizer, 1979; Bartke, 1980; Evans, Cronin and Thorner, 1982).

1.5 Aims of this Thesis

From the above account of the mechanisms known to be involved in the regulation of gonadotrophin and PRL secretion, it is clear that several questions remain unanswered. The unique anatomical arrangement of the hypophysial portal vasculature enables the study of the control of the release of adenohypophysial hormones since substances released into the portal vessels regulate the adenohypophysial secretions (Section 1.1). The development of techniques (Section 1.1.2) that have enabled the collection of hypophysial portal vessel blood samples in the living, although anaesthetised, rat have been decisive in establishing the role of LHRH as a hypothalamo-hypophysial releasing factor (Section 1.1.4). The aim of the experiments described in this thesis was to answer some of the questions regarding the control of gonadotrophin secretion by determining changes in the secretion hypothalamic peptides into the hypophysial portal vessel circulation. The key questions asked were whether

i) the negative feedback influence of the testicular steroids on gonadotrophin secretion (Section 1.3.2) is exerted, in part at least, by suppressing the release of LHRH into pituitary stalk blood - Chapters III and IV;

ii) the enzymatic conversion of the oestrogens to hydroxylated metabolites produces biologically active metabolites, as is found to occur when testosterone is reduced to 5α-dihyrotestosterone - Chapter V.

iii) prolonged hyperprolactinaemia affects the secretion of LHRH and DA into pituitary stalk blood, in view of the marked suppression of gonadotrophin release that is produced by hyperprolactin-

aemia (Section 1.4.4) and the known role of DA as a PIF (Section 1.4.2) - Chapter IV;

iv) during periods of prolonged hyperprolactinaemia, the ability of testosterone to suppress the release of LHRH into stalk blood is more pronounced than during periods when the concentration of PRL in the systemic circulation is not increased above normal - Chapter IV;

v) there is any basis for considering that CCK, gastrin and VIP, three peptides originally isolated in the gut and subsequently in the brain (Section 1.1.5), should be considered as physiological releasing factors as defined by the neurohumoural hypothesis (Section 1.1.1) - Chapters VI and VII.

CHAPTER II

Materials and Methods

CHAPTER II

2.1 Animals

Wistar COBS ('caesarian originated barrier sustained') rats used in the majority of the experiments were supplied by Charles River Ltd (Margate, Kent) or obtained from breeding colonies in the Department of Pharmacology, University of Edinburgh. For studies on rats with pituitary transplants (Chapter IV), rats of the inbred PVG strain were supplied by Bantin and Kingman Ltd (Hull).

The rats were housed in groups of 4 per cage in a room equipped with a programmed 14 h light - 10 h dark illumination cycle (lights on 06.00 - 20.00 h) at a constant temperature of 23 ± 1°C and a constant humidity. Tap water and food (rat diet Oxoid, modified 41B) were freely available. All animals used were adapted for a minimum period of one week before experiments.

2.2 Experimental and Surgical Procedures

2.2.1 Anaesthetics, Drugs and Steroids

The following anaesthetics were used:

(i) ALTHESIN (Glaxo, Middlesex)

9 mg alphaxolene (3α-hydroxy-5α-pregnane-11,20-dione) plus 3 mg alphadone acetate (21-acetoxy-3α-hydroxy-5α-pregnane-11,20-dione) per ml aqueous vehicle consisting of 20% polyoxyethylated castor oil made isotonic with sodium chloride. The dosage was 0.4 - 0.6 ml/100 g body weight (b.w.) for females and 1.0 - 1.5 ml/100 g b.w. for males, injected intraperitonially (i.p.).

(ii) ANAESTHETIC ETHER (Macfarlane Smith Ltd, Edinburgh)

Diethyl ether, dosage as required.

(iii) AVERTIN (Winthrop, Surrey)

1 g tribromoethanol per ml amylene hydrate diluted 2.0 : 8.0 ml in absolute alcohol, then 1.0 : 9.0 ml 0.9% sodium chloride solution. The dose used was 0.25 g/kg b.w., i.p.

(iv) KETALAR (Parke-Davis & Co. Ltd, Pontypool, Gwent)

Ketamine hydrochloride, diluted with 0.9% sodium chloride, to give a 15 mg/ml solution. The dosage was 100-150 mg/kg b.w. in a 2.5 - 3.8 ml i.p.

(v) SAGATAL (May & Baker Ltd, Dagenham)

60 mg/ml sodium pentobarbitone in alcohol. This was administered at a dose of 36 mg/kg b.w., i.p.

(vi) URETHANE (BDH Chemicals Ltd, Poole)

Ethyl carbamate made into a 10% solution with 0.9% sodium chloride solution. The dose was 1 mg/kg b.w., i.p.

(vii) XYLOCAINE (Astra Sweden)

Lignocaine hydrochloride 2.0%. A dose of 0.2 - 0.4 ml/rat was injected subcutaneously (s.c.).

The following drugs and steroids were used:

(i) HEPARIN ('Pularin', Weddel Pharmaceuticals Ltd, London)

2500 i.u./rat in a volume of 0.5 ml was injected into the external jugular vein in all experiments in which pituitary stalk blood was collected. Heparinised saline was made up by diluting stock heparin with 0.9% saline to give 1000 i.u./ml.

(ii) PREGNANT MARE SERUM GONADOTROPHIN ('Folligon', Intervent Laboratories Ltd, Cambridge)

Stock solutions of 200 i.u./ml solvent were diluted with saline (0.9%) and stored frozen in 0.5 ml aliquots of 100 i.u. A dose of 20 i.u./rat in 0.1 ml volume was injected i.p.

(iii) STEROIDS

5-dihydroxytestosterone propionate (Steraloids Inc., New Hampshire, U.S.A.)

The powder was dissolved in ethyl oleate and the dose required administered by s.c. injection.

2-hydroxyoestradiol and 2-hydroxyoestrone (Sigma, St. Louis, Missouri)

The required dose was made up just before use in propylene glycol containing 0.01% absorbic acid and injected s.c. in a volume of 0.25 ml. The crystalline powder was stored in the dark under nitrogen.

oestradiol benzoate ('Benztrone', Paines and Byrne, Middlesex); testosterone propionate ('Viromone', Paines and Byrne, Middlesex)

These were both supplied dissolved in ethyl oleate. The required concentration was obtained by diluting the stock with arachis oil (Hopkins and Williams).

oestrone (Sigma, Poole, Dorset)

The powder was dissolved in propylene glycol containing 0.01% ascorbic acid and the required dose administered by s.c. injection.

testosterone (Sigma, Poole, Dorset)

Crystalline testosterone was used to fill silastic tubing to make implants.

2.2.2 *Determination of the Oestrous Cycle, Ovulation and Uterine Weights*

The oestrous cycles were monitored by daily vaginal lavages. The following cytological characteristics were observed in the vaginal smears of 4-day cyclic rats.

Oestrus:	Cornified epithelial cells.
Metoestrus:	Predominantly leucocytes with nucleated and cornified epithelial cells.
Dioestrus:	Leucocytes only with occasional epithelial cells.
Pro-oestrus:	Nucleated epithelial cells, often in clumps.

Approximately 95% of the rats showed 4-day oestrous cycles and these animals were used for experiments only after at least two consecutive, regular cycles had been confirmed.

The uterotrophic effect of the catechol oestrogens (Chapter V) was assessed by weighing the uterus after removal from the animal. The uterus was removed and stripped of adhering fat and connective tissue, and any fluid present was expressed. The uterus was blotted before being weighed.

2.2.3 *Castration and Testosterone Implants*

Male rats were castrated under pentobarbitone (24 mg/rat) anaesthesia. A single midline incision in the ventral surface of the scrotal sac was used to expose the tunica vaginalis of each testis. A further incision here enabled complete exteriorisation of the testis which was ligated around the spermatic cord. Both testes and vas deferens were removed. Before suturing the incisions, the area was sprayed with antibiotic (Rikospray Antibiotic; Bacitracin, Polymyxin and Neomycin aerosol, Riker Laboratories, Loughborough). Sham-castration consisted of exteriorising and then replacing the gonad.

Implants of testosterone were prepared by the following method. Silastic tubing (Dow Corning Corp., Michigan) with an inner diameter of 1.57 mm, an outer diameter of 3.18 mm and 10 mm or 30 mm in length were filled with crystalline testosterone (Sigma, Poole). The ends were closed with Silastic glue (Dow Corning Corp., Michigan). The implants were then kept in 1 litre of 0.9% saline for 24 h at room temperature with 3 changes of saline 2, 6 and 12 h later. Any leakage from the implants can be detected at this stage by checking that the saline is not clouded. The implants were stored in dry, sealed plastic vials.

Before use, the implants were put in 0.9% saline for 4 h at room temperature. This prevented a massive leakage of testosterone which occurred if the implants were put straight into the animal. The implants were placed under the skin through a small incision in the back. Metal clips were used to close the incision.

2.2.4 Antrectomy and Vagotomy

Both these procedures were performed on anaesthetised animals. A midline incision was made to provide wide exposure of the abdominal organs. The stomach and duodenum were ligated and the gastric antrum dissected out.

Bilateral abdominal vagotomy was carried out by transecting between two ligatures tied one above the other around the oesophagus and the right and left vagal trunks. This procedure was carried out under a Zeiss operating microscope to ensure both vagal trunks to the gastric antrum were cut.

2.2.5 Pituitary Stalk Blood Collection

The hypophysial portal vessels were exposed and blood samples collected from the cut pituitary stalk, using the method developed by Worthington (1964), and modified by Fink and Jamieson (1976), based on the transpharyngeal approach of Green and Harris (1949).

The anaesthetised animals were placed supinely on an operating board and a midline incision made in the skin and subcutaneous tissues. These tissues, together with the salivary glands were separated in the midline along their entire length. After passing a silk suture under the trachea, a small transverse incision was made into which a polythene tube 2 cm long was inserted and secured in position. This airway was kept clear of mucous at all times using a suction pump. Silk sutures were then placed into the flaps of the divided lower lip and through the tip of the tongue. After tight ligatures had been secured around each mandibular ramus, the lower jaw was divided in the midline. The muscles of the floor of the mouth and oral mucosa were divided by incisions on the left and right sides of the mandible. To mobilise the tongue sufficiently to expose the soft palate and epiglottis, a further incision along the medial border of the mandible was extended posteriorly, taking care to avoid damaging the lingual arteries. The tongue was ligated at its base while the suture placed earlier in its tip was used to maintain the tongue retracted from the soft palate. Using an iris electro-cautery, a midline incision was made in the soft palate, extending from the posterior edge of the hard palate to about 1 mm anterior towards the epiglottis, and laterally to the pterygoid process of the sphenoid bone. The underlying mucosa of the posterior pharyngeal wall was scraped away with cotton wool. The rest of the procedure was carried out with the aid of a binocular

microscope. A hole was drilled in the outer table of the basispheroid bone, approximately 1 mm caudal to the transverse venous sinus. This was filled with bone wax (Ethicon Ltd, Edinburgh) and gradually extended anteriorly through the venous sinus to the basispheroid-prespheroid suture. Haemorrhage during this process was minimised by packing with bone wax at frequent intervals. Drilling was performed using a minimum amount of downward pressure. The inner palate was gradually exposed and drilled to uniform thinness which would yield to touch and enable its removal with watchmaker's forceps. Any bleeding beyond this stage was controlled using gentle pressure for a few minutes with cotton wool.

The dura mater overlying the median eminence and the pituitary stalk was incised in a V-shape with a triangular fragment of stainless steel razor blade held in a pinchuck. The apex of the incision was made to lie just anterior to the median eminence with base at the junction of the pituitary stalk and the anterior pituitary gland. The flap of the dura mater and similar flap of arachnoid mater were carefully retracted caudally with jeweller's forceps revealing the portal vessels (see Figure 2.2a). After ensuring no bleeding was taking place, 2500 i.u. of Heparin (Weddel Pharmaceuticals Ltd, London) were injected into the external jugular vein.

The pituitary stalk was cut with iridectomy scissors (Weiss Ltd, London, B1053R) at the junction with the pituitary gland in order to include as many portal vessels as possible. The blood pooled in the trough of the basi-spheroid bone was aspirated slowly, every 20-40 seconds, with a glass pasteur pipette (1.0 - 1.2 mm internal diameter) and stored in small plastic tubes (PT 15734, Luckhams Ltd, Sussex) on ice. In the majority of the animals, the pituitary stalk was cut and

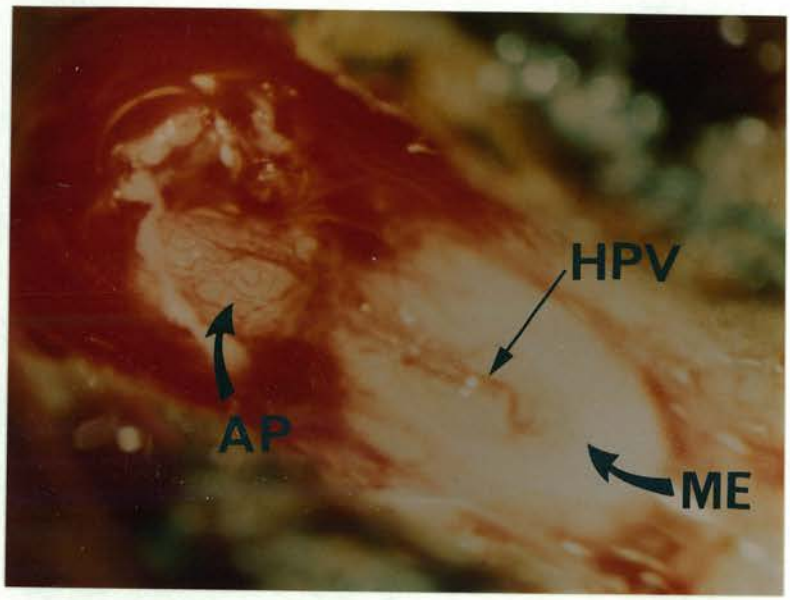


FIGURE 2.2a: The anterior pituitary gland (AP), hypophysial portal vessels (HPV) and median eminence (ME) after removal of the dural membranes, as seen under a Zeiss dissecting microscope. The transpharyngeal approach described by Worthington (1966) was used to expose the stalk.

collection of portal blood begun within 20-25 minutes of injection of the anaesthetic. In order to minimise protease inactivation of the peptides cholecytokinin and vasoactive intestinal peptide, blood samples were collected on ice in tubes in which the protease inhibitor Trasylol (5,000 Kallikrein Inactivator Units (KIU)/ml blood) (Bayer Pharmaceuticals Ltd) and ethylenediaminetetraacetic acid (EDTA) (5.4 mM/l blood) were added. At the end of the collection period, the volume of blood was recorded before centrifugation at 4°C at approximately 2500 g (MSE Mistral 2L Centrifuge) for 15 minutes. The plasma was stored at -40°C until assayed for peptides and monoamines.

2.2.6 Peripheral Blood Sampling and Storage

Peripheral blood samples were obtained from the external jugular vein of anaesthetised animals. To expose the vein, a longitudinal incision (length 2 cm) was placed 1.0 - 1.5 cm anterior to the right clavicle. The required amount of blood was withdrawn from the vein using syringes previously heparinised with 1000 i.u./ml heparinised saline, attached to 25 gauge hypodermic needles. The needle was introduced into the vein through the pectoralis major muscle. This method of blood sampling enables frequent collections without further surgery. In between collection periods the vein was covered with cotton wool moistened with saline. In experiments where plasma PRL levels were to be measured (Chapters IV & V), the volume of blood removed from the jugular vein was replaced immediately with warm isotonic saline. The blood samples obtained were immediately transferred from the syringes into plastic tubes (LP3, Luckham Ltd, Sussex) which had been cooled on ice and were thus maintained until centrifuged at 4°C. The plasma obtained was removed using pasteur pipettes into plastic tubes (Eppendorfs No. 72.690, Sarstedt, West Germany) and stored at -40°C until assayed.

2.2.7 Electrode Implantation and Electrical Stimulation Parameters

Manufacture: In all experiments involving electrical stimulation of various parts of the brain, platinum electrodes were used to avoid electrochemical stimulation of the brain by deposition of iron which occurs when steel electrodes are used. These were constructed from 10-13 mm lengths of platinum wire (Precious Metals Ltd, London), 0.125 mm in diameter. Glass tubing was drawn to an inside diameter sufficient to tightly encase the wire and fixed in place with cement (Araldite, Ciba-Geigy, Cambridge), leaving 0.3 mm bare at one end.

Insulated copper wire was soldered to the other free end of the platinum wire, and the whole assembly was mounted on a teflon jig, and fixed in place with acrylic cement (Dental Fillings Ltd, London). The overall length of the electrodes was 11-13 mm with a bared tip 0.3 mm in length. Both single and twin unipolar electrodes (parallel and separated by 1-2 mm) were used, depending on the area of the brain to be stimulated.

Implantation: Adult male rats (180-200 g) were anaesthetised with 10% Urethane (10 mg/kg i.p.). The head was fixed in a stereotaxic apparatus calibrated for use with the stereotaxic atlas of the rat forebrain by de Groot (1959); that is, the incisor bar was 5.0 mm higher than the intra-aural line. The scalp was incised in the midline to expose the dorsal surfaces of the frontal and parietal bone beneath the cranial periosteum which was removed before a hole (approximately 4 x 4 mm) was drilled in the required area of the skull. Bone fragments were carefully removed to avoid compression of the cerebral hemisphere. The cranial endosteum and dura mater were incised to facilitate electrode insertion, taking care to avoid laceration of the superior sagittal sinus. Any bleeding was controlled by light pressure with cotton wool. The electrode was then lowered into place using a triplanar micromanipulator (Prior, Bishop Stortford, Herts) and fixed in place with dental cement using stainless steel screws in the skull as anchors. The leads of the electrode were tucked subcutaneously as the wound was sutured and the animals were housed individually.

During the post-operative period, 300 mg of Aureomycin (Cyanamid (GB) Ltd, Gosport, chlorotetracyclin hydrochloride) and 25 mg of Glucose (BDH Chemicals Ltd) was added to each litre of the rats' drinking water.

Stimulation parameters: The electrical stimulus used was based on experiments by Fink and Aiyer (1974), and Jamieson and Fink (1976). The stimulus was applied for a period of 30 minutes using a simple relay circuit delivering accurately balanced, biphasic square wave pulses with a frequency of 60Hz, pulse duration of 1 ms and pulse amplitude of 500 µA (1 mA peak to peak). The stimulus was produced by a constant current stimulator (Neurolog, Digitimer Ltd, Herts) in a 30 sec on 30 sec off sequence, and monitored on a calibrated oscilloscope (Model S51B, Telequipment, London).

Electrode site verification: On completion of the experiment, the animals were decapitated and the skull placed in 10% buffered formalin. After fixation, the dental cement securing the electrodes was dissolved with chloroform (BDH Ltd, Poole) and the electrodes were carefully withdrawn. The brain was dissected out and placed in 30% sucrose overnight. Coronal sections 75-100 µm thick were cut on a freezing microtome ('Frigomobil' model 1206, Reichert-Jung, Germany) and stained in 1% cresyl fast violet (details in Appendix). Electrode tracks and their final placement were verified by examining the stained serial sections with the aid of an optical microscope.

2.2.8 Pituitary Transplantation

Adult male rats (130-150 days old, 200-240 g b.w.) of the inbred PVG strain supplied by Banting & Kingman Ltd (Hull) were used as donors and recipients of anterior pituitary glands. Anterior pituitary glands were dissected away from the posterior pituitary and intermediate lobe and placed in saline. Two anterior pituitary glands per rat were grafted under the kidney capsule of a recipient using light ether anaesthesia. After spraying with antibiotic (Rikospray, Riker

Laboratories, Loughborough) the flank incisions were closed by placing one stitch in the muscle and metal clips to hold the skin together.

Forty-two days later, when pituitary stalk blood was collected from the implanted rats, the adequacy of vascularisation of the grafted pituitary glands was determined by examination of the graft under a dissecting microscope. In addition, the rats were perfused with Indian ink at the end of the collection of pituitary stalk blood and the kidney with the pituitary transplant dissected out and fixed in buffered formalin. The kidneys were wax-embedded and 8-10 µm thick sections were stained with either haematoxylin-eosin or orange fuchsin green (OFG; Slidders, 1961). Elevated plasma PRL concentrations in the rats with anterior pituitary transplants (Chapter IV) confirmed that the grafts had been functional. Details of the methods and solutions used to prepare the histological sections are given in the Appendix.

2.3 Radioimmunoassays

2.3.1 Introduction

The basis of radioimmunoassays (RIA), in broad terms is competition between labelled and unlabelled molecules for a limited number of binding sites on a specific reactor (e.g. the specific antibody). A progressive inhibition of the binding of labelled molecules occurs as the concentration of the unlabelled molecules is increased. The concentration of a substance in an unknown sample can be determined by comparing the degree of inhibition observed in the sample with that produced by known standards. Several reviews have been published on the basic principles, theory and techniques of RIA (Midgley, Niswender and Rebar, 1969; Kirham and Hunter, 1971; Rodbard, 1971; Abraham, 1974; Ekins, 1974; Yalow, 1980). Therefore, only the methods which were

used to measure rat plasma concentrations of peptide hormones in the studies described in this thesis are discussed.

Ovine LH, rat FSH, rat PRL, synthetic LHRH, porcine VIP and synthetic unsulphated CCK-8 were labelled with I^{125} (NaI^{125}, Amersham International, Bucks.), using modifications of the chloramine-T method of Greenwood, Hunter and Glover (1963). The double antibody technique (Utiger, Parker and Daughaday, 1962) was used to separate the 'free' hormone from the antibody-hormone complex or 'bound' hormone in the assays of LH, FSH, PRL and LHRH. 'Free' VIP and CCK was separated from 'bound' hormones by selective adsorption to silicate and ion exchange resin respectively. These assays were done in the laboratories of Dr. W. Rotsztejn (Inserm U55, Service de Diabétiologie et d'Études Radioimmunologiques des Hormones Protéiques, Hôpital Saint-Antoine, Paris) and Professor G.J. Dockray (Department of Physiology, Liverpool University, England). Details of the methods for radioisotope labelling and preparation of stock solutions for LH, FSH, LHRH and PRL assays are given in the Appendix.

The 'bound' hormone was counted using an automatic Gamma Counter (Berthold Mag 310, Scotlab, Lanarkshire) and the concentration of the peptides was obtained by linear regression of the standard curves of logit B/B_0 on the ordinate against log concentration on the abcissa; B/B_0 = cpm of standard of sample minus background/cpm of total bound minus background; ie, logit $B/B_0 = \ln(B/B_0/1-B/B_0)$. The lower limit of sensitivity of the assay was determined by calculating mean counts per minute (cpm) minus 2 x standard deviation of the total bound tubes (Rodbard, 1971).

The coefficient of variation between assays for any one peptide was monitored by adding two pools (high and low) to each assay. The

pools were plasma from ovariectomised rats for both the high and low pools in the LH and FSH assays. Plasma from male rats with either an acid extract of rat hypothalamic tissue to give the high pool or synthetic LHRH to give the low pool, were used for the LHRH assay. In addition, the variation of the 'total counts' (labelled hormone), 'blanks' (zero antiserum added) and the 'total bound' (zero unlabelled hormone) as a percentage of the total counts to give % bound and the equivalent concentrations of the 20%, 50% and 80% B/B_0 was also recorded for each assay.

2.3.2 Radioimmunoassays of LH and FSH

The concentration of LH and FSH was measured in rat plasma and homogenates of anterior pituitary glands.

The ovine-ovine RIA developed by Niswender, Midgley, Monroe and Reichert (1968) was used to measure the LH concentration. Details of the method used have been described by Aiyer (1974), Aiyer and Fink (1974) and Henderson (1976) and are outlined in Appendix A1. The ovine pituitary LH (LER - 1056 - C2) used for iodination was provided by Dr. L.E. Reichert Jr. (National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases [NIADDK], Baltimore, U.S.A.) and ovine LH (NIH - LH - S18) used to prepare the standards (range 0.25 - 16 ng/ml) was provided by the NIADDK. The hormone-specific antibody, antiovine LH (GDN 15) was raised in rabbits and provided by Dr. G.D. Niswender (NIADDK). The antibody was used at a final dilution of 1 : 240,000. The second antibody, used to separate 'bound' hormone from 'free' hormone was anti-rabbit gamma globulin (ARGG), raised in donkeys against rabbit gamma globulins (Wellcome Reagents Ltd, Beckenham, Kent). The antibody was used at a final dilution of 1 : 150.

The concentration of FSH in rat plasma was determined by an RIA method based on the method used by Daane and Parlow (1971) and described in detail by Aiyer (1974), Jamieson (1974), Chiappa (1976) and Henderson (1976). An outline of the method used is given in Appendix A.1. The hormones used for the iodination and preparation of standards (range 62.5 - 4000 ng/ml) were NIADDK rat FSH-I-3 and NIADDK rat FSH-RP-1, respectively. The hormone-specific antibody was NIADDK anti-rat-FSH-56, raised in rabbits and used at a final dilution of 1 : 10,000. The ARGG was used at a final dilution of 1 : 100.

The standard curves of representative assays of LH and FSH are shown in Figure 2.3a and 2.3b, respectively. The quality control data for LH and FSH assays are shown in Figures 2.3c and 2.3d, respectively. The interassay coefficient of variation for the LH high pool was 10% (mean 39.7 ng/ml, n=4) and 10% for the LH low pool (mean 16.4 ng/ml, n=4). For the FSH high pool the coefficient of variation was 9% (mean 758.8 ng/ml, n=3) and 14% for the FSH low pool (mean 424.3 ng/ml, n=3). The lower limits of sensitivity for 20 µl plasma samples were between 4.0 and 6.0 ng/ml for the LH assay and between 2.0 and 3.0 µg/ml for the FSH assay.

2.3.3 Radioimmunoassay of LHRH

The double antibody RIA developed by Nett, Akbar, Niswender, Hedlund and White (1973) was used for measuring the concentration of LHRH in rat pituitary stalk plasma samples. The method has been described by Jamieson (1974), Fink and Jamieson (1976) and Chiappa (1976) and is outlined in Appendix A.2. Synthetic LHRH (ICI Pharmaceuticals, Macclesfield) was used for iodination and to prepare standards (range 7.8 pg/ml - 500 pg/ml). The hormone specific

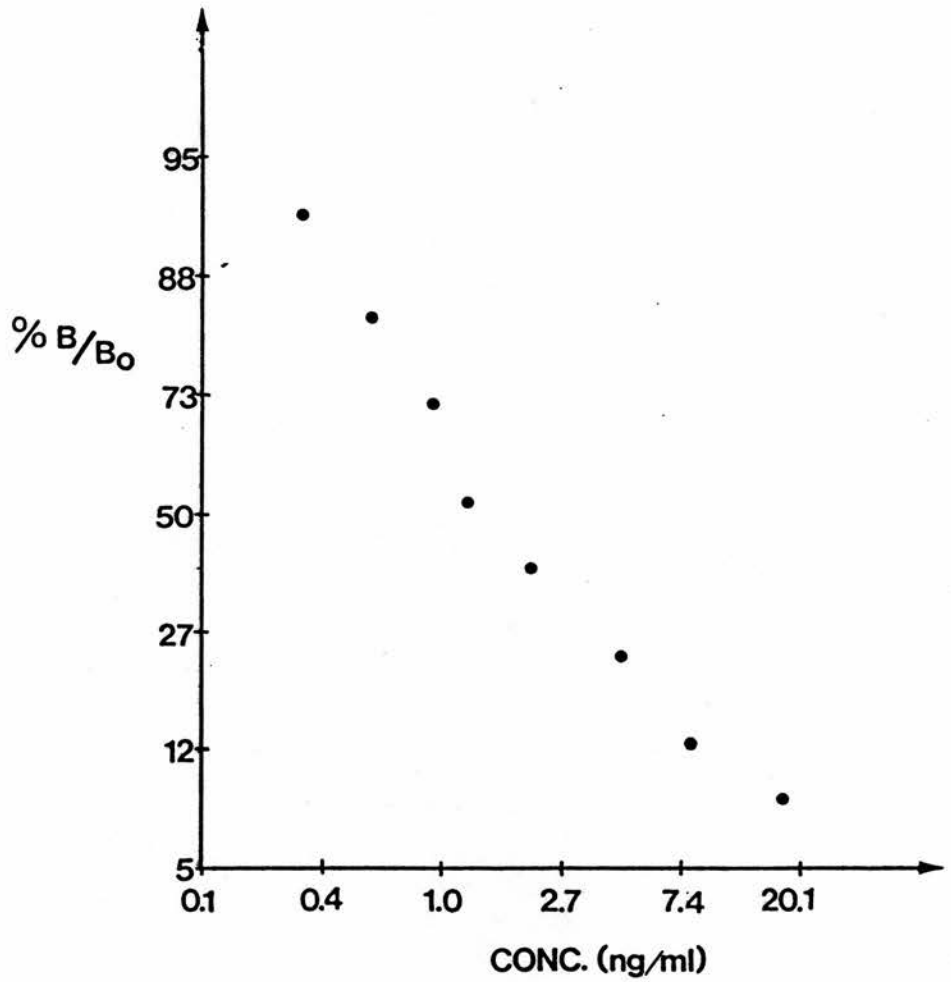


FIGURE 2.3a: Standard curve of a representative assay for luteinising hormone (LH). Each point represents the mean of 3 replicates.

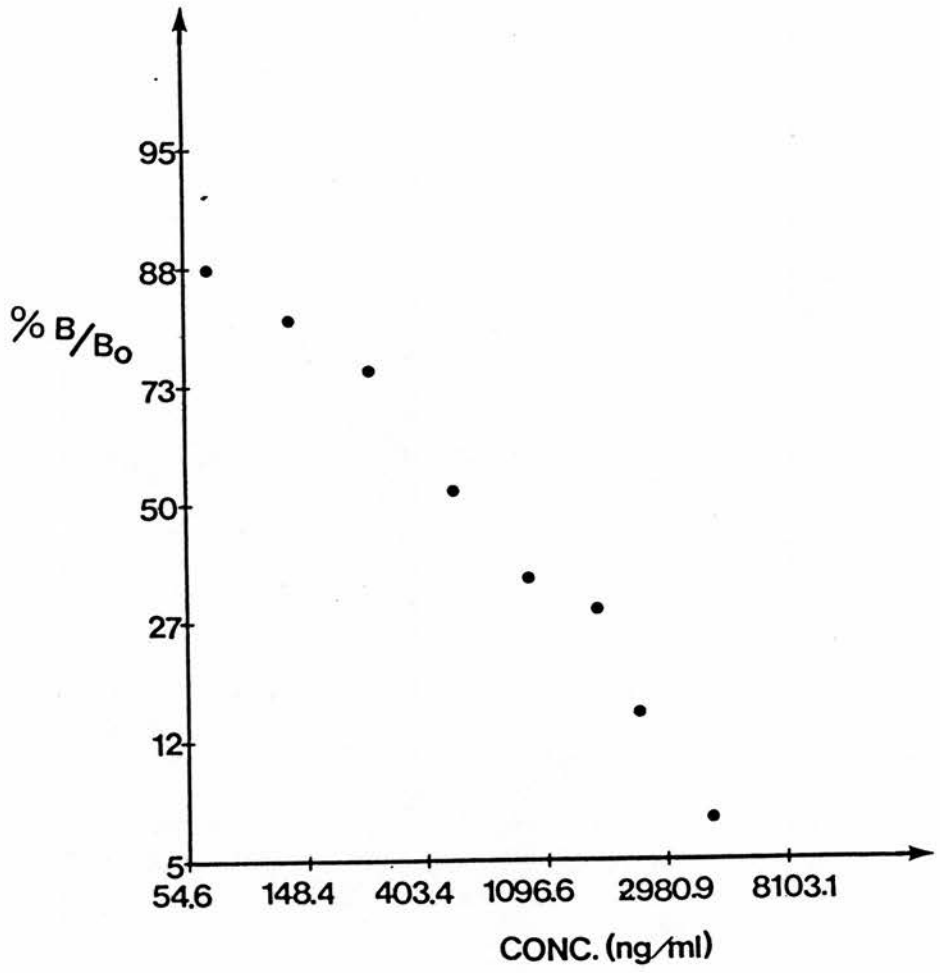


FIGURE 2.3b: Standard curve of a representative assay for follicle-stimulating hormone (FSH). Each point represents the mean of 3 replicates.

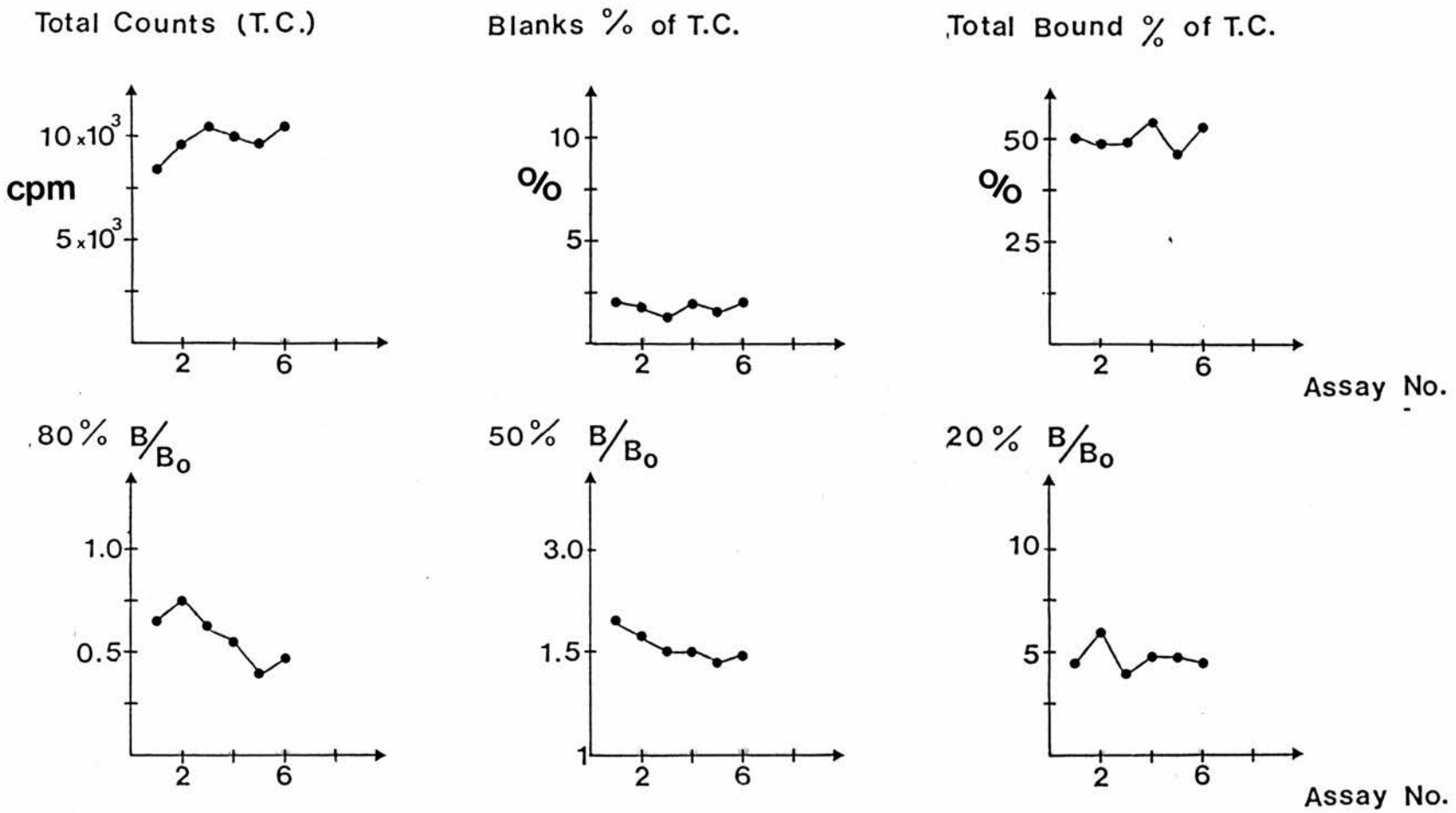


FIGURE 2.3c: Quality control data for the luteinising hormone (LH) assays.

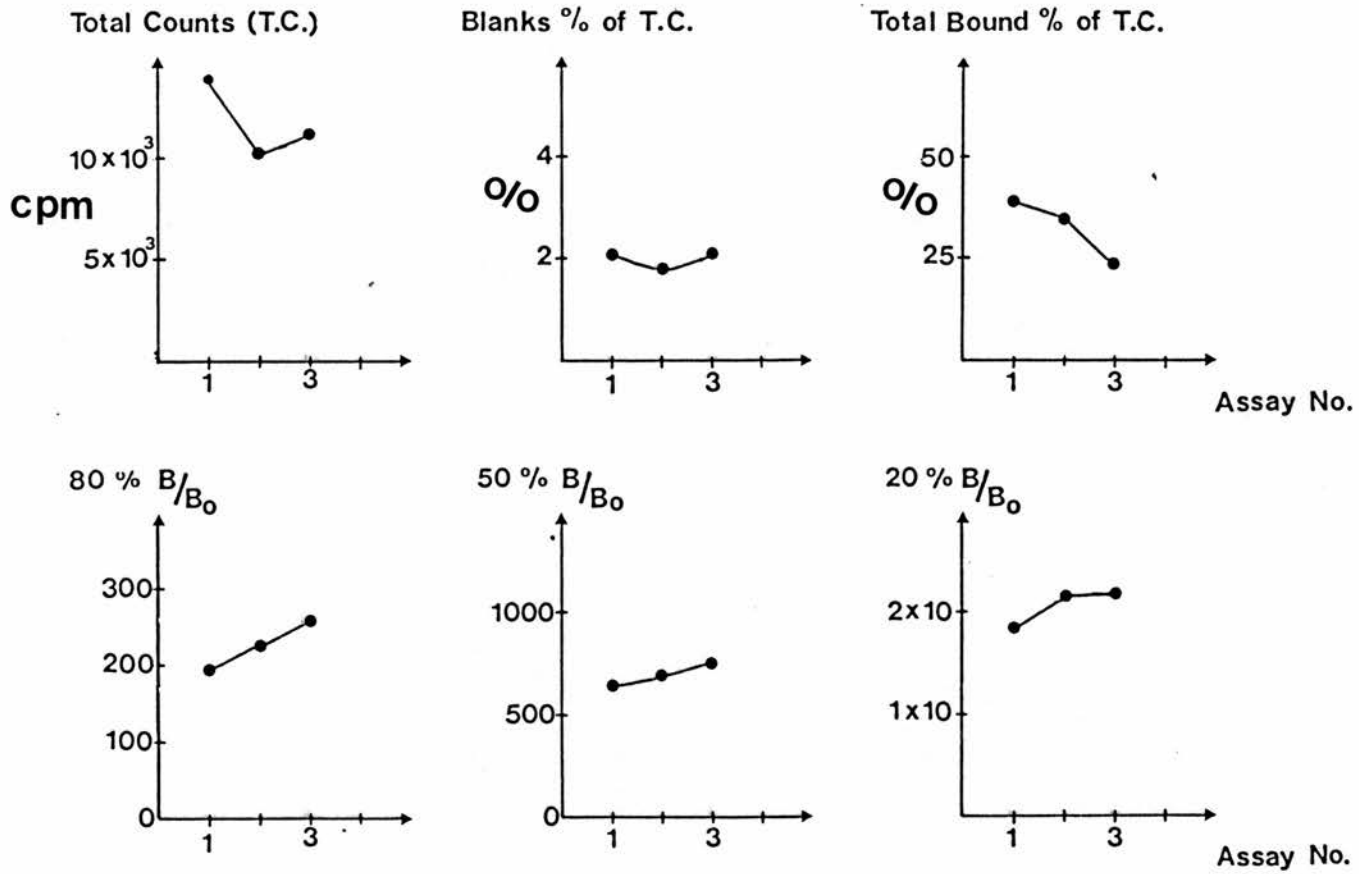


FIGURE 2.3d: Quality control data for the follicle-stimulating hormone (FSH) assays.

antibody (R-42-anti-GnRH), raised in rabbits was provided by Dr. G.D. Niswender (NIADDK) and used at a final dilution of 1 : 32,000. This antibody recognised the whole LHRH molecule and did not cross-react with LHRH analogues and LHRH fragments (Nett *et al.*, 1973) or various peptides and pituitary hormones (Nett *et al.*, 1973; Chiappa, 1976; Jamieson and Fink, 1976). The second antibody, ARGG (Wellcome Reagents Ltd, Beckenham, Kent) was used at a working dilution of 1 : 150 to separate the 'free' hormone from the 'bound' hormone.

A standard curve of a representative assay is shown in Figure 2.3e and the quality control data are shown in Figure 2.3f. The standards in assays number 5 and 6 displayed a shift in the 80, 50 and 20% B/B_0 values. In order to standardise the data, the values for LHRH concentration obtained in the plasma samples assayed in assays number 5 and 6 were divided by the ratio of the $\%B/B_0$ of the standard curve for assays 5 and 6 and those in the other assays. The interassay coefficients of variation were found to be 21.5% and 23.6%, respectively, using a high pool (mean 221.5 pg/ml, n=3) and a low pool (mean 36.6 pg/ml, n=4). The lower limit of sensitivity of this assay for 100 µl samples of plasma ranged from 10-12 pg/ml.

2.3.4 Radioimmunoassay of PRL

Double antibody RIA kits supplied by the NIAMDD were used to determine the concentration of PRL in rat plasma samples. The assay has been described by Pickering (1978) and is outlined in Appendix A.2. The hormone, rat-PRL-I-5 (NIADDK) was used for iodination and rat-PRL-RP-1 (NIADDK) used to prepare reference standards (range 0.5 - 64 ng/ml). The hormone specific antibodies were anti-rat-PRL-S5 (NIADDK) used at a final dilution of 1 : 20,000. The second antibody, ARGG (Wellcome Reagents Ltd, Beckenham) was used

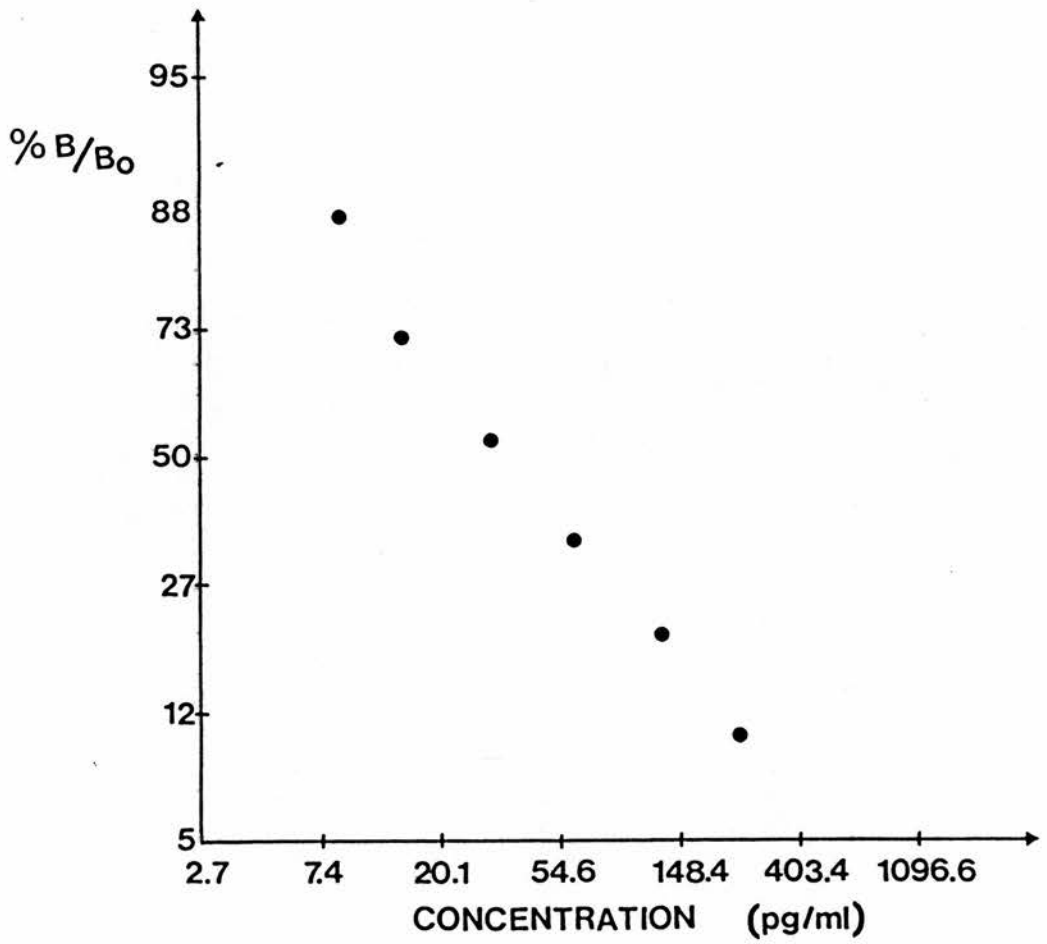


FIGURE 2.3e: Standard curve of a representative assay for luteinising hormone-releasing hormone (LHRH). Each point represents the mean of 3 replicates.

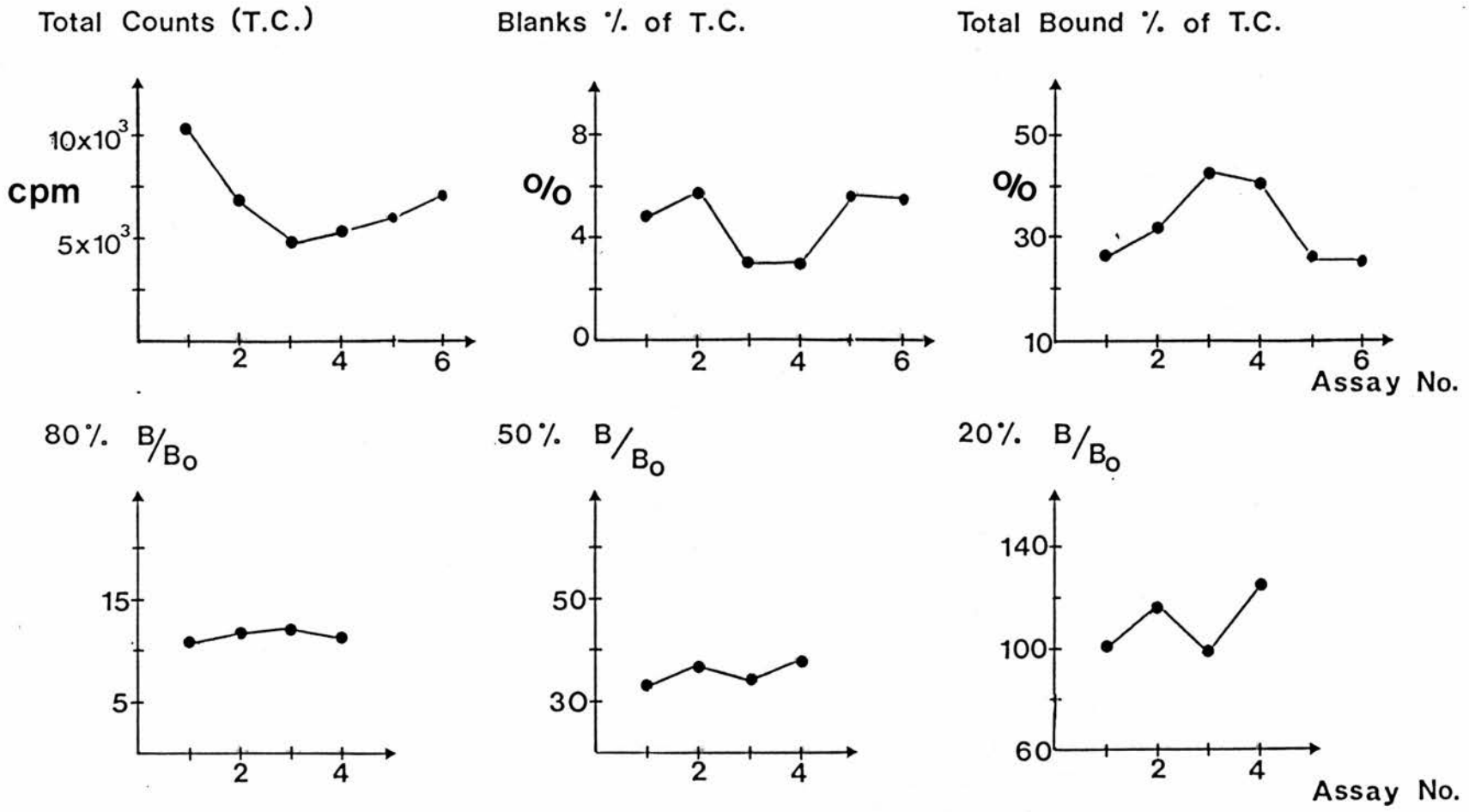


FIGURE 2.3f: Quality control data for the luteinising hormone-releasing hormone (LHRH) assays.

at a final dilution of 1 : 150. A representative standard curve and quality control data for the assay are shown in Figures 2.3g and 2.3h, respectively. Using a high pool (mean 242.3 ng/ml, n=3) and a low pool (mean 33.7 ng/ml, n=3), the interassay coefficients of variation were 12.0% and 13.0%, respectively. The lower limits of sensitivity of the assays for 20 µl samples of plasma ranged from 20.0 - 30.0 ng/ml.

2.3.5 Radioimmunoassay of VIP

The VIP concentration in rat plasma was determined by RIA based on the method described by Maletti, Besson, Bataille, Laburthe and Rosselin (1980) and Maletti, Rostene, Carr, Sherrer, Rotten, Kordon and Rosselin (1982), and done by Dr. W.H. Rotsztejn (Hôpital Saint-Antoine, Paris). Plasma samples collected in Edinburgh were sent by air freight to Paris packed in dry ice in insulated polystyrene containers. The samples remained frozen at all times.

The sensitivity of the assay was approximately 0.5 ng/ml. Figure 2.3i shows a typical standard curve for the VIP assay. The specificity of the VIP antibody was directed to a fragment of the carboxyl-terminal and to the medium part of the VIP molecule, as confirmed by the cross-reaction of the antibody with the VIP synthetic fragment 10-28 (Maletti *et al.*, 1982). There was approximately four orders of magnitude difference in the binding capacity of the antibody to VIP compared to the structural analogues of VIP, gastric inhibitory peptide (GIP) and porcine peptide having N-terminal histidine and C-terminal isoleucine-amide (PHI; Maletti *et al.*, 1982). The interassay variation was 15%.

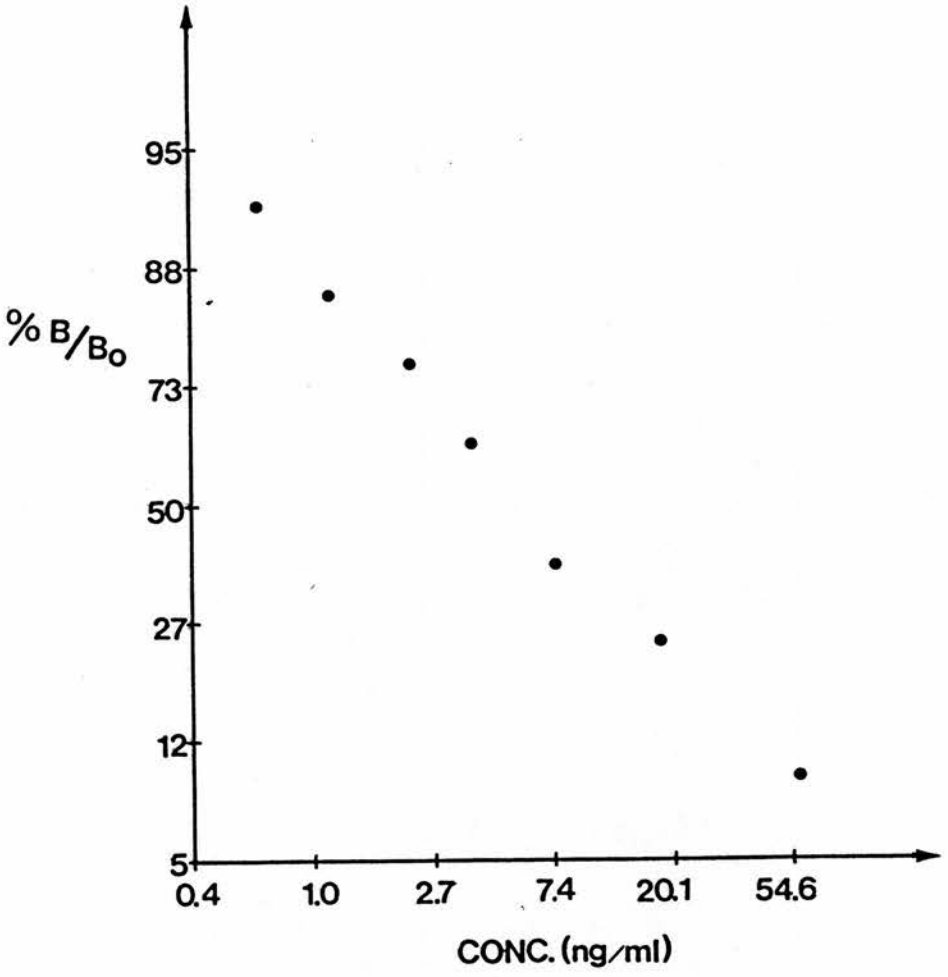


FIGURE 2.3g: Standard curve of a representative assay for prolactin (PRL). Each point represents the mean of 3 replicates.

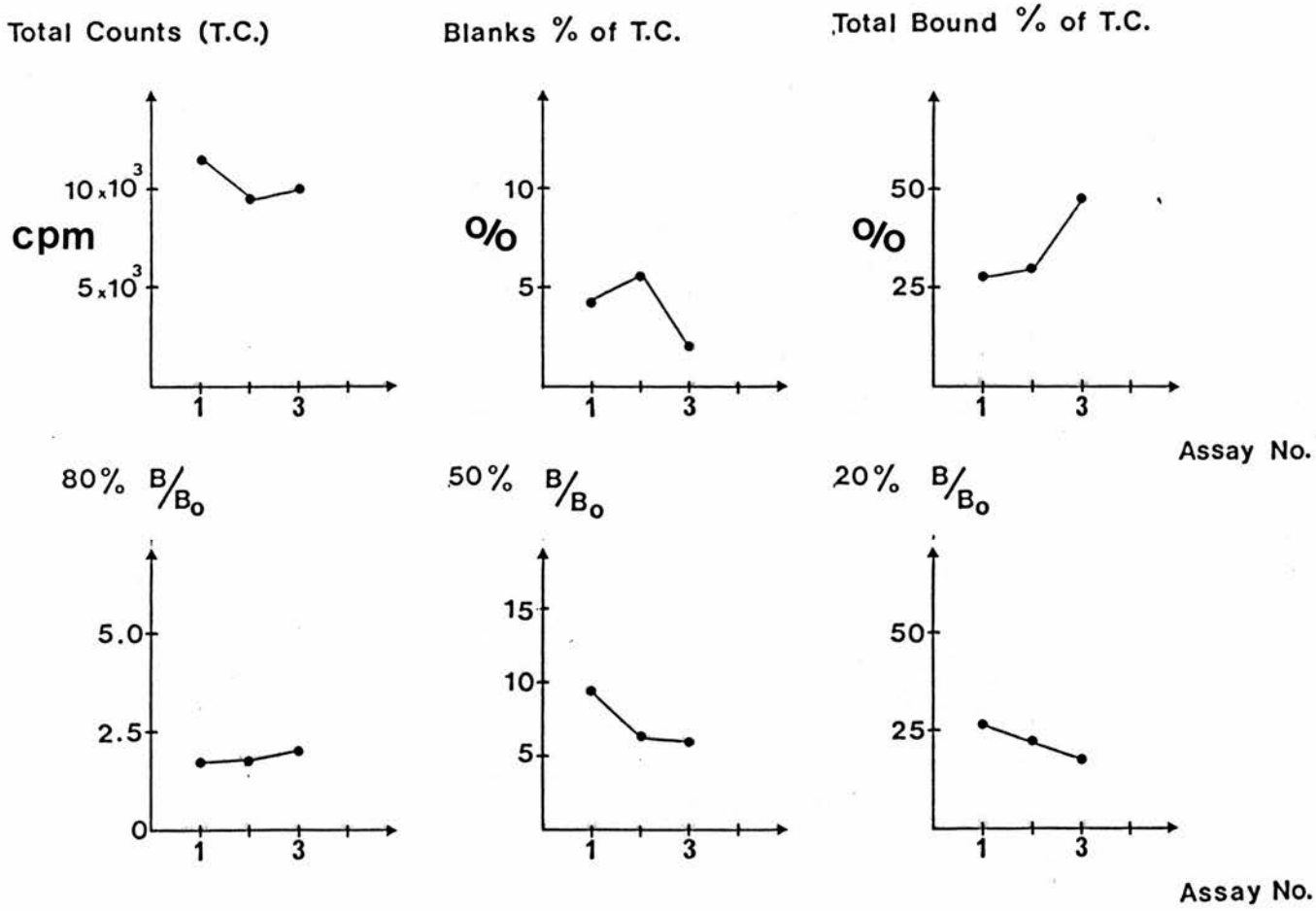


FIGURE 2.3h: Quality control data for the prolactin (PRL) assays.

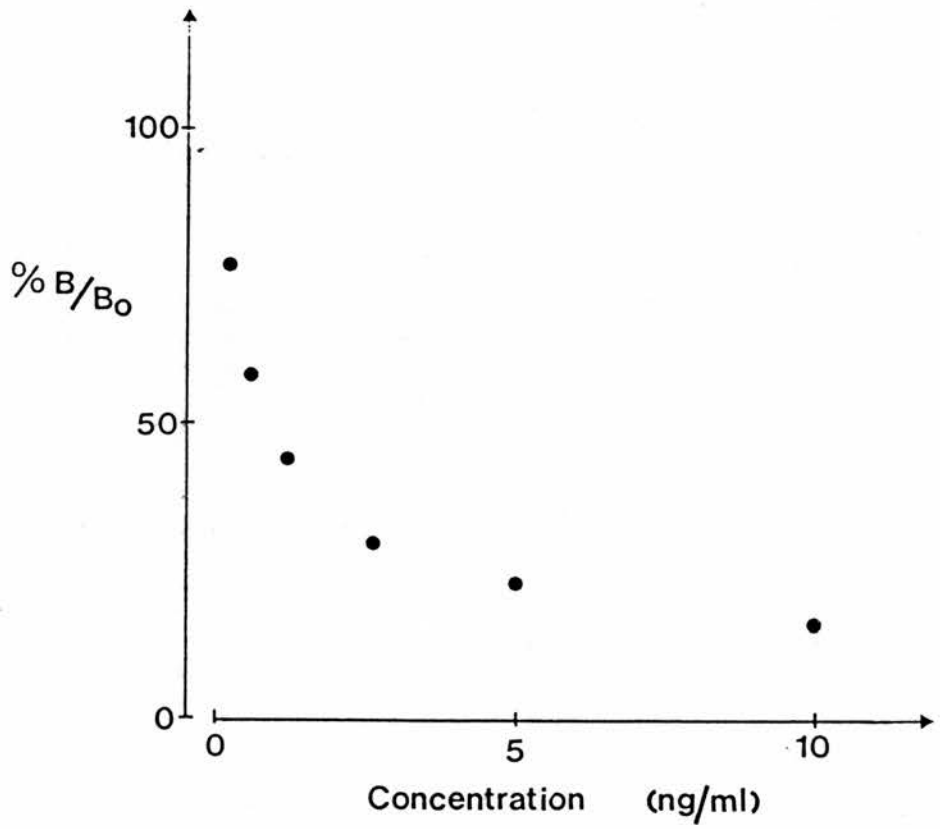


FIGURE 2.3i: Standard curve for a representative assay for vasoactive intestinal polypeptide (VIP; Rotsztejn, collaborative data).

2.3.6 *Radioimmunoassay of CCK and Gastrin*

These assays were done by Professor G.J. Dockray (Liverpool University). Plasma samples were sent to Liverpool packed in polystyrene containers containing dry ice. The samples remained frozen at all times. Antibodies with differing specificities for the C-terminal pentapeptide sequence of CCK and gastrin were used (discussed in Section 6.1). The method for the RIA of CCK and gastrin is described in detail by Dockray and Taylor (1976) and Dockray (1980), respectively. The standard curve of a representative assay of CCK is shown in Figure 2.3j. The standard curve of gastrin parallels that of CCK (Dockray, personal communication). The lower limits of the sensitivity of the CCK and gastrin assays were 20 pmol/l and 5 pmol/l respectively.

2.4 High Performance Liquid Chromatography (HPLC)

2.4.1 *Introduction*

Several groups have described the use of liquid chromatography with electrochemical detection (LCED) for the measurement of plasma catecholamines (Refshauge, Kissinger, Dreiling, Blank, Freeman and Adams, 1974; Hallman, Farnebo, Hamburger and Jonsson, 1978; Hjemdahl, Daleskog and Kahan, 1979; Mefford, Gilberg and Barchas, 1980). This technique has proved to be a highly selective, extremely sensitive and also a rapid, inexpensive, non-destructive analytical method. LCED also has the advantage of allowing the measurement of several catecholamines and their metabolites simultaneously from one sample. It has therefore almost replaced other assay techniques such as the previously extensively used radioenzymatic method (Coyle and Henry, 1973; da Prada and Zurcher, 1976; Peuler and Johnson, 1977) which is more time-consuming and expensive per sample compared with

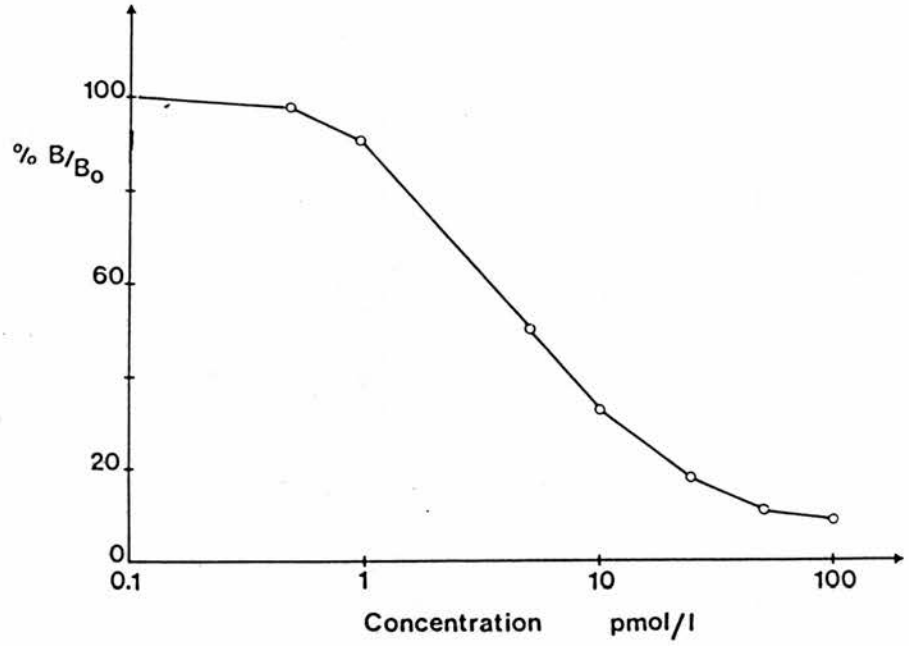
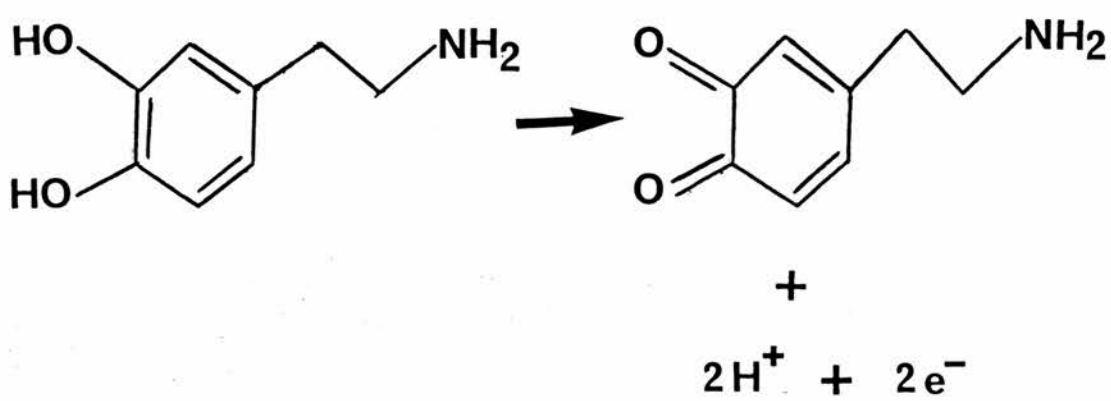


FIGURE 2.3j: Standard curve of a representative assay for cholecystokinin (CCK; Dockray, collaborative data).

LCED, although smaller volumes of plasma may be used. The principles and advantages of LCED have been extensively reviewed by Mefford (1981). The technique consists of a simple liquid-solid extraction of the catecholamines on alumina followed by their elution with dilute acid. The alumina is selective for catecholamines and enables their concentration prior to separation with liquid chromatography and subsequent quantification by electrochemical detection.

In order for a compound to be amenable to electrochemical detection, it must have electrochemical ability, i.e. it must undergo redox reactions in such a way as to accommodate the loss or addition of one or more electron. This rearrangement must occur within certain limits of electrical potentials. These limits are 'fixed' by the electrode material in which the electrode is placed. Carbon electrodes in aqueous solutions were used for the analysis of catecholamines in pituitary stalk plasma. Nearly all aromatic amines and phenols are oxidised between the potential limits of +0.5 and +0.7 volts with regerence to an Ag/AgCl reference electrode. The process of electrochemical oxidation converts the phenolic compounds to quinones. A typical two electron oxidative mechanism for catecholamines converting them to quinones can be represented by the following process:



The electrons are lost to the detector electrode, thus providing the basis for detection of these compounds. The current which is derived from these electrons is proportional to the concentration of the species in solution (Levich, 1962). The O-methylated metabolites of the catecholamines are more difficult to oxidase as the methoxy group must be cleared. This requires a higher energy of activation and therefore a higher electrochemical detection potential. These differences in oxidation potential add selectivity and enable the differentiation between substances which oxidise at different potentials.

2.4.2 Materials and Methods

(a) Standards and reagents:

Analytical reagent grade chemicals were used without further purification. All solutions were prepared with distilled water. Stock solutions (100 µg/ml free base) of dopamine (DA), hydrochloride (HCl), noradrenaline (NA) HCl, adrenaline (A) HCl, dihydroxyphenylacetic acid (DOPAC) HCl and the internal standard n-acetyl-dopamine (nADA) HCl were prepared in 0.1M hydrochloric acid and 10^{-6}M ascorbate and were freshly diluted on the day of the experiment. All the standards were purchased from Sigma (Poole). Stock solutions were stable for up to two months if stored at 4°C in the dark. Acid washed alumina (Al_2O_3) was prepared according to Anton and Sayre (1962).

(b) Sample preparation:

Pituitary stalk blood and jugular venous blood was collected (Sections 2.2.5 and 2.2.6, respectively) into tubes placed on ice containing EDTA (5.4 mM/l) and ascorbic acid (50 mM/l). After centrifugation (15 min, 4°C, 600 g), the plasma was aliquoted into 100 µl aliquots and 2 ng of nADA, the internal standard, was added to each aliquot. The samples were then stored frozen at -40°C until analysis.

The plasma catecholamines were concentrated and extracted with alumina according to Hallman *et al.* (1979), after thawing. A summary of the procedure is presented in Figure 2.4a. After elution of the catecholamines from the alumina, 20 µl of supernatant or 20 µl of standard solution containing each of the NA, A, DA, DOPAC and nADA were injected into the column.

(c) The electrochemical detection system:

The system used was that described by Keller, Oke, Mefford and Adams (1976), shown systematically in Figure 2.4b. The samples were injected into the column by a Rheodyne 7125 rotary injection valve with a 20 µl injection loop. The amines were separated by reverse pair ion exchange chromatography on a 25 cm-long Hypersil-ODS (5 µ particle size) column packed on a Magnus P6050 slurry packing unit at 5000 psi. The injector and analytical column were mounted in a Faraday cage. The mobile phase was 0.1M potassium dihydrogen orthophosphate buffer containing 30 mg/l sodium octyl sulphate, 10% HPLC-grade methanol and 0.1 mM EDTA (pH 4.0), and degassed by applying a vacuum for 2 min then bubbling for 30 min with helium gas. This buffer was pumped at a constant flow rate of 0.8 ml/min by a Gilson 301 pump. The effluent then passed through a transducer cell (Bioanalytic Systems Inc., Luton, LC-17) with a carbon paste working electrode and an Ag/AgCl reference electrode (Figure 2.4c). The working potential was fixed at +0.6V relative to the reference electrode. The current flow was measured by an electronic controller unit (Bioanalytical Systems Inc., Luton, LC-17) which amplified and converted the signal to a voltage. The chromatograms were recorded on a Bryans (Surrey) Model BS 600.

(a) 100 µl plasma + 20 µl nADA (2 ng)

↓ *thaw frozen samples*

(b) add 20 µl trichloroacetic acid

↓ *centrifuge (5 min, 4°C, 600 g)*

(c) transfer supernatant

+

add 50 µl Na metabisulphate (1M)

+

25 mg acid-washed alumina

↓ *vortex (5 sec)*

(d) add 400 µl EDTA (1M) Tris buffer (pH 8.6)

↓

(e) shake continuously for 10 min, 4°C

↓

(f) wash Al_2O_3

x 2 with 1 ml Tris (0.5M) +
100 µl Na Metabisulphite (1M) per 100 ml water

+

x 3 with 1 ml distilled water

↓

(g) elute catecholamines into 60 µl HCl (0.5M)

↓

(h) Inject 20 µl into LCED system

FIGURE 2.4a: Flowchart of the extraction procedure for plasma catecholamines (based on Hallman *et al.*, 1978).

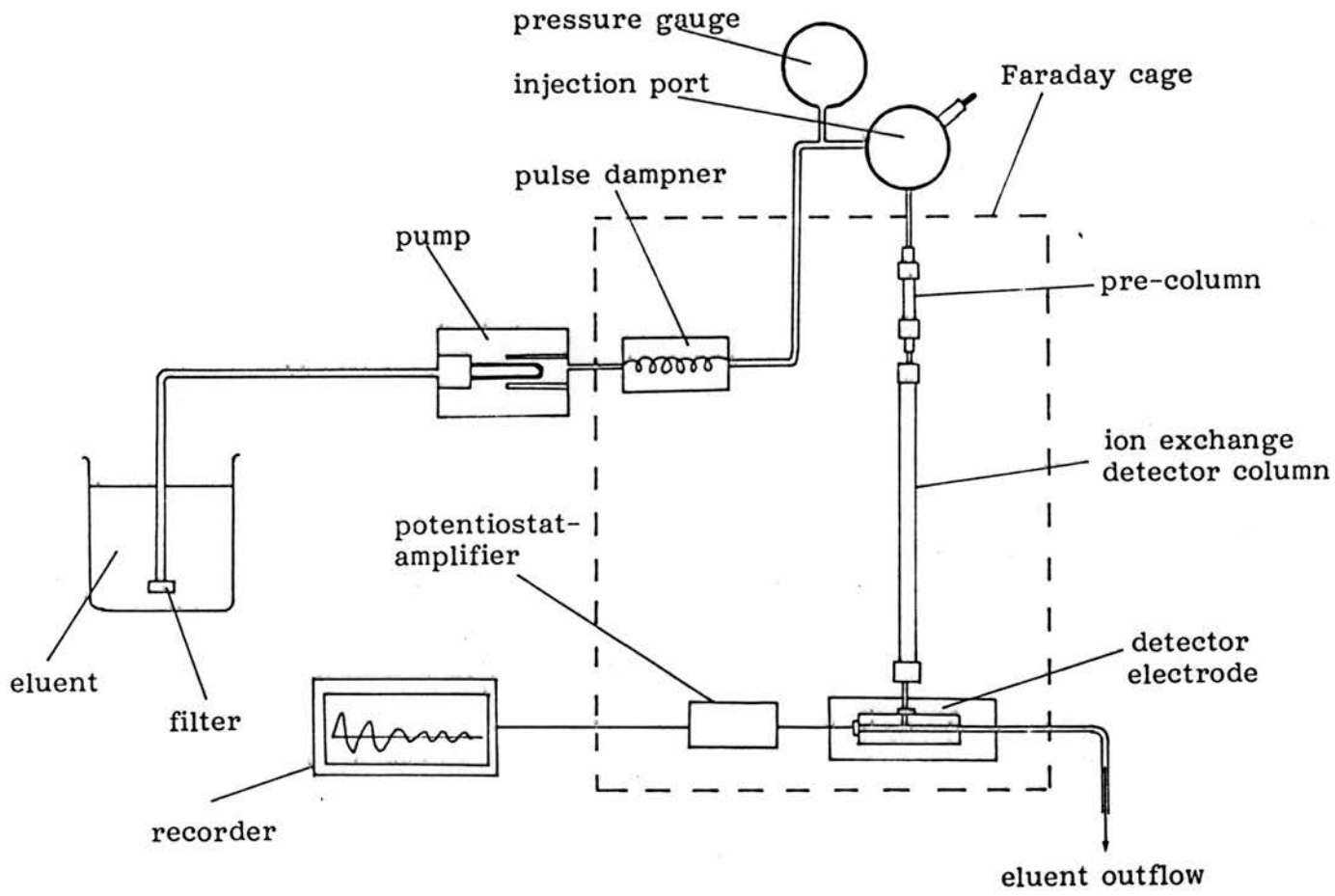


FIGURE 2.4b: A schematic diagram of the liquid chromatographic-electrochemical detector system.

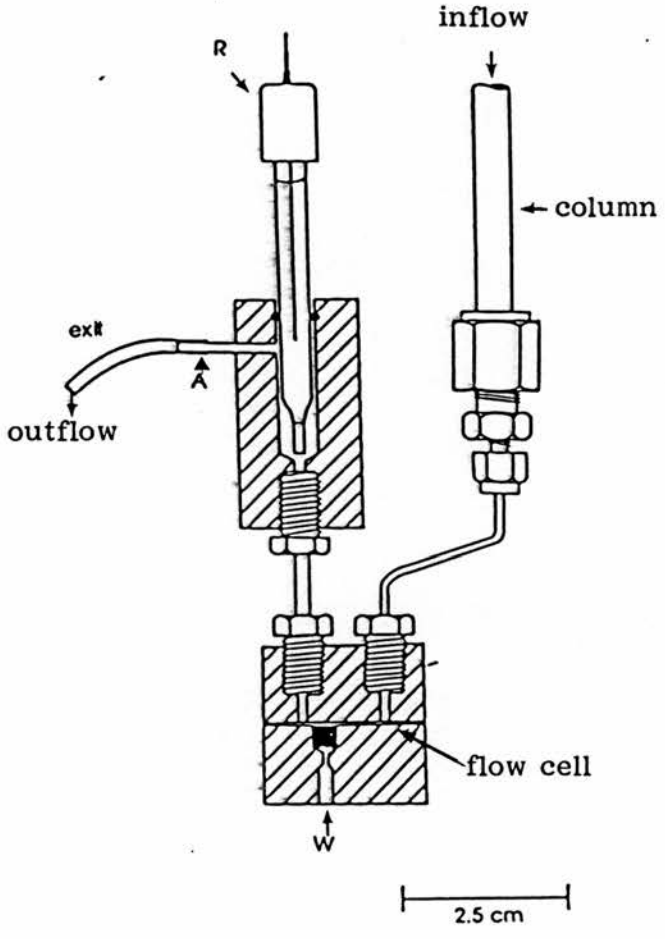


FIGURE 2.4c: Cross-section of a BAS LC-17 transducer cell;
W = working electrode; R = reference electrode;
A = auxiliary electrode.

2.4.3 *Calculations*

A typical chromatogram obtained after ion-pair separation of 2 ng each of DA, DOPAC and nADA is shown in Figure 2.4d. The retention times using the standard buffer at a flow rate of 0.8 ml/min were found to be approximately 7 min for DA, 10 min for DOPAC and 12 min for nADA. A constant amount of nADA (2 ng/20 µl) added to standard solutions and (2 ng/100 µl) aliquots of plasma before freezing, was used as the internal standard. Measurements of the peak height of each compound over a range of concentrations showed linearity (Figure 2.4e). The concentration of DA and DOPAC in rat plasma samples was determined by linear regression from the relevant standard curve, correcting for incomplete recovery (usually 60-70%) of the internal standard. External standards of various concentrations were run at various intervals to determine the sensitivity of the detector.

2.5 Statistical Analyses

All the data in this thesis have been given as the mean value with their standard error (± S.E.M.). Parametric statistical methods were then applied. The significance of increments was determined by the Students' paired t-test. The Students' unpaired t-test was used to assess the significance of a difference between two means. Differences between more than two means were compared by one-way analysis of variance and the multiple range test for heteroscadistic means (Duncan, 1955, 1957), with the modified tables of Harter (1960).

Non-parametric statistical methods were used (Chapters III and IV) when concentrations of LHRH in pituitary stalk blood samples were below the lower limit of the radioimmunoassay. A value equal to the lower limit

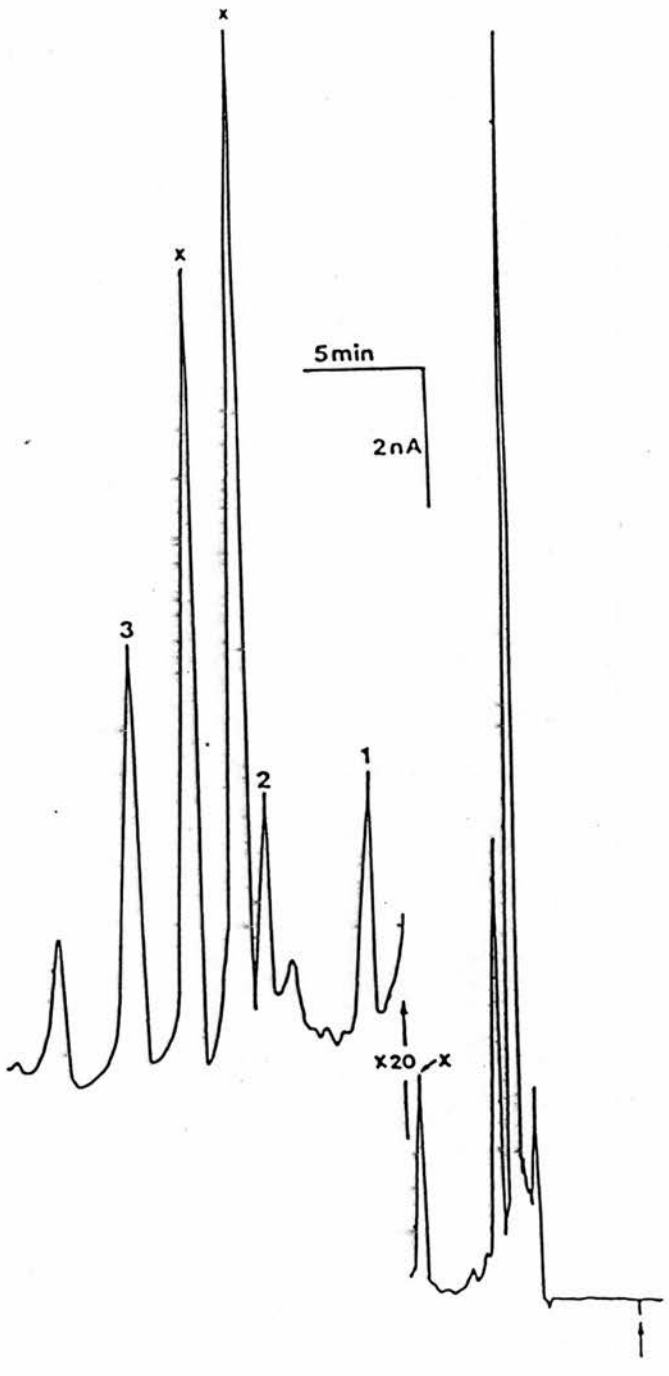


FIGURE 2.4d: A representative trace from the HPLC system using electrochemical detection. This trace shows the peaks obtained after injection of 20 µl of an alumina extract of pituitary stalk plasma.

Peak 1 = dopamine
Peak 2 = dihydroxyphenylacetic acid
Peak 3 = N-acetyl dopamine (internal standard)
Peak X = unknown peak, attributed to alumina extraction procedure.

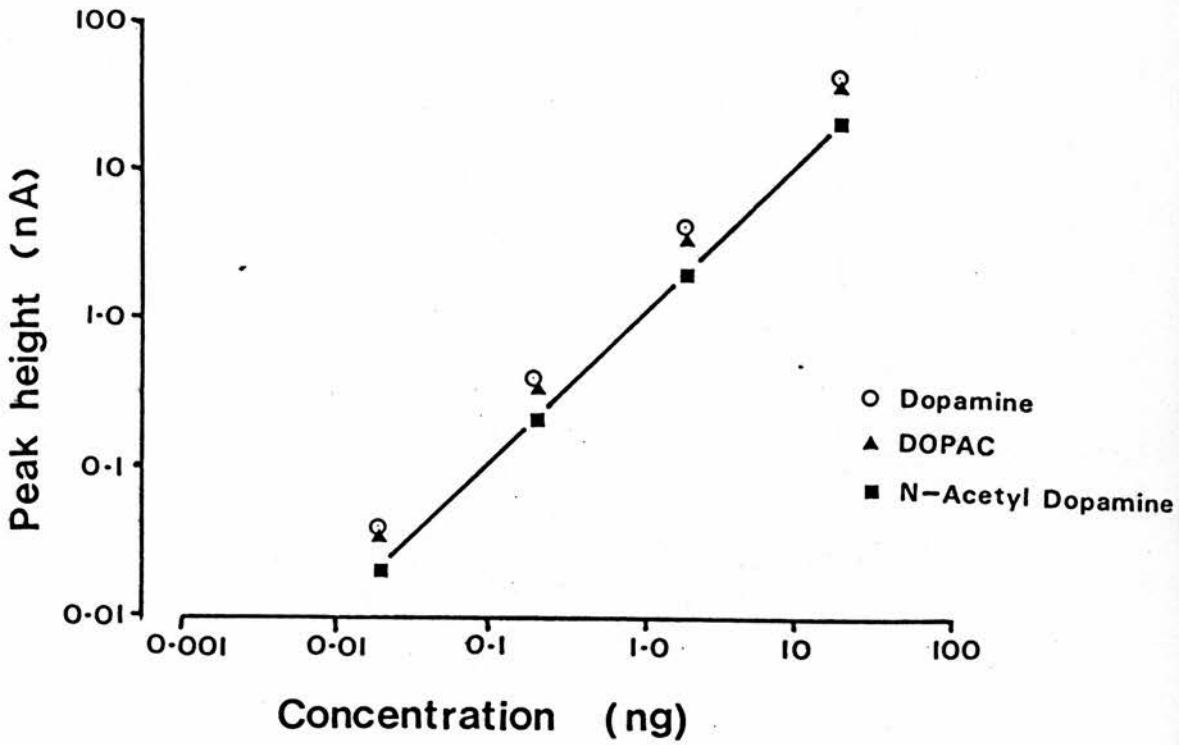


FIGURE 2.4e: Standard curves for dopamine, dihydroxyphenyl acetic acid (DOPAC) and N-acetyl dopamine (internal standard).

of the radioimmunoassay was assigned to the samples and the difference between the means of the groups containing these samples was determined by the Mann-Whitney U-test (Mann and Whitney, 1947), when two means were compared or the Kruskal-Wallis one-way analysis of variance by ranks (Kruskal and Wallis, 1952), when more than two means were compared.

Differences were accepted as being statistically significant if $p < 0.05$.

CHAPTER III

The Relationship Between Luteinising Hormone-Releasing Hormone (LHRH) Secretion and Pituitary LHRH Receptor Number in Long-Term Castrated Rats Following Gonadal Steroid Administration

CHAPTER III

3.1 Introduction

Gonadotrophin secretion in the male is controlled by the negative feedback of gonadal steroids (Moore and Price, 1932; Lee, Jaffe, Midgley, Kohen and Niswender, 1972; Kalra, Fawcett, Krulich and McCann, 1973; Damassa, Kobashigawa, Smith and Davidson, 1976; Cheung and Davidson, 1977; Kalra and Kalra, 1979). Castration markedly increases the release and synthesis of gonadotrophins (Gay and Midgley, 1969; Kingsley and Bogdanove, 1973; Badger, Wilcox, Meyer, Bell and Cicero, 1978; Nansel, Aiyer, Meinzer and Bogdanove, 1979), decreases the hypothalamic content of LHRH (Moguilevsky, Scacci, Debeljuk and Faigon, 1975a; Root, Reiter, Duckett and Sweetland, 1975; Shin and Howitt, 1976; Campbell and Ramaley, 1978; Rudenstein, Bigdeli, McDonald and Snyder, 1979; Gross, 1980; Kalra and Kalra, 1980) and increases the number of LHRH receptors in the anterior pituitary gland (Clayton and Catt, 1981; Frager, Pieper, Tonatta, Duncan and Marshall, 1981; Giguere, Lefabre and Labrie, 1981). The increase in LHRH binding after castration is due to an increase of the actual number of receptors, as opposed to an increase in receptor affinity (Frager *et al.*, 1979; Clayton, Solano, Garcia-Vela, Dufau and Catt, 1980).

Treatment of castrated rats with one of three gonadal steroids, testosterone (T), dihydrotestosterone (DHT) or 17β-oestradiol (OE_2), known to be present in intact males (Ewing, Desjardins, Irby and Robaire, 1977; Saksena, Lau and Chang, 1978; Saksena and Lau, 1979; Keel and Abney, 1980) reduced to normal, plasma LH and FSH concentrations (Cheung and Davidson, 1977; Badger *et al.*, 1978; Campbell and Ramaley, 1978; Clayton and Catt, 1981), pituitary LHRH receptor numbers (Clayton and Catt, 1981; Frager *et al.*, 1981)

and hypothalamic LHRH content (Kingsley and Bogdanove, 1973; Shin and Howitt, 1976; Campbell and Ramaley, 1978; Nansel *et al.*, 1979; Rudenstein *et al.*, 1979; Gross, 1980; Kalra and Kalra, 1980). A delay of one month before treatment of castrated rats with gonadal steroids, fully reduced plasma LH concentrations, but FSH secretion was only partially reduced towards the values in intact animals (Campbell and Ramaley, 1978; Conne, Scaglioni, Lang, Sizonenko and Aubert, 1982). Treatment of long-term castrated rats with gonadal steroids did not, however, suppress the post-castration increase in the number of pituitary LHRH receptors or the decrease in the hypothalamic content of LHRH (Conne *et al.*, 1982).

Although changes in hypothalamic LHRH content reflect a balance of synthesis, release and degradation, it has been suggested that a decrease in hypothalamic LHRH content after castration reflects an increase in secretion of LHRH from the hypothalamus (Root *et al.*, 1975; Shin and Howitt, 1976; Campbell and Ramaley, 1978; Gross, 1980). The demonstration that LHRH synthesis in hypothalamic slices incubated *in vitro* increased one month after castration (Moguilevsky, Enero, Szwarcfarb and Dosoretz, 1975), concomitant with a decrease in hypothalamic content of LHRH (Kingsley and Bogdanove, 1973; Shin and Howitt, 1976; Campbell and Ramaley, 1978; Rudenstein *et al.*, 1979; Gross, 1980; Kalra and Kalra, 1980), provides strong evidence for an increased post-castration release of endogenous LHRH. In addition, treatment with gonadal steroids reversed the castration-induced reduction in LHRH content in the median eminence (Campbell and Ramaley, 1978; Gross, 1980) and medial basal hypothalamus (Shin and Howitt, 1976; Chen, Geneau and Meites, 1977; Kalra and Kalra, 1980). Therefore, the negative feedback action of gonadal

steroids appears to be mediated, at least in part, by an action on LHRH neurones that terminate in the median eminence.

Data supporting the hypothesis that the gonadotroph response to castration is determined largely by an increase in LHRH secretion has recently received considerable support from investigations into the regulation of pituitary LHRH receptors. These studies have indicated that the interaction of endogenous LHRH with its receptor is essential for the normal maintenance of LHRH receptors and LH secretion. If endogenous LHRH secretion was abolished before orchidectomy, either by a lesion of the median eminence (Clayton, Channabasavaiah, Stewart and Catt, 1982a), subcutaneous injections of antiserum to LHRH (Clayton, Popkin and Fraser, 1982b) or administration of a specific antagonist to LHRH receptors (Clayton *et al.*, 1982a), the characteristic 2-fold post-castration increase in LHRH receptor number and 10-fold increase in serum LH concentration was prevented. Administration of LHRH or a potent LHRH agonist reversed the effects of removal of endogenous LHRH (Clayton *et al.*, 1982a, 1982b). Therefore, the post-castration increase in LHRH receptors and gonadotrophin secretion appear to depend upon increased hypothalamic LHRH secretion. However, estimations of the number of LHRH receptors during the oestrous cycle in the rat (Clayton *et al.*, 1980; Savoy-Moore, Schwartz, Duncan and Marshall, 1980; Barkan, Regiani, Duncan and Marshall, 1981) and hamster (Adams and Spies, 1981) showed that when plasma gonadotrophin and LHRH concentrations were high, pituitary LHRH receptors were reduced. Pituitary LHRH receptors increased through late meteostrus and dioestrus in the rat, when endogenous LHRH secretion has been shown to be low but increasing (Sarkar, Chiappa, Fink and Sherwood, 1976). On pro-oestrous afternoon,

however, LHRH binding declined by 50% for a 60-90 min period (Clayton *et al.*, 1980; Savoy-Moore *et al.*, 1980), at the time of a 5-6-fold increase in the LHRH concentration of pituitary stalk blood and immediately before the pre-ovulatory gonadotrophin surge (Sarkar *et al.*, 1976). The decrease in LHRH receptors at pro-oestrus has been suggested to be due to increased occupancy of the LHRH receptor by endogenous LHRH so that less binding occurs of the labelled analogue of LHRH used to measure receptor number (White and Ojeda, 1982). This hypothesis is disputed by Savoy-Moore *et al.* (1980) and Barkan *et al.* (1981) who were unable to demonstrate decreasing receptor binding capacity following exogenous LHRH administration (600 ng/rat, s.c.) to adult male rats or ovariectomised rats with OE_2 implants. The most plausible explanation put forward for the decrease in pituitary LHRH receptors, despite a marked increase in LHRH secretion at pro-oestrus, is that LHRH 'down-regulates' its receptors. 'Down-regulation' is a homeostatic mechanism in which the number of hormone receptors decrease in response to elevated levels of homologous hormone (Pollet and Levey, 1980). 'Down-regulation' of LHRH receptors has been proposed to explain changes in pituitary sensitivity during the oestrous cycles involving internalisation of LHRH receptors (Hazum, Cuatrecasas, Marian and Conne, 1980). 'Down-regulation' has been observed with large doses of other peptide hormones and their receptors, for example, Angiotensin 11 has been demonstrated to regulate the number of Angiotensin receptors on adrenal glomerulosa cells in situations of sodium depletion (Catt, Harwood, Aguilera and Dufau, 1979). The reason why 'down-regulation' of LHRH receptors does not occur in the long-term castrated rats is not yet clear.

During lactation and in male rats with pituitary transplants (Chapter IV), serum and pituitary levels of LH and FSH have been found to be suppressed (McCann, Graves and Taleisnik, 1967; Ford and Melampy, 1973; Hammons, Velasco and Rothchild, 1973; Bartke, Smith, Micheal, Peron and Datterio, 1977; McNeilly, Sharpe, Davidson and Frazer, 1978), pituitary LHRH receptor content was decreased (Clayton *et al.*, 1980; Fraser, Popkin, McNeilly and Sharpe, 1982b) and there was diminished pituitary responsiveness to LHRH (Lu *et al.*, 1976a; Muralidhar, Maneckjee and Moudgal, 1977; Hodson, Simpkins and Meites, 1978; Winters and Loriaux, 1978; Greeley and Kizer, 1979). However, there is no direct evidence that LHRH concentrations in the hypophysial portal vessels is reduced in the lactating rat or in the hyperprolactinaemic male rat.

The demonstration that, in the absence of ovarian steroids, the suckling stimulus and prolactin can effectively inhibit the post-castration rise in gonadotrophin secretion (Smith and Neill, 1977), questions the importance of gonadal steroids in the regulation of gonadotrophin secretion by prolactin. Serum oestradiol (OE_2) concentrations have been found to be positively correlated with LHRH binding capacity throughout the oestrous cycle (Savoy-Moore *et al.*, 1980; Clayton *et al.*, 1980). This raises the possibility that steroid-mediated receptor induction occurs before pro-oestrus, since a rise in circulating OE_2 concentration at this stage of the cycle has been demonstrated to initiate enhanced responsiveness of the pituitary to LHRH (Arimura and Schally, 1971; Aiyer and Fink, 1974). There is evidence to suggest that the potentiation of pituitary responsiveness to LHRH by OE_2 is mediated by synthesis of a protein (Kamel and Krey, 1982; Peegel and Menon, 1982). It has been proposed that OE_2 either exerts

a direct effect on the pituitary gland to increase LHRH receptor synthesis or acts indirectly on the hypothalamus to increase the release of endogenous LHRH (Speight, Popkin, Watts and Fink, 1981) which subsequently induces its own receptors in pituitary gonadotrophs (Clayton *et al.*, 1980). However, measurements of pituitary LHRH receptors in long-term ovariectomised rats with OE_2 implants demonstrated an acute fall in pituitary LHRH receptors before the daily afternoon LH surge, in the presence of a constant concentration of OE_2 (Barkan *et al.*, 1981). In addition, pentobarbital administration to these rats abolished both the LH surge and the fall in pituitary LHRH receptors while three injections of LHRH (225 ng/rat, i.v., every 30 min) given at 09.00 h, induced a marked increased in serum LH concentration but did not produce a fall in LHRH binding capacity (Barkan *et al.*, 1981). These data suggest that other factors, for example the opioid peptides (Barkin, Regianis, Duncan, Papavasiliou and Marshall, 1983), secreted by the hypothalamus before the LH surge, may also be involved in mediating the abrupt fall in LHRH receptors at pro-oestrus. Both OE_2 and androgen receptors are found in the hypothalamus and pituitary of the adult male rat (Kato, 1975; Lieberburg and McEwen, 1977; Kalra and Kalra, 1980).

The central actions of T have been suggested to occur after it is reduced to DHT (Massa, Stupnicka, Kniewald and Martini, 1972) or aromatised to OE_2 (Naftolin, Ryan, Davis, Reddy, Flores, Petro, Kuh, White, Wolin and Takaoka, 1975; Figure 3.1), although the relative importance of OE_2, DHT and T in regulating LH secretion remains uncertain (Kato, 1975; Mahesh, Muldoon, Eldridge and Korach, 1975; Martini, 1982).

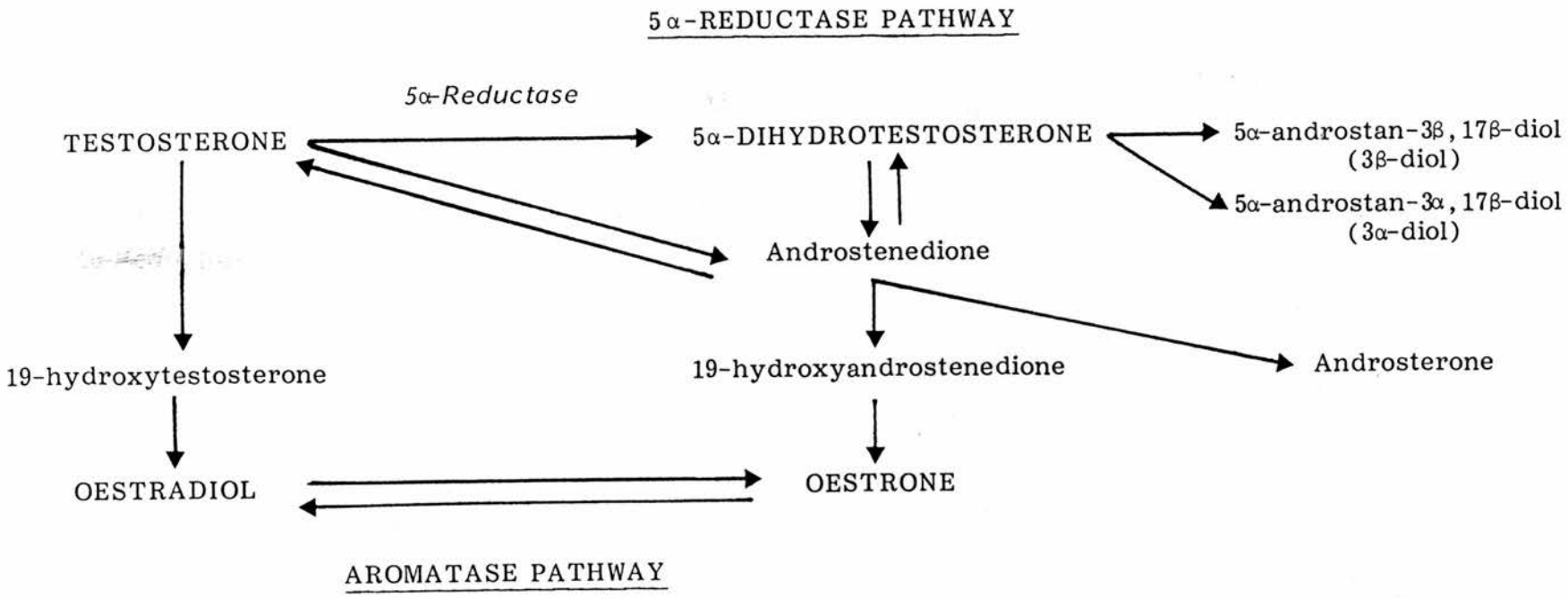


Figure 3.1: Schematic representation of the conversion of testosterone to 5α-dihydrotestosterone and oestradiol in central neuroendocrine tissue (based on Massa *et al.*, 1972; Naftolin *et al.*, 1975).

Purpose of present studies:

The main aims of the experiments described in this chapter were to determine whether

i) the castration-induced increases in gonadotrophin secretion, number of pituitary LHRH receptors and content of LH in the pituitary are due primarily to increased secretion of LHRH into stalk blood;

ii) the treatment of long-term castrated rats with OE_2, DHT or T, which suppresses LH secretion but has no effect of the number of pituitary LHRH receptors or the pituitary content of LH, alters the secretion of LHRH into stalk blood;

iii) OE_2, DHT and T have different effects on the release of LHRH into stalk plasma of long-term castrated rats.

3.2 Materials and Methods

3.2.1 Animals and Experimental Procedures

All the animals used were male rats of the Wistar strain which had been maintained under the conditions described in Section 2.1. Bilateral castration or sham-castration was carried out on 60-day old rats under pentobarbitone (24 mg/rat), as outlined in Section 2.2.3. All the rats were left without treatment for 30 days after surgery, before being injected for 8 days with gonadal steroids. The steroids injected were testosterone propionate (TP: 'Viromone', Paines and Byrne, Middlesex) and oestradiol benzoate (OE_2B: 'Benztrone', Paines and Byrne, Middlesex) which were supplied dissolved in ethyl oleate. 5α-dihydroxytestosterone propionate (DHTP: Steraloids Inc.,

New Hampshire, U.S.A.) was obtained in powder form. All the steroids were made up to the required concentration with arachis oil (Hopkins & Williams, Essex). The doses of the steroids injected in a volume of 0.25 ml, were 20 µg/rat/day of OE_2B, 300 µg/rat/day of DHTP and 300 µg/rat/day of TP. The two groups of control rats (sham-castrated and control, castrated) were injected with 0.25 ml arachis oil injections subcutaneously. The steroids or oil were injected at 16.00 h daily, 30 days after castration. On the 9th day of treatment the rats were divided into two groups (A and B) and the following parameters investigated in each group:

GROUP A

(a) Peripheral plasma concentrations of LH and FSH:

The animals were decapitated and trunk blood collected into heparinised glass tubes kept on ice. The blood was then centrifuged at 2500 g for 15 min at 4°C and the plasma stored at -40°C until assayed for LH and FSH concentrations (Section 2.3). The plasma and pituitary samples (see below) were sent to be assayed by airfreight to Dr. M.L. Aubert (University of Geneva, Switzerland), packed in dry ice which kept them frozen at all times.

(b) Pituitary LHRH receptor content and LH concentration:

The anterior pituitary glands from the rats were dissected out on ice immediately after decapitation, snap frozen on dry ice and stored at -40°C. The concentration and content of LHRH receptors in crude pituitary homogenates was determined by saturation analysis using the method of Conne, Aubert and Sizenonko (1979), using a radioiodinated superactive analog of LHRH as tracer. Two pituitary glands were pooled to make one receptor measurement. The results are expressed

as fmoles/mg protein (concentration) and fmoles receptor sites/ pituitary (content). The pituitary content of LH was determined by radioimmunoassay (Section 2.3).

GROUP B

(a) Release of LHRH into pituitary stalk blood:

The hypophysial portal vessels were exposed (method in Section 2.2.5) under Althesin anaesthesia (1.0 - 1.5 ml/100 g b.w.). After cutting the pituitary stalk, blood samples were collected from the cut end for two consecutive 30 min periods. After centrifugation (2500 g, 4°C, 15 min) the plasma was stored frozen until assayed for LHRH (Section 2.3). The results are expressed as the concentration (pg/ml) of LHRH in stalk plasma and the content (pg/30 min) of LHRH in stalk blood. The values of LHRH given (Section 3.3) are the means of the two 30 min collection periods.

(b) LH concentration in peripheral plasma:

A sample (0.5 ml) of blood was obtained from the external jugular vein just before cutting the pituitary stalk. The plasma was stored frozen at -40°C until the concentration of LH was determined by radioimmunoassay (Section 2.3).

3.2.2 Calculations

The results were expressed as mean ± S.E.M. (n). Statistical comparisons were made using the unpaired Students t-test. More than two means were compared by a one-way analysis of variance and the multiple range test for heteroscadistic means (Duncan, 1957; Harter, 1960). Non-parametric statistics were applied to determine the significance of differences between means of LHRH concentrations in stalk

plasma since, in some cases, the concentration of LHRH in the sample was below the lower limit of detection of the assay (Section 2.3.3). A value equal to the lower limit of the assay was designated to these samples and the significance between groups determined by the Mann-Whitney U test if two groups were compared. More than two groups were compared using the Kruskal-Wallis one-way analysis of variance by ranks.

The level of significance was taken as $p < 0.05$.

3.3 Results

3.3.1 Experiment 1

(a) Effects of castration:

The peripheral plasma concentrations of LH and FSH and the pituitary content of LH in control, castrated rats were significantly ($p < 0.001$) greater than in sham-castrated rats (Figure 3.2a). There was no significant difference between the control, castrated rats and the sham-castrated rats in the concentration of LHRH in pituitary stalk plasma, or the total amount of LHRH released into stalk blood (content, pg/30 min), Figure 3.2b. The concentration and content of LHRH receptors in the pituitary were significantly ($p < 0.001$) increased in the castrate rats compared to the sham-castrate rats, Figure 3.2b.

(b) Effects of treatment with DHTP:

Treatment of castrate rats with DHTP (300 µg/rat) for 8 days suppressed the post-castration increases in the concentrations of LH in peripheral plasma (Figure 3.2a). The castration-induced increase in the concentration of FSH in peripheral plasma was suppressed by DHTP treatment but remained higher than the values in sham-castrated rats. The pituitary content of LH, the number of pituitary LHRH

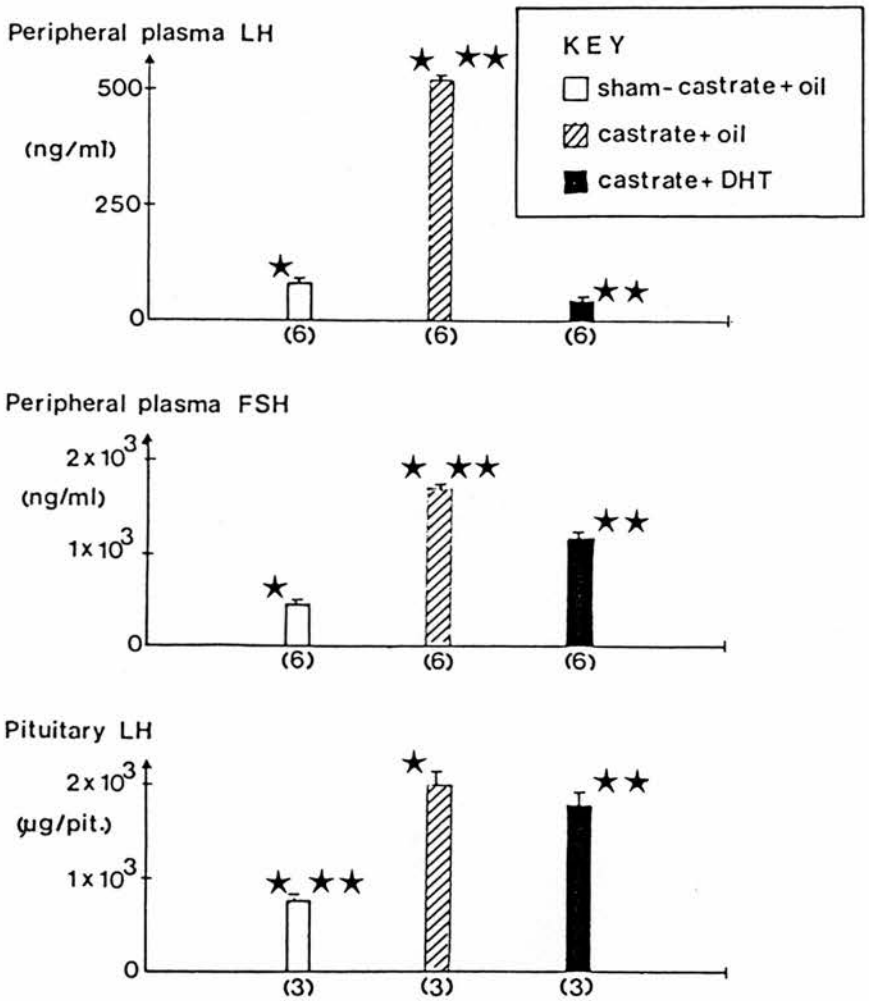


FIGURE 3.2a: The effects of castration and treatment of the castrate rats with arachis oil (0.25 ml/rat) or 5α-dihydrotestosterone propionate (DHTP, 300 µg/rat) for 6 days. The number of rats in each group is shown in parenthesis. The values given are the mean ± S.E.M. *, ** = $p < 0.001$ when the values within each parameter are compared with each other.

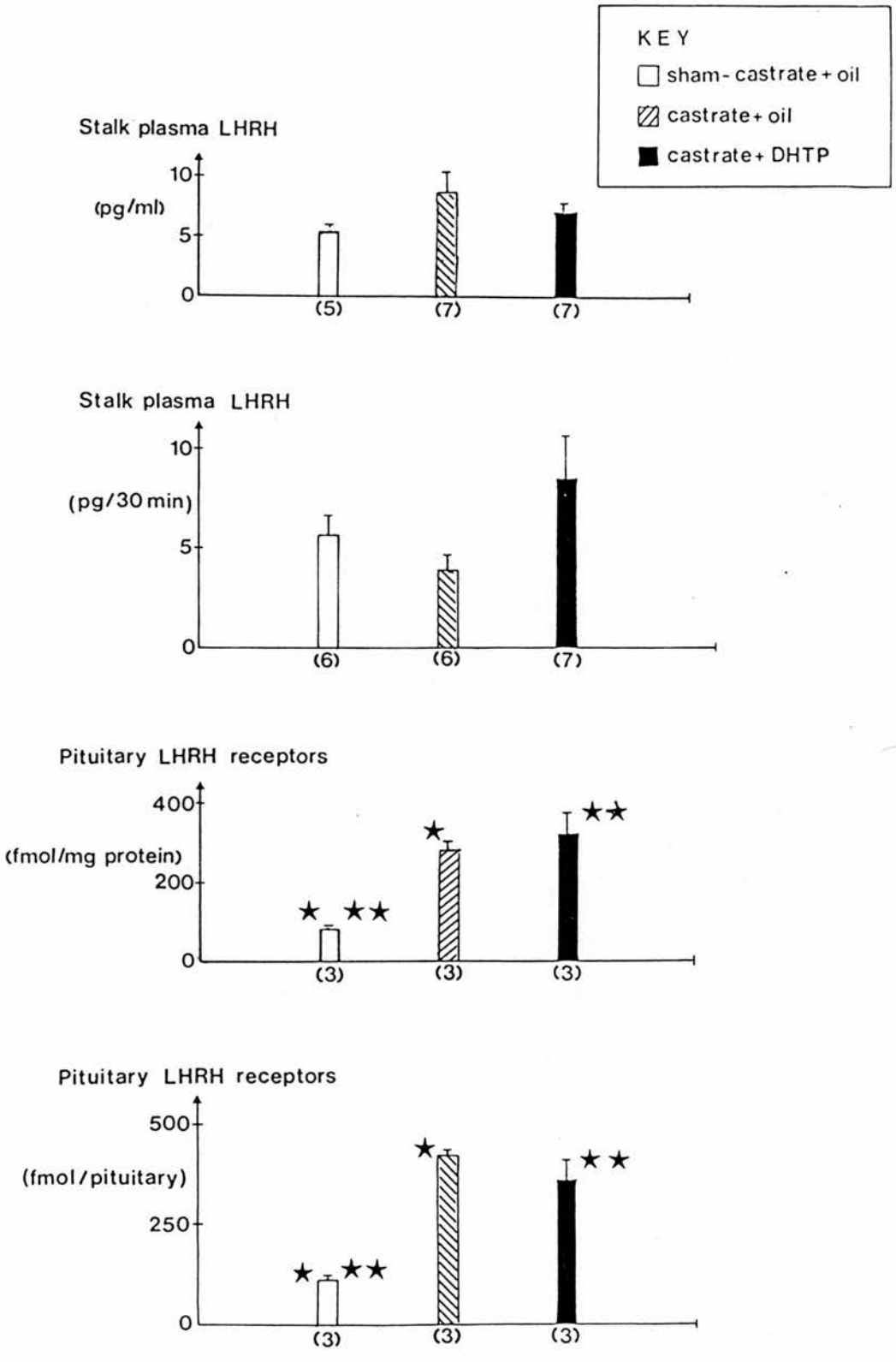


FIGURE 3.2b: The effects of castration and treatment of the castrate rats with arachis oil (0.25 ml/rat) or 5α-dihydrotestosterone propionate (DHTP; 300 μg/rat) for 8 days. The number of rats in each group is shown in parenthesis. The values given are the mean ± S.E.M. Two pituitary glands were pooled for each analysis of pituitary LHRH receptors. *, ** = $p < 0.001$ when the values in each parameter are compared with each other.

receptors and the concentration and content LHRH in stalk plasma in castrate rats treated with DHTP were similar to the values found in castrate rats injected with oil and significantly ($p < 0.001$) greater than the values in sham-castrated rats (Figure 3.2b). The changes in the number of LHRH binding sites in the pituitary gland were not accompanied by any alteration in the affinity constant of the LHRH analogue (Table 3.1).

3.3.2 Experiment 2

Groups of rats were treated as in Experiment 1 (i.e. sham-castrate injected with oil, castrate injected with oil and castrate injected with DHTP) and in addition two groups of rats were castrated and injected with either OE_2B or TP. The values obtained for the parameters investigated are shown in Figure 3.3a and 3.3b.

(a) Effects of castration:

Castration induced a significant ($p < 0.001$) increase in concentrations of plasma LH and FSH, pituitary LH and concentration and content of LHRH receptors in the pituitary (Figures 3.3a, 3.3b), as found in Experiment 1. In addition, there was no significant difference between the castrate rats and the sham-castrate rats in the content of LHRH in stalk blood (Figure 3.3b), as found in Experiment 1.

(b) Effects of treatment with OE_2B, DHTP or TP:

Treatment of castrate rats with OE_2B (20 µg/rat), DHTP (300 µg/rat) or TP (300 µg/rat) for 8 days completely restored the concentration of LH in peripheral plasma to the values found in sham-castrate rats treated with oil (Figure 3.3a). In the group of rats treated with OE_2B which were used to obtain samples of stalk blood under Althesin anaesthesia (Figure 3.4, Group B), the concentrations of LH in peripheral

TABLE 3.1: The mean ± S.E.M. values of the affinity constant (K_A) of the LHRH agonist for LHRH binding sites in the anterior pituitary glands of groups (n=6) of sham-castrated and castrated rats injected for 8 days with oil (0.25 ml/rat/day) and long-term castrated rats injected for 8 days with 5α-dihydrotestosterone propionate (DHTP; 300 µg/rat/day). Two anterior pituitary glands were pooled for each estimation of the K_A value (collaborative data, Aubert).

Treatment	No. of samples	Affinity constant (K_A) x $10^{10}M^{-1}$
Sham-castrated + oil	3	1.13 ± 0.18
Castrated + oil	3	0.99 ± 0.09
Castrated + DHTP	3	1.38 ± 0.16
Combined mean ± S.E.M.	9	1.17 ± 0.09

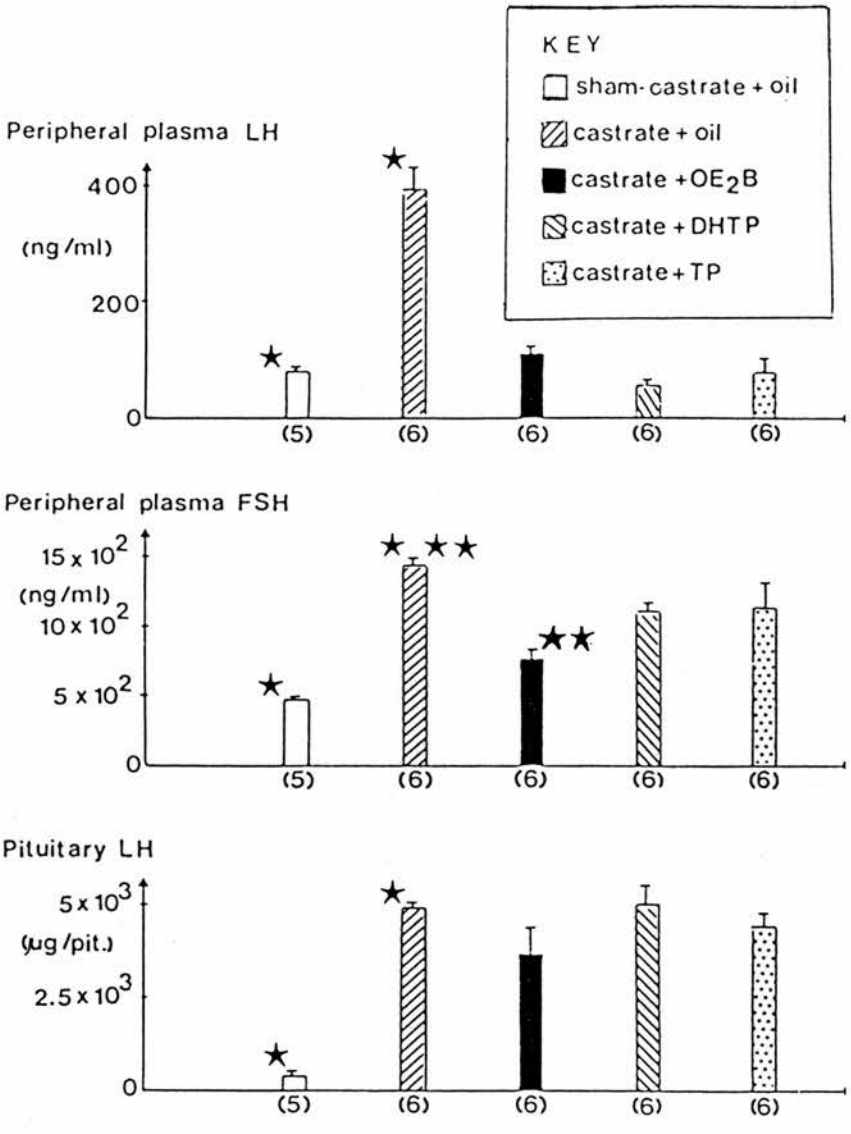
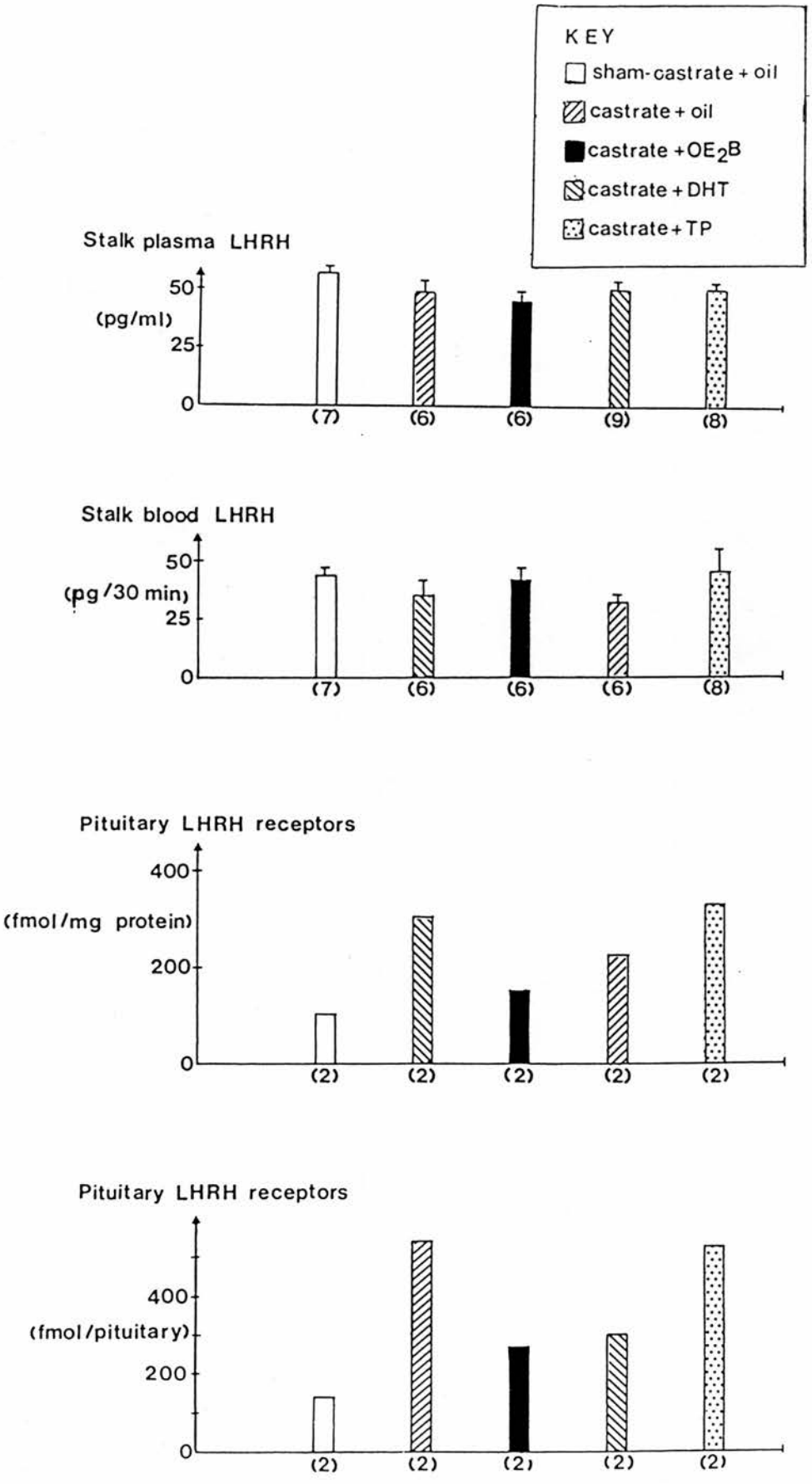


FIGURE 3.3a: The effects of castration and treatment of castrate rats with oestradiol benzoate (OE_2B) (20 µg/rat), 5α-dihydrotestosterone propionate (DHTP; 300 µg/rat) or testosterone propionate (TP; 300 µg/rat) for 8 days. Sham-castrated and control, castrated rats were injected with arachis oil (0.25 ml/rat for 8 days. The number of rats in each group is shown in parenthesis. The values given are the mean ± S.E.M. *, ** = $p < 0.001$ when the values in each parameter are compared with each other.

FIGURE 3.3b: The effects of castration and treatment of castrate rats with oestradiol benzoate (OE_2B; 20 µg/rat), 5α-dihydrotestosterone propionate (DHTP; 300 µg/rat) or testosterone propionate (TP; 300 µg/rat) for 8 days. Sham-castrated and control, castrated rats were injected with arachis oil (0.25 ml/rat) for 8 days. The number of rats in each group is shown in parenthesis. The values given are the mean ± S.E.M. Pituitary LHRH receptors were determined in 2 pools of 2 pituitary glands each.


KEY
sham-castrate + oil
castrate + oil
castrate + OE_2B
castrate + DHT
castrate + TP
Stalk plasma LHRH
(pg/ml)
50
25
0
(7)
(6)
(6)
(9)
(8)
Stalk blood LHRH
(pg/30 min)
50
25
0
(7)
(6)
(6)
(6)
(8)
Pituitary LHRH receptors
(fmol/mg protein)
400
200
0
(2)
(2)
(2)
(2)
(2)
Pituitary LHRH receptors
(fmol/pituitary)
400
200
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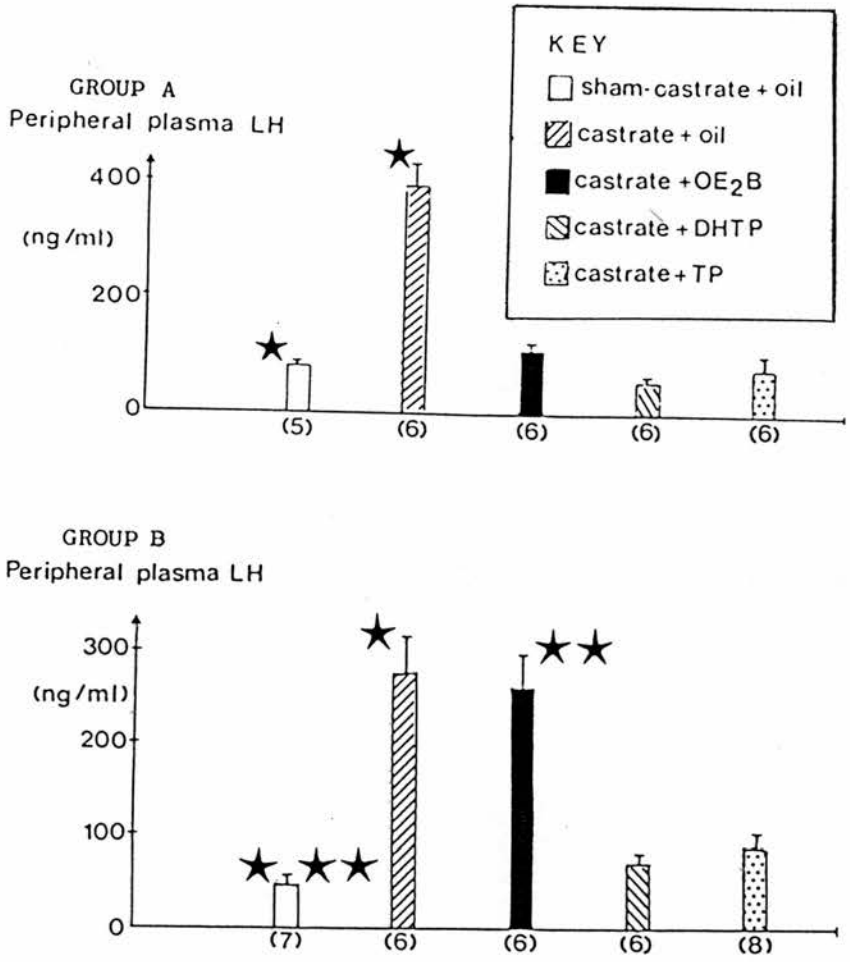

FIGURE 3.4: The effects of castration and treatment of castrate rats with oestradiol benzoate (OE_2B; 20 µg/rat), 5α-dihydrotestosterone propionate (DHTP; 300 µg/rat) or testosterone propionate (TP; 300 µg/rat) for 8 days. Sham-castrated and control, castrated rats were injected with arachis oil (0.25 ml/rat) for 8 days. Peripheral blood samples were obtained either after decapitation (Group A) or from the external jugular vein under Althesin anaesthesia, just before cutting the pituitary stalk (Group B). The number of rats in each group is shown in parenthesis. The values given are the mean ± S.E.M. * = $p < 0.001$ when the values are compared with each other in either Group A or Group B.

plasma collected before cutting the pituitary stalk were similar to the values in castrate rats injected with oil, as opposed to those treated with DHTP and TP.

The concentration of FSH in peripheral plasma was significantly ($p < 0.005$) suppressed, but not completely restored to sham-castrate values by treatment with OE_2B (Figure 3.3a). Neither DHTP nor TP treatments were effective in suppressing the castration-induced increase in the concentration of FSH in the peripheral plasma.

The increase in the content of pituitary LH induced by castration was not suppressed by treatment with OE_2B, DHTP or TP (Figure 3.3a).

The concentration and content of LHRH in stalk plasma was not altered by treatment of castrated rats with OE_2B, DHTP or TP, and the values were similar to those in sham-castrate rats (Figure 3.3b).

The content and concentration of pituitary LHRH receptors remained elevated despite treatment with OE_2B, DHTP or TP (Figure 3.3b). However, out of the three steroids injected, OE_2B was the most effective in suppressing the post-castration rise in the number of pituitary LHRH numbers.

3.4 Discussion and Conclusions

These studies support previous findings that secretions from the testes maintain an inhibitory influence on plasma concentrations of the gonadotrophins (Kalra *et al.*, 1973; Damassa *et al.*, 1976; Badger *et al.*, 1978), the pituitary content of LH (Gay and Midgley, 1969; Conne *et al.*, 1982) and the number of LHRH receptors in the pituitary (Frager *et al.*, 1979; Clayton *et al.*, 1980; Conne *et al.*, 1982). In agreement with other studies (Campbell and Ramaley, 1978; Conne *et al.*, 1982), treatment of long-term castrated rats with the gonadal

steroids, OE_2B or TP, completely suppressed the post-castration rise in plasma LH concentrations, despite the continued elevation in pituitary LH content and pituitary LHRH receptors (Figures 3.3a, 3.3b). In addition, the present studies also showed that treatment with DHTP, a 5α-reduced metabolite of TP, produced similar effects to TP on the changes induced by long-term castration in the hypothalamic-pituitary axis, supporting that theory that the conversion of TP to DHTP is important for TP to exert its negative feedback effects (Martini, 1976; Kalra and Kalra, 1980; McEwen, 1980; Martini, 1982). The demonstration that TP or DHTP was ineffective in completely reducing the concentration of plasma FSH to the values intact rats (Figure 3.3a), supports the suggestion that testicular inhibin is also necessary for the maintenance of tonic, low concentrations of plasma FSH (Setchell, Davies and Main, 1977; Morris and Jackson, 1978; Franchimont, Verstraelen-Proyard, Hazee-Huelstein, Renard, Demoulin, Bourguignon and Hustin, 1979). Although in the experiments in this chapter (Figure 3.3a), OE_2B was completely effective in suppressing the post-castration rise in plasma FSH concentrations, the plasma concentrations of OE_2B likely to occur by the subcutaneous injection of 20 µg/rat would be considerably higher than the concentrations of plasma OE_2B (20-45 pg/ml) that occur in the intact male rat (Butcher, Inskeep and Pope, 1978; Saksena *et al.*, 1978; Saksena and Lau, 1979; Keel and Abney, 1980). The relative importance of OE_2 in suppressing FSH secretion is therefore not clearly established.

The post-castration rises in plasma gonadotrophin secretion, pituitary LH synthesis and the number of pituitary LHRH receptors cannot, on the basis of the present studies, be attributed to increased secretion of LHRH into pituitary stalk blood (Figure 3.3). The marked

depletion of the LHRH content in the median eminence and mediobasal hypothalamus induced by castration (Shin and Howitt, 1976; Conne *et al.*, 1980; Gross, 1980; Kalra and Kalra, 1980) may therefore not be due to increased release of LHRH. Whether short-term castration increases LHRH secretion into stalk blood remains to be investigated.

The first suggestion that an increased secretion of LHRH occurred after castration was based on the demonstration that hemi-pituitaries superfused with portal vessel blood from castrate rats induced greater concentrations of LH in the superfusate than portal blood from intact male rats (Ben-Jonathan, Mical and Porter, 1973). Direct measurements of LHRH concentrations in pituitary stalk blood under pentobarbitone anaesthesia, an anaesthetic which blocks the pro-oestrus LHRH surge (Sarkar *et al.*, 1976), did not show a significant difference between intact and castrate rats (Eskay, Mical and Porter, 1977). LHRH in stalk plasma in the present experiments (Figures 3.2b, 3.3) was collected under Althesin anaesthesia, an anaesthetic which does not completely block the pro-oestrus LH surge, although it is reduced (Sarkar *et al.*, 1976). Therefore, it is possible that the reasons that castration did not induce an increase in LHRH secretion (Figures 3.2b, 3.3b) were that in the male rat, Althesin anaesthesia and the trauma of surgery done to expose the portal vessels, masked any increases in LHRH secretion. However, using Althesin anaesthesia and similar surgery to expose the portal vessels, Sarkar and Fink (1980) and Sherwood and Fink (1980) demonstrated that ovariectomy increased LHRH release into stalk blood.

The secretion of a constant amount of LHRH into stalk blood in intact and castrate rats was unexpected, firstly, in view of the numerous studies (Section 3.1) which suggest that endogenous LHRH regulates

the number of pituitary LHRH receptors, and, secondly, present results (Figures 3.2b, 3.3b) and those by Conne *et al*. (1982) showed that castration induced an increase in the number of pituitary LHRH receptors. Since the number of pituitary LHRH receptors remained elevated even after steroid treatment (Figures 3.2, 3.3b; Conne *et al*., 1982), it appears that prolonged elevations of gonadotrophin secretion, and possibly other changes induced by long-term castration, prevent down-regulation of LHRH receptors, which may require elevated LHRH release. However, since in fact there was no increase in LHRH release in the castrate rats (Figures 3.2b, 3.3b), pituitary LHRH receptor numbers remained elevated. This hypothesis is supported by the report that immunoneutralisation of LHRH but not treatment with TP was effective in suppressing the castration-induced increase in pituitary LHRH receptors (Aubert, Conne, Nawrati, Lang and Sizonenko, 1981).

In conclusion, the measurement of the release of LHRH into stalk plasma in long-term castrated rats in the experiments described in this chapter has shown that the removal of the inhibitory influence of the testes, which increases the release of LH and FSH, the synthesis of LH and the number of LHRH receptors in the pituitary, is not accompanied by an increase in the release of LHRH. Furthermore, the suppression of post-castration increases in plasma LH and FSH concentrations by OE_2B, DHTP or TP cannot be attributed to a decrease in the release of LHRH into stalk plasma, as there was no significant change in the release of LHRH into stalk plasma after injections of these gonadal steroids.

CHAPTER IV

Effects of Long-Term Hyperprolactinaemia, Castration and Testosterone Replacement on Dopamine (DA) and Luteinizing Hormone-Releasing Hormone (LHRH) Secretion into Pituitary Stalk Blood

CHAPTER IV

4.1 Introduction

Hyperprolactinaemia has been implicated in the pathogenesis of human infertility (Bohnet, Dahlen, Wuttke and Schneider, 1976; Quigley, Judd, Gilliland and Yen, 1979; Evans, Cronin and Thorner, 1982). Between 13-30% of women with secondary amenorrhea have increased plasma PRL concentrations (Franks, Murray, Jequier, Steele, Nabarro and Jacobs, 1975). Infertility and absence of cyclic ovarian activity during the immediate post-partum period in mammals is also directly associated with increased PRL concentration in the circulation (McNeilly, 1980).

The mechanism(s) responsible for the anti-fertility effects of hyperprolactinaemia are not fully understood. Experimental and lactational hyperprolactinaemia in the rat are accompanied by decreased plasma and pituitary gonadotrophin concentrations (Ford and Melampy, 1973; Bartke, Smith, Micheal, Peron and Dalterio, 1977; Smith and Neill, 1977; McNeilly, Sharpe, Davidson and Fraser, 1978). Hyperprolactinaemia also significantly delayed and attenuated the characteristic post-castration increases in plasma LH and FSH and pituitary LH concentration (Grandison, Hodson, Chen, Advis, Simpkins and Meites, 1977; Smith and Neill, 1977; Winters and Loriaux, 1978; McNeilly, Sharpe and Fraser, 1980a). An increase in plasma PRL concentration, induced by pituitary implants, has also been reported to prevent the increase in pituitary LHRH receptor number after castration or ovariectomy and on the morning of pro-oestrus (Clayton and Bailey, 1982c; Marchetti and Labrie, 1982), as well as the decrease in hypothalamic LHRH content after castration (Grandison *et al.*, 1977). In addition, a reduction in pituitary LH release in response to exogenous LHRH administration has been reported to occur in the lactating rat (Lu, Chen, Grandison,

Huang and Meites, 1976; Muraldhar, Maneckjee and Mougdal, 1977; Smith, 1982) and in intact male rats and castrated rats (Winters and Loriaux, 1978; Greeley and Kizer, 1979; Tresguerres and Esquifino, 1981), which suggests that PRL also acts at the pituitary level to alter LH release by reducing pituitary responsiveness to LHRH. During lactation and pathological hyperprolactinaemia, the LH pulse frequency has been demonstrated to be significantly reduced (Bohnet *et al.*, 1975). All these data are consistent with reduced endogenous LHRH secretion in the hyperprolactinaemic condition together with an alteration in the responsiveness of the pituitary gland to LHRH.

The inhibitory effect of hyperprolactinaemia on the post-castration LH rise may result from increased dopamine (DA) turnover induced by PRL in the hypothalamus. DA turnover in the tuberinfundibular (TIF) system was accelerated by systemic or intracerebroventricular injection of ovine PRL (Hökfelt and Fuxe, 1972; Gudelsky, Simpkins, Mueller, Meites and Moore, 1976; Annunciato and Moore, 1978) and by hyperprolactinaemia induced either by anterior pituitary transplants under the kidney capsule (Olson, Fuxe and Hökfelt, 1972; Hohn and Wuttke, 1978; Morgan and Herbert, 1980) or by subcutaneous implants of pituitary tumour tissue (Perkins, Westfall, Paul, MacLeod and Rogol, 1979). In addition, DA receptor activation has been reported to inhibit LH release (Fuxe and Hökfelt, 1969; Drouva and Gallo, 1977). The DA agonist, 2α-bromoergocriptine (CB-154), completely reversed the inhibitory effects associated with pituitary transplants, on pituitary LHRH receptor numbers and gonadotrophin secretion (Marchetti and Labrie, 1982). Moreover, specific dopamine receptor antagonists (e.g. Pimozide) increased LHRH concentration in stalk blood, while blockers of DA synthesis (e.g. α-methyl-p-tyrosine) decreased LHRH release

(Sarkar and Fink, 1981). A decrease in DA turnover in the TIF neurones at pro-oestrus has been proposed to result in the removal of an inhibitory dopaminergic influence on LHRH neurones in the median eminence (Ahren, Fuxe, Hamberg and Hökfelt, 1971; Fuxe, Hökfelt and Nilsson, 1972). This removal of the inhibitory effect on LHRH release is proposed to result in the LHRH surge on pro-oestrus (Ahren *et al.*, 1971; Fuxe *et al.*, 1972).

Although plasma concentrations of testosterone did not differ significantly in intact controls and hyperprolactinaemic rats (Bartke *et al.*, 1977; McNeilly, 1980), the presence of the gonads, but not the adrenal gland, appears to be necessary for PRL to exert an inhibitory effect on gonadotrophin secretion (McNeilly *et al.*, 1980). Furthermore, PRL increased the sensitivity of the hypothalamus to negative feedback effects of gonadal steroids on gonadotrophin secretion (McNeilly *et al.*, 1978, 1980; Marchetti and Labrie, 1982). Administration of oestradiol-17β to ovariectomised rats with pituitary transplants has been reported to increase further the plasma PRL concentrations and potentiate the inhibitory effects of the pituitary transplant on pituitary LHRH receptor content and gonadotrophin secretion (Marchetti and Labrie, 1982).

Purpose of present study:

The present study was undertaken to test the hypotheses:

i) that the increased concentrations of plasma gonadotrophins after castration are due to increased release of LHRH and decreased release of DA into pituitary stalk blood, which can be reversed by testosterone (T) administered by silastic implants;

ii) that long-term hyperprolactinaemia reduces the secretion of LHRH and increases the secretion of DA into pituitary stalk blood;

iii) that the suppression of the post-castration rise in plasma gonadotrophin secretion in the hyperprolactinaemic rat is due to a reduction in the secretion of LHRH into stalk blood in hyperprolactinaemic rats compared with that in control rats;

iv) that the hyperprolactinaemic rat is more sensitive to the negative feedback effects of T at the level of the hypothalamus, as seen by the changes in the concentration of LHRH in stalk blood in castrated rats with and without T implants.

4.2 Materials and Methods

4.2.1 Animals and Treatments

Adult male rats (130-150 days old, 240-280 g b.w.) of the inbred PVG strain were used in all experiments. The animals were divided into two groups; animals in one group had two anterior pituitary glands transplanted under the kidney capsule under ether anaesthesia, while animals in the other group were used as control animals. After 28 days the two groups of rats were divided into the following groups:

i) intact, untreated controls;
ii) castrated, untreated group;
iii) castrated, implanted with 10 mm T capsules;
iv) castrated, implanted with 30 mm T capsules.

The T capsules were implanted subcutaneously in the back of the animals at the time of castration, under ether anaesthesia. Metal clips were used to suture all surgical cuts.

4.2.2 Collection of Blood Samples

Blood samples were collected from the rats 14 days after the implantation of testosterone capsules. The rats were administered Althesin (0.5 - 1.5 ml/100 g b.w.) and the hypophysial portal vessels exposed as described in Section 2.2.5. After an intravenous injection of heparin (2500 IU/rat in 0.5 ml volume), a 1.0 ml sample of blood was obtained from the external jugular vein. The pituitary stalk was then cut and blood samples collected for 30 min periods before, during and after the application of an electrical stimulus to the median eminence. The stimulus was applied for 30 min by placing a unipolar platinum electrode on the surface of the median eminence. The stimulus parameters consisted of 30 sec trains of biphasic square wave pulses of 60 Hz, 1 mA peak to peak and 1 msec duration (Section 2.2.7). These parameters have been shown to be optimum for LH release (Jamieson and Fink, 1976).

Pituitary stalk blood samples of 10-20 µl were collected alternately every 30-45 sec into two sets of tubes kept on ice. One set of tubes contained Trasylol (20 KIU/l) and EDTA (5.4 mM/l) into which the blood was collected and immediately mixed. These samples were centrifuged at 2500 g for 15 min at 4°C at the end of the collection period of 30 min. The plasma was divided into 100 µl aliquots and n-Acetyl dopamine (2 ng in 20 µl 0.1N HCl) added before the samples were stored at -40°C. The concentration of DA and one of its metabolites, dihydroxyphenylacetic acid (DOPAC) in these samples was subsequently determined by HPLC (Section 2.4). Blood samples collected into the second set of tubes and the peripheral blood samples were centrifuged at 2500 g for 15 min at 4°C. The plasma was stored at -40°C until analysed for LH, FSH, PRL and LHRH by double antibody radioimmunoassay (Section 2.3).

4.2.3 *Treatment of Pituitary Grafts*

At the end of the collection of pituitary stalk blood the vascularisation of the pituitary transplants was verified by examination of the transplant under the kidney capsule using the dissecting microscope. The rats were perfused with Indian ink, the kidney with the transplanted pituitary dissected out and kept in buffered formaldehyde until embedded in paraffin wax (Appendix A5). Serial sections (20 µm thick) of the kidney and pituitary transplant were cut. Some sections were stained with the orange fuchsin (Appendix A5; Slidders, 1961) green (OFG) stain and some were left unstained and examined under the optical microscope.

4.2.4 *Calculations and Statistics*

The concentrations of DA, DOPAC and the hormones LHRH, LH, FSH and PRL in plasma samples from each group of rats was expressed as the mean ± S.E.M. The amount of LHRH and DA released into stalk blood during each 30 min collection period was calculated by multiplying the concentration of LHRH or DA by the volume of stalk blood collected. This value is referred to as the content. The Student's t-test was used to determine differences between two means. When more than two means were compared, an analysis of variance and multiple range test (Duncan, 1957; Harter, 1960) was used. In cases where the value of LHRH determined by radioimmunoassay was below detection, the value equal to the lower limit of the assay was assigned and the difference between groups determined by non-parametric statistics, using either the Mann-Whitney or Kruskal-Wallis test. The level of significance was taken as $p < 0.05$.

4.3 Results

4.3.1 Experiment 1

(a) Concentrations of LH, FSH and PRL in peripheral plasma and LHRH secretion into pituitary stalk plasma:

- *Effects of pituitary transplants*

The implantation of pituitary transplants into intact rats for 42 days induced about a 2-fold increase in the peripheral plasma concentration of PRL (Figure 4.1). Hyperprolactinaemia was associated with a significant ($p < 0.05$) decrease in the concentration of plasma FSH (Figure 4.1). The concentration of LH in peripheral plasma (Figure 4.1) and the release of LHRH into pituitary stalk plasma (Figures 4.2a, 4.2b) was not significantly altered by implantation of pituitary transplants compared to control rats.

Electrical stimulation of the ME of all the animals induced a significant ($p < 0.05$) increase in the release of LHRH into stalk plasma in the control rats (Group A) and the rats with pituitary transplants (Group B) (Figures 4.2a, 4.2b). The concentration and content of LHRH in stalk plasma of control rats and rats with pituitary transplants were of a similar magnitude during the period of stimulation. During the post-stimulation period, the concentration and content of LHRH in stalk blood were approximately equal to the values measured in the pre-stimulation period (Figures 4.2a, 4.2b). The volume of pituitary stalk blood collected during each 30 min period is shown in Table 4.1. Electrical stimulation of the ME of two groups (intact, control and castrate, hyperprolactinaemic with 30 mm T implants) only induced a significant ($p < 0.05$) increase in the mean volume of stalk blood collected. However, there was no significant difference between groups, in the volume of stalk blood collected before, during or after stimulation.

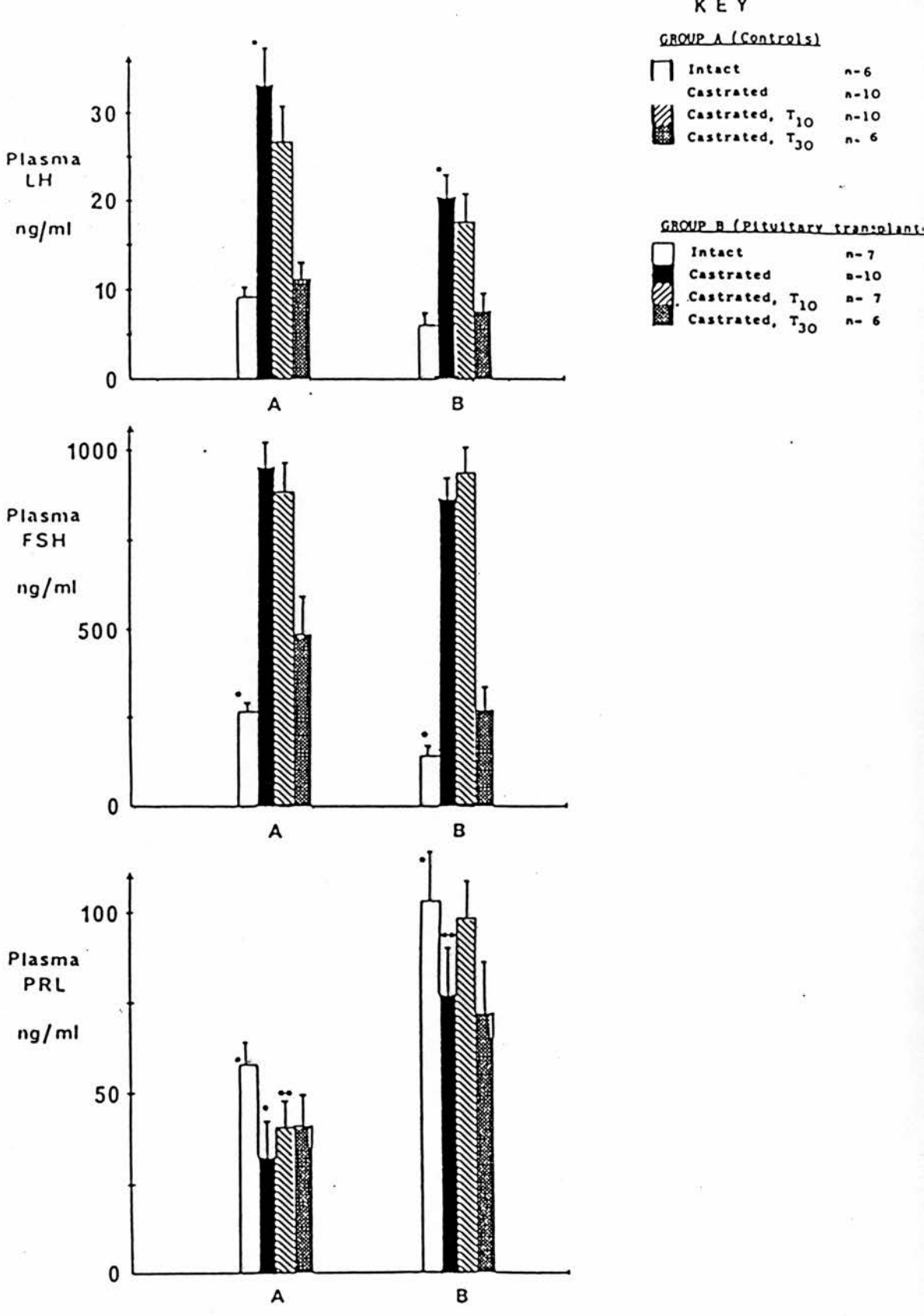

FIGURE 4.1: Peripheral plasma concentration of LH, FSH and PRL in control rats (Group A) and rats with two anterior pituitary glands transplanted under the kidney capsule (Group B). The values shown are the mean ± S.E.M.; peripheral blood samples were collected under Althesin anaesthesia, just before the pituitary stalk was cut to collect stalk blood.

T_{10} = 10 mm and T_{30} = 30 mm testosterone implants.

*, ** = $p < 0.05$ when the values in Group A are compared to Group B and when plasma PRL concentrations in intact and castrated rats in Group A are compared with each other.

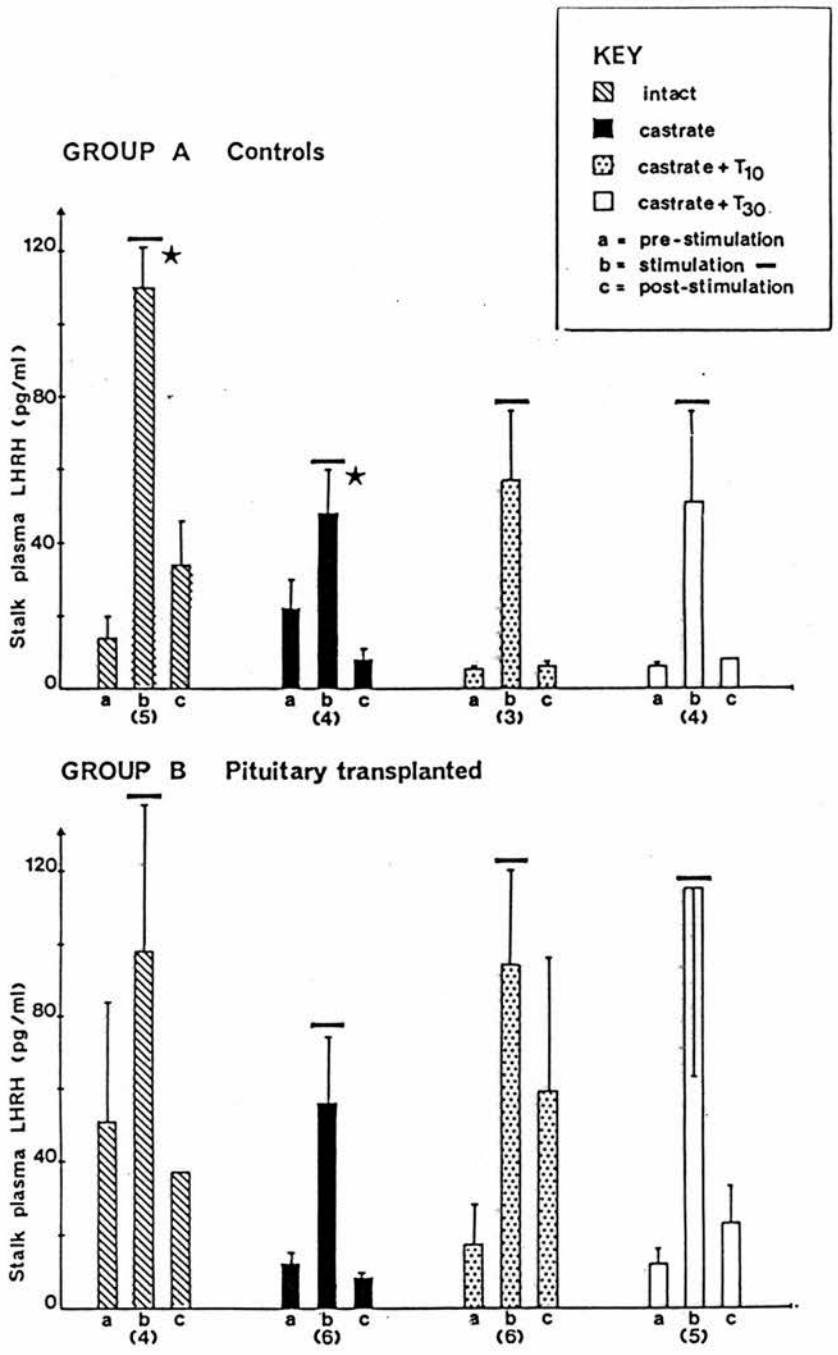

FIGURE 4.2a: The concentration of LHRH in pituitary stalk plasma collected under Althesin anaesthesia from control rats (Group A) and rats with pituitary transplants (Group B) before, during and after electrical stimulation of the median eminence. The values given are the mean ± S.E.M. (n).

T_{10} = 10 mm and T_{30} = 30 mm testosterone implants.

* = $p < 0.05$ when the values are compared with each other.

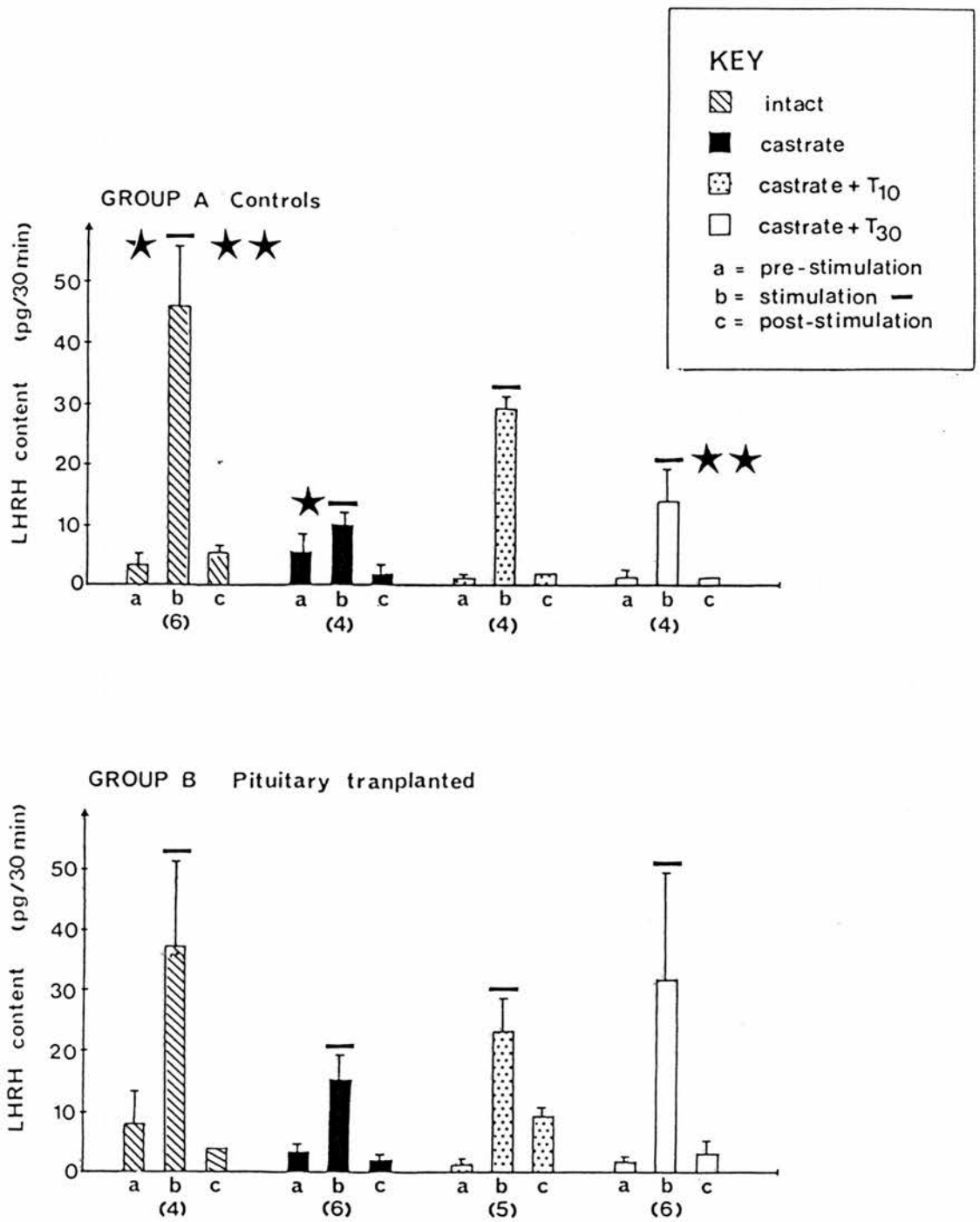

FIGURE 4.2b: The content of LHRH in pituitary stalk blood of control rats (Group A) and rats with pituitary transplants (Group B), under Althesin anaesthesia, before, during and after electrical stimulation of the median eminence. The values given are the mean ± S.E.M. (n).

T_{10} = 10 mm and T_{30} = 30 mm testosterone implants.

* = $p < 0.05$ when the values are compared with each other, within Group A or Group B.

TABLE 4.1: The volume (µl/30 min) of pituitary stalk blood obtained in the collection periods before, during and after electrical stimulation of the median eminence of groups of control rats and rats with pituitary transplants. The values given are the mean ± S.E.M. (n). * = $p < 0.05$ when the volume of stalk blood collected during the pre-stimulation period is compared with that collected during the stimulation period.

Group	Pre-stimulation	Stimulation	Post-stimulation
EXPERIMENT 1			
Control:			
Intact	*185 ± 27 (6)	*400 ± 60 (6)	130 ± 10 (6)
Castrate	236 ± 40 (6)	333 ± 80 (6)	240 ± 65 (5)
Castrate + testosterone (10 mm)	200 ± 30 (6)	380 ± 60 (7)	240 ± 75 (5)
Castrate + testosterone (30 mm)	265 ± 35 (8)	385 ± 60 (6)	200 ± 35 (4)
Pituitary transplanted			
Intact	185 ± 40 (4)	378 ± 101 (4)	130 ± 75 (3)
Castrate	240 ± 50 (7)	405 ± 90 (7)	195 ± 30 (5)
Castrate + testosterone (10 mm)	195 ± 50 (5)	320 ± 85 (6)	175 ± 25 (6)
Castrate + testosterone (30 mm)	*145 ± 10 (8)	*280 ± 50 (6)	168 ± 10 (5)
EXPERIMENT 2			
Intact:			
Control	410 ± 80 (3)	970 ± 230 (3)	260 ± 30 (3)
Pituitary transplanted	*150 ± 40 (6)	*540 ± 75 (6)	165 ± 20 (6)

The values of LHRH in stalk plasma of the individual rats in the group with pituitary transplants is shown in Table 4.2. Rat number 3 had a similar concentration and content of LHRH during the pre-stimulation period to rats 1 and 2, but on stimulation of the ME an increase in the release of LHRH did not occur despite an increase in the flow of stalk blood seen in all the rats, which suggested that all rats had received an electrical stimulus. In addition, rat number 4 had a significantly ($p < 0.05$) higher concentration and content of LHRH during the pre-stimulation period than the other rats. In view of the small number of animals in this group, a further group of 6 animals with pituitary transplants was studied (Experiment 2).

Table 4.3 shows the mean ± S.E.M. dose of Althesin that was required to induce anaesthesia before surgery to expose the pituitary stalk vessels. For rats with pituitary transplants the dose of Althesin required was almost half that required for the control male rats, and is comparable to the doses required to induce deep anaesthesia in female rats.

- *Effects of castration*

Castration for 14 days induced a significant ($p < 0.05$) and equivalent increase in the concentration of LH and FSH in peripheral plasma of both control rats and rats with pituitary transplants (Figure 4.1). Plasma PRL concentrations in the control group (A) and in the group with pituitary transplants (B) were significantly ($p < 0.05$) decreased by castration when compared to values in intact animals.

There was no difference in the concentration or content of LHRH in pituitary stalk plasma after castration of the control rats or the rats with pituitary transplants (Figures 4.2a, 4.2b).

TABLE 4.2: The concentration (pg/ml) and content (pg/30 min) of LHRH in pituitary stalk plasma of the rats in Experiment 1 with pituitary transplants, before and during electrical stimulation of the median eminence. The volume of pituitary stalk blood collected over the 30 min pre-stimulation and stimulation periods is also shown.

	Pre-stimulation			Stimulation		
Rat No.	LHRH concentration (pg/ml)	LHRH content (pg/30 min)	Volume (µl/30 min)	LHRH concentration (pg/ml)	LHRH content (pg/30 min)	Volume (µl/30 min)
1	19	3	150	193	41	210
2	12	4	300	115	71	460
3	22	3	140	6	1	210
4	152	23	150	79	37	470

TABLE 4.3: Comparison of the doses of Althesin (ml/100 g body weight) required to induce anaesthesia for surgery to collect pituitary stalk blood from male rats of the PVG strain (controls and hyperprolactinaemic rats) and female rats of the Albino Wistar strain (at 16.00 h on pro-oestrus or oestrus). Values given are mean ± S.E.M. * = $p < 0.05$ when the values are compared with each other.

Group	n	Body weight (g)	Dose injected (ml/100 g)
Males:			
Control	11	238.0 ± 14.0	*1.9 ± 0.2
Hyperprolactinaemic	11	269.0 ± 7.0	*0.7 ± 0.0
Females:			
Pro-oestrus	8	246.0 ± 8.0	0.6 ± 0.0
Oestrus	8	290.0 ± 8.0	0.5 ± 0.0

Electrical stimulation of the ME of castrated, control rats induced a significantly ($p < 0.05$) lower release of LHRH into stalk plasma compared with that in intact, control rats (Figures 4.2a, 4.2b). This difference in the release of LHRH during stimulation of the ME between the castrated and intact rats was not seen in the rats with pituitary transplants or after administration of T to rats in Group A or Group B.

- *Effects of testosterone implants*

Testosterone (T) implants of 10 mm lengths did not suppress the post-castration rises in plasma LH and FSH in either the control or hyperprolactinaemic rats. However, the 30 mm implants of T were effective in restoring plasma LH concentrations to values in intact rats, in both the control group and the group of animals with pituitary transplants. The T implants (10 mm or 30 mm lengths) had no effect on the concentration of PRL in peripheral plasma (Figure 4.1) and LHRH in stalk plasma (Figures 4.2a, 4.2b) in either the control rats (Group A) or those with pituitary transplants (Group B) compared with that in intact rats.

Electrical stimulation of the ME of the rats with T implants produced a significant ($p < 0.05$) increase in the release of LHRH into stalk plasma compared with that before stimulation (Figures 4.2a, 4.2b). The increase in LHRH concentration in stalk plasma of castrated rats with 10 mm and 30 mm implants of T was equal to that in intact control rats and hyperprolactinaemic rats. However, the LHRH content in stalk blood of control rats with 30 mm implants of T was significantly ($p < 0.05$) lower than in intact, control rats during the stimulation period.

(b) DA and DOPAC secretion into pituitary stalk plasma:

The concentration of DA in peripheral plasma was undetectable (<100 pg/ml) in all groups of rats.

The concentrations (Figure 4.3a) and content (Figure 4.3b) of DA and DOPAC in pituitary stalk blood from intact, control rats was similar to those of rats castrated for 14 days and rats with chronic hyperprolactinaemia (pituitary transplanted rats; Figure 4.1). Implantation of implants of T into castrated rats had no effect on either the concentration or content of DA or DOPAC in pituitary stalk plasma.

Electrical stimulation of the median eminence did not alter the release of DA into stalk plasma, but there was a significant ($p < 0.05$) increase in DOPAC concentrations and content during stimulation compared to values before stimulation in intact, control rats.

4.3.2 Experiment 2

(a) Concentrations of LH, FSH and PRL in peripheral plasma:

The concentrations of LH, FSH and PRL in peripheral plasma of groups of intact, control rats and intact rats with pituitary transplants are shown in Figures 4.4 and 4.5. The values obtained in Experiment 1 (Figures 4.1 and 4.2) and a mean of the values from Experiments 1 and 2 are also shown.

The concentration of plasma PRL was significantly ($p < 0.05$) higher in the rats with pituitary transplants than in the control rats in Experiment 2, as found in Experiment 1. In contrast to Experiment 1, the hyperprolactinaemia induced by the pituitary transplants in Experiment 2 was associated with a significant ($p < 0.05$) decrease in the plasma concentration of LH. The plasma LH concentrations in the combined mean of the two experiments was not significantly different in the hyperprolactinaemic rats compared with the control rats.

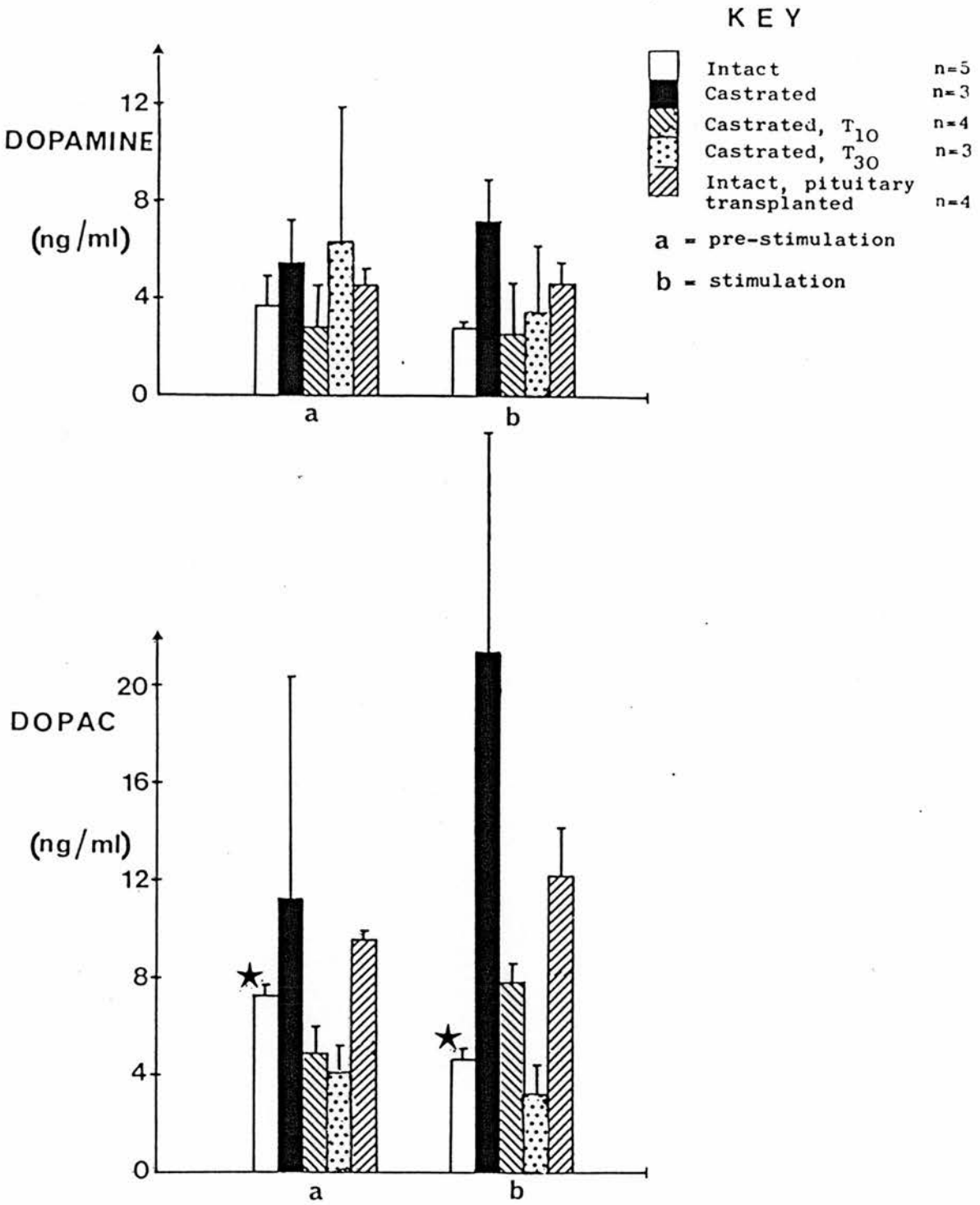

FIGURE 4.3a: The contentration of dopamine and dihydroxyphenyl-acetic acid (DOPAC) in pituitary stalk plasma of intact rats, castrated rats with and without testosterone implants and rats with pituitary transplants, measured by HPLC with electrochemical detection. The blood samples were collected under Althesin anaesthesia for 30 min periods before and during electrical stimulation of the median eminence.

T_{10} = 10 mm and T_{30} = 30 mm testosterone implants.

* = $p < 0.05$ when the values are compared with each other.

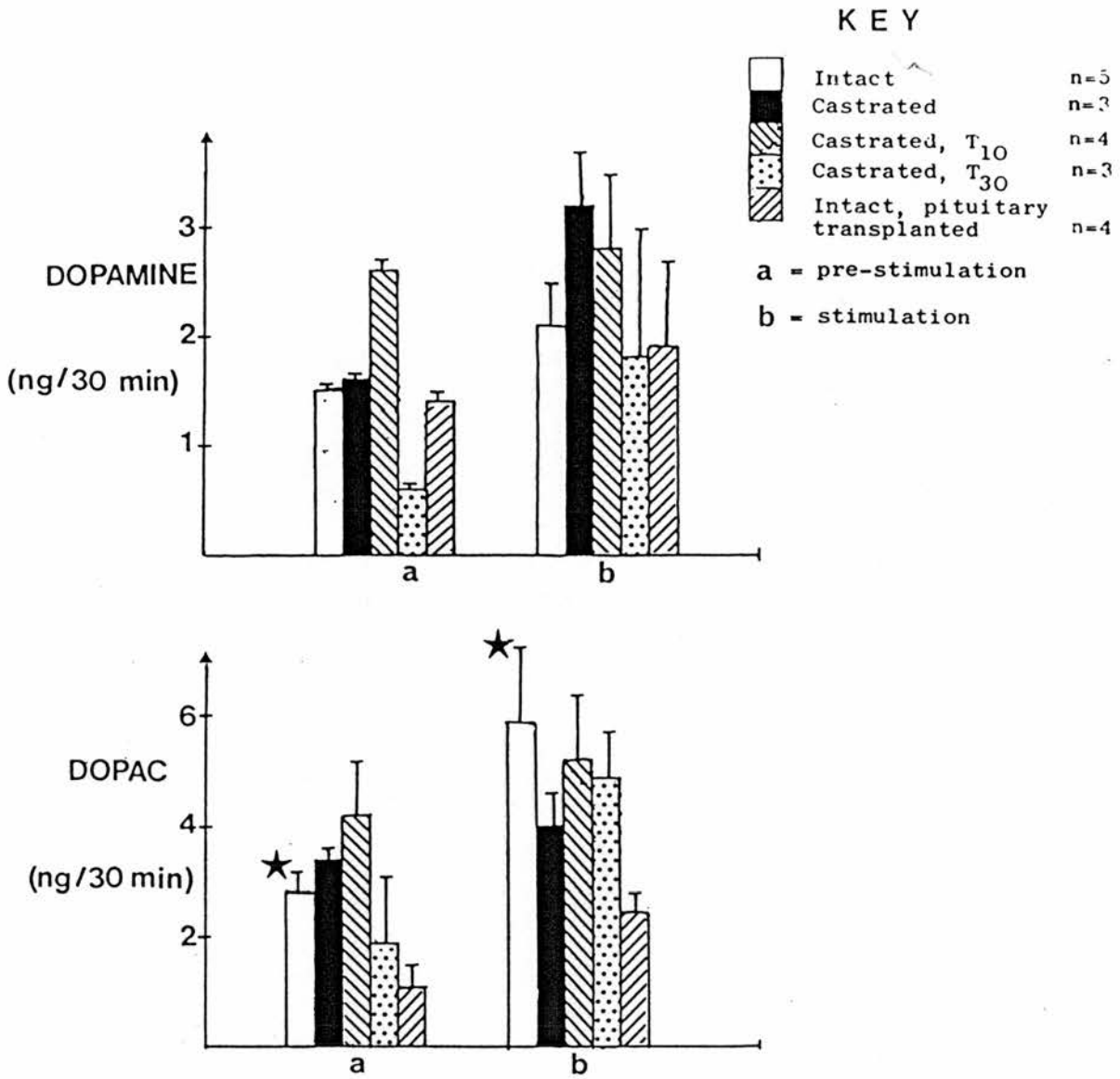

FIGURE 4.3b: The content of dopamine and dihydroxyphenyl-acetic (DOPAC) in pituitary stalk blood of intact rats, castrated rats with and without testosterone implants, and rats with pituitary transplants, measured by HPLC with electrochemical detection. The blood samples were collected under Althesin anaesthesia for 30 min periods before and during electrical stimulation of the median eminence.

T_{10} = 10 mm and T_{30} = 30 mm testosterone implants.

* = $p < 0.05$ when the values are compared with each other.

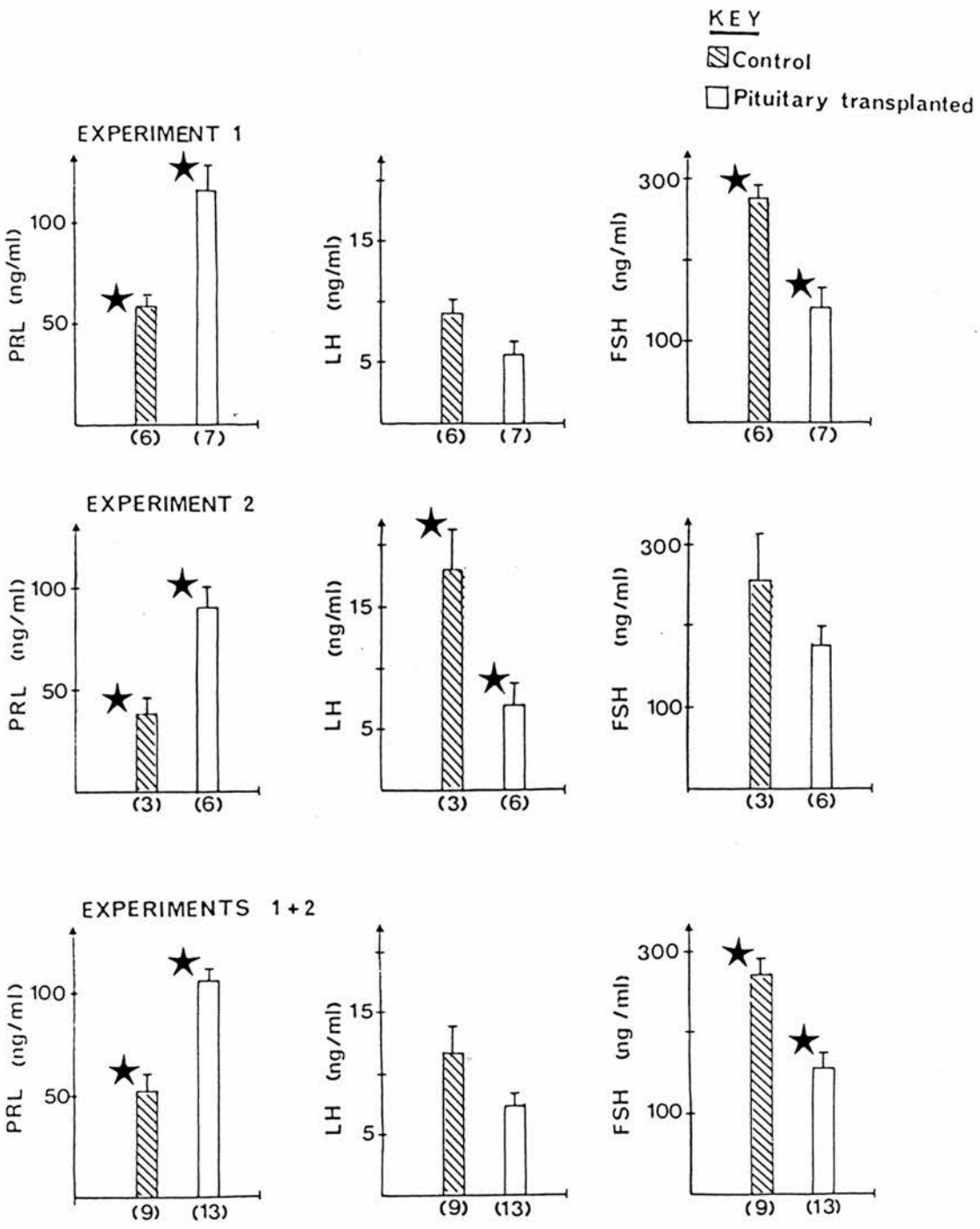

FIGURE 4.4: Peripheral plasma concentrations of PRL, LH and FSH in groups of control rats and rats with pituitary transplants. Blood samples were collected under Althesin anaesthesia in two separate experiments. The values given are the mean ± S.E.M. (n) obtained in each experiment and a mean of the combined experiments. The concentration and content of LHRH in the stalk blood of these groups of rats is shown in Figure 4.5.

* = $p < 0.05$ when the values within each parameter are compared with each other.

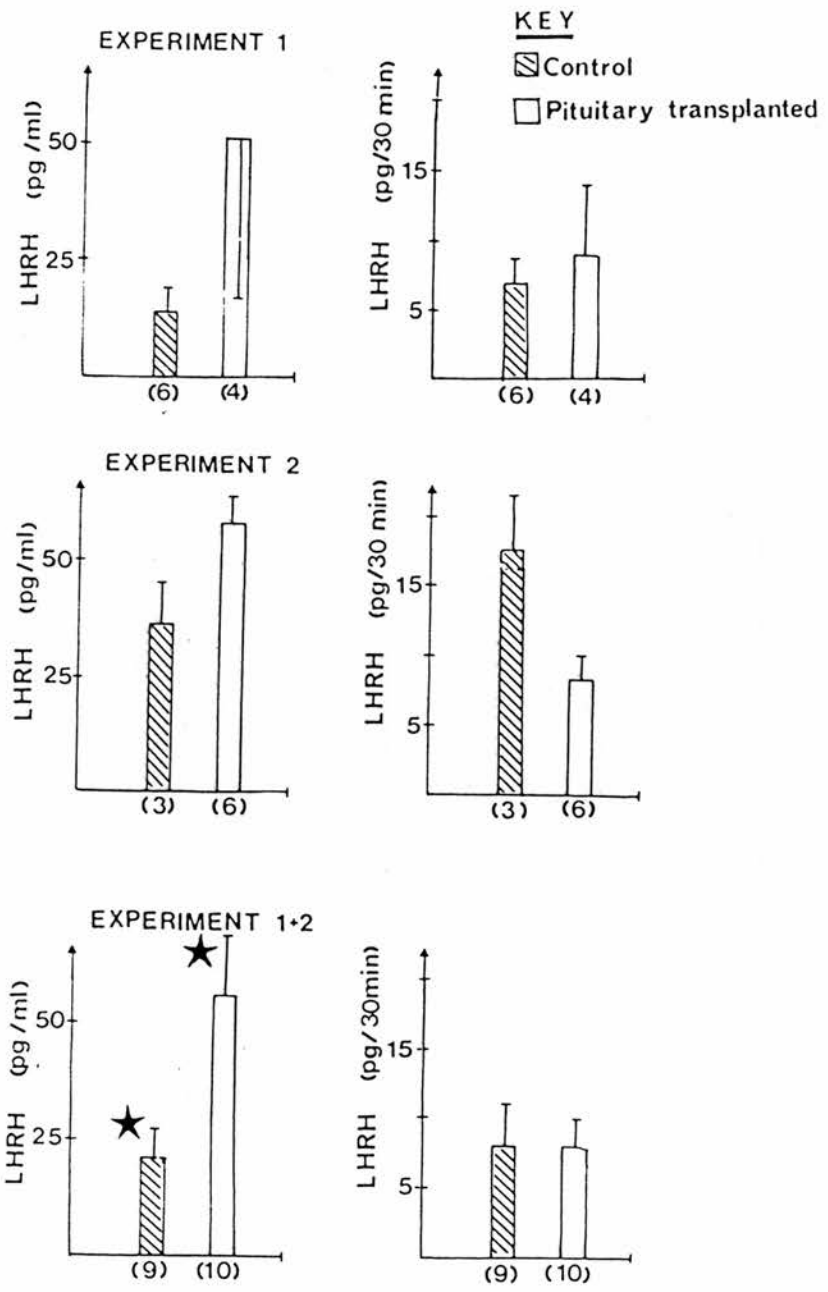

FIGURE 4.5: The concentration and content of LHRH in pituitary stalk blood of control rats and rats with pituitary transplants. Blood samples were collected under Althesin anaesthesia in two separate experiments. The mean ± S.E.M. (n) values in each experiment and the combined mean of the experiments is given. The peripheral plasma concentrations of PRL, LH and FSH in these rats are shown in Figure 4.4.

* = $p < 0.05$ when the values are compared with each other.

The plasma FSH concentration of the control rats in Experiment 2 was not significantly different from the control rats in Experiment 1 (Figure 4.4), but, in contrast to Experiment 1, hyperprolactinaemia did not suppress plasma FSH concentrations. The mean of the combined experiments showed that plasma FSH concentrations were suppressed by hyperprolactinaemia (Figure 4.4).

(b) LHRH concentration in pituitary stalk plasma:

The mean concentration and content of LHRH in stalk plasma of the rats in Experiment 1 or the mean of the combined experiments (1 + 2; Figure 4.5) were not altered by the implantation of pituitary transplants compared with that in control rats.

As in Experiment 1, electrical stimulation of the median eminence of the rats in Experiment 2 induced a significant increase in the stalk plasma concentration and content of LHRH in control rats and rats with pituitary transplants (Figure 4.6). Although the increase in LHRH concentration in stalk plasma in rats with pituitary transplants was significantly ($p < 0.05$) greater than in the control rats, this difference was not seen when the contents of LHRH during the stimulation period were compared.

4.3.3 Some Observations on the Pituitary Grafts

Examinations under the dissecting microscope (Figure 4.7a) showed that the pituitary grafts were well vascularised around 42 days after implantation under the kidney capsule, when pituitary stalk blood samples were collected. Wax-embedded sections of the kidney and the pituitary grafts taken from rats that had been perfused with Indian ink (Figures 4.7b, 4.7c) also confirmed that the pituitary transplants were vascularised after implantation. Some cell death occurred in the pituitary grafts, indicated by the yellow cells in unstained sections of the grafts (Figure 4.7d).

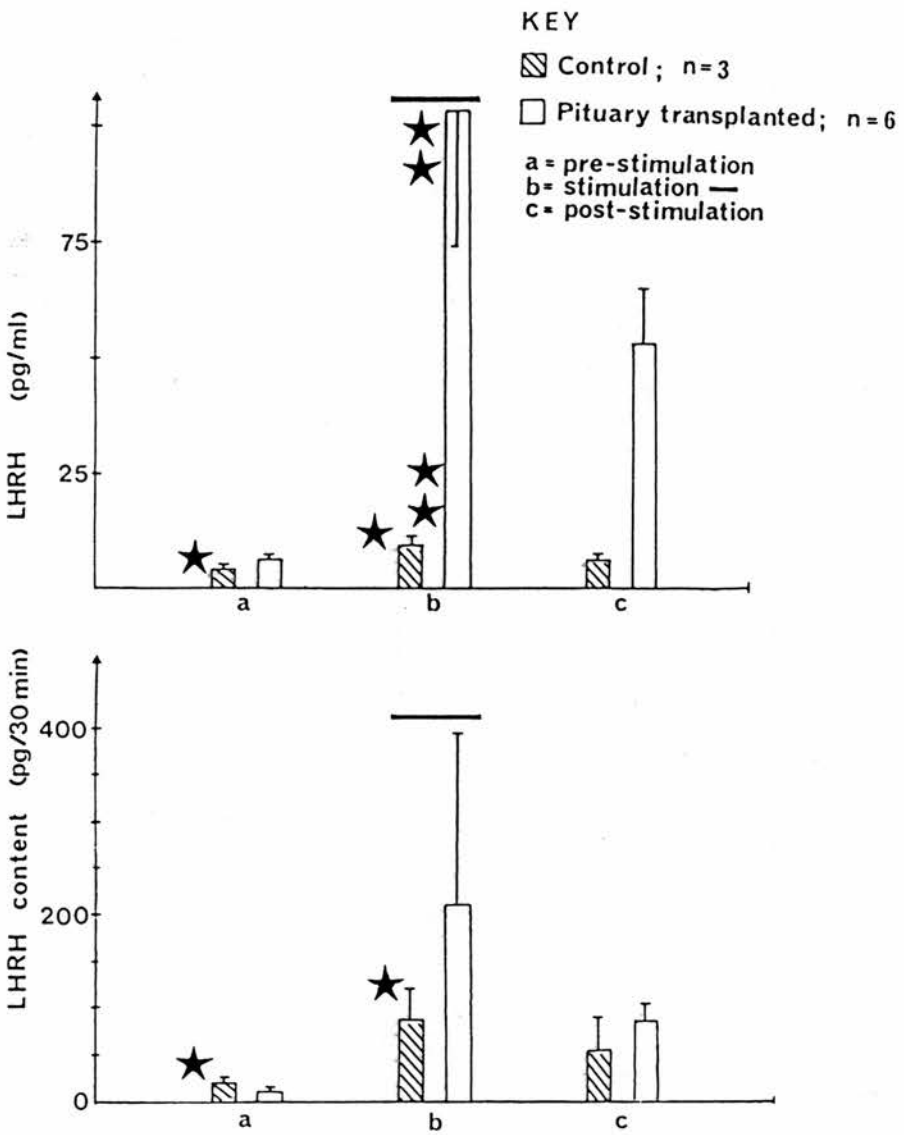

FIGURE 4.6: The concentration and content of LHRH in stalk blood of control rats and rats with pituitary transplants before, during and after electrical stimulation of the median eminence, under Althesin anaesthesia. The values shown are the mean ± S.E.M.

*, ** = $p < 0.05$ when the values within each parameter are compared with each other.

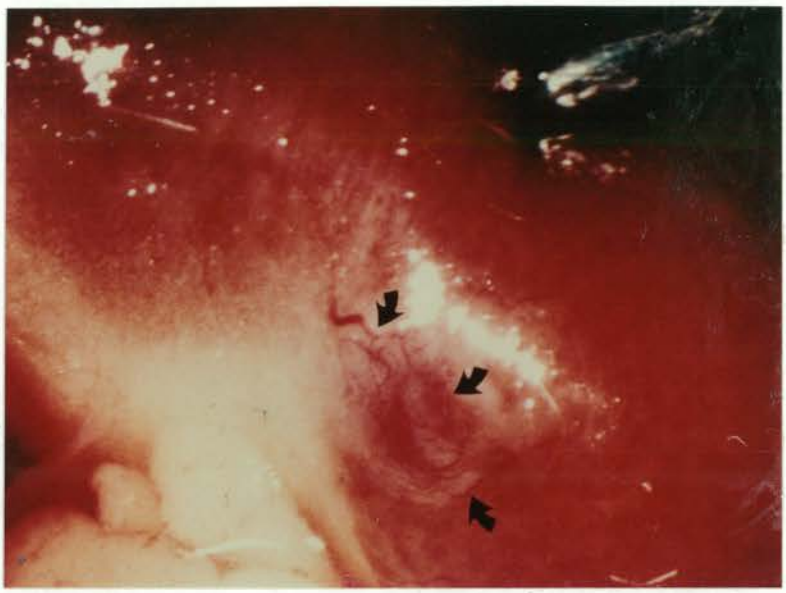

FIGURE 4.7a: The anterior pituitary transplant 42 days after implantation under the kidney capsule, as seen under the dissecting microscope. The pituitary transplant is well-vascularised (blood vessels indicated by arrows).

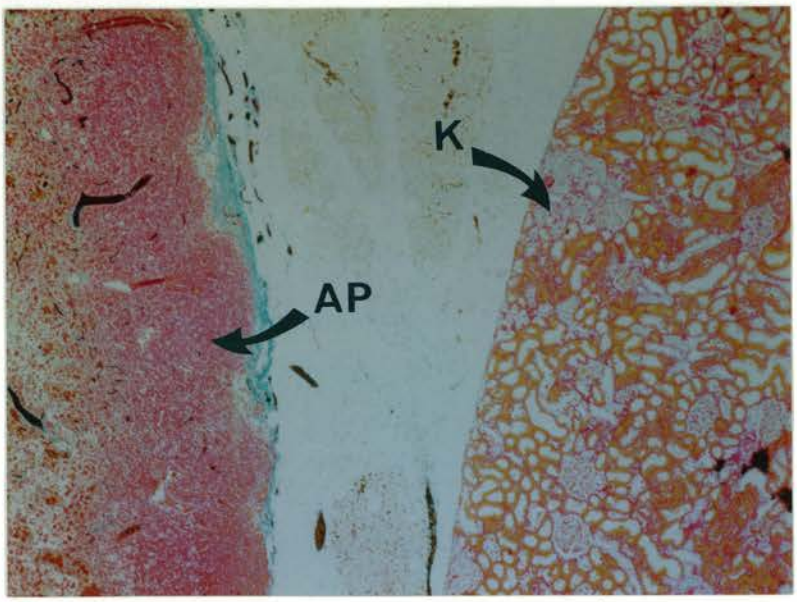

FIGURE 4.7b: Transverse section of the pituitary transplant (AP) which was transplanted under the kidney (K) capsule. Blood vessels containing indian ink with which the animal was perfused before removing the kidney are indicated by arrows.

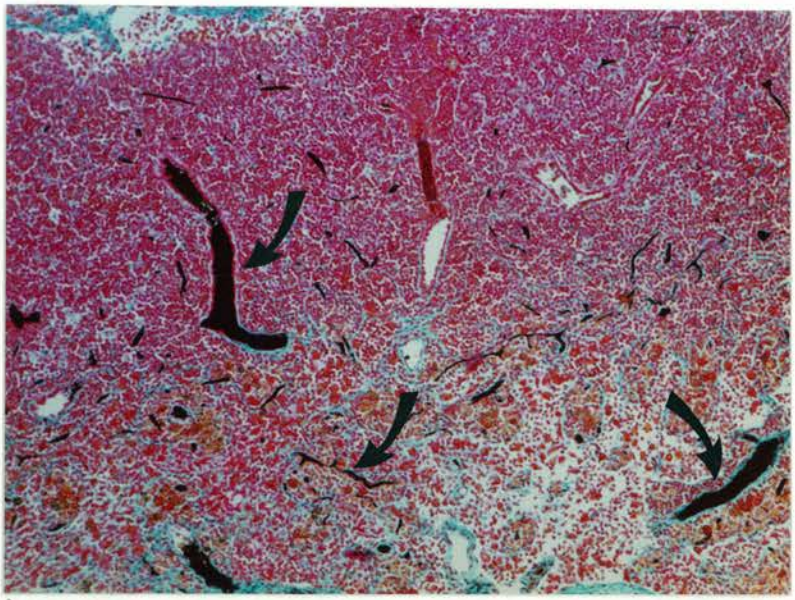

FIGURE 4.7c: Transverse section of the pituitary transplant at a high magnification showing indian ink in blood vessels (indicated by arrows).

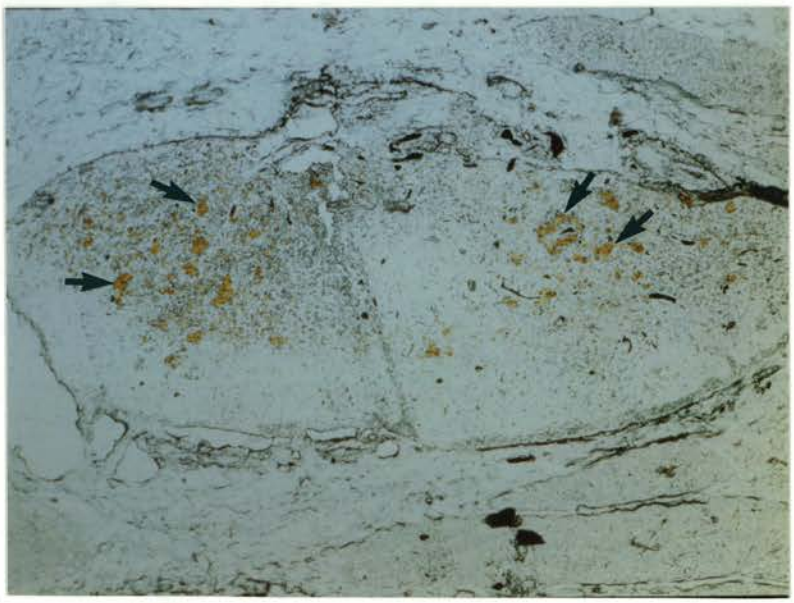

FIGURE 4.7d: An unstained section of the pituitary transplant. Arrows indicate dead tissue (yellow) in the pituitary which was transplanted 42 days earlier.

4.4 Discussion and Conclusions

Pituitary transplants under the kidney induced an approximately 2-fold increase in plasma PRL concentrations and suppressed the release of pituitary gonadotrophins, as shown previously (Bartke *et al.*, 1978; McNeilly *et al.*, 1978; Winters and Loriaux, 1978). The plasma concentration of PRL induced by the pituitary transplants are at the lower range of those found in post-partum lactating rats (Lu, Chen, Grandison, Huang and Meites, 1976b) and were therefore within the physiological range. The post-castration rise in plasma LH and FSH was inhibited by hyperprolactinaemia, in agreement with the findings of Grandison *et al.* (1977), McNeilly *et al.* (1978), Winters and Loriaux (1978) and Greeley and Kizer (1979).

The implantation of T capsules into castrated rats suppressed the post-castration rise of plasma LH and FSH in the hyperprolactinaemic rats and in the control rats as previously reported (McNeilly *et al.*, 1978, 1980). The plasma T concentrations achieved by the capsules of T in control and hyperprolactinaemic rats have been reported to be 2.1 ± 0.2 v. 1.8 ± 0.1 ng/ml for the 10 mm implants and 4.0 ± 0.2 v. 5.0 ± 0.3 ng/ml for the 30 mm implants (McNeilly *et al.*, 1980). The data in Figure 4.1 therefore suggest that plasma T concentrations greater than or equal to 4 ng/ml are required to completely suppress the post-castration rises of plasma LH and FSH. This plasma T concentration is considerably higher than the value of 1.6 ng/ml found by Damassa, Kobashigawa, Smith and Davidson (1976) to maintain LH concentrations at or below those found in intact animals. The plasma concentrations of T in intact and hyperprolactinaemic intact rats have been reported to be 8.3 ± 0.4 and 3.7 ± 0.4 ng/ml respectively (McNeilly *et al.*, 1980). The fact that the 10 mm implants of T

were ineffective in reducing plasma LH or FSH concentrations to intact values in the hyperprolactinaemic rats compared to the control rats does not support the hypothesis that hyperprolactinaemia sensitises the hypothalamic-pituitary axis to the negative feedback effects of testosterone (McNeilly *et al.*, 1980b, 1983). The reason why the 10 mm implants of T did not inhibit the post-castration rises in plasma LH and FSH in the hyperprolactinaemic rats (Figure 4.1) is not clear since the plasma concentrations of PRL in the rats used in this study (Figure 4.1) were similar to the values of plasma PRL reported by McNeilly *et al.* (1980a), although the basal plasma concentrations of LH and FSH of the hyperprolactinaemic rats (Figure 4.1) were overall lower than in the studies by McNeilly *et al.* (1980b).

The release of LHRH into pituitary stalk plasma was not altered significantly, either by inducing hyperprolactinaemia or by castration (Figures 4.2a, 4.2b). Therefore, neither the suppression of gonadotrophin secretion induced by hyperprolactinaemia nor the post-castration rises in gonadotrophin secretion can be attributed to a decrease and increase, respectively, in the release of LHRH into stalk blood. An increase in the concentration of LHRH in pituitary stalk blood has been reported to occur in ovariectomised rats that were also adrenalectomised (Sherwood and Fink, 1980). In the adult male rat it has been demonstrated that the gonads are required for PRL to exert an inhibitory influence on pituitary LH and FSH release (Grandison *et al.*, 1977; Winters and Loriaux, 1978; McNeilly *et al.*, 1980a). The role of the adrenal gland in modulating gonadotrophin secretion in the hyperprolactinaemic rat is still controversial (Bartke *et al.*, 1977; Greeley and Kizer, 1979; McNeilly *et al.*, 1980a; Weber, Ooms and Vreeburg, 1982). However, removal of the testes or replacement of T in castrated

rats did not alter the concentration of LHRH release in stalk plasma (Figures 4.2a, 4.2b). In addition, the rats with hyperprolactinaemia were not more sensitive to the feedback effects of T, as assessed by the LHRH concentration in stalk plasma.

Electrical stimulation of the ME of castrated, control rats did not increase the concentration of LHRH in stalk plasma to intact, control values. This significant ($p < 0.05$) difference between the castrated and intact control rats in the release of LHRH after electrical stimulation was not seen in the hyperprolactinaemic rats, suggests that PRL changes the response of the LHRH neurones to castration may sensitize LHRH neurones to input from electrical stimulii in castrated rats. The hypothalamic content of LHRH does not differ between intact, control rats and rats with pituitary transplants (McNeilly *et al.*, 1978). However, the demonstration that castration induced a decrease in hypothalamic content of LHRH (Shin and Howitt, 1976; Badger, Wilcox, Meyer, Bell and Cicero, 1978) which was reversed by replacement of T in the castrated rats (Shin and Howitt, 1976; Kalra and Kalra, 1978) suggests that the reason for less release of LHRH into stalk plasma of castrated rats following electrical stimulation of the ME, may be that there is less LHRH available for release in the castrated, control rats.

The number of LHRH receptors in the anterior pituitary gland was reduced below normal in rats immunised against LHRH (Fraser, Popkin, McNeilly and Sharpe, 1982b) and in rats with hyperprolactinaemia induced either by transplantation of pituitary glands for 98 days (Fraser *et al.*, 1982b) or by suckling in the lactating rat (Clayton *et al.*, 1980). From these studies it has been proposed that a reduction in endogenous LHRH secretion accompanies the decrease in the number of LHRH receptors in the pituitary gland of hyperprolactinaemic rats

(Clayton *et al.*, 1980; Fraser *et al.*, 1982b). This view, however, is not substantiated by the demonstration that the release of LHRH into pituitary stalk blood of hyperprolactinaemic rats was not significantly different from control rats (Figure 4.5).

The secretion of DA into pituitary stalk blood was not increased by long-term hyperprolactinaemia or castration compared to intact controls (Figures 4.3a, 4.3b). These concentrations of DA were significantly ($p < 0.05$) higher than those previously published by Ben-Jonathan, Oliver, Weiner, Mical and Porter (1977) and Gudelsky and Porter (1980) who, using pentobarbitone anaesthesia and a radio-enzymatic assay, found that the DA concentrations in pituitary stalk were only 0.2 - 0.6 ng/ml in intact and castrated rats, and 1.1 ng/ml in rats given PRL by intracerebroventricular injections. The results presented here are the first data on DA concentrations in pituitary stalk plasma collected under Althesin (an anaesthetic that does not suppress the pre-ovulatory LHRH surge; Sarkar *et al.*, 1976), and measured using HPLC with electrochemical detection. The higher concentration of DA measured in the present studies shows that pento-barbitone anaesthesia result in lower plasma DA concentrations than those found under Althesin anaesthesia.

The lack of a significant difference in the DA concentration in pituitary stalk plasma of hyperprolactinaemic rats has also been reported by Cramer, Parker and Porter (1979a), who found that the DA concentration in pituitary stalk plasma of female rats bearing anterior pituitary transplants were similar to dieoestrus control rats. Electrical stimulation of the ME did not alter the release of DA into stalk plasma in any of the groups of rats, although DOPAC release was increased. Direct electrical stimulation of the ME has been reported

(Fekete, Herman, Kanyicska and Makar, 1980) to reduce the DA content of the ME, although ME stimulation did not alter the plasma PRL concentrations in the study by Fekete *et al.* (1980).

A recent report (Weber, de Greef, de Koning and Vreeburg, 1983) that male rats bearing PRL- and adrenocorticoid (ACTH)-secreting pituitary tumours showed an increased rate of secretion of DA and a decreased rate of secretion of LHRH into stalk plasma suggests that a significant increase in plasma PRL as well as ACTH may be necessary to observe changes in DA and LHRH release. Adrenalectomised, tumour-bearing rats did not show the decreases in plasma LH and FSH or LHRH secretion seen in intact rats (Weber *et al.*, 1983). Furthermore, the plasma concentrations of PRL induced by the tumours are 12-fold greater than those found in rats with pituitary transplants (Figure 4.1; Bartke *et al.*, 1977; McNeilly *et al.*, 1980b), post-partum lactating rats (Whitworth, Grosvenor and Mena, 1981) or after electrical stimulation of the mammary nerve trunk of lactating rats (de Greef, Plotsky and Neill, 1981). The physiological relevance of the study by Weber *et al.* (1983) is therefore debatable.

Hyperprolactinaemia sensitized the brain to the anaesthetic effects of the steroid, Althesin (Table 4.3). The response to the anaesthetic effects if Althesin has been found to differ between male and female rats and appeared to be due mainly to plasma oestrogen concentrations (Fink, Sarkar, Dow, Dick, Borthwick, Malnick and Twine, 1982). Elevated concentrations of oestradiol in the circulation would induce high plasma PRL concentrations (Ratner, Talwalker and Meites, 1963; Meites and Clemens, 1972; Gudelsky, Nansel and Porter, 1981). Therefore, the sex difference in the response to Althesin (Fink *et al.*, 1982) cannot be due solely to increased plasma concentrations of oestrogen, but is probably due to elevated plasma concentrations of PRL.

The morphology of the pituitary grafts 15, 45 and 90 days after implantation under the kidney capsule has been examined by Everett (1954); Aguado, Alvial and Rodriguez (1977, 1981). Vascularisation of the transplanted pituitary gland, observed at around 42 days after implantation (Figures 4.7a, 4.7b), has been observed as early as 8 days after implantation (Everett, 1954), although some cell death was visualised in the implants (Figure 4.7d).

Electron microscopical studies on the pituitary grafts showed that the number of LH and FSH cells had decreased 45 days after implantation of the pituitary grafts compared to implantation for 15 days (Aguado *et al.*, 1981). The number of prolactin cells in the pituitary transplants 45 days after implantation was significantly less than at either 15 or 90 days after implantation, although the prolactotrophs displayed hypertrophied organelles which is indicative of high secretory activity (Aguado *et al.*, 1981).

The main conclusions that can be drawn from the studies described in this chapter are that,

i) the post-castration rises in plasma gonadotrophins cannot be attributed to an increased release of LHRH or a decreased release of DA into pituitary stalk blood compared to control rats, although electrical stimulation of the median eminence of castrated rats induced lower LHRH release than in control rats;

ii) T administration to control rats or hyperprolactinaemic rats had no effect on the concentration of LHRH in stalk plasma which suggests that the negative feedback effects of testosterone on gonadotrophin secretion, in castrated control rats or in castrated hyperprolactinaemic rats, cannot be attributed to a suppression of LHRH release into stalk blood;

iii) long-term hyperprolactinaemia did not alter the secretion of LHRH or DA into stalk blood although pituitary gonadotrophin secretion was suppressed, compared to control rats;

iv) the suppression of the post-castration rises in plasma gonadotrophins in hyperprolactinaemic rats was not due to a reduction in the secretion of LHRH into stalk blood in hyperprolactinaemic rats compared to control rats.

CHAPTER V

The Effects of Acute Administration of 2-Hydroxylated Metabolites of Oestrogens on LH and PRL Secretion in Pre-pubertal Rats

CHAPTER V:

5.1 Introduction

5.1.1 Formation and Distribution of Oestrogen Metabolites

The exact neuroendocrine mechanisms underlying the biphasic regulation of pituitary gonadotrophin and PRL secretion by oestrogens are still not fully understood. There is evidence that suggests the involvement of oestrogen metabolites. The biotransformation of oestrogens in mammals has been demonstrated to be almost exclusively limited to oxidative reactions (Figure 5.1a). The first step involving oxidation of the 17β-hydroxy group of oestradiol to the ketone oestrone is followed by competitive hydroxylations in the five carbon D ring or in the aromatic A ring (Fishman, Bradlow and Gallagher, 1960). The ring D hydroxylation is predominantly at the 16 α-position, forming oestriol (Marrian, 1930) and 16 α-hydroxyoestrone (Marrian, Loke, Watson and Panattoni, 1957). The major end-products of the competitive ring A hydroxylation at either the C-2 or C-4 position are, respectively, the 2- and 4-hydroxyoestrogens (Fishman, Cox and Gallagher, 1960; Ball and Knuppen, 1978; Emons, Hoppen, Ball and Knuppen, 1980). The term "catechol oestrogen" (CAE) is applied to these hydroxylated metabolites of oestrogen which have the 18C structure of oestrogens with a phenolic hydroxyl group at C-3 and an additional one at C-2 or C-4, resulting in a catechol group (an aromatic ring bearing two hydroxyl groups) at one end of the oestrogen molecule (Figure 5.1b). The 2-hydroxyoestrogens, 2-hydroxyoestradiol and 2-hydroxyoestrone will be referred to as CAE_2 and CAE_1, respectively†.

The catechol oestrogens are partially O-methylated to 2-methoxy-oestrogens by a catechol-O-methyl transferase (COMT), which is

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†CAE_1 = 1,3,5(10)-estratriene-2,3-diol-17-one
CAE_2 = 1,3,5(10)-estratriene-2,3,17-triol

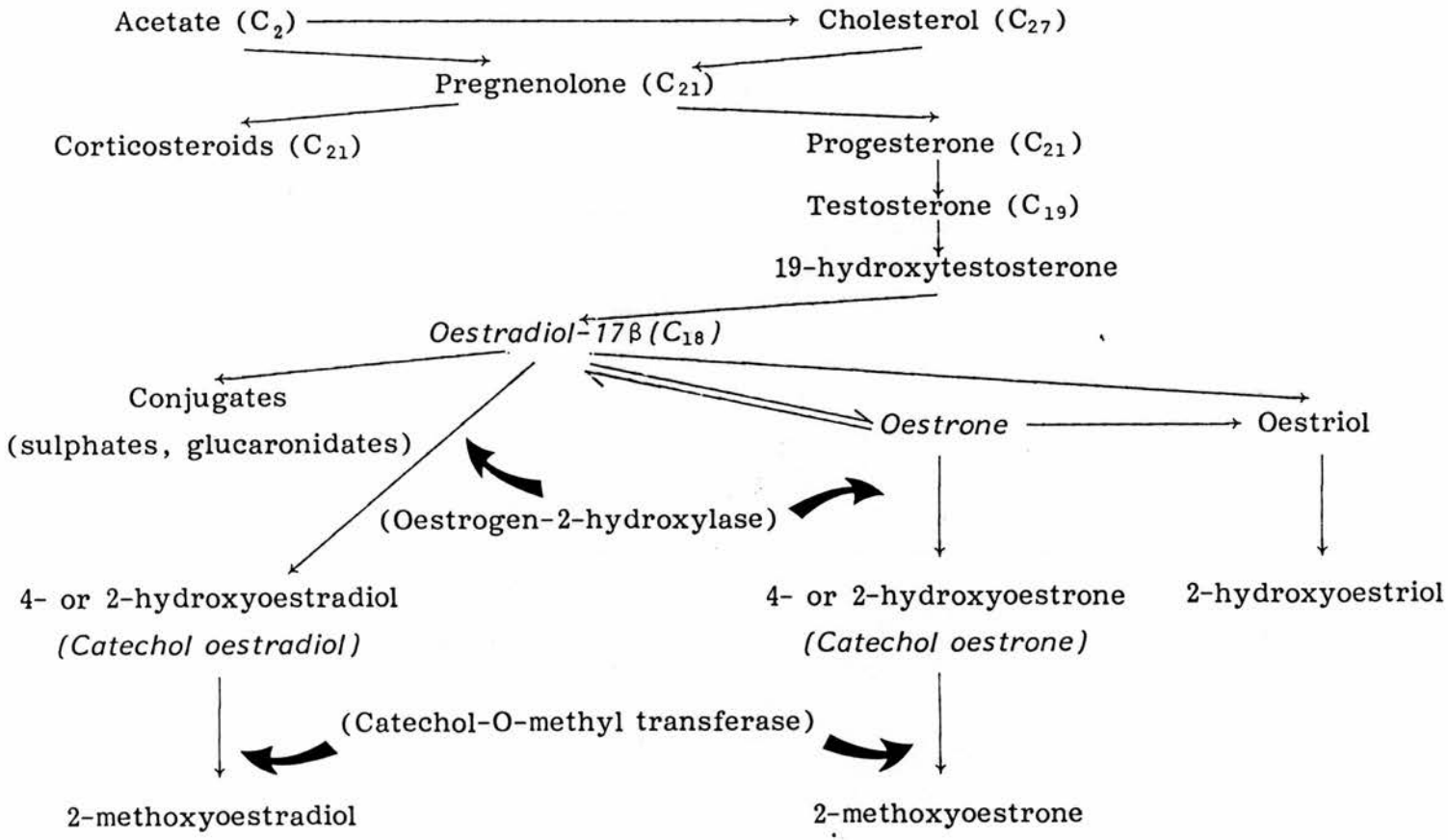


FIGURE 5.1a: Main pathway in the metabolism of oestrogens and catechol oestrogens (based on Dorfman, 1963; Ball, Hoppen and Knuppen, 1974).

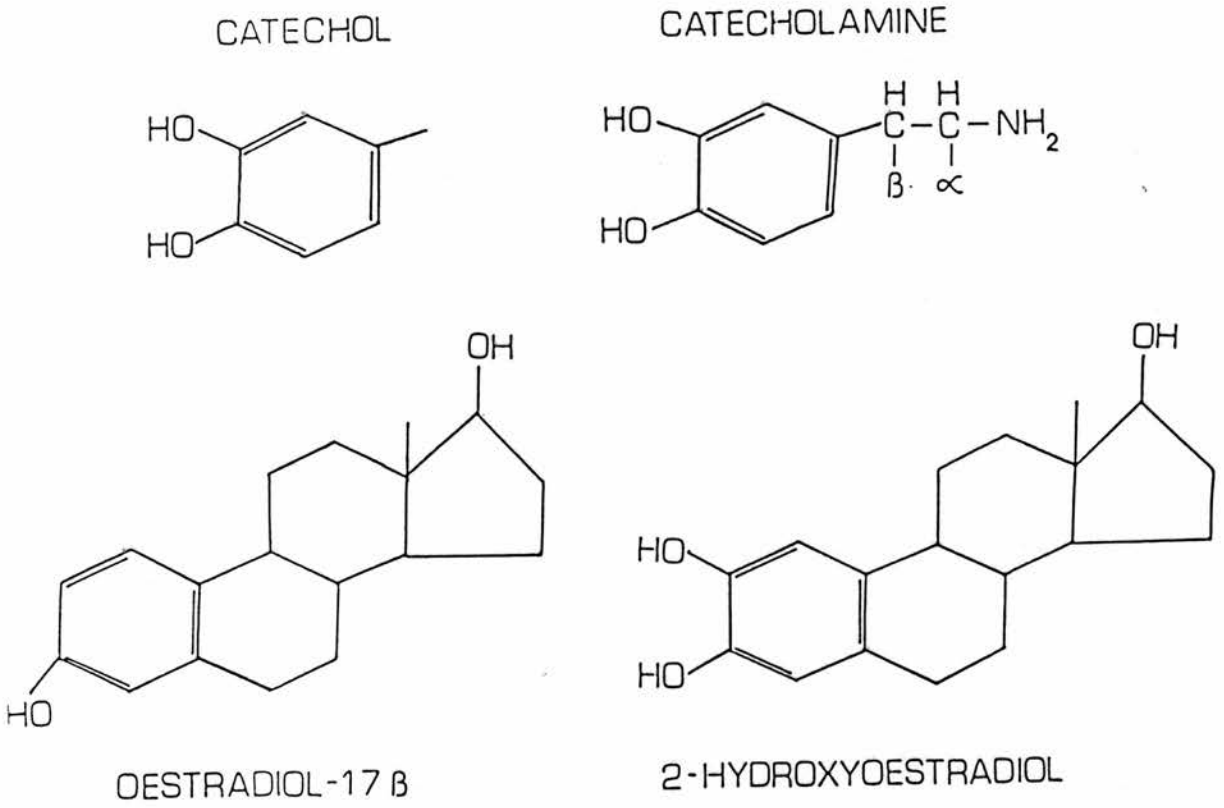


FIGURE 5.1b: Formulae of the catechol group, catecholamines, oestradiol-17β and 2-hydroxyoestradiol (catechol oestradiol, CAE_2) showing the structural similarity of 2-hydroxyoestradiol to both the catecholamines and oestradiol-17β.

identical with the enzyme responsible for the O-methylation of the catecholamines (Kraychy and Gallagher, 1957; Ball, Knuppen, Haupt and Breuer, 1972; Hoffman, Paul and Axelrod, 1980a). It has been demonstrated that CAE_2 is an effective inhibitor of both tyrosine hydroxylase (TH) (Lloyd and Weisz, 1978; Foreman and Porter, 1980), the rate-limiting enzyme in catecholamine biosynthesis (Levitt, Spector, Sjoerdsma and Udenfriend, 1965), and COMT, an enzyme involved in the metabolism of the catecholamines (Ball *et al.*, 1972; Breuer and Koester, 1974). Inhibition of the activity of these enzymes by micromolar concentrations of CAE_2 has been found to depend on the "catechol" part of the molecule rather than the aliphatic side chain (Lloyd and Ebersole, 1980; Lloyd and Weisz, 1978). Although the enzymes, TH and COMT, from steroid-target areas of the brain (e.g. hypothalamus, amygdala) are not more sensitive to inhibition by CAE_2 than other areas of the brain investigated (e.g. striatum), the inhibition of enzyme activity by CAE_2 was comparable to that of equimolar levels of dihydrophenylalanine (DOPA), the precursor of endogenous catecholamines, when subsaturating levels of the endogenous cofactor tetrahydrobiopterin were used (Lloyd and Ebersole, 1980). In a recent study, Parvizi and Wuttke (1983) reported that CAE_1 and CAE_2 reduced catecholamine turnover in the anterior medial hypothalamus and pre-optic area in castrated male and female rats. These interactions with key enzymes in the aminergic system have been postulated to act as a "biochemical link" between the oestrogens and the neurotransmitters found to be most directly involved in the hypothalamic control of gonadotrophin and PRL release (McCann, Ojeda, Fawcett and Krulich, 1974; Weiner and Ganong, 1978; Barraclough and Wise, 1982).

The formation of the catechol oestrogens, CAE_1 and CAE_2 is catalysed by oestradiol-2-hydroxylase which is widely distributed in body tissues, although the highest activity has been found to occur in the liver (King, 1961; Barbieri, Canick and Ryan, 1978; Hoffman, Paul and Axelrod, 1980b). Neither the ovary, a site of oestradiol synthesis, nor the virgin uterus, a major oestradiol target, had significant oestradiol-2-hydroxylase activity. The discovery that this enzyme is active in the brain and pituitary gland of man (Ball and Knuppen, 1978) and rats (Fishman and Norton, 1975; Ball, Haupt and Knuppen, 1978; Hoffman *et al.*, 1980b) has stimulated speculation that the transformation of oestrogens to catechol oestrogens was involved in the physiological regulation of gonadotrophin and PRL release by oestrogens. This concept received support from subsequent demonstrations that the catechol oestrogens are present in the pituitary and hypothalamus in concentrations at least tenfold higher than the parent oestrogens (Paul and Axelrod, 1977). In addition, CAE_2 has been shown to compete with oestrogens for binding to soluble oestrogen receptors in the anterior pituitary and hypothalamus (Davies, Naftolin, Ryan, Fishman and Siu, 1975; Clarke and Findlay, 1980), as well as with dopaminergic ligands for dopamine receptors in rat anterior pituitary membranes (Schaeffer and Hseuh, 1979).

5.1.2 Catechol Oestrogens and PRL Release

A role for CAE_2 as a dopamine antagonist which removes dopaminergic inhibition of PRL release has been proposed to account for the observation that oestrogens stimulate PRL secretion (Chen and Meites, 1970; Frantz, Kleinberg and Noel, 1972; de Léan, Garon, Kelly and Labrie, 1977). CAE_2 administration has been reported to suppress PRL release in immature rats (Barbieri, Todd, Morishita,

Ryan, Fishman and Naftolin, 1980) and stimulate PRL release in hypogonadal women (Adashi, Casper, Fishman and Yen, 1980), ovariectomised rats (Yanai and Nagasawa, 1979), male rats bearing atrial cannulae (Shin, Bates and Jellinck, 1981) and 25-day old female rats (Rodriguez-Sierra and Blake, 1982a). CAE_1 on the other hand, suppressed PRL secretion in normal young women (Fishman and Tulchinsky, 1980) and oestrogen-primed post-menopausal women (Adashi, Rakoff and Fishman, 1979; Schinfeld, Tulchinsky, Schiff and Fishman, 1980), adult male rats bearing oestradiol implants (Shin *et al.*, 1981) and inhibited the pre-ovulatory PRL surge in cycling rats (Katayama and Fishman, 1982). *In vitro* studies showed that CAE_2, like dopamine, inhibited PRL release from the superfused rat pituitary gland (Linton, White, de Tineo and Jeffcoate, 1981) and both CAE_1 and CAE_2 inhibited the growth of PRL-secreting tumours (Lambert, Nagy, Utterlinden and MacLeod, 1982). Normal PRL synthesis by the pituitary gland has been found to be inhibited by CAE_1 while CAE_2 stimulated PRL synthesis and release from male rat pituitary glands and inhibited release from female rat pituitary glands (Lambert *et al.*, 1982).

5.1.3 Catechol Oestrogens and LH Secretion

Studies on the effect of the catechol oestrogens on gonadotrophin release has also produced a vast amount of conflicting data. The demonstration that CAE_1 induced a dose- and time-dependent rise in serum LH concentration in immature male rats (Naftolin, Morishita, Davies, Todd, Ryan and Fishman, 1975) provided the first evidence for a biological action of the catechol oestrogens on a physiological parameter. The primary oestrogens, oestradiol and oestrone, lowered LH and FSH in this model (Morishita, Adachi, Naftolin, Ryan and Fishman, 1976). This positive feedback effect of CAE_1 in the male rat after acute

administration has since then been challenged (Rodriguez-Sierra and Blake, 1980b). In addition, a potent inhibition of LH release by CAE_1 and CAE_2 has been demonstrated in 35-40-day old male rats (Rodriguez-Sierra and Blake, 1980b), suggesting that these catechol oestrogens could instead play a role in the negative feedback effect on LH secretion. In ovariectomised, oestrogen-primed rats the administration of CAE_1 and 4-hydroxyoestradiol in the morning potentiated the cyclical release of LH and FSH in the afternoon (Gethmann and Knuppen, 1976; Ball, Emons, Klingebiel, Gruhn and Knuppen, 1981). Continuous administration for up to 72 h of a number of oestrogen metabolites to 22-day old ovariectomised rats, by means of implanted osmotic pumps, showed that CAE_2 and 4-hydroxyoestrone induced a suppression of plasma LH in morning samples, 2-methoxyoestrone had no effect, while CAE_1 produced a statistically significant increase in plasma LH after 48 hours of infusion (Martucci and Fishman, 1979). The stimulation of LH secretion by CAE_1 suggested that CAE_1 may be involved in the positive feedback effect on gonadotrophin secretion in the female rat. However, a recent study demonstrated that CAE_2, not CAE_1, induced a surge in LH secretion 48 hours after administration to 25-day old female rats (Rodriguez-Sierra and Blake, 1982b). The administration of CAE_1, CAE_2 or 4-hydroxyestrone together with oestradiol to ovariectomised ewes failed to alter the negative feedback action of oestradiol on LH secretion (Clarke and Findlay, 1980), which suggests that these catechol oestrogens lack anti-oestrogenic properties in this model. In the adult castrated rat, 4-hydroxyestradiol induced a potent decrease in plasma LH concentration while CAE_2 had no effect even at a high dose, although CAE_2 inhibited the suppression of LH release by 4-hydroxy-

estradiol and oestradiol (Foreman, 1980; Parvizi, Ellendorf and Wuttke, 1981). Acutely administered CAE_1 to hypogonadal women inhibited the release of LH and FSH provided they were oestrogen-primed (Adashi *et al.*, 1979).

5.1.4 Purpose of the Present Study

From the above account, it is clear that a firm conclusion cannot be reached regarding the role of the catechol oestrogens in neuroendocrine reproductive mechanisms. At the time this thesis was begun (October 1979) it seemed reasonable, therefore, to carry out controlled studies to establish whether CAE_1 or CAE_2

(i) modulated the release of LH and PRL;

(ii) exerted oestrogen-like effects on the uterus and ovaries;

(iii) altered the release of LH into peripheral plasma by modulating the release of LHRH into pituitary stalk blood.

The two models in which these effects of the catechol oestrogens were investigated were the immature male rat (Section 5.2) and the immature female rat treated with pregnant mare serum gonadotrophin, to induce ovulation (Section 5.3). On completion of the studies on the effects of CAE_1 and CAE_2 on LH and PRL release and the effects of CAE_2 on uterine and ovarian weights, it was not possible to obtain the required amounts of pure catechol oestrogens. Therefore, the effect of CAE_1 and CAE_2 on the release of LHRH into pituitary stalk blood was not investigated as originally planned.

5.2 The Effects of the Catechol Oestrogens on LH and PRL Secretion in the Immature Male Rat

5.2.1 Materials and Methods

All experiments were carried out using male Albino Wistar COBS (Charles River Ltd, Margate, Kent) which were aged 35 d and had been maintained under conditions described in Section 2.1 for one week. Groups of rats were injected s.c. with 0.25 ml of either the vehicle, CAE_1, CAE_2, oestradiol (OE_2) or oestrone (OE_1). The steroids were purchased from Sigma (Poole). Due to the exceptional instability of the catechol oestrogens they were stored in powder form under nitrogen and protected from light. The catechol oestrogen to be injected was made up to the required concentration in propylene glycol containing 0.1% ascorbic acid, immediately before use.

The animals were decapitated for the collection of trunk blood and the anterior pituitary, 6 h after treatment (18.00 h). Blood samples were centrifuged at 4°C and stored frozen until assayed for LH and PRL by RIA (described in Section 2.3).

The protein concentration of the anterior pituitary homogenates was estimated by the Lowry, Rosenbrough, Farr and Randall (1951) method (Appendix A.3) and the amount (ng) of LH per µg protein calculated.

The data were analysed by one-way analysis of variance, followed by the multiple range test (Duncan, 1957; Harter, 1960) to determine the significance of the differences between the various treatments.

5.2.2 Results

(a) Plasma LH concentrations:

The plasma LH concentration 6 h after s.c. injections of four doses (10 µg, 50 µg, 100 µg and 200 µg) of OE_1, OE_2, CAE_1, CAE_2 or

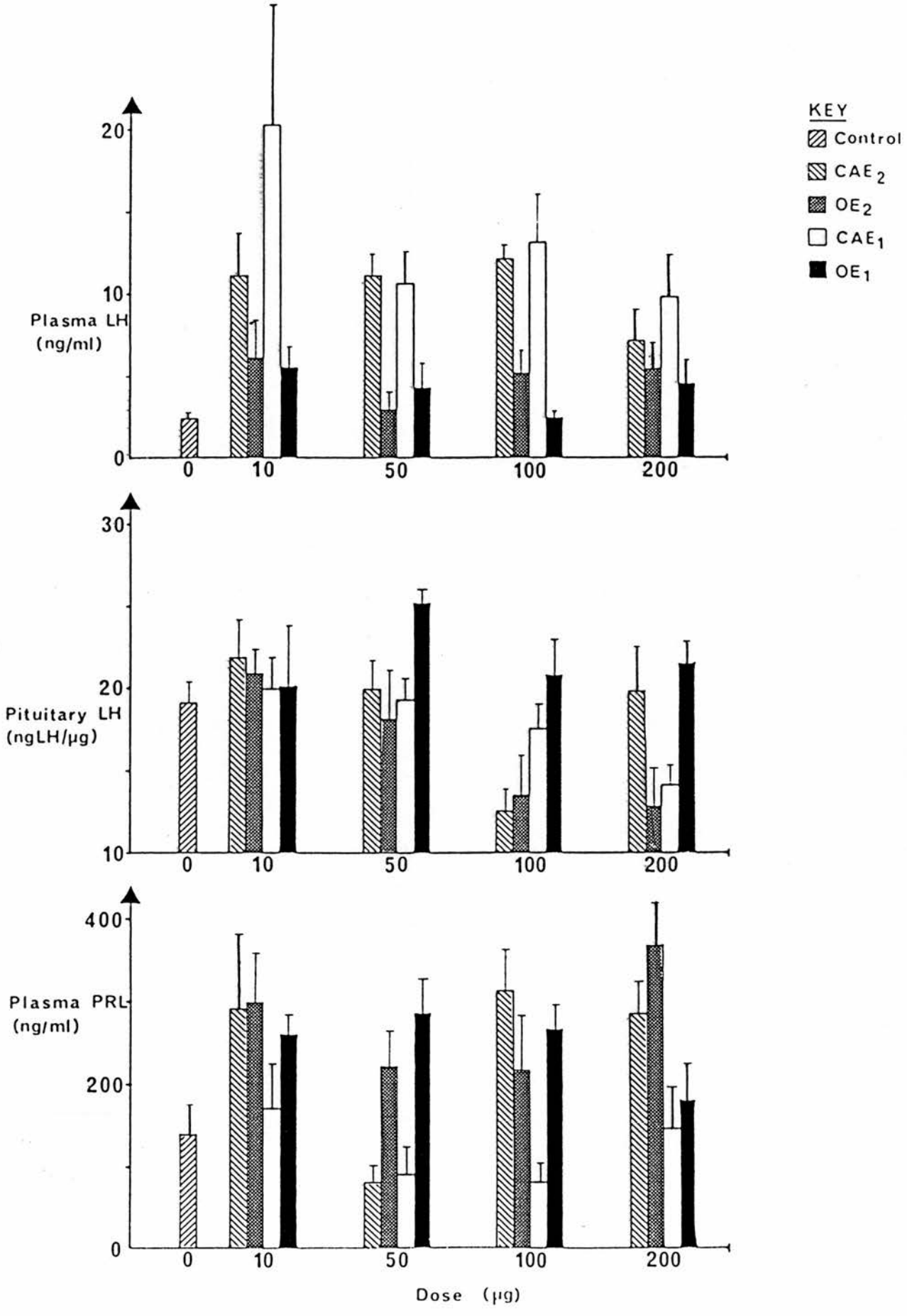
the vehicle are shown in Figure 5.2a. There was no statistically significant difference in plasma LH concentrations after either OE_1 or OE_2 administration compared to the concentrations in vehicle treated control animals. All doses of CAE_1 and CAE_2 injected induced a significant ($p < 0.05$) increase in plasma LH concentration compared to the control rats. The maximum LH concentration was seen after a single injection of CAE_1. The dose-response experiment showed that peak elevation occurred with the 10 µg dose of CAE_1 while increasing doses produced smaller rises. A similar effect on plasma LH levels after CAE_2 administration was also seen.

Changes in plasma LH concentration 2, 6, 24 and 48 h after the administration of CAE_2 (50 µg) are shown in Table 5.2a. A 10-fold increase in plasma LH release occurred within 2 h which was maintained for as long as 48 h (duration of experiment).

TABLE 5.2a: Peripheral plasma concentration of PRL and LH (ng/ml) in 35-day old male rats at various intervals after a subcutaneous injection of 50 µg 2-hydroxy-oestradiol (CAE_2) or 0.25 ml oil. Values are given as mean ± SEM (n).

	Peripheral plasma			
	LH concentration (ng/ml)		PRL concentration (ng/ml)	
Oil (0.25 ml/rat)				
6 h	2.4 ± 0.4	*(29)*	138.0 ± 36.0	*(8)*
24 h	2.0 ± 0.4	*(8)*	235.0 ± 26.0	*(4)*
48 h	1.4 ± 0.8	*(8)*	126.0 ± 88.0	*(4)*
CAE_2 (50 µg/rat)				
2 h	27.3 ± 5.2	*(4)*	223.0 ± 8.0	*(4)*
6 h	11.1 ± 1.2	*(17)*	80.0 ± 21.0	*(4)*
24 h	19.8 ± 3.2	*(4)*	177.0 ± 84.0	*(4)*
48 h	15.7 ± 1.9	*(8)*	176.0 ± 93.0	*(4)*

FIGURE 5.2a: LH concentrations in peripheral plasma and anterior pituitary gland and PRL concentrations in peripheral plasma from 35-day old rats 6 h after injections of various doses of 2-hydroxyoestradiol (CAE_2), oestradiol (OE_2), 2-hydroxyoestrone (CAE_1) or oestrone (OE_1) or the vehicle (control). Values given are the mean ± SEM.


KEY
Control
CAE2
OE2
CAE1
OE1
Plasma LH (ng/ml)
20
10
0
Pituitary LH (ngLH/µg)
30
20
10
Plasma PRL (ng/ml)
400
200
0
0
10
50
100
200
Dose (µg)

(b) Pituitary LH content:

Figure 5.2a shows anterior pituitary LH content (ng LH/μg protein) 6 h after the injection of various doses of the steroids and the vehicle. Injections of CAE_2 (100 μg) and OE_1 (50 μg) produced a significant ($p < 0.05$) decrease and increase, respectively, in pituitary LH content compared to the value in vehicle-treated control animals. There was no other significant difference in pituitary LH content between any of the treatment groups.

(c) Plasma PRL concentration:

The effect of subcutaneous injections of the steroids and the vehicle on PRL release 6 h later is shown in Figure 5.2a. Injection of the primary oestrogens OE_1 and OE_2 stimulated PRL release; the increase in plasma PRL concentration was significantly ($p < 0.05$) greater than in vehicle-injected control animals after the highest dose (200 μg) of OE_2 and the lowest dose (10 μg) of OE_1.

None of the four doses of CAE_1 injected 6 h previously produced a significant change in plasma PRL concentrations compared with those in vehicle-treated control animals. Only the 100 μg dose of CAE_2 significantly ($p < 0.05$) stimulated PRL release.

Table 5.2a shows the peripheral plasma PRL concentration at various time intervals after the injection of CAE_2 (50 μg). Although this dose of CAE_2 produced a significant ($P < 0.05$) increase in LH secretion compared to oil-treated control animals, it produced no significant changes in plasma PRL concentration, either 2, 6, 24 or 48 h after its administration.

5.2.3 Discussion and Conclusions

These results are the first to support the study by Naftolin *et al.* (1975) in which 50 and 100 µg doses of CAE_1 elevated serum LH by 4-7 fold. In addition, the results presented in this section demonstrated that CAE_2, another naturally occurring catechol oestrogen, at similar doses to CAE_1 also stimulated LH release. The failure of CAE_2 to stimulate LH secretion in experiments with pre-pubertal male rats (Rodriguez-Sierra and Blake, 1980, 1982b) and castrated male rats (Foreman, 1980; Parvizi *et al.*, 1981) raised the possibility that the increase in plasma LH concentration demonstrated by Naftolin *et al.* (1975) was stress-induced. This seemed likely in view of the fact that their animals were anaesthetised by inhalation of a 50% $O_2 : CO_2$ mixture and blood samples were obtained by cardiac puncture. In the study presented here, the rats were decapitated as in the studies by Rodriguez-Sierra and Blake (1980, 1982b), in which catechol oestrogens (100 µg doses) either did not alter LH release or else reduced plasma LH concentrations. The reason for this discrepancy between the results obtained by different workers is not clear.

Plasma LH concentrations were determined at various intervals after the subcutaneous injections of CAE_2 (50 µg; Table 5.2a). Within 2 h a 10-fold increase in plasma LH concentration occurred, compared to vehicle-treated controls. Plasma LH concentration remained higher than controls up to 48 h after a single injection of CAE_2 (50 µg). The half-life of CAE_1 and CAE_2 in plasma is 180s and 90s, respectively (Kono, Merriam, Brandon, Loriaux and Lipsett, 1980). Therefore, it is unlikely that the injected CAE_2 remained in the circulation for 48 h. However, since the purity of the catechol oestrogens supplied by Sigma (Poole) was not checked, it can be argued that the stimulatory

effects of these metabolites of the oestrogens is due to contamination of the catechol oestrogen preparations with the parent oestrogen. Neither OE_1 nor OE_2 significantly altered plasma LH concentrations compared with those in vehicle-treated control animals. It is unlikely, therefore, that contamination of the catechol oestrogen preparation OE_1 or OE_2 would significantly affect the potency of CAE_1 and CAE_2 to stimulate LH release.

The finding that OE_1 and OE_2 administration did not alter the plasma LH concentration in 35-day old male rats (Figure 5.1c) was also observed by Naftolin *et al.* (1975). Administration of an intravenously injected bolus of CAE_2 (100 µg) to chronically castrated adult rats did not alter the plasma LH concentration, but it was able to inhibit the suppression of LH secretion by OE_2 (Franks, Maclusky, Naish and Naftolin, 1981). In addition, OE_2 did not alter LH release from superfused male rat pituitary gland (Linton *et al.*, 1981). Although it has been shown that OE_2 is a potent inhibitor of LH secretion in the long-term castrated rat (Swerdloff and Walsh, 1973; Franks *et al.*, 1981), the suppression of LH secretion in the intact adult male (Verjan, de Jong, Cooke, van der Molen and Eik-Nes, 1974; van Beurden, Mulder, de Jong and van der Molen, 1977; Franks *et al.*, 1981) is not a consistent finding (Tcholakian, Chowdhury and Steinberger, 1974).

The physiological role of OE_1 in modulating LH secretion is not yet fully known. In studies on pre-pubertal male rats similar to those described in this section (in which OE_1 had no effect on plasma LH concentration), OE_1 (100 µg) injected s.c. reduced serum LH concentration 6 h later but this effect was not observed 24 or 48 h later (Rodriguez-Sierra and Blake, 1982b). Plasma concentrations

of both OE_1 and LH found to be elevated in post-menopausal women and in women with polycystic ovarian syndrome (Sitterii and MacDonald, 1974; Rebar, Judd, Yen, Rakoff, Vandenberg and Naftolin, 1976), suggesting a possible link between OE_1 and LH.

The mechanism by which CAE_1 and CAE_2 stimulate the release of LH secretion has not been resolved. The demonstration that CAE_2 (10^{-7} to 10^{-10} mol/l) did not significantly alter the release of LH from superfused rat pituitary glands (Linton *et al.*, 1981) suggests that CAE_2 did not act at the pituitary level. The pituitary content of LH determined 6 h after injections of CAE_1, CAE_2, OE_1 or OE_2 were similar to vehicle-injected control rats (Figure 5.2a) which suggests that pituitary LH synthesis was not increased despite the marked increase in pituitary LH release induced by CAE_1 and CAE_2 (Figure 5.2a). An alternative mechanism by which CAE_1 and CAE_2 may stimulate the release of LH could be by increasing the sensitivity of the pituitary to LHRH. Hseuh, Erickson and Yen (1979) showed that CAE_2 increased the sensitivity of cultured pituitary cells taken from ovariectomised rats, to LHRH-induced LH release. CAE_2 was less effective than OE_2 while CAE_1 had no effect on the sensitivity of the pituitary gonadotrophs to LHRH (Hseuh *et al.*, 1979). These studies by Hseuh *et al.* (1979) do not explain why OE_2 did not alter plasma LH concentrations and why CAE_1 stimulated LH release in the 35-day old rats (Figure 5.2a). The possibility that the catechol oestrogens increase the release of LHRH into pituitary stalk blood and so stimulate LH release remains to be investigated.

A physiological role for CAE_1 in PRL release in the pre-pubertal male rat appears to be unlikely. Doses up to 200 µg CAE_1 did not significantly alter PRL secretion compared to vehicle-treated controls

(Figure 5.2a), in agreement with other similar studies (Rodriguez-Sierra and Blake, 1982). In contrast, CAE_1 has been shown to suppress PRL in normal young women (Fishman and Tulchinsky, 1980) and in oestrogen-primed post-menopausal women (Schinfield *et al.*, 1980). Administration of CAE_2 (100 µg/rat), OE_2 (200 µg/rat) and OE_1 (10 µg/rat) significantly increased PRL concentrations in peripheral plasma (Figure 5.2a). A stimulatory effect on PRL secretion by CAE_2 and OE_1 in the pre-pubertal male rat has been found in recently published studies (Rodriguez-Sierra and Blake, 1982b). Although Rodriguez-Sierra and Blake (1982b) found OE_2 did not alter PRL release, there is evidence from several studies that OE_2 stimulates PRL release (Frantz, Kleinberg and Noel, 1972; MacLeod; 1976; De Léan, Ferland, Drouin, Kelly and Labrie, 1977) and from anterior pituitary cell cultures (Raymond, Beaulieu and Labrie, 1978).

Since CAE_2 has the capacity to compete with dopaminergic ligands for dopamine receptors in the anterior pituitary (Schaeffer and Hseuh, 1979), it is possible that CAE_2 stimulated PRL release by preventing dopamine from inhibiting PRL release from the pituitary (Shaar and Clemens, 1974; Neill, 1980). This proposal is, however, not supported by experiments showing that exposure of superfused male rat pituitary glands to CAE_2 alone or with dopamine reduced PRL secretion by 51-60% (Linton *et al.*, 1981). In addition, it has been argued that the delayed effect (2-4 hours) of CAE_2 on PRL concentration (Adashi *et al.*, 1980; Shin *et al.*, 1981) and the relatively small magnitude of the response suggest that CAE_2 does not act in the same manner as known dopamine antagonists (e.g. metoclopramide: Judd, Lazarus and Smythe, 1976). Alternatively, a hypothalamic site of action of CAE_2 to inhibit DA synthesis is also possible. A decrease in

DA turnover in the anterior medio-basal hypothalamus and preoptic area after CAE_2 administration to gonadectomised rats has been associated with the increase PRL secretion induced by CAE_2 (Parvizi and Wuttke, 1983). CAE_2 has also been shown to inhibit the activity of tyrosine hydroxylase in the hypothalamus, at physiological concentrations of CAE_2 (Lloyd and Weiz, 1978). This would reduce the synthesis of dopamine and therefore the dopaminergic inhibition of PRL. The ability of CAE_2 to alter catecholamine synthesis significantly would depend on two main conditions. Firstly, that CAE_2 had access to the appropriate neuronal sites and, secondly, that a mechanism existed for concentrating CAE_2 in catecholamine neurones. These conditions are necessary since CAE_2 is very labile and rapidly o-methylated by the COMT in hypothalamic extracts (Breuer and Koester, 1974). It has been demonstrated that high concentrations of CAE_2 as well as the enzyme which converts OE_2 to CAE_2 exist in the hypothalamus (Fishman and Norton, 1975; Paul and Axelrod, 1977) and that the DA neurones in the TIF region, but not the substantia nigra, can concentrate tritiated OE_2 (Pfaff and Keiner, 1974; Stumpf, Sar and Keefer, 1975). The two conditions mentioned above are, therefore, fulfilled which suggests that CAE_2 could be stimulating PRL release by acting at the hypothalamus. The duration of the effect of CAE_2 may be controlled by the degree of access of catechol oestrogens to COMT. The methylated metabolites or OE_1 and OE_2 do not inhibit tyrosine hydroxylase activity (Foreman and Porter, 1980), suggesting that these steroids could act as reservoirs of the catechol oestrogens.

In conclusion, the studies presented in this section showed that CAE_1 stimulated LH secretion while CAE_2 stimulated both LH and PRL secretion in the pre-pubertal male rat. This demonstrates that the

enzymatic conversion of OE_1 and OE_2 to 2-hydroxylated derivatives does not produce inactive metabolites.

5.3 The Effect of Acute Catechol Oestrogen Administration to Immature Female Rats Treated With Pregnant Mare Serum Gonadotrophin (PMSG)

5.3.1 Introduction

Several studies suggest that the catechol oestrogens may play a role in inducing hormonal changes (Fishman, 1976; Gelbke, Ball and Knuppen, 1977; Paul, Hoffman and Axelrod, 1980). The formation of CAE_1 and CAE_2 is catalysed by oestradiol-2-hydroxylase, a microsomal cytochrome P450-dependent mono-oxygenase (Gelbke *et al.*, 1977; Paul, Axelrod and Diliberto, 1977). Radioenzymatic measurements of the activity of oestradiol-2-hydroxylase in the brain of female rats during the oestrous cycle showed a sharp rise in activity occurred at pro-oestrus, just before the ovulatory LH surge (Fishman, Norton and Krey, 1980). The enzyme activity in the liver remained unchanged throughout the cycle. Measurements of plasma CAE_1 concentrations during the human menstrual cycle demonstrated that a 6-fold increase occurred at ovulation (Ball, Gelbke and Knuppen, 1975), suggesting that a specific increase in oestradiol-2-hydroxylase activity occurred at ovulation. These cyclic changes in enzyme activity in conjunction with evidence for a positive feedback effect of exogenous CAE_2 on pituitary LH release (Naftolin *et al.*, 1975; Gethman and Knuppen, 1976; results presented in Section 5.1.3) raises the possibility that endogenous CAE_2 is involved in the physiological induction of the pre-ovulatory LH surge. There is evidence that oestradiol-2-hydroxylase activity in the brain and liver of male rats also responds

to hormonal manipulation. Castration induced a reversible fall in enzyme activity in both the brain and liver while cytochrome P450-inducers (e.g. barbiturates) had no effect (Hoffman *et al.*, 1980b).

Using cultured pituitary gonadotrophs it has been demonstrated that CAE_2, but not CAE_1, sensitises the cultures to LHRH although CAE_2 was less potent than either OE_1 or OE_2 (Hseuh *et al.*, 1979). This suggests that the metabolism of OE_2 to CAE_2 (Figure 5.1a) could be important in prolonging its effect whereas the conversion of OE_2 to CAE_1 may be important in terminating its activity.

The administration of catechol oestrogens to adult female rats reduced fertility and prevented the development of oestrous cycles in neonatal female rats (Parvizi and Naftolin, 1977). The effects of CAE_1 and CAE_2 administration to the neonatal female rats resembled those seen in androgenised female rats (Barraclough, 1961), suggesting that these catechol oestrogens cause defeminisation. Evidence suggesting that the catechol oestrogens could act as endogenous anti-oestrogens include the demonstration that CAE_2 competes with OE_2 for soluble oestrogen receptors in the pituitary and hypothalamus (Davies *et al.*, 1975; Clarke and Findlay, 1980) and inhibit oestrogen-elicited accumulation of hypothalamic cAMP (Paul and Skolnick, 1977).

The timing of the first gonotrophin surge in pre-pubertal rats treated with PMSG has been demonstrated to depend on the capacity of the ovaries to secrete E_2 in the form of a surge (Wilson, Horth, Endersby and McDonald, 1974). There was a positive correlation between the dose of PMSG, the peak concentration of plasma OE_2 and the average number of ova shed (Wilson *et al.*, 1974). Plasma OE_2 concentrations reached a maximum 42-52 h after PMSG treatment and then fell significantly (Wilson *et al.*, 1974). Both the pre-ovulatory

surge of LH and the time of ovulation have been shown to be advanced by up to 24 h by the simultaneous administradion of PMSG and OE_2 (Park and Zarrow, 1971). This stimulatory effect of OE_2 was used to compare the effects of CAE_1 and CAE_2 in the PMSG-treated 30-day old female rat. In the immature female rat treated with PMSG, the hormonal changes (Park and Zarrow, 1971; Sorrentino, Reiter, Lee and Schalch, 1972; Wilson *et al.*, 1974; Sarkar and Fink, 1979) and neural pathways that mediate the neuroendocrine signal for ovulation (Quinn and Zarrow, 1965; Sorrentino *et al* ., 1972) are similar to those in the adult female rat (Everett, 1964; Halasz and Pupp, 1965; Koves and Halsz, 1970; Butler and Donovan, 1971; Fink, 1979). However, unlike the adult female rat, no significant increase in pituitary responsiveness to LHRH appears to occur in PMSG-treated rats (Sarkar and Fink, 1979) and this may explain, at least in part, the fact that the LH surge in PMSG-treated rats is only about a third that of the spontaneous surge at puberty (Wilson *et al.*, 1974; Sarkar and Fink, 1979).

Purpose of present study:

The aim of the experiments described in this section was to determine the effects of CAE_2 on PMSG-induced ovulation compared to the effects of OE_1 and OE_2 by measuring the magnitude of the pre-ovulatory LH surge and plasma PRL concentrations. In addition, the effects of the oestrogens and CAE_2 on the reproductive tract were compared by determining the weights of the uterus and ovaries.

5.3.2 Materials and Methods

Female Wistar rats (Charles River, U.K.) were obtained on day 24 of life and kept on a lighting schedule of 14 hours light (lights on between 06.00 h and 20.00 h). At 11.00 h on day 30 the rats were injected subcutaneously (s.c.) with either 20 iu PMSG ("Folligon", Intervet Laboratories Ltd, Cambridge) or 0.9% saline. On day 31, 28 h

later, the rats were injected with either 2.5 µg OE_2, 2.5 µg OE_1, 50 µg CAE_2 or the vehicle (propylene glycol containing 0.01% ascorbic acid), in a volume of 0.25 ml by an s.c. injection given at 16.00 h. At 17.00 h on day 32, blood samples (0.3 ml each) were collected from the external jugular vein under ether. The plasma obtained was kept frozen at -20°C until assayed for LH and PRL by radioimmunoassay (Section 2.3). On the morning of day 33, between 08.30 h and 10.00 h the animals were decapitated. The uteri were removed and stripped of adhering fat and connective tissue. Any fluid present was expressed then the uteri were blotted and weighed.

Differences between the mean values of the five groups were compared by analysis variance and the multiple range test for heteroscedastic means and unequal numbers of replicates (Duncan, 1957; Harter, 1960). Values of $p < 0.05$ were considered as significant.

5.3.3 Results

(a) Effects on plasma hormone concentrations:

The concentration of LH and PRL in peripheral plasma samples obtained at approximately 17.00 h on day 32 after various injections on day 30 and 31 are shown in Table 5.3a.

A single injection of 20 iu PMSG produced a significant ($p < 0.05$) increase, 56 h later, in plasma LH concentrations compared to those in the other groups. The administration of 50 µg CAE_2 28 h after an injection of 20 iu PMSG significantly ($p < 0.05$) blocked this increase in plasma LH concentration. Plasma LH concentrations in animals treated with saline followed by either OE_2 or CAE_2 were similar to each other and significantly lower ($p < 0.05$) than those in animals treated with PMSG followed by saline. In 4 out of 12 rats the injection of 2.5 µg OE_1 stimulated a marked increase in plasma LH concentration (mean ±

TABLE 5.3a: Plasma LH (ng/ml) and PRL (pg/ml) concentrations, mean ± SEM (n) at approximately 17.00 h in groups of 32-day old female rats, subcutaneously administered either 20 iu PMSG, saline, 2.5 µg oestradiol-17β (OE_2), 2.5 µg oestrone (OE_1) or 50 µg CAE_2 on day 30 and 31 as indicated. Blood samples were obtained, under ether anaesthesia, from the external jugular vein.

		Peripheral plasma	
Day 30	Day 31	LH concentration (ng/ml)	PRL concentration (pg/ml)
PMSG (20iu)	Saline (0.25 ml)	131.5 ± 18.7 *(16)*	387.0 ± 42.0 *(4)*
Saline (0.25 ml)	CAE_2 (50µg)	12.9 ± 4.4 *(17)*	271.0 ± 78.0 *(3)*
PMSG (20iu)	CAE_2 (50µg)	27.1 ± 6.7 *(14)*	145.0 ± 79.0 *(3)*
Saline (0.25 ml)	OE_2 (2.5µg)	5.9 ± 2.3 *(16)*	251.0 ± 100.0 *(3)*
Saline (0.25 ml)	OE_1 (2.5µg)	49.7 ± 22.3 *(12)*	291.0 ± 114.0 *(5)*

TABLE 5.3b: Plasma LH concentration in individual rats 25 h after a s.c. injection of 2.5 µg oestrone to 30-day old females.

Rat	Plasma LH concentration (ng/ml)
1	50.2
2	140.7
3	170.3
4	210.6
5	4.8
6	4.7
7	5.1
8	4.5
9	3.8
10	3.9
11	3.2
12	4.3
Mean	50.5
SEM	21.2
n	12.0

SEM = 142.9 ± 34.1 ng/ml); in the other 8 rats plasma LH concentrations remained low (mean ± SEM = 4.3 ± 0.2 ng/ml). The plasma LH concentrations in the individual rats injected with 2.5 μg OE_1 are shown in Table 5.3b.

There was no significant difference between any of the groups with respect to the plasma PRL concentrations (Table 5.3a).

(b) Effects on the reproductive tract:

Table 5.3c shows the weights (mg) of uteri and ovaries of 33-day old female rats treated with PMSG, saline or steroids. A significant ($p < 0.05$) increase in uterine weight occurred after the injection of 20 iu PMSG compared with that of any of the other treatments. The injection of 2.5 μg OE_2 in animals previously injected with saline resulted in an increase in the weight of the uterus which was significantly ($p < 0.05$) less than after injecting 20 iu PMSG followed by saline and significantly ($p < 0.05$) greater than after injecting saline followed by 50 μg CAE_2. The uterotrophic effect of 20 iu PMSG injected on day 30 was significantly ($p < 0.05$) reduced by the injection of 50 μg CAE_2 on day 31. The mean weight of the uterii in the group of rats treated with both 20 iu PMSG and 50 μg CAE_2 was approximately the same as that in animals injected with saline followed by 2.5 μg E_1.

Table 5.3c shows that treatment of 30-day old rats with 20 iu PMSG significantly ($p < 0.05$) increased the weight of the ovaries compared to any of the other treatments. The injection of 50 μg CAE_2 28 h after 20 iu PMSG given on day 30 produced a significantly ($p < 0.05$) greater increase in ovarian weight compared with that of treatment with 20 iu PMSG alone. The administration of 50 μg CAE_2 or 2.5 μg OE_2 produced an equivalent increase in the weight of the ovaries. This was significantly ($p < 0.05$) greater than that after injecting 2.5 μg OE_1

TABLE 5.3c: The weight (mg) of uterii and ovaries of 33-day old female rats administered after 20 iu PMSG, saline, 2.5 µg oestradiol-17β (OE_2), 2.5 µg oestrone (OE_1) or 50 µg CAE_2 on day 30 and 31 as indicated. Values are given as mean ± SEM (n).

Treatment		Weight of uterus (mg)	Weight of ovaries (mg)
Day 30	Day 31		
PMSG (20iu)	Saline (0.25 ml)	155.9 ± 12.7 *(18)*	63.8 ± 3.5 *(19)*
Saline (0.25 ml)	CAE_2 (50µg)	64.9 ± 13.7 *(14)*	24.6 ± 3.1 *(14)*
PMSG (20iu)	CAE_2 (50µg)	91.4 ± 7.0 *(15)*	95.3 ± 11.4 *(15)*
Saline (0.25 ml)	OE_2 (2.5µg)	112.2 ± 12.2 *(13)*	28.8 ± 6.2 *(12)*
Saline (0.25 ml)	OE_1 (2.5µg)	98.8 ± 12.0 *(12)*	18.1 ± 2.3 *(11)*

and significantly ($p < 0.05$) less than the mean ovarian weight produced by injection of 20 iu PMSG alone or followed by 50 µg CAE_2.

5.3.4 *Discussion and Conclusions*

Administration of 20 iu PMSG to pre-pubertal female rats induced an increase in the circulating LH concentration approximately 56 h later had a uterotrophic effect, increased the weight of the ovaries and induced ovulation. These effects of PMSG are in agreement with previous findings (e.g. Park and Zarrow, 1971; Wilson *et al.*, 1974; Sarkar and Fink, 1979). Experiments described in this section demonstrated that while 2.5 µg OE_2 had a potent uterotrophic effect, it was not as effective as PMSG in inducing the release of LH and increasing ovarian growth in immature female rats. In contrast, 2.5 µg OE_1 produced plasma LH concentrations which were about 9-fold greater than those produced by OE_2. The uterotrophic activity of OE_1 was equivalent to that of OE_2. These results provide new data on the oestrogenic nature of OE_1 and are the first to demonstrate a stimulation of LH release by OE_1.

The principal metabolite of OE_2, CAE_2, which was more potent than OE_2 in increasing plasma LH concentrations, suppressed the plasma LH surge induced by PMSG. Franks, Ball, Naftolin and Ruf (1980) have demonstrated that administration of doses of CAE_2 up to 100 µg did not alter the time of ovulation or the number of ova induced by PMSG. Therefore, the magnitude of the suppression of the pre-ovulatory LH surge induced by PMSG after CAE_2 administration does not apparently affect the ability of PMSG to induce ovulation.

The biological action of the catechol oestrogens was initially distinguished by their lack of uterotrophic activity (Gordon, Cantrall, Cekleniak, Albers, Maner, Stolar and Bernstein, 1964). CAE_1 and CAE_2 were found to retain less than 0.1% of the uterotrophic activity of their parent compounds and this was attributed to their reduced binding to uterine OE_2 receptors (Martucci and Fishman, 1976). In later studies, wax pellets (Martucci and Fishman, 1979) or miniature osmotic pumps (Jellinck, Davis, Krey, Luine, Roy and McEwen, 1979) were used to infuse CAE_1 at a rate of approximately 25 µg/day. These studies as well as those in which CAE_1 were injected daily (Kono *et al.*, 1981) demonstrated that CAE_1 did not have any significant uterotrophic activity. The low uterotrophic effect of CAE_2 was confirmed in the studies described (Section 5.2.3, Table 5.3c). In addition, these studies (Table 5.3c) provide the first evidence that CAE_2 inhibit the uterotrophic activity of PMSG.

CAE_1 administered to adult rats at noon on pro-oestrus has been demonstrated to abolish the pre-ovulatory PRL surge (Katayama and Fishman, 1982). After CAE_2 administration, in contrast, the plasma PRL concentration 12-15 h before ovulation (Table 5.2) was not significantly different from OE_2- or PMSG-treated rats. This suggests

that CAE_1 and CAE_2 differ in their effects on PRL release. In addition, CAE_2 has an equipotent effect to its parent oestrogen in inducing PRL release.

In conclusion, data from experiments on pre-pubertal female rats showed that CAE_2 induced a larger rise in the pre-ovulatory LH surge than its parent oestrogen, although its uterotrophic activity was lower than OE_2. CAE_2 also reduced the uterotrophic effect of PMSG as well as the magnitude of the pre-ovulatory LH surge induced by PMSG. Whether the changes in plasma LH concentration produced after CAE_2 administration are brought about by modulation of the release of LHRH into pituitary stalk blood remains to be investigated.

CHAPTER VI

Immunoreactive Cholecystokinin and Gastrin in Pituitary Stalk Blood

CHAPTER VI

6.1 Introduction

Cholecystokinin (CCK) was originally isolated from hog intestine as a 33-amino acid peptide (Jorpes and Mutt, 1973). It strongly stimulates gall bladder contraction and release of digestive enzymes (e.g. amylase) from the exocrine pancreas (Jorpes and Mutt, 1973; Mutt, 1980; Williams, 1980). The presence of a CCK-like gastrointestinal peptide has also been demonstrated in the central nervous system. Gastrin-like material in brain extracts of several vertebrates was first described by Vandergaegen, Signeau and Gepts (1975). Studies using immunological techniques combined with fractionation procedures have indicated that this gastrin-like material is distinct from heptadecapeptide gastrin and is identical to the biologically active carboxy terminal octapeptide of CCK (CCK-8) (Dockray, 1976; Muller, Straus and Yalow, 1977; Dockray, Gregory and Hutchison, 1978; Rehfeld, 1978; Robberecht, Deschodt-Lanckman and Vanderhaeghen, 1978). Subsequent studies have demonstrated that while CCK exists in at least five forms in the brain (viz. a component larger than CCK-39, components similar in size to CCK-33, CCK-12, CCK-8 and a smaller molecular form), the predominant form is CCK-8 (60-70%) and approximately 15% is CCK-33 (Muller *et al.*, 1977; Dockray *et al.*, 1978; Larsson and Rehfeld, 1979; Rehfeld, Gottermann, Larsson, Emson and Lee, 1979; Lamers, Morley, Poitras, Sharp, Carlson, Hershman and Walsh, 1980; Ryder, Eng, Straus and Yalow, 1981). The partial amino acid sequence of CCK-33 is shown in Figure 6.1a. CCK-8 in the brain is formed by selective endopeptidase cleavage of CCK-33 (Deschodt-Lanckman, Bui, Noyer and Christopher, 1981; Straus, Malesci and Yalow, 1981) and since this enzyme system is more efficient in the brain than the gut (Eng, Shiina, Straus and Yalow, 1982), there

FIGURE 6.1a: Partial amino acid sequence of CCK 22-33. Area in ⬚ represents the area of homology with gastrin (G). Arrows (↓) show the cleavage sites of CCK-converting enzymes.

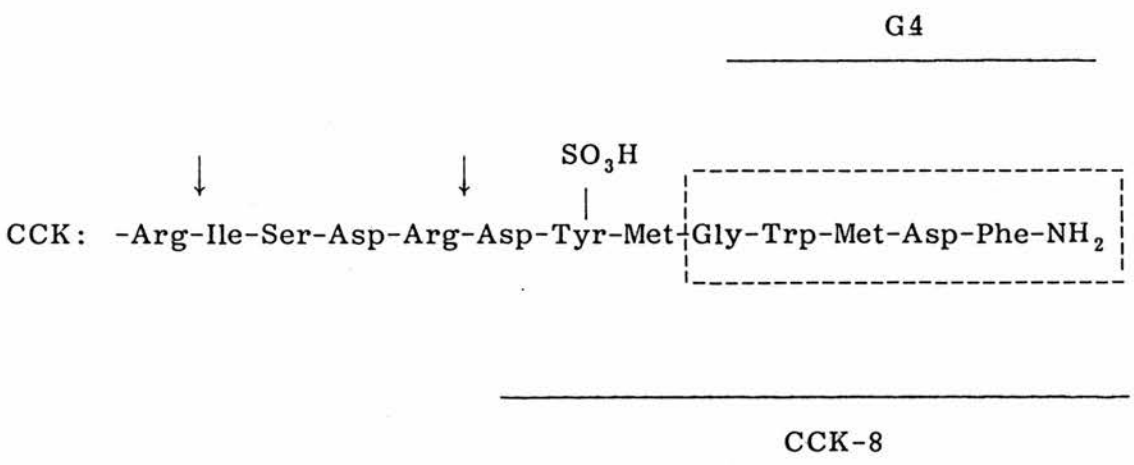
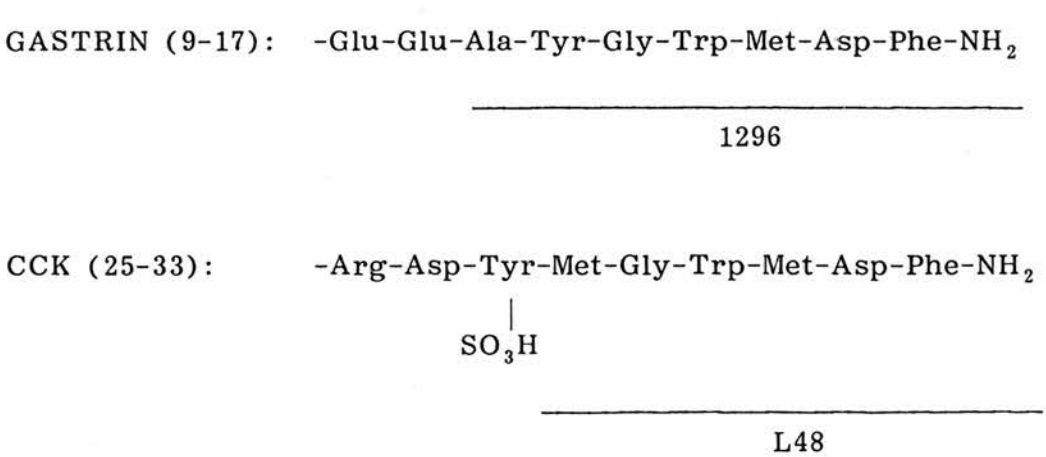


FIGURE 6.1b: Antigenic determination of two COOH-terminal specific antisera, L48 and 1296. Antiserum L48 raised to CCK-8 coupled to bovine serum albumin has a 50 times higher affinity for CCK-8 and G-17 than G-4 and G-5. Antiserum 1296 cross-reacts about 20 times more with G-17 than with CCK-8.



is very little CCK-33 in the brain. In the gut, on the other hand, there are roughly equal amounts of CCK-8 and CCK-33 (Rehfeld, 1978; Dockray, 1980). The major form of CCK in peripheral nerves, such as those innervating pancreatic islets, has been reported to be C-terminal tetrapeptide (CCK-4) which is common to gastrin (Figure 6.1) (Rehfeld, Larsson, Gottermann, Schwartz, Holst, Jensen and Morley, 1980), although Dockray and Gregory (1980) have disputed the existence of free CCK-4 in the pancreas. CCK has structural and functional homologies to gastrin. Their identical C-terminal sequence is responsible for their biological activity while the rest of each molecule modifies activity quantitatively and determines relative potencies for various targets (Rehfeld, 1981). These characteristics together with immunocytochemical and radioimmunochemical studies of the occurrence of gastrin and CCK in different vertebrate species suggest these two peptides have evolved from a common ancestral molecule (Larsson and Rehfeld, 1977; Dockray, 1979).

The development of a range of antisera specific for different sequences of CCK and gastrin has enabled the use of immunohistochemical methods to localise these peptides in the brain (Innes, Correa, Uhl, Schneider, Snyder, 1979; Larsson and Rehfeld, 1979; Loren, Alumet, Hakanson and Sundler, 1979; Vanderhaeghen, Lotstra, De Mey and Giles, 1978) and gut (Dockray, 1976; Buffa, Solicia and Go, 1976; Rehfeld *et al.*, 1979).

6.1.1 CCK and the Pituitary

Gastrin-17 and gastrin-34 have only been located in the pig pituitary gland (Rehfeld, 1978) although the pituitary of several other species including the rat has been surveyed (Beinfeld, Meyer and Brownstein, 1980). Substantial amounts of CCK-like immunoreactivity

have been shown to be present in fibres in the pars nervosa (PN) (Loren *et al.*, 1979; Vanderhaeghen *et al.*, 1980; Beinfeld, Meyer and Brownstein, 1980) but not in the pars intermedia (PI) or pars distalis (PD). Section of the pituitary stalk resulted in the disappearance of CCK from the PN while destruction of both paraventricular nuclei resulted in a 60% fall in PN CCK (Beinfeld *et al.*, 1980). It has therefore been suggested that CCK in the PN originates in neuronal cell bodies in or near the paraventricular nuclei, and that these neurones project to the PN by way of the median eminence. Presumably 40% of the CCK in the PN originates in supraoptic nucleus where, as in the paraventricular nucleus, CCK cell bodies have been seen to coexist with oxytocin (Loren *et al.*, 1979; Vanderhaeghen *et al.*, 1980). The reduction of CCK content of PN under physiological conditions such as lactation and hypernatremia, which stimulate oxytocin and vasopressin release, respectively, suggests CCK may be co-secreted with oxytocin and vasopressin (Beinfeld *et al.*, 1980). Scattered CCK cell bodies and fibres have been seen in all hypothalamic nuclei except the mammillary bodies (Vanderhaeghen *et al.*, 1980; Loren *et al.*, 1979).

Numerous CCK immunoreactive fibres have been found in the external layer of the median eminence of dogs (Vanderhaeghen, Lotstra, Vierendeels, Gilles, Deschepper and Verbanck, 1981), rats (Vanderhaeghen *et al.*, 1980; Hökfelt, Elde, Fuxe *et al.*, 1978; Loren *et al.*, 1979; Anhut, Meyer and Knepel, 1983) and frogs (Larsson and Rehfeld, 1977). The distribution of CCK within the hypothalamo-hypophysial system suggests that CCK could have a role in control of pituitary secretion.

There is evidence that both CCK-8 and gastrin alter the release of a number of hormones from the anterior pituitary. Central administra-

of CCK-8 and gastrin stimulated the release of growth hormone (GH) and inhibited the release of thyrotropin (TSH) and luteinizing hormone (LH) (Vijayan, Samson and McCann, 1978a, 1979a). The release of follicle-stimulating hormone (FSH) was not altered by either CCK or gastrin. Prolactin (PRL) release was stimulated by CCK-8 and inhibited by gastrin (Vijayan *et al.*, 1978a, 1979a). The modulation of LH, PRL and GH release by CCK-8 and gastrin appear to be by way of a hypothalmic action rather than a direct action on the pituitary while the stimulation of TSH by gastrin has been attributed to an effect of gastrin on the hypothalamus and pituitary (Vijayan *et al.*, 1978a). Intravenous injections of CCK-8 and gastrin into rats, or the incubation of hemi-pituitaries with these peptides, did not produce the changes in the release of anterior pituitary hormones seen after intraventricular administration, except for the inhibitory effect of gastrin on TSH release (Vijayan *et al.*, 1978a, 1979a). Presumably CCK-8 in ng doses and gastrin in µg doses injected intravenously acted to inhibit the release of LHRH and TRH which resulted in reduced circulating levels of LH and TSH. There is evidence in humans that CCK can decrease the TSH response to TRH (Morley, 1981), suggesting this may be a mechanism by which CCK modulates anterior pituitary TSH concentrations. CCK has also been demonstrated to reverse the inhibitory effect of somatostatin on GH release in monolayer cultures of a GH tumour cell line (Morley, Melmed, Briggs, Carlson, Hershman, Solomon, Lamers and Damassa, 1979). This may explain the finding that intravenous injection of CCK to humans increased GH secretion (Domschke, Lux and Domschke, 1980), an effect not seen when gastrin is administred to rats by the same route (Vijayan *et al.*, 1978a). Intracerebroventricular and intraperitoneal injections of crude CCK

extracts lead to pronounced increases in plasma corticosterone levels in intact but not hypophysectomised rats (Fekete, Bokor, Penke, Kovács and Telgdy, 1981; Itoh, Hirota, Katsuura and Odaguchi, 1981).

6.1.2 CCK and the Brain (Other than Hypothalamus)

Substantial concentrations of CCK have also been found in brain areas outside the hypothalamus except for the pons, medulla and cerebellum; the highest concentrations were in the cortex and striatum (Brownstein *et al.*, 1981; Innis *et al.*, 1979; Vanderhaeghen *et al.*, 1980). In the ventral tegmental area and in the zona compacta of the substantia nigra, CCK immunoreactivity has been demonstrated to coexist in a subpopulation of dopamine-containing neurons that project to the amygdala and cortex (Innes *et al.*, 1979; Hokfelt, Skirbol, Rehfeld, Goldstein, Markey and Dann, 1980; Meyer, Beinfeld, Oertel and Brownstein, 1981). The degeneration of these cell bodies and terminals may explain the diminution of CCK and its receptors in the basal ganglia and cortex in Huntington's disease (Hokfelt *et al.*, 1980; Emson, Rehfeld, Langerin and Rossor, 1980; Hays, Meyer and Paul, 1981) and has been taken as evidence that CCK may be involved in extrapyramidal function. Numerous CCK-containing cell bodies and nerve terminals have also been localised throughout the hippocampus (Loren *et al.*, 1979; Handelmann, Meyer, Oertel and Beinfeld, 1981), in several amygdaloid nuclei, the mesencephalon and medulla oblongata (Innes *et al.*, 1979; Loren *et al.*, 1979; Vanderhaeghen *et al.*, 1980). Several major pathways, such as the medial forebrain bundle and the stria terminalis, contain CCK immunoreactive fibres.

(ob/ob) mice (Straus and Yalow, 1979; Saito, Williams and Goldfine, 1981) and normal mice after a 2-5 day fast (Straus and Yalow, 1980). Fasting in mice also increased the number of CCK receptors in the olfactory lobe and hypothalamus (Saito *et al.*, 1981). These studies relating CCK levels and CCK receptor numbers to fasting have, however, not been confirmed by other workers (Lamers *et al.*, 1979; Ho and Hansky, 1979; Schneider, Monohan and Hirsch, 1979; Oku, Glick, Shimonura, Inoue, Bray and Walsh, 1980).

6.1.3 Interactions between CCK and Monoaminergic Neurones

Evidence that CCK can alter monoamine levels in various brain areas suggests another mechanism by which CCK could regulate anterior pituitary hormone release. Intraventricular administration of CCK antiserum has been found to decrease the dopamine (DA) and noradrenaline (NA) content of the hypothalamus, mesencephalon, amygdala and septum, while an increase in DA and a decrease in NA content occurred in the striatum (Kadar, Fekete, Lonovics and Telegdy, 1981). In the same experiments, the 5-HT content of mesencephalon, amygdala and septum was reduced, and increased in the striatum. The reduction in DA content has been attributed to a decrease in DA synthesis due to an inhibition of tyrosine hydroxylase activity, while the sulfate ester on the tyrosine residue of the CCK molecule (Figure 6.1a) has been found to inhibit the production and release of DA at the nerve terminal (Itoh and Katsuura, 1981). The failure of CCK-8 to interfere with the behavioural effects of apomorphine and haloperidol (Itoh and Katsuura, 1981) supports a presynaptic site of action to explain the antagonism of DA by CCK-8. The inhibition of DA by CCK-8 may explain the PRL-release stimulating action of CCK-8 (Section 6.1.1, Vijayan *et al.*, 1979). There is evidence that CCK and

Administration of CCK has been reported to produce a variety of effects on central nervous function. For example, intracerebroventricular administration of CCK-8 (20-250 ng) induced dose-related hypothermia and hypoglycaemia (Katsuura, Hirota and Itoh, 1981; Morley, Levine and Lindbland, 1981). The hypothermia was antagonised by throtrophin releasing hormone (TRH: Katsuura *et al.*, 1981) and similar in magnitude to that produced by equimolar doses of neurotensin (Yehuda and Kastin, 1980). Pharmacological doses (150-100 µg/kg) CCK administered parentally have been shown to induce sedation, catalepsy, ptosis and inhibition of rearing activity in mice (Zetler, 1980a; Katsuura and Itoh, 1982). CCK-8 and the frog skin decapeptide, caerulein (which shares seven of eight residues with CCK-8) delayed the onset of convulsions that followed the administration of strychnine, penetetrazol, picrotoxin and bicuculline (Zetler, 1980b). Intraventricular CCK-8 (400-1800 ng) markedly suppressed L-dopa-induced behavioural effects in rats (Katsuura and Itoh, 1982) and antagonised the potentiation of L-dopa by TRH (Metcalf and Dettmar, 1981).

The satiety produced by introduction of food into the intestine can be mimicked by either systemic injections of CCK and its analogs (Gibbs, Young and Smith, 1973; Houpt, Anika and Wolff, 1978) or by injections of CCK into the cerebral ventricles (Della-Ferra and Baile, 1979) and ventromedial hypothalamus (Stern, Cudillo and Kruper, 1976). CCK-antibody injected into the cerebral ventricles stimulated feeding in sheep but not in rats (Della-Ferra, Baile, Schneider and Grinken, 1981) while continuous injections of picomole quantities of CCK-8 into the cerebral ventricles of sheep decreased feeding (Della-Ferra and Baile, 1979). The concentration of CCK and the number of CCK receptors were reduced in the cerebral cortex of both genetically obese

DA co-exist in the same neurons (Innes *et al.*, 1979; Hokfelt *et al.*, 1980; Meyer *et al.*, 1981) and that schizophrenia may be related to an excess of central dopaminergic neuronal activity (Mackay and Crow, 1980; Snyder, 1982). This has led to speculation that CCK-8 could also act as an anti-psychotic agent and by decreasing central dopaminergic transmission, have a therapeutic effect in schizophrenia. It has been claimed that an analog of CCK-8 was beneficial in a large proportion of patients suffering from schizophrenia for 2 - 27 years (Itoh and Katsuura, 1981). This study has not yet been confirmed.

6.1.4 Purpose of Present Study

Whether CCK and gastrin play a major role in modulating the release of hormones from the anterior pituitary is still largely undecided. However, in view of the localisation of these peptides in brain areas which project to the median eminence and the demonstration that CCK can be released from hypothalamic synaptosomes by depolarisation (Pinget, Straus and Yalow, 1978; Sheppard, Klaff, Hudson and Tyler, 1980; Dodd, Edwardson and Dockray, 1980), the aims of the present study were to determine:

1. whether the concentrations of CCK and gastrin in pituitary stalk blood were greater than in the peripheral circulation;

2. whether electrical stimulation of various areas of the brain known to contain immunoreactive CCK-gastrin-like material modulated the release of CCK or gastrin into stalk plasma, since electrically-stimulated release of a substance provides indirect evidence for physiological release of that substance;

3. to what extent the peripheral sources of CCK and gastrin, notably the gastric antrum, contributed to the concentrations of CCK and gastrin measured in stalk plasma.

To avoid problems with immunological cross-reactivity in radioimmunoassay procedures to determine the concentrations of CCK and gastrin in plasma and tissue samples, antisera (L/48 and 1296) prepared with differing specificities for mid- and C-terminal regions were used (Figure 6.1b). These antisera do not, therefore, cross-react with the inactive NH_3-terminal fragments (Dockray and Walsh, 1975; Dockray, Vaillant and Hutchison, 1981).

6.2 The Effect of Electrical Stimulation of Various Brain Areas on CCK and Gastrin Release into Pituitary Stalk Blood

6.2.1 Methods

(a) Animals: Adult male rates of the Albino Wistar COB strain were used in all experiments (180-200 g body weight). Urethane (1 g/kg b.w.) administered intraperitoneally was used to induce anaesthesia. Pituitary stalk blood was collected as described in Section 2.2.5 for consecutive periods of 30 min each before, during and after the application of the electrical stimulus. Peripheral blood samples were collected from the external jugular vein before the pituitary stalk was cut and at the end of each 30 min period of pituitary stalk blood collection. All blood samples were collected into tubes kept on ice and containing EDTA (5.4 mM/l blood) and Trasylol (5 KIU/l). The plasma was stored frozen at -40°C and sent to Dockray (Liverpool) packed in dry ice. The samples remained frozen until assayed.

(b) Implantation of electrodes and stimulation parameters: Bipolar glass-insulated platinum electrodes were prepared and implanted as described in Section 2.2.7 under Avertin anaesthesia. The electrode tips were 1 mm apart for implantation in the suprachiasmatic nucleus (SCN), ventral hippocampus (VH) and amygdala (AMY) and paraventricular nucleus (PVN). For the preoptic area (POA) and dorsal hippocampus (DH), electrodes with tips separated by 2 mm were implanted. The co-ordinates used were taken from de Groot's atlas (1959) and are shown in Table 6.2a.

TABLE 6.2a: Co-ordinates used for the implantation of electrodes with reference to the stereotaxic co-ordinates described by de Groot (1959).

Brain area	Co-ordinates Anterior	Ventral	Lateral	
AMY	5.4	-2.5	3.5	
POA	7.8	-1.5	1.0	— either side of midline
PVN	6.4	-1.3	0.3	
SCN	7.4	-2.3	0.5	— either side of midline
DH	3.0	+2.0	1.5	
VH	3.0	-3.2	3.5	

All the rats implanted with electrodes appeared healthy and were feeding and drinking 4-5 days after electrode implantation when pituitary stalk blood samples were obtained.

For stimulation of the median eminence (ME), a unipolar glass-insulated platinum electrode was placed on the surface of the ME just before heparin was injected and the pituitary stalk cut.

The parameters of the electrical stimulus used were those shown to produce the optimum release of LH (Fink and Aiyer, 1974; Jamieson

and Fink, 1976); trains (30 sec on; 30 sec off) of biphasic square wave pulses with a frequency of 60 Hz, duration of 1 msec and amplitude of 500 μA (1 mA peak-to-peak). The electrode was left in place for the duration of the entire experiment. At the end of each experiment the heads were fixed in 10% formalin. Subsequently, serial frozen sections of the brain were prepared, stained with Cresyl fast violet and examined (Section 2.2.7).

(c) Radioimmunoassay and calculations: The concentration of CCK and gastrin in plasma samples was determined by radioimmunoassay using antibodies with differing specificities for the COOH-terminal of these two peptides (Dockray, 1980; Dockray, Vaillant and Hutchison, 1981; Section 2.3; Figure 6.1b). The sensitivity of the CCK assay was 20 pmol/l and for gastrin it was 5 pmol/l.

Radioimmunoassays using the two antibodies L48, which reacts almost equally with gastrin and CCK, and 1296, which is specific for the N-terminus of gastrin-17 and reacts poorly with CCK, were done simultaneously on all the samples. Since the CCK concentration in peripheral plasma is <10 fmol/ml, the difference between L48 and 1296 was used to determine the concentration of hypothalamus-derived CCK in pituitary stalk plasma.

The content (concentration x volume/unit time) of CCK and gastrin in pituitary stalk blood collected over a 30 min period was calculated. The significance of differences between means was determined by the unpaired t-test. The differences between more than two means was determined by analysis of variance and the multiple range test (Duncan, 1957; Harter, 1960).

6.2.2 Results

(a) Characteristics of CCK in pituitary stalk blood: Figure 6.2a shows that serial dilutions of pituitary stalk plasma yielded a displacement curve which had a slope similar to the standard curve obtained with synthetic CCK-8. Gastrin in rat plasma diluted in parallel with standard gastrin (Dockray, unpublished data).

The mean ± SEM CCK concentration in pituitary stalk blood in unstimulated control rats was significantly ($p < 0.05$) greater than the CCK concentration in peripheral plasma (Table 6.2b)

TABLE 6.2b: The effect of Trasylol (5 KIU/l) and EDTA (5.4 mM/l) addition to whole blood on CCK concentration (pmol/l) in peripheral and pituitary stalk plasma, and CCK content (fmol/30 min). Values are given as mean ± SEM; n=7 in each group.

	Without Trasylol/ EDTA	With Trasylol/ EDTA
Peripheral plasma		
Concentration (pmol/l)	43.10 ± 10.0	130.80 ± 23.0
Pituitary stalk plasma		
Concentration (pmol/l)	75.00 ± 13.0	221.40 ± 35.0
Content (fmol/30 min)	31.00 ± 18.0	208.40 ± 58.0

Rapid enzymatic degradation of CCK occurred if blood samples were simply collected into cold tubes kept on ice and frozen at 20°C until assayed (Table 6.2b). The addition of EDTA (5.4 mM/l) and Trasylol (5 KIU/l) to whole blood at the time of collection was found to be extremely effective in minimising peptide breakdown; CCK values

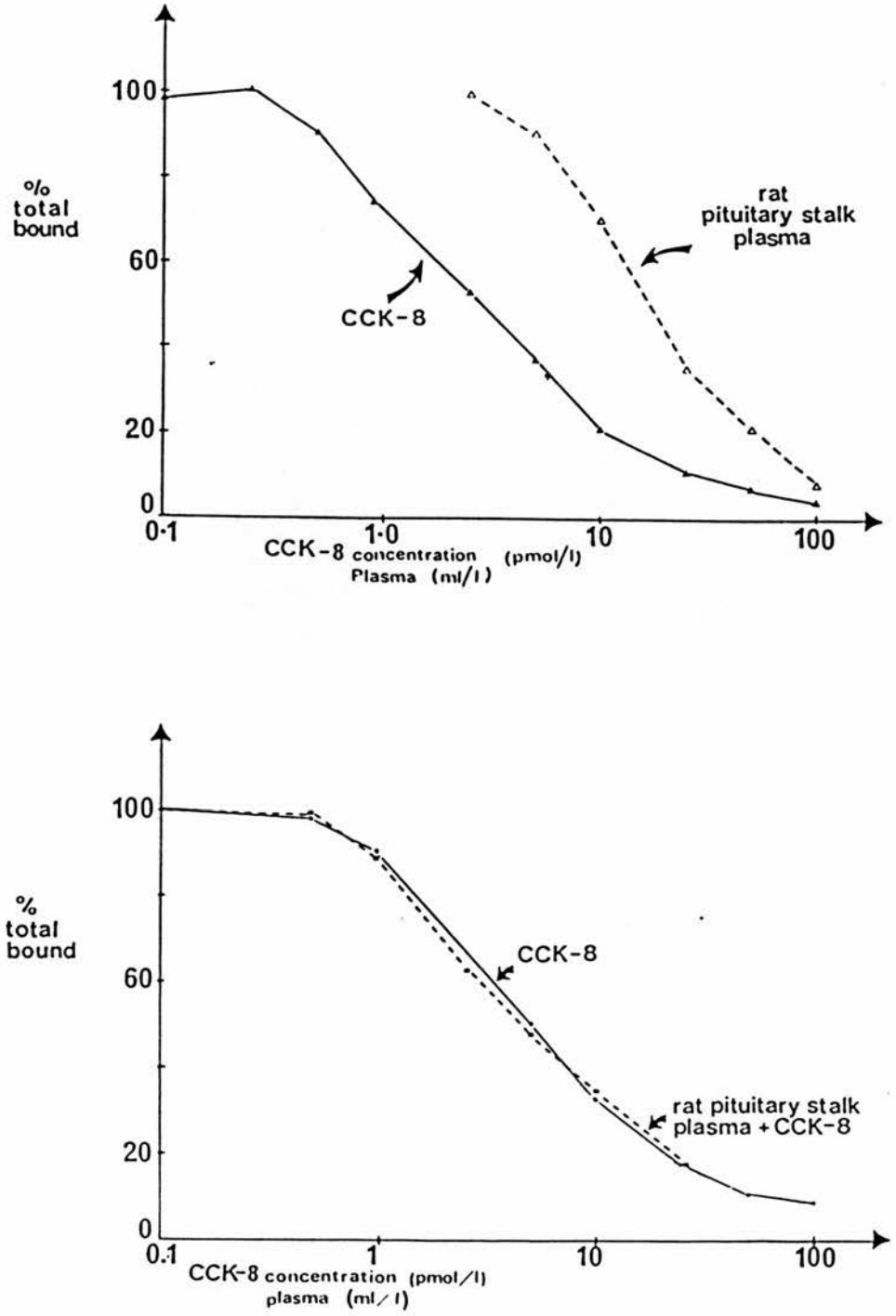


FIGURE 6.2a: Inhibition of binding curves in the CCK radioimmunoassay for synthetic CCK-8, pituitary stalk plasma, and plasma containing synthetic CCK-8 (collaborative data, Dockray). The lower limit of the assay was 0.5 pmol/l.

in both peripheral and pituitary stalk plasma was significantly ($p < 0.05$) higher after EDTA and Trasylol addition.

(b) The effect of electrical stimulation of the brain on CCK and gastrin release into pituitary stalk blood: Sections of the rat brain showing the positions of the electrodes are shown in Figure 6.2b. Stimulation of all areas except AMY, DH and VH produced a marked increase in the depth of respiration.

Cholecystokinin: The values of CCK (pmol/l) in peripheral and pituitary stalk plasma collected before, during and after the application of an electrical stimulus to various brain areas are shown in Figure 6.2c. Details of these data, including the number of rats in each group, are presented in Table 6.2c. Peripheral plasma 'control' values refer to the CCK concentration of the blood obtained from the external jugular vein shortly before the pituitary stalk was cut in order to collect pituitary stalk blood. The 'control' group listed in the column of the brain areas stimulated refers to the group of rats which were not implanted with electrodes and did not receive any electrical stimulation.

Pituitary stalk plasma CCK concentration was significantly ($p < 0.05$) higher than peripheral plasma in the unstimulated 'control' group of rats, both before ('control' sample) and 30 min after ('pre-stimulation' sample) the pituitary stalk was cut (Table 6.2c). Over the next hour of collection, the CCK concentration of both pituitary stalk plasma and peripheral plasma was found to fall progressively.

Electrical stimulation did not produce any statistically significant differences in the CCK concentration of peripheral plasma compared to the pre-stimulation period in any of the groups.

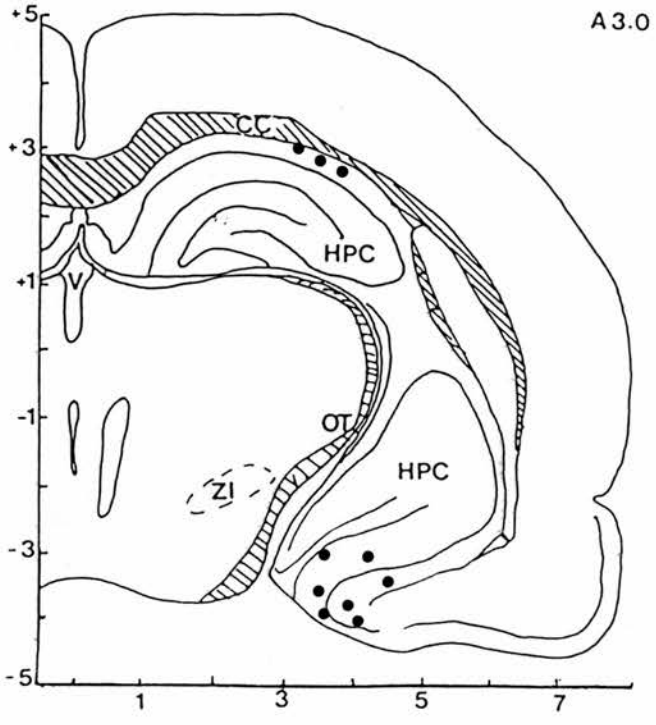
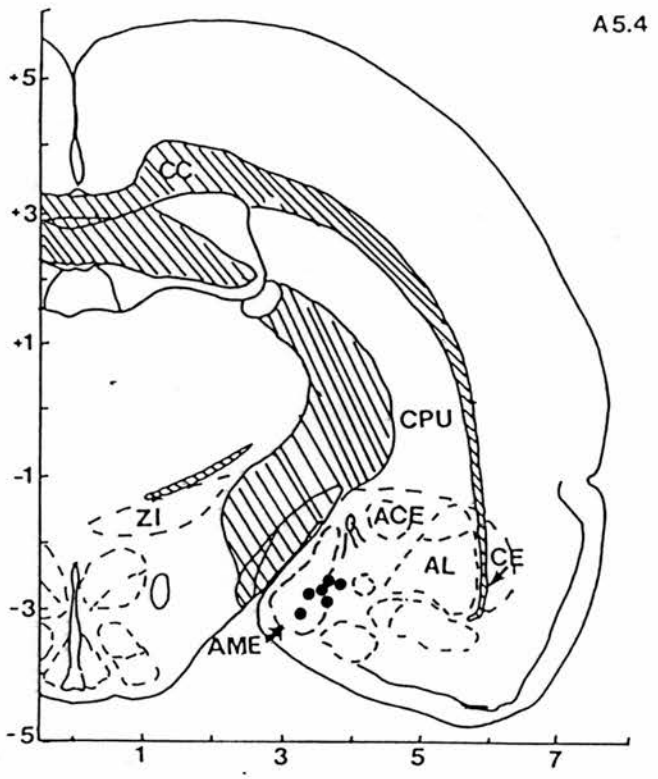


FIGURE : 6.2b(i) Coronal sections of the rat brain taken from de Groot's atlas (1959) showing positions of the electrode tips (●) in the following areas:

A3.0 dorsal and ventral hippocampus (HPC)
A5.4 central amygdaloid nucleus (AME)
A6.2 paraventricular nucleus (PVH)
A7.4 suprachiasmatic nucleus (SC)
A7.8 preoptic nucleus (POA)

Abbreviations

AAA	anterior amygdaloid area	ACE	central amygdaloid nucleus
AH	anterior hypothalamic area	AL	lateral amygdaloid nucleus
AME	medial amygdaloid nucleus	CA	anterior commissure
CC	corpus callosum	CE	external capsule
CO	optic chiasm	CPU	caudate-putamen nucleus
FX	fornix	HPC	hippocampus
MFB	medial forebrain bundle	OT	optic tract
POA	hypothalamic preoptic area	PVH	hypothalamic paraventricular nucleus
SC	suprachiasmatic nuclei		
SO	supraoptic nuclei	V	cerebral ventricle
VH	hypothalamic ventromedial nucleus	ZI	zona incertica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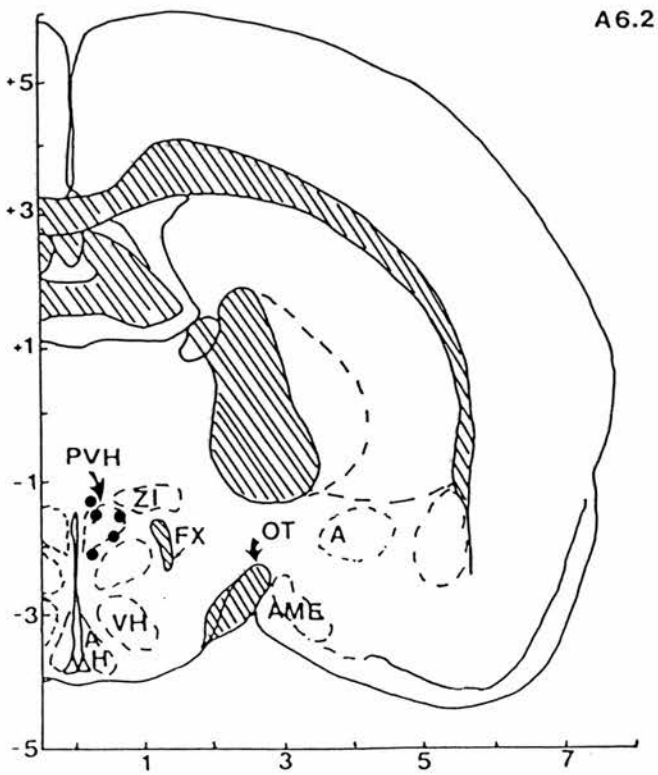
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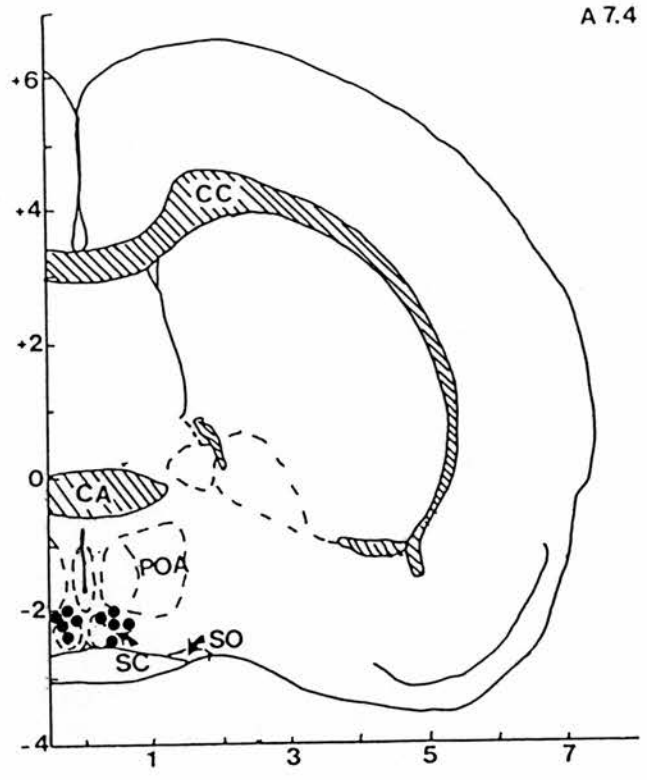
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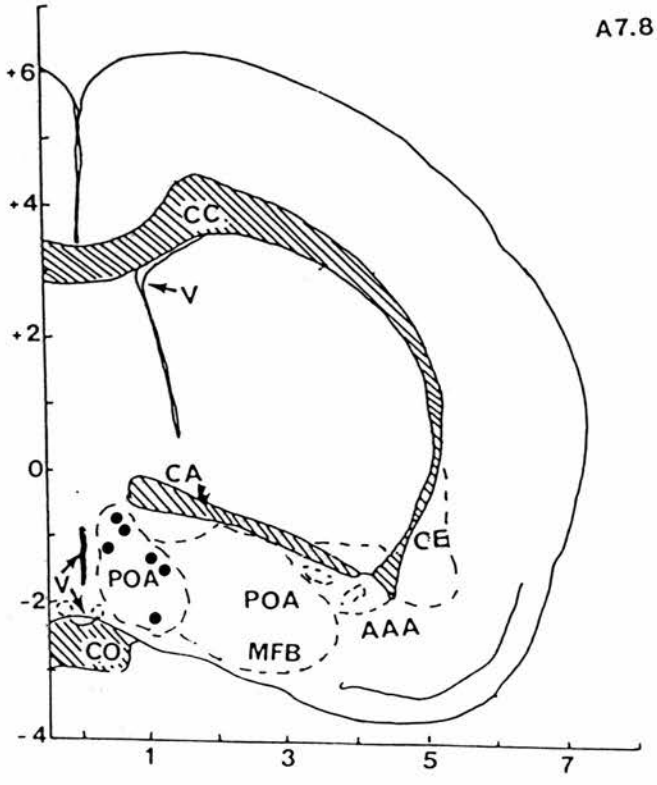
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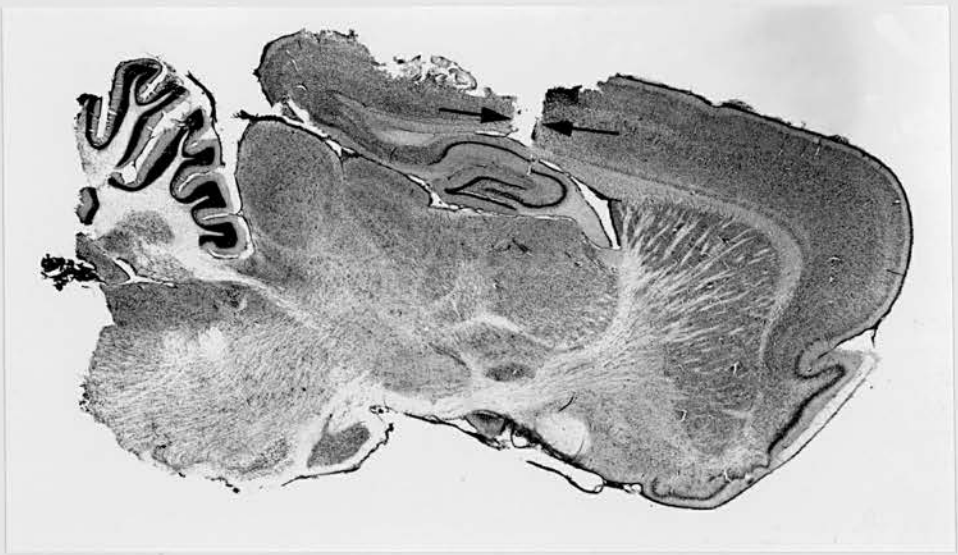
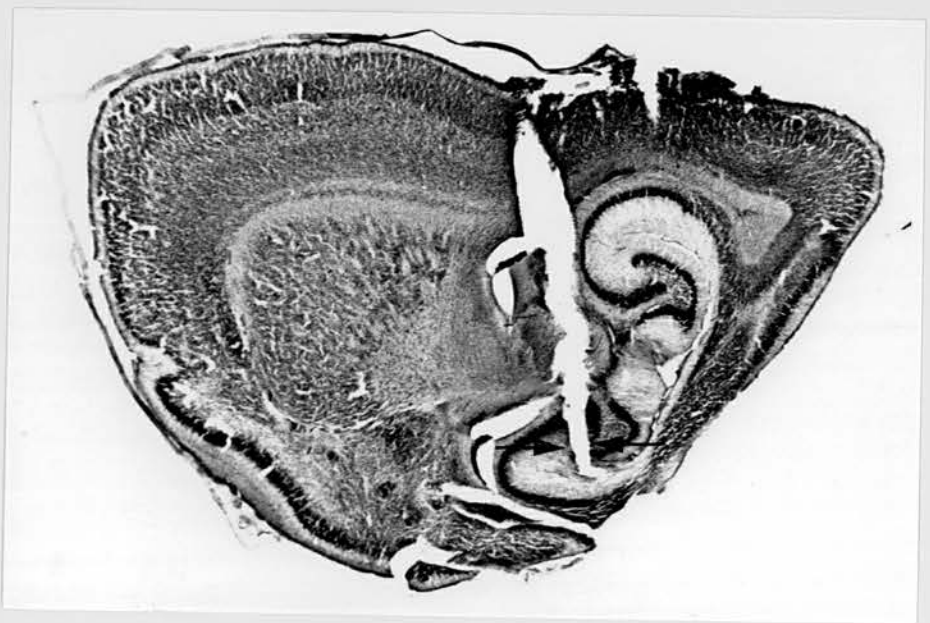
FIGURE 6.2b(ii): Representative photomicrographs of brain sections (A - F) showing positions of electrode tracts in the dorsal and ventral hippocampus, central amygdaloid nucleus, paraventricular nucleus, suprachiasmatic nuclei and preoptic area.

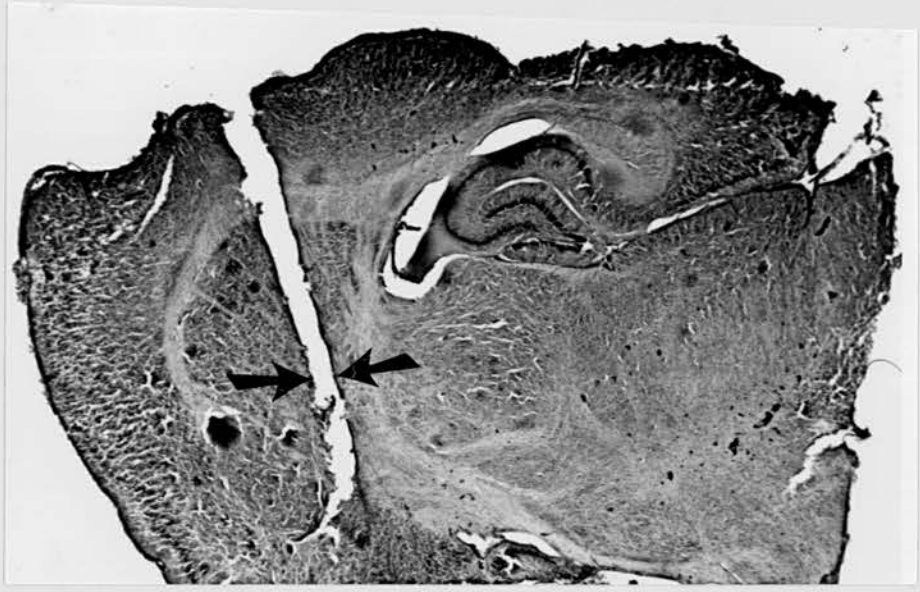
A. Dorsal Hippocampus



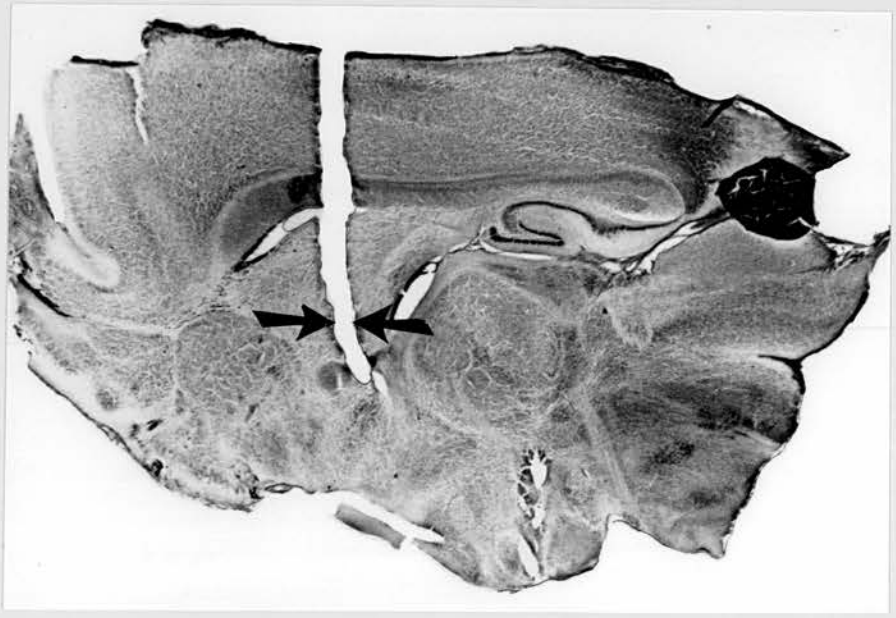
B. Ventral Hippocampus



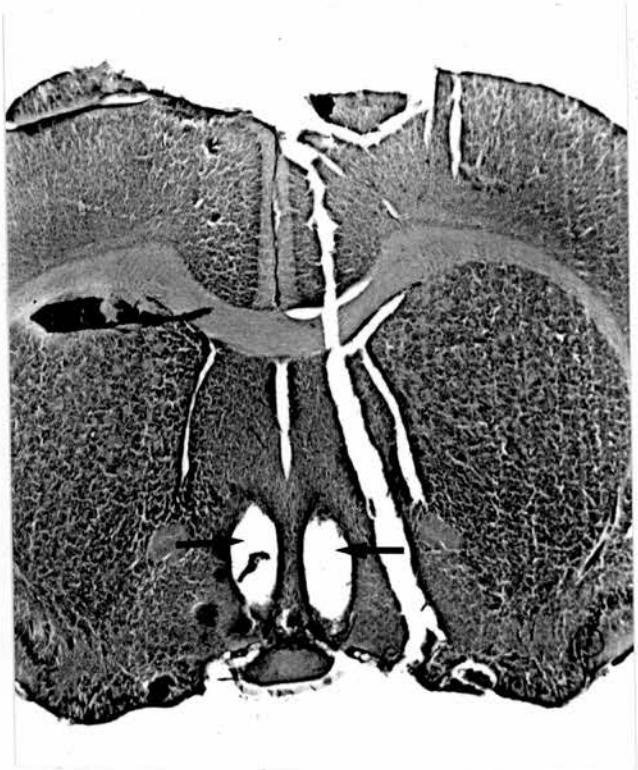
C. Central Amygdaloid Nucleus



D. Paraventricular Nucleus



E. Suprachiasmatic Nuclei



F. Preoptic Area

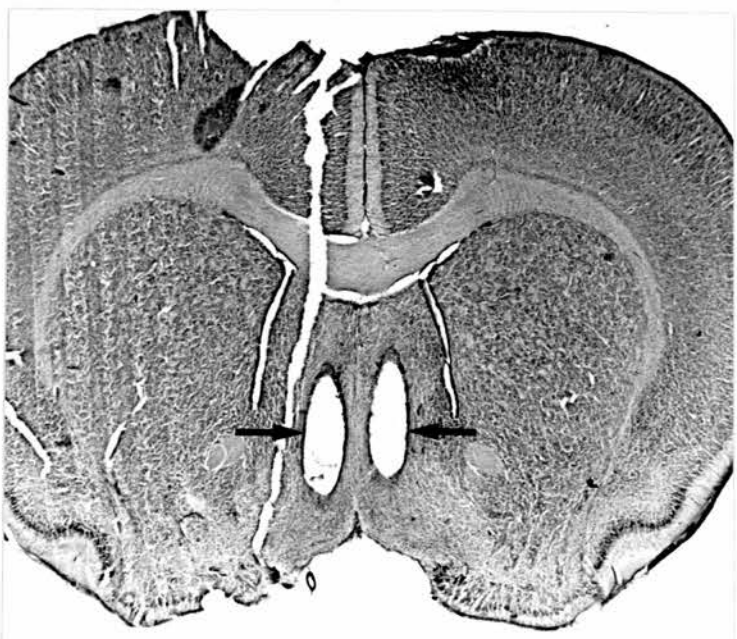


FIGURE 6.2c: CCK concentration (pmol/l) in peripheral ■ and pituitary stalk plasma □

a = peripheral plasma samples before stalk sectioned

b = pre-stimulation period

c = stimulation period (indicated by a bar)

d = post-stimulation period

Pituitary stalk blood was collected for 30 min during each period.

Abbreviations

AMY	amygdala
ME	median eminence
POA	preoptic area
PVN	paraventricular nucleus
SCN	suprachiasmatic nucleus
DH	dorsal hippocampus
VH	ventral hippocampus
C	unstimulated controls

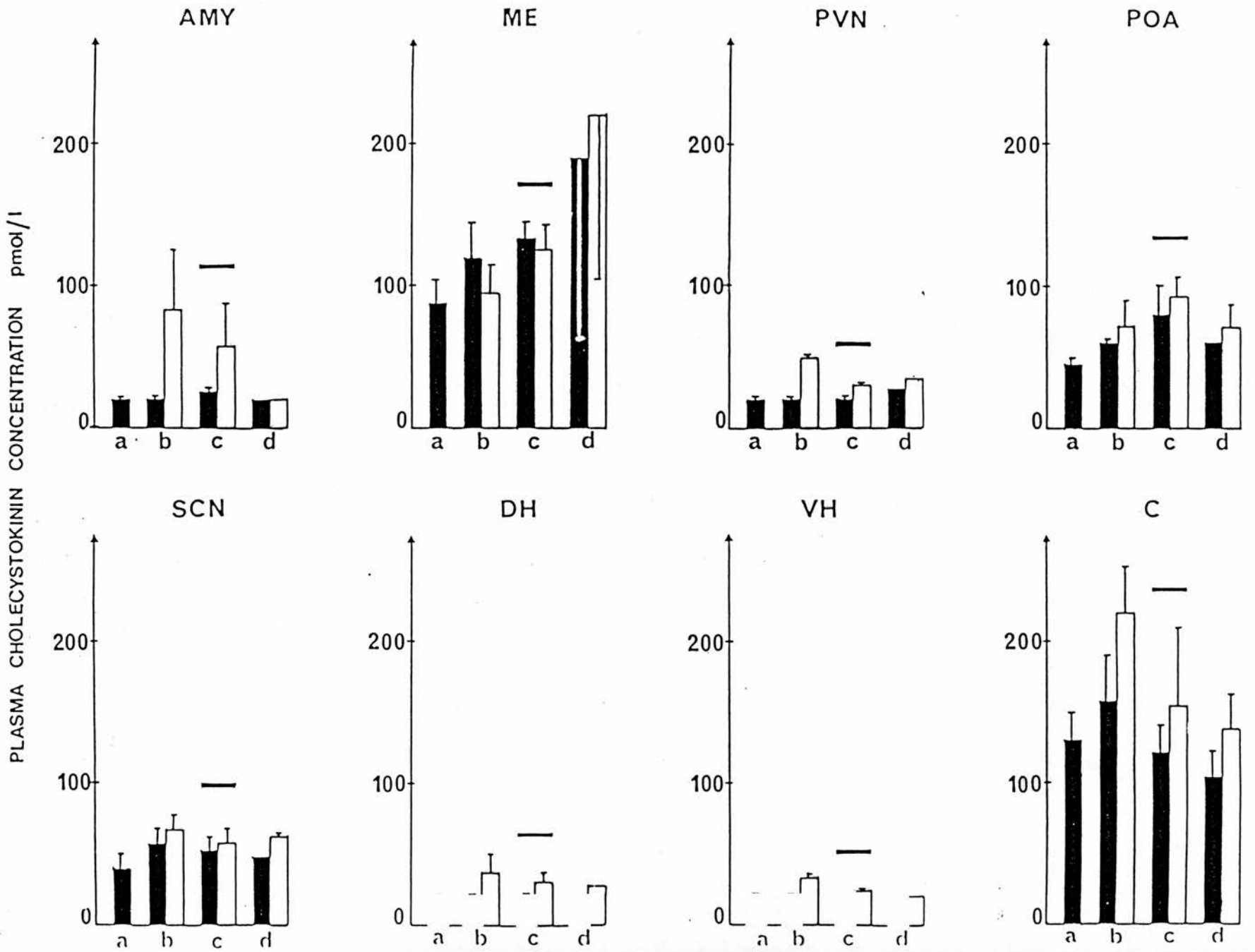

AMY
ME
PVN
POA
SCN
DH
VH
C
PLASMA CHOLECYSTOKININ CONCENTRATION pmol/l
200
100
0
a
b
c
d

TABLE 6.2c: The CCK concentration (pg/ml) in peripheral plasma and pituitary stalk plasma before, during and after electrical stimulation of various brain areas (see Figure 6.2c for abbreviations). The 'control' group (C) represents unstimulated rats. Peripheral 'control' samples are those obtained before the pituitary stalk was cut. The values are given as mean ± SEM; the number of rats in each group is given in parenthesis.

Brain area	Peripheral plasma				Pituitary stalk plasma		
	Control	Pre-stim	Stim	Post-stim	Pre-stim	Stim	Post-stim
AMY	20.0 ± 0.0 (6)	20.0 ± 0.0 (6)	26.7 ± 7.0 (6)	20.0 20.0 (2)	38.2 ± 10.0 (5)	24.4 ± 3.0 (5)	20.0 20.0 (2)
ME	88.0 ± 15.0 (6)	119.7 ± 25.0 (6)	134.4 ± 11.0 (5)	98.0 280.0 (2)	96.3 ± 24.0 (6)	127.3 ± 24.0 (6)	219.0 ± 106.0 (4)
POA	43.7 ± 7.0 (6)	61.8 ± 5.0 (6)	84.2 ± 19.0 (6)	60.0 42.0 (2)	74.7 ± 19.0 (6)	93.0 ± 14.0 (6)	73.6 ± 16.0 (5)
PVN	20.9 ± 1.0 (7)	20.4 ± 0.0 (7)	20.6 ± 1.0 (5)	29.0 (1)	49.3 ± 8.0 (7)	29.3 ± 3.0 (7)	36.0 (1)
SCN	40.7 ± 10.0 (7)	58.7 ± 11.0 (7)	52.8 ± 14.0 (5)	70.0 27.0 (2)	65.4 ± 11.0 (7)	58.4 ± 10.0 (7)	66.3 ± 4.0 (3)
DH	20.0 ± 0.0 (3)	20.0 ± 0.0 (3)	21.7 ± 1.0 (3)	34.0 20.0 (2)	37.3 ± 14.0 (9)	31.7 ± 8.0 (8)	23.0 20.0 (2)
VH	20.0 ± 0.0 (9)	20.7 ± 1.0 (9)	22.9 ± 3.0 (7)	20.0 20.0 (2)	32.2 ± 6.0 (9)	23.9 ± 3.0 (8)	20.0 20.0 (2)
C	130.8 ± 23.0 (10)	158.2 ± 38.0 (9)	122.0 ± 24.0 (6)	104.8 ± 16.0 (5)	221.4 ± 35.0 (7)	153.6 ± 55.0 (5)	139.5 ± 28.0 (4)

The application of the stimulus to the ME, POA, SCN and DH produced a slight increase in CCK release into pituitary stalk blood. These increments in CCK concentration and content over 30 min were not, however, significantly different from the values found in pituitary stalk plasma during the pre-stimulation period in each group. Similarly, a reduction of CCK concentration and content in pituitary stalk blood on electrical stimulation of the PVN was not statistically significant. Less marked reductions of CCK concentration in pituitary stalk plasma, thought not statistically significant, were also seen on stimulation of the AMY and VH. However, in the unstimulated control group (C) there was also a reduction (although not significant) in the content and concentration of CCK pituitary stalk blood over the three successive 30 min collection periods. This suggests that a time-related decline of CCK release into pituitary stalk blood occurs after the pituitary stalk has been cut. The amount of CCK released into pituitary stalk blood over a 30 min period (content, fmol/30 min) for each group is represented in Figure 6.2d. These values reflect the changes in CCK concentration (pmol/l) in pituitary stalk blood seen in these groups.

The volume of pituitary stalk blood collected together with CCK concentration and content/30 min are shown in Table 6.2d. The volume of blood was increased by 200-300 µl in all groups in which an electrical stimulus was applied to the brain. In the unstimulated group, by comparison, a fall of a similar volume occurred in the second 30 min period of pituitary stalk blood collection.

FIGURE 6.2d: CCK content (fmol/30 min) of pituitary stalk plasma samples collected for 30 min periods before, during and after electrical stimulation of various brain areas (see Figure 6.2b for abbreviations used).

a = pre-stimulation period

b = stimulation period (indicated by bar)

c = post-stimulation period

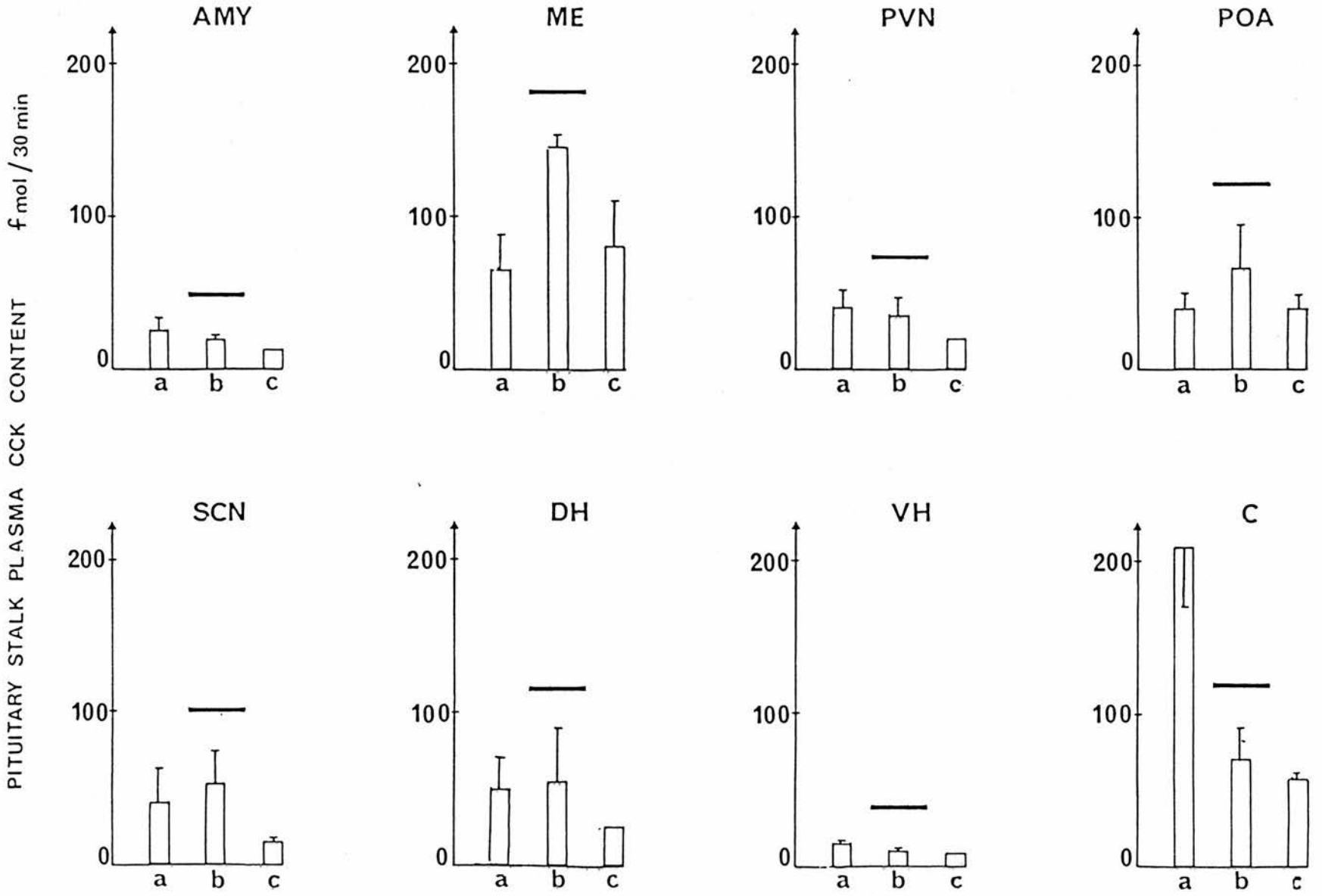

AMY
ME
PVN
POA
SCN
DH
VH
C
200
100
0
a
b
c
PITUITARY STALK PLASMA CCK CONTENT f mol / 30 min

TABLE 6.2d: Changes in pituitary stalk blood volume, CCK concentration and content before and during the application of an electrical stimulus to various brain areas (see Figure 6.2c for abbreviations used). The 'control' group (C) represents unstimulated rats. Values are given as mean ± SEM; the number of rats in each group is given in parenthesis.

Brain area	Pre-stimulation			Stimulation		
	Volume (µl)	Concentration (pmol/l)	Content (pmol/30 min)	Volume (µl)	Concentration (pmol/l)	Content (pmol/30 min)
AMY	485 ± 108 *(6)*	83.8 ± 46.0 *(6)*	25.4 ± 9.0 *(6)*	740 ± 55 *(4)*	55.0 ± 31.0 *(6)*	19.8 ± 5.0 *(6)*
ME	625 ± 140 *(6)*	96.3 ± 27.0 *(6)*	65.8 ± 25.0 *(6)*	943 ± 281 *(6)*	127.3 ± 24.0 *(6)*	145.8 ± 9.0 *(6)*
PVN	486 ± 112 *(5)*	49.3 ± 8.0 *(7)*	41.4 ± 11.0 *(7)*	716 ± 151 *(5)*	29.3 ± 3.0 *(7)*	35.2 ± 14.0 *(7)*
POA	572 ± 102 *(6)*	74.7 ± 19.0 *(6)*	40.7 ± 12.0 *(6)*	786 ± 165 *(5)*	93.0 ± 14.0 *(6)*	66.9 ± 28.0 *(6)*
SCN	611 ± 177 *(7)*	65.4 ± 11.0 *(7)*	41.5 ± 22.0 *(7)*	996 ± 290 *(5)*	58.4 ± 10.0 *(7)*	53.8 ± 22.0 *(7)*
DH	1057 ± 288 *(3)*	37.3 ± 14.0 *(3)*	51.3 ± 22.0 *(3)*	1263 ± 739 *(3)*	31.7 ± 8.0 *(3)*	54.3 ± 35.0 *(3)*
VH	429 ± 46 *(7)*	32.2 ± 6.0 *(8)*	16.0 ± 3.0 *(9)*	517 ± 59 *(6)*	23.9 ± 3.0 *(8)*	10.5 ± 2.0 *(8)*
C	799 ± 125 *(9)*	221.4 ± 35.0 *(7)*	208.4 ± 58.0 *(8)*	564 ± 79 *(5)*	122.0 ± 24.0 *(6)*	84.4 ± 23.0 *(4)*

Gastrin: Gastrin concentrations were measured in the same plasma samples as those used to measure CCK from groups of rats in which the electrical stimulus was applied to the ME, POA or SCN. The data obtained from this experiment are shown in histogram form (Figure 6.2e) while additional details, such as the number of rats per group, are given in Table 6.2e.

Though the concentration of gastrin in pituitary stalk blood collected during the pre-stimulation period was found to be higher than in peripheral plasma, this difference was not statistically significant.

Electrical stimulation of the ME, POA or SCN had no appreciable effect on the concentration or content/30 min of gastrin in pituitary stalk blood, while the increase in peripheral plasma concentration was also not statistically significant.

The mean ± SEM volume of pituitary stalk blood collected and the concentration and content (30 fmol/min) of gastrin in these samples is shown in Table 6.2f. Changes in gastrin content (fmol/30 min) are shown graphically in Figure 6.2f. There were no significant differences in pituitary stalk blood volumes or increments in these volumes in each group.

6.2.3 Discussion and Conclusions

The first criteria for defining a substance as a releasing factor in the hypothalamo-hypophysial axis is that its concentration in portal vessel blood should be greater than in the peripheral circulation (Harris, 1972). The second requirement is that the substance should be extractable from the hypothalamus, stalk and median eminence. Measurements of CCK concentration in pituitary stalk blood showed that

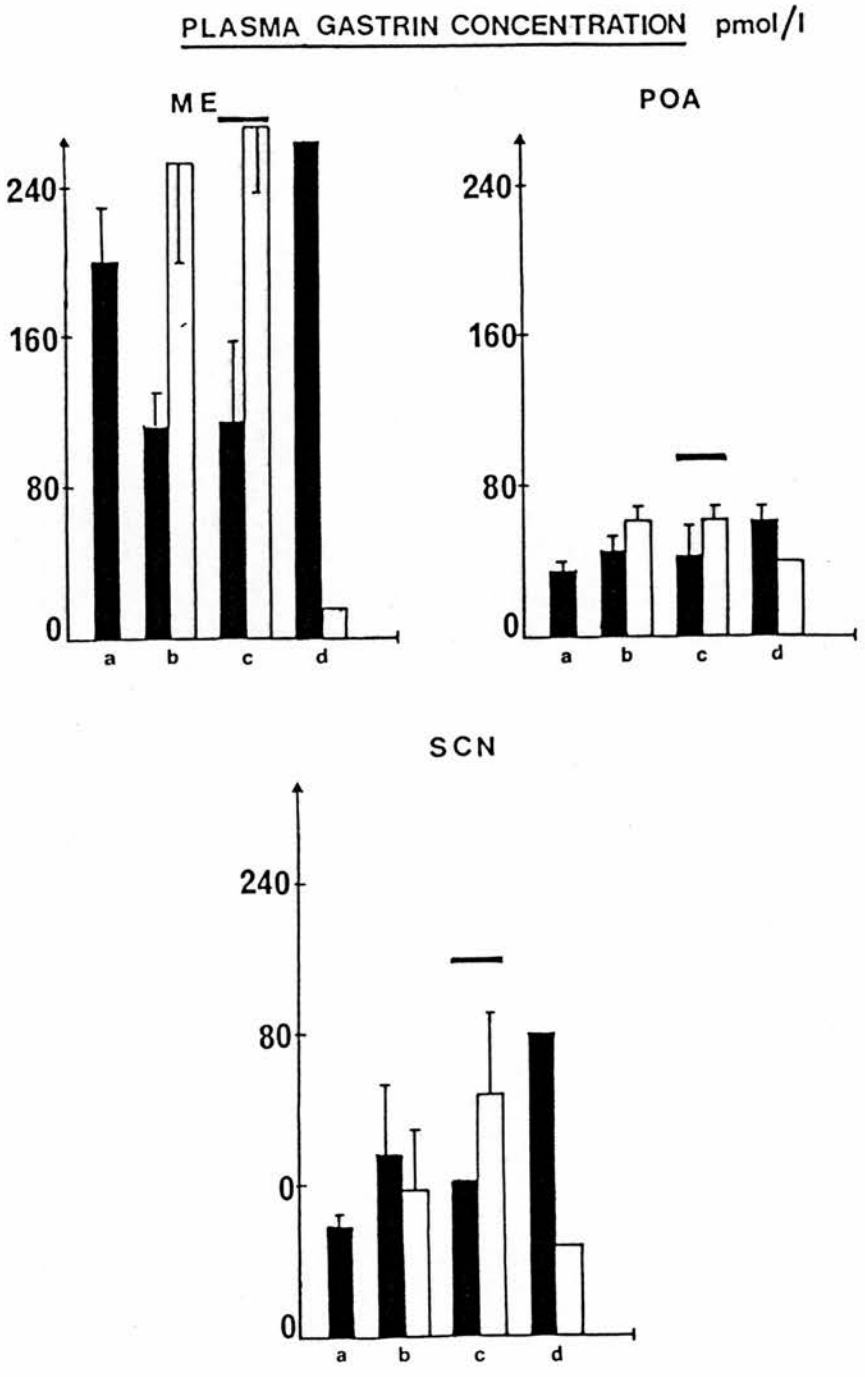


FIGURE 6.2e: Gastrin concentration (pmol/l) in peripheral and pituitary stalk plasma.

b = pre-stimulation period
c = stimulation period (indicated by bar)
d = post-stimulation period

Pituitary stalk blood was collected for 30 min periods (see Figure 6.2b for abbreviations used).

TABLE 6.2e: Gastrin concentration (pmol/l) in peripheral and pituitary stalk plasma obtained before, during and after electrical stimulation of the median eminence (ME), preoptic area (POA) and suprachiasmatic nucleus (SCN). The values given are mean ± SEM; the number of rats in each group is given in parenthesis.

Brain area	Peripheral plasma				Pituitary stalk plasma		
	Control	Pre-stim	Stim	Post-stim	Pre-stim	Stim	Post-stim
ME	199.0 ± 32.0 *(4)*	110.7 ± 19.0 *(3)*	114.8 ± 43.0 *(5)*	264.0 *(1)*	252.0 ± 57.0 *(4)*	272.0 ± 25.0 *(3)*	18.0 *(1)*
POA	35.7 ± 6.0 *(3)*	45.3 ± 7.0 *(3)*	71.0 ± 17.0 *(4)*	92.0 32.0 *(2)*	59.5 ± 10.0 *(4)*	59.6 ± 11.0 *(5)*	42.0 ± 13.0 *(3)*
SCN	57.0 ± 8.0 *(4)*	98.0 ± 36.0 *(3)*	74.0 92.0 *(2)*	312.0 10.0 *(2)*	76.0 ± 33.0 *(5)*	128.5 ± 44.0 *(4)*	48.0 *(1)*

TABLE 6.2f: Changes in pituitary stalk blood volume, gastrin concentration and content before and during the application of an electrical stimulus to various areas of the brain (for abbreviations see Figure 6.2e). Values are given as mean ± SEM; the number of rats in each group is given in parenthesis.

Brain area	Pre-stimulation			Stimulation		
	Volume (µl)	Concentration (pmol/l)	Content (fmol/30 min)	Volume (µl)	Concentration (pmol/l)	Content (fmol/30 min)
ME	625 ± 140 *(6)*	252.0 ± 57.0 *(4)*	214.4 ± 40.0 *(4)*	943 ± 281 *(6)*	272.0 ± 25.0 *(3)*	252.5 ± 46.0 *(3)*
POA	572 ± 102 *(6)*	59.5 ± 10.0 *(4)*	44.5 ± 11.0 *(4)*	786 ± 165 *(5)*	59.6 ± 11.0 *(5)*	53.9 ± 23.0 *(5)*
SCN	552 ± 156 *(5)*	76.4 ± 33.0 *(5)*	79.8 ± 40.0 *(5)*	996 ± 290 *(4)*	128.5 ± 44.0 *(4)*	188.4 ± 95.0 *(4)*

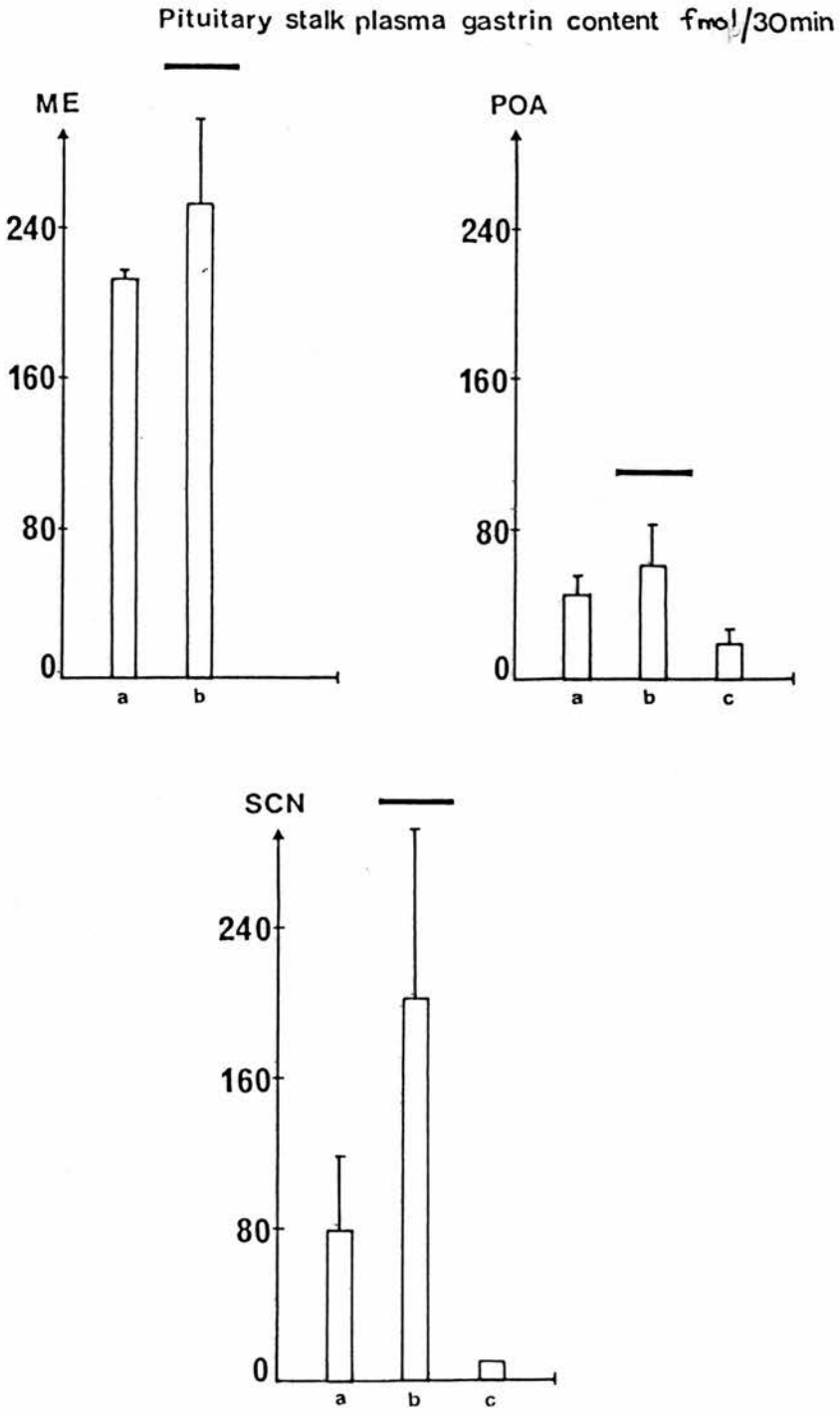


FIGURE 6.2f: Gastrin content (fmol/30 min) of pituitary stalk plasma samples collected for 30 min periods before, during and after electrical stimulation of various brain areas (see Figure 6.2b for abbreviations used).

a = pre-stimulation period
b = stimulation period (indicated by bar)
c = post-stimulation period

these values were indeed significantly ($p < 0.05$) greater than peripheral blood (Tables 6.2b and 6.2c), 'control' group. Although gastrin concentration in pituitary stalk blood was also greater than peripheral blood, the difference was not statistically significant. The presence of immunoreactive CCK-8 in the hypothalamus, stalk and median eminence has been confirmed by several studies, mentioned in the Introduction to this Chapter. The concentration of CCK in the hypothalamus is approximately 250 pmol/g which is only about 50 pmol/g more than found in the jejunum and about half as much as in the cerebral cortex (Dockray, 1982). It has been shown that the predominant form of CCK in the brain is CCK-8 (Muller *et al.*, 1977; Dockray *et al.*, 1978; Rehfeld *et al.*, 1980; Ryden *et al.*, 1981). The presence of CCK-8 in pituitary stalk blood suggests that this is also the major form released. These data receive support from the fact that CCK-8 was released from synaptosome fractions isolated from the hypothalamus, cortex, striatum and thalamus in response to depolarisation (Dodd *et al.*, 1980; Sheppard *et al.*, 1980). The sulfated form of CCK-8 is more active than the non-sulfated fragments (Fekete *et al.*, 1980; Saito *et al.*, 1981). Immunoreactive gastrins have been found in pituitary extracts only (Rehfeld, 1978) and include a large component as well as G-34 and G-17. The immunoreactive forms of CCK or gastrin in pituitary stalk blood were not determined. There is no published evidence demonstrating the release of gastrin from nerve endings. Therefore, CCK, but not gastrin, appears to fulfil the first two criteria to establish them as releasing factors.

Further investigations to demonstrate that these peptides could have physiological roles as neurotransmitters or neuromodulators in the hypothalamic-pituitary system, along the lines used to establish

LHRH as a releasing factor (Fink and Jamieson, 1976; Eskay, Mical and Porter, 1977) produced some interesting and unexpected results. Electrical stimulation of several brain areas shown to contain immunoreactive CCK cell bodies and nerve fibres was coupled with measurements of the peptide in portal vessel blood, to provide direct evidence for a relationship between increased activity of these neurones and release of CCK. Since blood flow in portal vessels increases with stimulation (Fink and Jamieson, 1976) and pituitary stalk blood collections are likely to be contaminated with blood from sources other than portal (Fink and Harris, 1970), the concentration provides an unreliable estimate of the amount of the substance released. Data from studies on the effects of electrical stimulation are therefore also evaluated on the basis of increments in content.

The stimulus parameters were the same as those found to be optimal for the release of LH (Jamieson and Fink, 1976) and similar to that found to be optimal for oxytocin release in the rabbit (Harris, Manabe and Ruf, 1969) and suckling induced release of oxytocin, which is produced by a burst of about 50 Hz in the supraoptic hypophysial system (Wakerly and Lincoln, 1973). Due to constraints on time and expense, an extensive study to determine the optimum parameters for CCK release was not undertaken. However, a marked increase in depth of respiration with a fall in respiratory rate (Fink and Jamieson, 1976) was observed on stimulation of POA, SCN, PVN and ME, suggesting these brain areas were stimulated on application of the stimulus.

The absence of a statistically significant difference in the concentration of CCK and gastrin in pituitary stalk blood on electrical stimulation of any of the selected brain areas, suggests that these

peptides are not physiologically released into the hypophysial portal vessel circulation. The data suggest that electrical stimulation of the ME, SCN, POA and DH merely slowed down the drop in CCK levels that are found after cutting the pituitary stalk. The concentration of CCK in peripheral plasma in the unstimulated 'control' group was significantly ($p < 0.05$) greater than in any of the other groups. A wide range of CCK concentrations was found in the stimulated groups. This variability could be attributed to a phasic release of CCK as well as a post-operative alteration in the release of CCK.

Interpretation of the differences in CCK and gastrin levels in pituitary stalk blood on stimulation of several areas of the brain is made difficult by the fact that intrinsic connections of these peptide-containing cell bodies and fibres are still not clear. The fact that electrical stimulation of the POA, SCN and ME did not alter gastrin values was not surprising in view of the lack of immunohistochemical evidence for the presence of gastrin in these areas. However, the absence of a significant increase in CCK release into pituitary stalk blood on stimulation of the ME, despite evidence for the presence of CCK-immunoreactive fibres in the external layer of the ME (Hökfelt *et al.*, 1978; Loren *et al.*, 1979; Vanderhaeghen *et al.*, 1980), was unexpected and is difficult to explain. It has been demonstrated that a large proportion of CCK fibres from the PVN and a smaller proportion from the SCN project to the external layer of the median eminence (Larsson and Rehfeld, 1977; Loren *et al.*, 1979; Beinfeld *et al.*, 1980). The absence of an increase in CCK release into pituitary stalk blood on stimulation of the ME, PVN and SCN could be due to the fact that there are no CCK nerve terminals originating from the PVN or SCN that terminate on hypophysial portal vessels. There is evidence

that CCK is released from nerve terminals (Pinget *et al.*, 1978; Sheppard *et al.*, 1980) by a calcium-dependent depolarisation (Rehfeld *et al.*, 1979; Dodd *et al.*, 1980). Therefore, despite the fact that in order to collect pituitary stalk blood the pituitary stalk was cut, it is unlikely to have been an appreciable release of CCK into the blood samples collected from the severed fibres to the posterior pituitary. Electrical stimulation of the POA, which has been demonstrated to contain CCK immunoreactivity (Loren *et al.*, 1979; Vanderhaeghen *et al.*, 1980), also did not alter the release of CCK into pituitary stalk blood. The recent finding that the CCK content of the hippocampus was unchanged by knife cuts which disrupted its afferent and efferent connections (Handelmann *et al.*, 1981) suggests that CCK immunoreactivity is intrinsic to this area. The fact that electrical stimulation of either the DH or the VH did not alter the CCK concentration in pituitary stalk blood shows that there is no direct projection of CCK neurones in the hippocampus to the ME.

These experiments do not rule out the possibility that electrical stimulation of one of these areas could influence stimulation-induced release from a different area. For example, hippocampal stimulation reduced LHRH output induced by stimulation of the POA (Chiappa, Fink and Sherwood, 1977). In addition, the CCK immunoreactive fibres and cell bodies in the amygdala (Innes *et al.*, 1979; Loren *et al.*, 1979; Vanderhaeghen *et al.*, 1980) probably project to areas other than the hypophysial portal vessels. Knife cuts that separated amygdala from the caudate reduced the CCK content in the caudate by 30% (Meyer *et al.*, 1981), indicating that the caudate was one of the areas to which CCK fibres from the amygdala project.

In conclusion, although these data show that CCK-8 concentration in pituitary stalk blood is significantly ($p < 0.05$) greater than in peripheral blood, the role of CCK-8 as a physiological releasing factor is doubtful. Electrical stimulation of several brain areas that have been demonstrated to contain CCK-immunoreactivity did not alter the release of CCK-8 into pituitary stalk blood. The gastrin concentration of pituitary stalk blood is not significantly higher than peripheral blood. Electrical stimulation of the PVN, SCN and ME did not alter these levels of gastrin. It is possible that the optimal parameters for the release of CCK and gastrin were not used.

6.3 Changes in Plasma CCK and Gastrin with Gastric Antrectomy

6.3.1 Introduction

The series of experiments described in this section were carried out after those described in Section 6.2. Since circulating gastrin originates almost exclusively from the antrum and duodenum (Malstrom, Stadil and Rehfeld, 1976; Dockray, 1979), whereas the brain is the main source of CCK in the mammalian system (Dockray, 1976; Rehfeld, 1978), and since serum gastrin concentrations are reduced more than 10-fold by duoantrectomy (Rehfeld, Stadil, Baden and Fischerman, 1975), changes in values of peripheral and portal vessel blood CCK and gastrin after duoantrectomy were studied. The fact that CCK shares the biologically active COOH-terminal amino acids with gastrin (Figure 6.1) and that the radioimmunoassay used to measure CCK and gastrin values involves using antibodies with differing specificities for these two peptides (Figure 6.1), suggests that experimental reduction of gastrin would lower the total effective cross reaction in the assay.

6.3.2 Methods

(a) Animals and treatments:

Male rats (180-200 g) of the Albino Wistar COB strain were used in all experiments. Urethane (1 mg/kg i.p.) or Althesin (1 m l/ 100 g) were used to induce anaesthesia. Pituitary stalk blood was collected as described in Section 2.2.5 during three consecutive 30 min periods before, during and after the application of an electrical stimulus to the ME (as in Section 6.2.1). Peripheral blood samples were collected from the external jugular vein. All blood samples were collected on ice into tubes containing EDTA 5.4 (mM/l) and Trasylol (5 KIU/l). The plasma was frozen until assayed for CCK and gastrin by radioimmunoassay (Section 2.3). The lower limit of the CCK assay was 20 pmol/l and for gastrin it was 5 pmol/l.

Gastric antrectomy and vagotomy were carried out as described in Section 2.2.4, approximately 1 h before samples of peripheral and portal blood were collected. To determine the time course of the clearance of CCK and gastrin from peripheral plasma after antrectomy, external jugular venous samples (0.4 - 0.5 ml) were collected at frequent intervals before and from 5 min up to 90 min after the removal of the antrum.

(b) Calculations:

The concentrations of CCK and gastrin in the plasma were determined by radioimmunoassay (Section 2.3). The content (concentration x volume per unit time) of CCK was calculated for pituitary stalk blood. The significance of the differences between means was assessed by the paired and unpaired students' t-test.

6.3.3 Results

(a) Changes in circulating CCK and gastrin concentrations after gastric antrectomy:

Figure 6.3a shows that within the first 5 min there was a 48% reduction in the plasma concentration of gastrin and a 51% reduction in the plasma concentration of CCK; after 5 min, CCK values remained undetectable (<20 pmol/l). Since blood samples were not collected during the fast phase (0-5 min) of the clearance of the peptides, the half-life cannot be accurately calculated. The concentration of both CCK and gastrin before antrectomy was 46% and 40% lower, respectively, under Althesin compared with urethane anaesthesia (Figure 6.3a). Urethane anaesthesia was therefore used in all other experiments described in this Chapter.

(b) CCK and gastrin release into pituitary stalk blood after gastric antrectomy and electrical stimulation of the median eminence:

Table 6.3a shows the gastrin and CCK concentration of peripheral and pituitary stalk blood sampled for 30 min periods, beginning 1 h after gastric antrectomy. Removal of the antrum significantly ($p < 0.05$) reduced circulating gastrin levels by half during the pre-stimulation period. Pituitary stalk blood gastrin concentrations were lowered even more dramatically ($p < 0.05$); gastrin content of pituitary stalk blood was also significantly ($p < 0.05$) lower after removal of the antrum. This suggests that the gastric antrum is a major source of gastrin measured in both peripheral and pituitary stalk blood.

The effect of antrectomy on circulating CCK concentration was almost as great as for gastrin. The concentrations of CCK in samples of peripheral blood samples taken before ME stimulation were reduced significantly ($p < 0.05$). The reduction in CCK content of pituitary

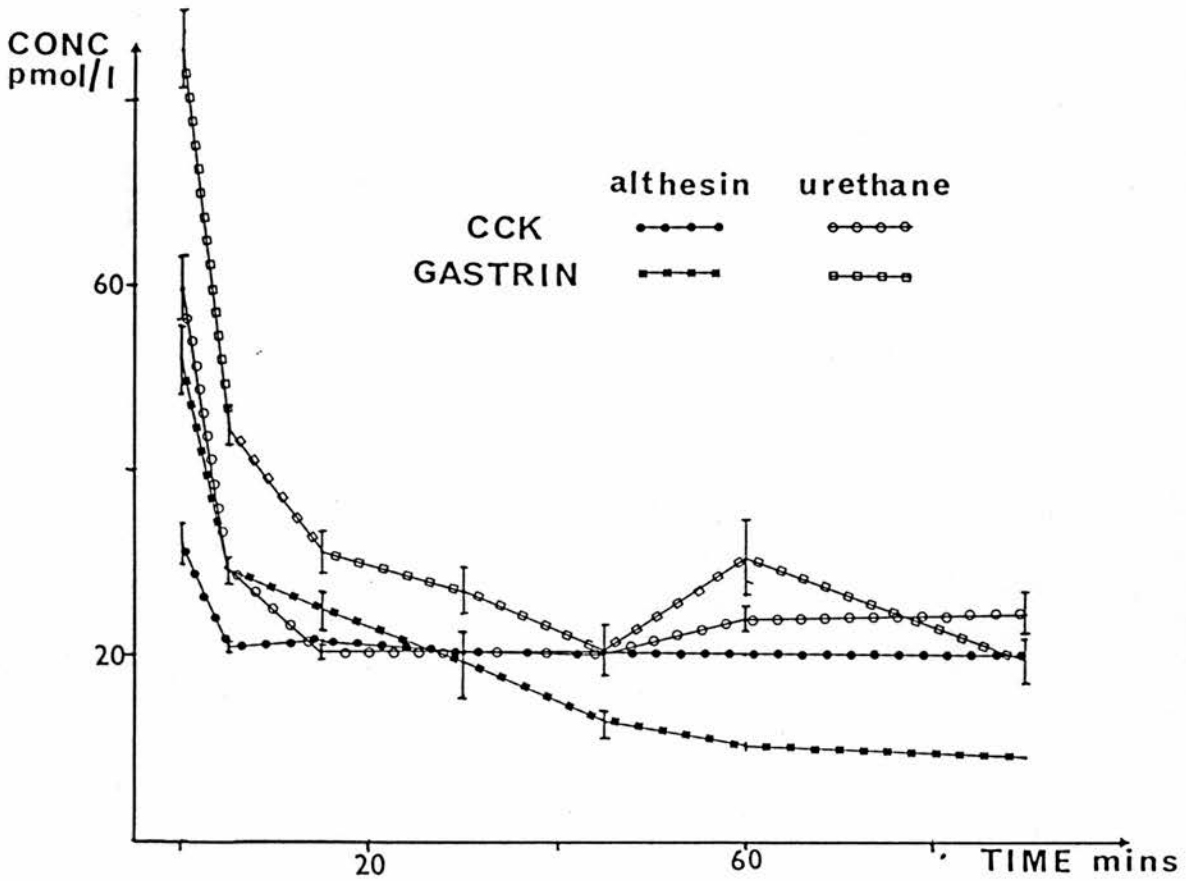


FIGURE 6.3a: CCK and gastrin concentration in the peripheral circulation before and up to 90 min after the removal of the gastrin antrum. Blood samples were collected from the external jugular vein under Urethane or Althesin anaesthesia. Groups of 5 rats each were used. The lower limit of the assays were 20 pmol/l for CCK and 5 pmol/l for gastrin.

TABLE 6.3a: Effect of gastric antrectomy and electrical stimulation of the median eminence (ME) on CCK and gastrin concentration and content in peripheral and pituitary stalk plasma. Values are given as mean ± SEM; the number of rats in each group is given in parenthesis.

	Peripheral plasma			Pituitary stalk plasma		
	Pre-stim	Stim	Post-stim	Pre-stim	Stim	Post-stim
ME stimulation only:						
CCK concentration (pmol/l)	119.7 ± 10.0 *(6)*	134.4 ± 5.0 *(6)*	98.0 280.0 *(2)*	96.3 ± 10.0 *(6)*	127.3 ± 10.0 *(6)*	219.0 ± 53.0 *(4)*
content (fmol/30 min)	-	-	-	65.8 ± 25.0 *(6)*	146.3 ± 69.0 *(6)*	93.4 ± 29.0 *(4)*
Gastrin concentration (pmol/l)	110.7 ± 19.0 *(3)*	114.8 ± 43.0 *(5)*	264.0 *(1)*	252.0 ± 57.0 *(4)*	272.0 ± 25.0 *(3)*	18.0 *(1)*
content (fmol/30 min)	-	-	-	214.4 ± 40.0 *(4)*	252.5 ± 46.0 *(3)*	-
ME stimulation + antrectomy:						
CCK concentration (pmol/l)	62.4 ± 9.0 *(5)*	36.3 ± 7.0 *(6)*	20.0 56.0 *(2)*	67.0 ± 22.0 *(6)*	51.5 ± 14.0 *(6)*	30.0 118.0 *(2)*
content (fmol/30 min)	-	-	-	27.8 ± 9.0 *(6)*	14.6 ± 4.0 *(6)*	5.7 35.0 *(2)*
Gastrin concentration (pmol/l)	70.0 ± 14.0 *(6)*	52.4 ± 20.0 *(5)*	15.0 38.0 *(2)*	75.2 ± 25.0 *(4)*	56.3 ± 14.0 *(5)*	48.0 18.0 *(2)*
content (fmol/30 min)	-	-	-	38.5 ± 10.0 *(4)*	20.8 ± 6.0 *(5)*	3.4 14.0 *(2)*

stalk blood was less marked, but CCK content of stalk blood was significantly ($p < 0.05$) lower after antrectomy.

Electrical stimulation of the ME did not significantly alter the release of gastrin into pituitary stalk plasma or peripheral plasma either in the intact group or 90-120 min after the removal of the antrum. Similar results were obtained for CCK concentrations, though there was a significant ($p < 0.05$) increase with ME stimulation in pituitary stalk plasma content over the 30 min period in the intact group (Table 6.3a). After removal of the antrum, this difference was not found. However, a significant ($p < 0.05$) reduction in pituitary stalk plasma CCK content/ 30 min was found after antrectomy.

(c) Changes in CCK release following vagotomy compared to gastric antrectomy

In order to rule out the possibility that removal of vagal release of CCK (Dockray, 1981) or the trauma of surgery on removal of the gastric antrum accounted for the reduced values of CCK found after antrectomy, the concentration and content of CCK was determined after cutting the vagii by cutting the oesophagus (Method; Section 2.2.4).

Table 6.3b shows that cutting the vagii had no significant effect on the CCK concentration in pituitary stalk blood compared with the values in control animals. Antrectomy, on the other hand, significantly ($p < 0.025$) reduced CCK concentration in the peripheral circulation, as found in the experiments described in Section 6.3.3b. Pituitary stalk plasma concentration was reduced by 65% and the CCK content of pituitary stalk blood collected over 30 min was reduced by 76% after gastric antrectomy.

TABLE 6.3b: The effect of antrectomy or cutting the oesophagus on CCK concentration (pmol/l) and content (fmol/30 min) in peripheral and pituitary stalk plasma compared to intact controls. Values are given as mean ± SEM; * and ** p <0.05 when plasma CCK concentration after antrectomy was compared with intact controls.

	No. of rats	Concentration (pmol/l)	Content (fmol/30 min)
Intact control			
Peripheral plasma	6	* 66.30 ± 7.0	
Pituitary stalk plasma	6	**134.70 ± 24.0	76.70 ± 21.0
Oesophagus cut			
Peripheral	9	78.30 ± 14.0	
Pituitary stalk plasma	7	125.50 ± 23.0	82.54 ± 28.0
Antrectomy			
Peripheral	8	* 34.10 ± 7.0	
Pituitary stalk plasma	6	** 46.30 ± 9.0	20.04 ± 4.0

6.3.4 *Discussion and Conclusions*

These results provide strong evidence that a large proportion of the CCK and gastrin in pituitary stalk blood originates from the gastric antrum. The first series of experiments (Section 6.3.3a) demonstrated that removal of the antrum reduced the peripheral plasma concentration of both gastrin and CCK by approximately 50%. An equivalent reduction of both CCK and gastrin was unexpected in view of the fact that the gastric antrum is known to be the major source of gastrin (Malstrom *et al.*, 1976), while the chief source of peripheral CCK is the intestines (Jorpes and Mutt, 1973; Larsson and Rehfeld, 1979) which were left untouched in this experiment.

That a statistically significant ($p < 0.05$) decrease in CCK and gastrin released into pituitary stalk blood also occurred after gastric

antrectomy was demonstrated by the second series of experiments (Section 6.3.3b). Electrical stimulation of the ME did not significantly alter the release of CCK or gastrin either in the intact group or after removal of the antrum. This suggests that even after removal of the peripheral source of CCK and gastrin, an electrical stimulus applied to the brain does not increase the release of the peptides into hypophysial portal vessel circulation; the lack of a significant effect of ME stimulation on the release of these peptides cannot be due to a masking of the effect due to peripheral release of CCK and gastrin.

The results of the third series of experiments (Section 6.3.3c) confirmed that gastric antrectomy consistently lowered the peripheral plasma CCK concentration and content/30 min of pituitary stalk plasma by 65-75%. In addition, these experiments showed that removal of the antrum rather than the trauma of surgery or sectioning of the vagii, which have been demonstrated to release CCK (Lundberg *et al.*, 1978; Dockray *et al.*, 1981), could account for the decrease in CCK concentration in peripheral and pituitary stalk plasma.

The demonstration that CCK concentration in plasma are reduced after gastric antrectomy suggests that either the antibodies used in the radioimmunoassays used to measure the concentrations of these peptides did not differentiate between the two structurally similar peptides, gastrin and CCK, or that the gastric antrum does in fact release a large amount of CCK. These experiments underline the difficulties that arise in interpreting data from investigations of a complex association between central and peripheral peptide pools. However, the measurement of CCK and gastrin concentrations after antrectomy provide evidence that suggests that CCK and gastrin measured in pituitary stalk plasma originate largely from the periphery.

CHAPTER VII

Vasoactive Intestinal Polypeptide (VIP) in Rat Pituitary Stalk Blood: Investigations into the Effects of Electrical Stimulation of Various Brain Areas and Possible Changes During the Rat Oestrous Cycle

CHAPTER VII

7.1 Vasoactive Intestinal Polypeptide (VIP) in Rat Pituitary Stalk Blood: Effects of Electrical Stimulation of Brain Areas Containing Immunoreactive VIP

7.1.1 Introduction

Vasoactive intestinal polypeptide (VIP) is a 28-amino acid residue polypeptide, first isolated from porcine duodenum (Said and Mutt, 1970). It has powerful hypotensive and vasodilatory effects and has been found in large quantities throughout the mammalian digestive system, from the oesophagus to the rectum (Polak, Pearse, Garaud and Bloom, 1974; Larsson, Fahrenkrug, Schaffalitsky de Muckadell, Syndler, Håkanson and Rehfeld, 1976; Besson, Laburthe, Bataille, Dupont and Rosselin, 1978; Hutchison, Dimaline and Dockray, 1981). In the rat, the highest concentration of VIP in the gut occurs between the duodenum (1676 ± 186 ng/g of tissue) and the colon (1214 ± 214 ng/g of tissue) with smaller amounts (180 ± 26 ng/g of tissue) in the pancreas (Besson *et al.*, 1978). Immunohistochemical studies show that VIP immunoreactivity is present in gastrointestinal nerves and forms a major part of the nervous system intrinsic to the gut wall (Larsson *et al.*, 1976). In addition, vagal nerve stimulation induces VIP release *in vivo* (Fahrenkrug, Galbo, Holst, Schaffalitzky de Muckadell, 1978) and *in vitro* from the isolated perfused stomach (Pederson, O'Dorisio, Howe, McIntoch, Mueller, Brown and Cataland, 1981).

Immunoreactive VIP has also been demonstrated in neurones of the peripheral and central nervous system by immunohistochemical and radioimmunoassay techniques (Larsson *et al.*, 1976; Besson, Rotsztejn, Laburthe, Epelbaum, Beaudet, Kordon and Rosselin, 1979a; Loren, Emson, Fahrenkrug, Björklund, Alumets, Håkanson and Sundler, 1979b; Roberts, Woodhams, Bryant, Crow, Bloom and Polak, 1980; Sims,

Donald, Hoffman, Said and Zimmerman, 1980; Hökfelt, Schultzberg, Lundberg, Fuxe, Mutt, Fahrenkrug and Said, 1982; Fahrenkrug and Emson, 1982). Based on light microscopic immunohistochemical studies, the distribution of VIP in the rat and mouse brain can be divided into four major systems (Sims *et al.*, 1980). These include:

1. An intracerebral cortical system (Fuxe, Hökfelt, Said and Mutt, 1977; Besson *et al.*, 1979a; Sims *et al.*, 1980). VIP immunoreactivity has been demonstrated in all regions of the cortex including the limbic cortex and subiculum (Kohler, 1982). The highest concentration of VIP in the brain is found in the occipital cortex (Besson *et al.*, 1979a).

2. An area of extremely dense fibres and terminals between the stria terminalis and its bed nucleus (Loren, 1979b; Roberts *et al.*, 1980; Sims *et al.*, 1980). These fibres also appear to form a VIP-containing pathway which links the amygdaloid complex with the hypothalamus (Roberts *et al.*, 1980; Sims *et al.*, 1980; Palkovits, Besson and Rotsztejn, 1981).

3. A system originating in the midbrain central grey (Larsson *et al.*, 1976; Fuxe *et al.*, 1977; Loren *et al.*, 1979b; Sims *et al.*, 1980; Marley, Emson, Hunt and Fahrenkrug, 1981).

4. A hypothalamic system with cell bodies localised primarily within the basal portion of the suprachiasmatic nucleus, medial preoptic area and anterior hypothalamus, with only a few VIP-containing fibres in the median eminence (Larsson *et al.*, 1976; Fuxe *et al.*, 1977; Loren *et al.*, 1979b; Samson, Said and McCann, 1979; Roberts *et al.*, 1980; Sims *et al.*, 1980; Card, Brecha, Karten and Moore, 1981; Rostène, Léránth, Maletti, Mezey, Besson, Eiden, Rosselin and Palkovits, 1982).

So far, no group has detected VIP in the cerebellum (Besson *et al.*, 1978; Loren *et al.*, 1979; Sims *et al.*, 1980). VIP has been detected in the pars nervosa of the dog but not several other mammalian species, by Van Noorden, Polak, Bloom and Bryant (1979) and in the rat by Samson *et al.* (1979). However, radioimmunological investigations by Besson *et al.* (1978) and immunohistochemical studies by Loren *et al.* (1979) have been unable to demonstrate VIP in the pars nervosa of the rat.

The selective distribution of VIP in the brain, its presence in nerve terminals and its calcium-dependant release from synaptosomes from the cerebral cortex and hypothalamus (Giachetti, Said, Reynolds and Koniges, 1977; Emson, Fahrenkrug, Schaffalitsky de Muckadell, Jessel and Iversen, 1978) and rat brain cortex and amygdala slices (Besson, Rotsztejn, Poussin, Lhiaubet and Rosselin, 1982), as well as a specific enzyme system which degrades this peptide in the brain (Keltz, Straus and Yalow, 1980), all suggest a role for VIP as a neurotransmitter or neuromodulator in the brain. Furthermore, VIP has a potent excitatory effect on neurones of the hippocampus, cerebral cortex, preoptic area, septum and midbrain central grey neurons (Phillis, Kilpatrick and Said, 1978; Dodd, Kelly and Said, 1979; Haskins, Samson and Moss, 1982) and binds to specific VIP receptors found only in VIP-rich areas of the brain (Deschodt-Lanckman, Robberecht and Christophe, 1977; Taylor and Pert, 1979; Staun-Olsen, Ottsen, Bartels, Nielsen, Gammeltoft and Fahrenkrug, 1982). In addition, the presence of immunoreactive VIP in the limbic system, in several hypothalamic nuclei and their linking pathways, as well as high concentrations of VIP in hypophysial portal vessel blood (Said and Porter, 1979; Shimatsu, Kato, Matsushita, Katakami, Yanaihara

and Imura, 1981) suggest that VIP may play a role in neuroendocrine regulation.

Purpose of the present study:

The main aims of the experiments described in this section were to determine:

1. whether the release of VIP into pituitary stalk blood is modulated by electrical stimulation of various areas of the brain known to contain immunoreactive VIP;
2. the effect of complete removal of the gut on the concentration of VIP in pituitary stalk blood to assess the contribution of VIP in the peripheral system to the stalk blood concentrations.

7.1.2 Materials and Methods

(a) Animals:

Adult male Wistar COB rats weighing 180-200 g were used in the experiments described in this section. Urethane (1 mg/kg b.w.) was administered intraperitoneally to induce anaesthesia. In the first series of experiments, pituitary stalk blood was collected (method described in Section 2.2.5) over consecutive 30 min periods before, during and after the application of the electrical stimulus to the brain. Peripheral blood samples from the external jugular vein were obtained before the pituitary stalk was cut and at the end of each 30 min period of pituitary stalk blood collection. In the second series of experiments the peripheral blood samples were obtained from the external jugular vein before and at 30 min intervals after the removal of the gut. Pituitary stalk samples were collected for a 30 min period which began 30 min after the gut was removed. The blood samples were collected into tubes kept on ice; the tubes contained Trasylol (5000

KIU/ml). The plasma was kept frozen at -40°C until packed in dry ice and sent by airfreight to Paris to be assayed for VIP. The samples remained frozen at all times.

(b) Implantation of electrodes and stimulation parameters:

The methods used to prepare and implant the bipolar glass-insulated platinum electrodes are described in Section 2.2.7. Implantation of the electrodes was carried out under Avertin anaesthesia, 4-5 days before collection of pituitary stalk blood samples. All the rats appeared healthy at the time of stalk blood collection. Electrodes were implanted in the amygdala (AMY), the suprachiasmatic nucleus (SCN), preoptic area (POA), paraventricular nucleus (PVN), ventral hippocampus (VH) and dorsal hippocampus (DH). The co-ordinates used were based on de Groot's atlas (1959) and are given in Table 6.2a. The median eminence (ME) was stimulated by way of a unipolar glass-insulated platinum electrode which had been placed on the surface of the ME with the aid of a micro-manipulator, after exposure of the portal vessels and removal of brain membranes, just before heparin was injected and the pituitary stalk cut. In all cases, the stimulus was applied for 30 min and consisted of biphasic square wave pulses in trains of 30 sec on and 30 sec off, frequency of 60Hz, amplitude of 500 µA (1 mA peak-to-peak) and pulse duration of 1 msec. The parameters of the electrical stimulus used were those shown to produce the optimum release of LH (Fink and Aiyer, 1974; Jamieson and Fink, 1976). The electrodes were left in place until the end of the experiment when rats were decapitated. The head was trimmed and fixed in buffered formalin. After decalcification of the skull the electrodes were carefully removed. The brain was frozen, serial sections were cut, stained with cresyl fast violet and placement of the electrodes

determined (Section 2.2.7). Brain sections indicating the positions of the electrode are shown in Figure 6.2b.

(c) Radioimmunoassay and calculations:

VIP immunoreactivity in the plasma samples was measured by a specific radioimmunoassay, as described in Section 2.3, according to the method of Maletti, Rostène, Carr, Scherrer, Rotten, Kordon and Rosselin (1982). The specificity of the VIP antibody was directed to a fragment of the carboxyl-terminal and the medium part of the molecule, as indicated by the cross-reaction of the antibody with the synthetic fragments 10-28 of VIP (Maletti *et al.*, 1982).

The concentration of VIP in the plasma samples was expressed in pg/ml. In addition, the content (concentration x volume per 30 min) was calculated for the pituitary stalk blood samples. The significance of differences between means was determined by the paired or unpaired t-test as appropriate. The differences between more than two means was determined by analysis of variance and Duncan's multiple range test for heteroscadistic means (Duncan, 1957; Harter, 1960).

7.1.3 Results

(a) Characteristics of VIP in pituitary stalk blood:

The mean (± SEM) concentration of VIP in pituitary stalk plasma was significantly ($p < 0.001$) greater than the concentration in peripheral plasma (Table 7.1a) before stimulation of any brain areas. The VIP content in pituitary stalk plasma in the 30 min collection period was 423 ± 68 pg/30 min.

Degradation of VIP during the collection period was found to be effectively minimised by the addition of the peptidase inhibitor, Trasylol (5000 KIU/ml whole blood) at the time of collection of the samples

TABLE 7.1a: VIP peripheral and pituitary stalk plasma from adult male rats showing the effect of Trasylol addition (5000 KIU/ml) to whole blood. Values given are mean ± SEM (n). *p <0.001 when peripheral and pituitary stalk plasma VIP concentration was compared.

	Peripheral plasma	Pituitary stalk plasma
With Trasylol:		
Concentration (pg/ml)	452.9 ± 124.6 (8)*	1281.0 ± 157.6 (7)*
Content (pg/30 min)		422.4 ± 68.6 (7)
Without Trasylol:		
Concentration (pg/ml)	321.4 ± 48.8 (6)	552.8 ± 119.7 (6)
Content (pg/30 min)		328.8 ± 66.5 (5)

(Table 7.1a). The VIP concentration in pituitary stalk plasma without Trasylol was approximately 60% of the concentration in samples to which Trasylol had been added. Peripheral plasma VIP concentrations were not significantly decreased in the samples without Trasylol.

(b) Effect of electrical stimulation on VIP concentrations in peripheral plasma and pituitary stalk plasma:

VIP concentrations in peripheral and pituitary stalk plasma before, during and after the application of an electrical stimulus to various areas of the brain are shown in Table 7.1b and represented in histogram form in Figure 7.1a. Peripheral 'control' values represent VIP concentrations in the peripheral circulation before the pituitary stalk was cut in order to collect pituitary stalk blood.

(i) Peripheral plasma VIP concentration

The VIP concentration in peripheral plasma varied considerably between groups. In the group of rats with electrodes in the POA, the

TABLE 7.1b: The VIP concentration (mean ± S.E.M.) in pg/ml of peripheral plasma and pituitary stalk plasma before, during and after electrical stimulation of various brain areas (see Figure 7.1a for abbreviations). The *control* data are values from rats that were not implanted or stimulated. Peripheral *control* values are those in samples taken before the pituitary stalk was cut.

	Peripheral plasma				Pituitary stalk plasma		
Brain area	Control	Pre-stim	Stim	Post-stim	Pre-stim	Stim	Post-stim
AMY	54.7 ± 11.6 *n=5*	42.8 ± 3.0 *n=4*	19.6 ± 5.5 *n=6*	5.0 42.9 *n=2*	244.4 ± 60.5 *n=6*	233.5 ± 79.6 *n=4*	283.0 ± 100.6 *n=3*
ME	22.7 ± 13.6 *n=4*	10.8 ± 2.9 *n=5*	21.9 ± 9.6 *n=6*	37.4 6.8 *n=2*	109.1 ± 30.6 *n=5*	92.2 ± 22.1 *n=4*	60.5 43.9 *n=2*
POA	157.8 ± 86.8 *n=5*	38.6 ± 12.5 *n=5*	30.9 ± 5.8 *n=5*	20.0 ± 9.2 *n=3*	173.0 ± 34.2 *n=6*	173.0 ± 47.2 *n=6*	105.5 ± 59.0 *n=4*
PVN	53.1 ± 23.0 *n=7*	31.4 ± 12.0 *n=7*	19.6 ± 3.1 *n=3*	10.5 *n=1*	174.0 ± 36.8 *n=7*	97.7 ± 25.6 *n=7*	66.4 *n=1*
SCN	36.8 ± 6.1 *n=7*	21.4 ± 6.9 *n=7*	20.0 ± 6.4 *n=4*	60.0 26.5 *n=2*	261.1 ± 115.9 *n=5*	91.2 ± 18.2 *n=5*	94.6 ± 26.0 *n=3*
DH	166.0 52.0 *n=2*	47.6 ± 20.2 *n=3*	8.5 15.0 *n=2*	-	79.2 ± 44.3 *n=3*	129.2 ± 54.9 *n=3*	121.0 68.9 *n=2*
VH	34.5 ± 4.5 *n=9*	11.4 ± 1.9 *n=9*	12.2 ± 3.2 *n=6*	6.6 14.7 *n=2*	263.4 ± 34.9 *n=9*	173.8 ± 30.0 *n=7*	336.0 61.9 *n=2*
Control	50.2 ± 4.2 *n=9*	47.1 ± 14.9 *n=10*	38.0 ± 3.4 *n=7*	38.6 ± 7.9 *n=6*	598.6 ± 334.9 *n=10*	550.0 ± 154.3 *n=8*	390.5 ± 115.4 *n=6*

peripheral plasma VIP concentration was 3 times greater ($p < 0.05$) than in the group of rats in which was not implanted or stimulated (the 'control' group). All the other groups had peripheral plasma VIP concentrations similar or lower than the unstimulated 'control' group. Cutting the pituitary stalk slightly lowered the peripheral plasma VIP concentration in all groups. Electrical stimulation produced no statistically significant changes in the peripheral plasma VIP concentration of any of the groups compared to the concentration in the unstimulated 'control' group.

(ii) Pituitary stalk plasma VIP concentrations

The VIP concentrations in pituitary stalk plasma within the different groups also showed considerable variability (pre-stimulation values, Table 7.1c). Electrical stimulation of seven areas of the brain did not produce any statistically significant changes in the VIP concentration of the pituitary stalk plasma (Figure 7.1a).

The amount of VIP released into pituitary stalk plasma over the 30 min before stimulation, 'content' in Table 7.1c, showed a similarly large intra-group variation as found for VIP concentration. Stimulation of the different areas of the brain produced no statistically significant changes in pituitary stalk plasma VIP content (Figure 7.1b).

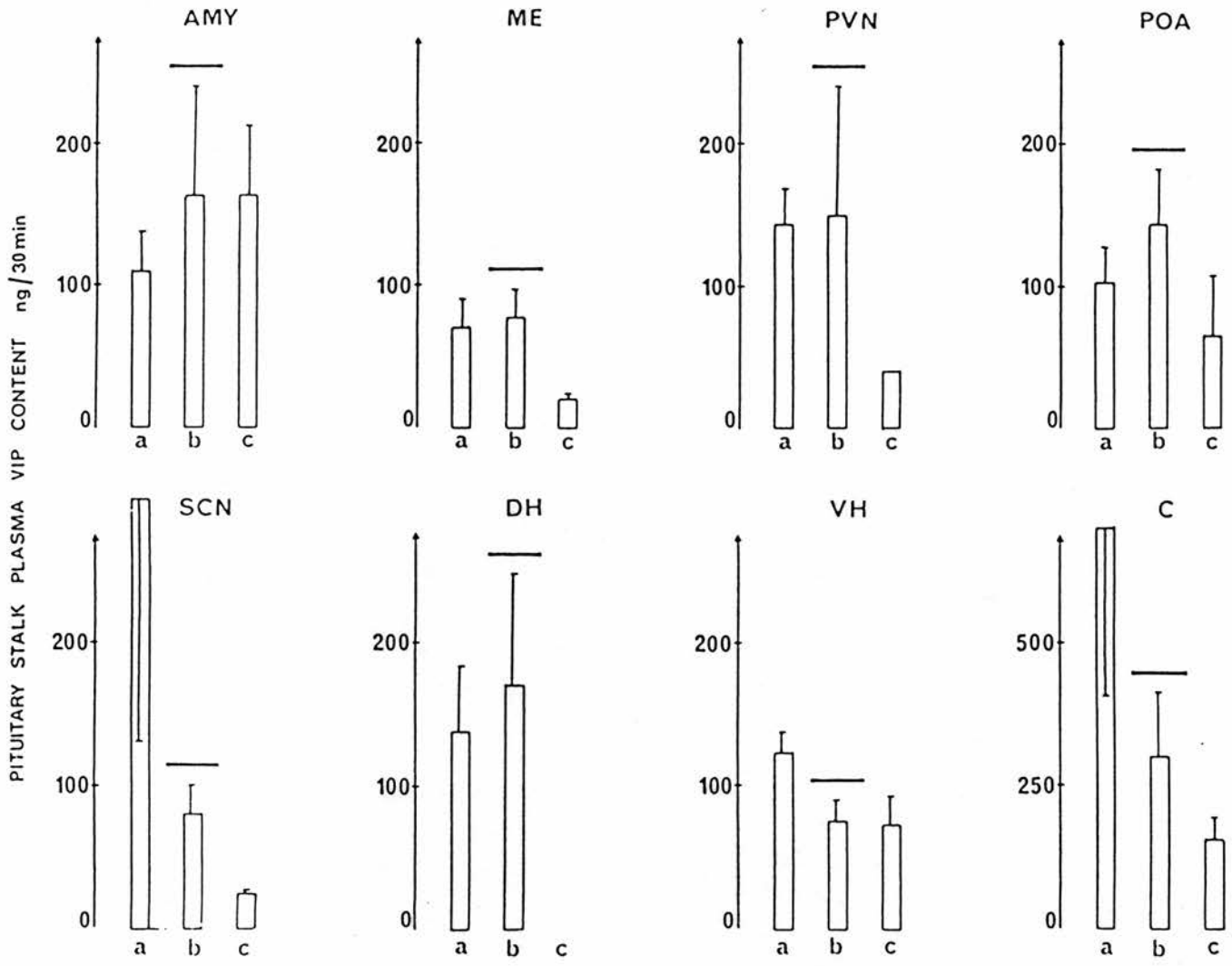
The volumes of pituitary stalk blood collected over each 30 min period are shown in Table 7.1c. An increase in blood flow was found to occur in all groups on application of the electrical stimulus. The relative increments in blood volume varied according to the area stimulated. However, there were no significant differences between groups after stimulation in terms of the blood volumes or increments in blood volumes.

TABLE 7.1c: Changes in VIP concentration (pg/ml) content (pg/30 min) and volume (µl) of pituitary stalk blood on electrical stimulation of various brain (for abbreviations see Figure 7.1a). The *Control* group represents unstimulated rats. Values are given as mean ± S.E.M.

	Pre-stimulation				Stimulation			
Brain area	No. of rats	Volume (µl)	Concentration (pg/ml)	Content (pg/30 min)	No. of rats	Volume (µl)	Concentration (pg/ml)	Content (pg/30 min)
AMY	6	485 ± 108	244.4 ± 60.5	107.9 ± 33.1	4	578 ± 120	233.5 ± 79.6	163.1 ± 83.0
ME	5	625 ± 140	109.1 ± 30.6	70.0 ± 21.9	4	943 ± 281	92.2 ± 22.1	78.6 ± 23.9
POA	6	572 ± 102	173.0 ± 34.2	103.7 ± 27.5	6	698 ± 66	173.0 ± 47.2	144.9 ± 40.9
PVN	7	999 ± 364	174.0 ± 36.8	140.0 ± 37.5	7	1022 ± 35	97.7 ± 25.6	149.2 ± 90.2
SCN	5	611 ± 177	261.1 ± 115.9	303.3 ± 171.7	5	863 ± 272	91.2 ± 18.2	80.7 ± 23.4
DH	3	1057 ± 288	79.2 ± 44.3	138.6 ± 46.7	3	1263 ± 739	129.2 ± 54.9	161.8 ± 79.3
VH	9	527 ± 78	263.4 ± 34.9	123.2 ± 15.2	7	548 ± 60	173.8 ± 30.0	76.6 ± 15.9
Control	10	749 ± 127	598.6 ± 334.9	581.9 ± 376.8	8	438 ± 56	550.0 ± 154.3	300.0 ± 113.5

FIGURE 7.1b: VIP content (pg/30 min) of pituitary stalk plasma samples collected for 30 min periods before, during and after electrical stimulation of various brain areas (see Figure 7.1a for abbreviations used).

a = pre-stimulation period;
b = stimulation period (indicated by bar);
c = post-stimulation.


AMY
ME
PVN
POA
SCN
DH
VH
C
PITUITARY STALK PLASMA VIP CONTENT ng/30 min
0
100
200
250
500
a
b
c

(c) VIP concentration in pituitary stalk blood after removal of the gut:

Table 7.1d shows that removal of the gut from the lower end of the oesophagus up to and including the rectum, significantly ($p < 0.01$) decreased the concentration of VIP in peripheral plasma compared to intact controls. Within 30 min the VIP concentration in peripheral plasma fell significantly ($p < 0.01$) and continued to fall over the next 60 min. There was no significant difference in the peripheral VIP concentration of the intact controls compared to the experimental group, before the gut was removed.

The concentration (pg/ml) and content (pg/30 min) of VIP in pituitary stalk blood was not significantly altered by removal of the gut (Table 7.1d).

7.1.4 Discussion and Conclusions

The significantly ($p < 0.05$) higher concentration of VIP in pituitary stalk blood compared to the peripheral circulation suggests a preferential release of VIP of central origin occurs into the hypophysial portal vessel circulation. This conclusion received support from the fact that the VIP concentration in the pituitary stalk concentration was not significantly altered by removal of the gut, which is the main source of VIP in the peripheral circulation (Polak *et al.*, 1974; Larssen *et al.*, 1976; Besson *et al.*, 1978; Hutchison *et al.*, 1981; Table 7.1d). This allows for a possible involvement of VIP in the control of anterior pituitary function. Electrical stimulation of several regions that have been demonstrated to contain VIP-immunoreactivity produced no significant change in the VIP concentration of pituitary stalk blood (Figure 7.1a).

TABLE 7.1d: VIP concentration and content in peripheral plasma and pituitary stalk plasma in intact control rats and rats with the gut removed. Peripheral plasma samples were obtained, a = before gut removed, stalk intact; b = 30 min after gut removed, stalk intact; c and d = 60 min and 90 min after gut removed, stalk cut. Pituitary stalk samples were collected in 30 min periods (I and II) starting 30 min after the removal of the gut; I = 30-60 min; II = 60-90 min. The values are given as mean ± S.E.M. *,** = $p < 0.01$ when the values are compared with each other.

	No. of rats	Peripheral plasma				Pituitary stalk plasma	
		a	b	c	d	I	II
Intact controls:							
Concentration (pg/ml)	5		15.4 ± 3.0*	19.1 ± 4.0	12.9 ± 2.4**	139.8 ± 17.9	134.8 ± 21.5
Content (pg/30 min)						74.6 ± 11.5	68.2 ± 17.6
Gut removed:							
Concentration (pg/ml)	9	11.8 ± 1.2	7.6 ± 0.7*	11.7 ± 1.6	5.2 ± 1.3**	125.5 ± 8.8	117.1 ± 18.7
Content (pg/30 min)						38.9 ± 6.7	22.8 ± 7.1

Electrical stimulation of the central nucleus of AMY did not change VIP concentrations in peripheral or pituitary stalk plasma (Figure 7.1a). There is immunohistochemical evidence that VIP immunoreactive fibres in the stria terminalis and its bed nucleus form a major pathway linking the AMY and the hypothalamus (Fuxe *et al.*, 1977; Roberts *et al.*, 1980; Sims *et al.*, 1980) and unilateral transection of the stria terminalis produced a significant fall in VIP content of the SCN and AMY (Palkovits *et al.*, 1981). There is, however, no published evidence that VIP fibres from the AMY project to the portal vessels.

VIP-containing cell bodies in the hippocampal complex are located mainly around the subicular complex (Loren *et al.*, 1969; Roberts *et al.*, 1981; Köhler, 1982). Electrical stimulation of either the DH or the VH produced no change in the VIP concentration of pituitary stalk plasma (Figure 7.1a). This suggests that VIP fibres from the hippocampal complex do not project to the portal vessels. Indeed, immunohistochemical studies showed that while VIP-containing fibres were distributed throughout the complex, their processes could not be traced for any great distances and appeared to be intrinsic to the hippocampus (Loren *et al.*, 1980; Roberts *et al.*, 1980; Köhler, 1982).

Electrical stimulations of the PVN, POA, ME or SCN produced no significant change in the VIP concentration of pituitary stalk plasma (Figure 7.1a). The SCN has been demonstrated to contain a dense population of VIP cell bodies and a rich supply of VIP-containing fibres (Loren *et al.*, 1979; Sims *et al.*, 1980; Card *et al.*, 1981). A moderately dense VIP fibre system has been found in the ventral border of the PVN and in the POA (Sims *et al.*, 1980; Card *et al.*, 1981). While there is immunohistochemical evidence for the presence of a few scattered VIP fibres in the ME (Roberts *et al.*, 1980; Sims *et al.*,

1980; Hökfelt *et al.*, 1982), the fact that electrical stimulation of the SCN, PVN, POA or ME did not alter VIP release into stalk plasma suggests that there is no significant VIP-innervation of the portal vessels originating from these areas. From experiments involving anterolateral deafferentation of the medial basal hypothalamus (Besson *et al.*, 1979a), transection of the periventicular VIP fibres from the SCN (Rostène *et al.*, 1982) and bilateral lesions of the SCN (Rotsztejn, personal communication), it has been proposed by Rotsztejn and his colleagues (Rostène *et al.*, 1982) that VIP fibres in the ME originate either from the medial basal hypothalamus (arcuate or ventromedial nucleii) or from caudal projections.

The regional overlap of VIP distribution with a number of peptides suggests that VIP may have a neuromodulatory role in the brain (Sims *et al.*, 1980; Card *et al.*, 1981). Iontophoretic application of VIP has been reported to alter the electrical activity of POA neurones whose axons project to the ME (Haskins *et al.*, 1982). VIP was found to be excitatory when applied to cell bodies of the rat hippocampus of the CA1 region (Dodd *et al.*, 1979). Although intraventicular injections of VIP have been reported to stimulate the release of LH in ovariectomised, conscious rats (Vijayan, Samson, Said and McCann, 1979b), VIP had no effect on LH release from monolayer cultures of gonadotrophs (Denef, Hautekeete and Dewals, 1978) or from enriched preparations of pituitary cells (Rotsztejn *et al.*, 1980a). The incubation of VIP with ME synaptosomes has been reported to stimulate the release of LHRH (Samson, Burton, Reeves and McCann, 1981). VIP was, however, ineffective in stimulating the release of LHRH from incubated hypothalamic slices (Besson, Rotsztejn and Ruberg, 1979b; Vijayan *et al.*, 1979b; Samson, Snyder, Said and McCann, 1980; Drouva, Epelbaum, Tapia-Arancibia, Laplante

and Kordon, 1981), in contrast to its ability to inhibit the release of another hypothalamic peptide, Somatostatin (Epelbaum, Tapia-Arancibia, Besson, Rotsztejn and Kordon, 1979). While these data support a role for VIP in the hypothalamus as a neuromodulator and suggest an indirect involvement of VIP in the regulation of some adenohypophysial hormones, the ability of VIP to act directly at the pituitary level to stimulate the release of PRL (Ruberg, Rotsztejn, Arancibia, Besson and Enjalbert, 1978; Shaar, Clemens and Dininger, 1979; Gourdji, Bataille, Vauclin, Grouselle, Rosselin and Tixier-Vidal, 1979; Rotsztejn, Benoist, Besson, Beraud, Bluet-Pajot, Kordon, Rosselin and Duval, 1980a) suggests that the greater secretion of VIP into pituitary stalk blood compared to peripheral blood (Figures 7.1a and 7.1b; Said and Porter, 1979; Shimatsu *et al.*, 1981) has a physiological function. The role of VIP as a putative PRL-releasing factor is discussed in Section 7.2. A recent report that 5-HT injections into the ventricles stimulated VIP release into pituitary stalk blood (Shimatsu, Kato, Matsura, Katakami, Yanaihara and Imura, 1982) provides strong support for the hypothesis that the stimulatory effect of 5-HT on PRL secretion is due to the release of a PRL-releasing factor rather than inhibition of dopamine release into pituitary stalk blood (Clemens, Rousch and Fuller, 1978; Lamberts and MacLeod, 1978; Pilotte and Porter, 1981). Therefore, although it has been shown (Table 7.1d) that VIP in the periphery does not make a significant contribution to the concentration of VIP measured in pituitary stalk blood and the origin of VIP in stalk blood is not yet resolved, it is likely that high concentrations of VIP are involved in the modulation of PRL secretion. The presence of VIP in cerebrospinal fluid (CSF; Fahrenkrug, Schaffalitsky de Muckadell and Fahrenkrug, 1977) does raise the

possibility that tanycyctes (special ependymal cells present in large numbers in the median eminence) may transport VIP, amongst other substances, from the CSF to pituitary stalk portal vessels (Knigge and Scott, 1970). However, the role, if any, of tanycytes in transporting substances between the CSF and pituitary stalk vessels is unclear (Bergland and Page, 1979; Mezey and Palkovits, 1982). It is also possible that the optimal parameters for the release of VIP were not used in this study (Figure 7.1a, Section 7.1.2) and that is why there was no significant difference in the concentration of VIP in stalk plasma after electrical stimulation of the brain. Finally, a VIP-like peptide, PHI (PHI-27; the peptide (P) having NH_2-terminal histidine (H) and COOH-terminal isoleucine (I) amide and 27 amino acid residues) has been found in the external layer of the ME (Hökfelt, Fahrenkrug, Tatemoto, Mutt, Werner, Hulting, Terenius and Chang, 1983). Although the cross-reactivity of PHI with the VIP-antibody used in the experiments described in this section is fairly low (Maletti *et al.*, 1982), it is possible that part of immunoreactivity in stalk blood attributed to VIP, is due to PHI.

7.2 VIP in Pituitary Stalk Blood: Concentration During the Rat Oestrous Cycle

7.2.1 Introduction

It has been established for nearly a decade that the hypothalamic tripeptide, thyrotrophin-releasing hormone (TRH) is a potent stimulator of PRL secretion (Bowers, Friesen, Hwang, Guyda and Folkers, 1971; Jacobs, Snyder, Wilber, Utiger and Daughaday, 1971; Tashijan, Barowsky and Tensen, 1971; Valverde, Chieffo and Reichlin 1972). The absence of an increase in plasma thyrotrophin (TSH) concentrations in

post-partum women in whom suckling induced high concentrations of PRL in the circulation (Gautvik, Tashijan, Kourides, Weintraub, Graeber, Maloof, Suzuki and Zuckerman, 1974), raised the possibility of the existence of a PRL-releasing factor (PRF) distinct from TRH. Pharmacological and experimental manipulations (Section 1.4.3) have confirmed that TRH can stimulate the release of PRL *in vitro* and *in vivo* (Jackson and Reichlin, 1974; Boyd, Spencer, Jackson and Reichlin, 1976; Meites and Sonntag, 1982). However, while anti-TRH serum induced a 50% fall in plasma PRL concentrations in the rat (Koch, Goldhaber, Firemen, Zor, Shaw and Tal, 1977) but not in the ewe (Fraser and McNeilly, 1982a), hypothalamic extracts do possess PRF activity that cannot be attributed to TRH (Boyd *et al.*, 1976; Szabo and Frohman, 1976; Grosvenor and Mena, 1980). Moreover, the dose of TRH required to induce the release of PRL is relatively high (Meites and Sonntag, 1982).

There is growing evidence to support the role of VIP as a PRF. The evidence includes the following:

i) VIP stimulated the release of PRL from enriched preparations of normal PRL-secreting cells (Rotsztejn *et al.*, 1980a), rat hemi-pituitaries *in vitro* (Ruberg *et al.*, 1978; Shaar *et al.*, 1979; Samson *et al.*, 1980) and normal human pituitary tissue (Malarkey, O'Dorisio, Kennedy and Cataland, 1981).

ii) Intraventricular and intravenous injections of VIP stimulated the release of PRL *in vivo* in various species (Kato, Iwasaki, Abe, Yanaihara and Imura, 1978; Vijayan *et al.*, 1979b; Bataille, Talbot, Milhaud, Mutt and Rosselin, 1981; Frawley and Neill, 1981).

iii) The presence of immunoreactive VIP in nerve endings of the mediobasal hypothalamus (Emson *et al.*, 1978; Besson *et al.*, 1979a), in prolactotrophs (Morel, Besson, Rosselin and Dubois, 1982) and in high concentrations in pituitary stalk blood (Section 7.1; Said and Porter, 1979; Shimatsu *et al.*, 1981).

iv) The existence of VIP receptors in human and rat PRL-secreting cells (Nicosia, Spada, Borghi, Cartelazzi and Gianattasio, 1980; Rotsztejn *et al.*, 1980a).

v) The stimulation of cAMP accumulation by low doses of VIP in the pituitary gland (Deschodt-Lanckman *et al.*, 1977; Quik, Iversen and Bloom, 1978; Borghi, Nicosia, Giachetti and Said, 1979; Rotsztejn, Dussaillant, Nobou and Rosselin, 1981) and in rat clonal PRL cells (Gourdji *et al.*, 1979) together with PRL release from the rat clonal PRL cells (Gourdji *et al.*, 1979). While the PRL-stimulating action of VIP was additive to TRH, the accumulation of cAMP was not (Gourdji *et al.*, 1979; Enjalbert, Arancibia, Ruberg, Priam, Bluet-Pajot, Rotsztejn and Kordon, 1980).

Apart from a direct effect of VIP on pituitary cells to stimulate the release of PRL, it has been proposed that VIP blocks the inhibitory effect of dopamine on PRL release. This was suggested by the demonstration that VIP significantly attenuated the inhibition of PRL release by dopamine from cultured pituitary cells (Kato *et al.*, 1978). Moreover, L-dopa the precursor of dopamine inhibited the release of PRL by VIP in anaesthetised rats (Kato *et al.*, 1978) and dopamine agonists specifically inhibited the activation of cAMP in the pituitary by VIP (Onali, Schwartz and Costa, 1981). The blockade of dopamine receptors with neuroleptics did not modify VIP-induced PRL release (Enjalbert

et al., 1980) which suggests that VIP does not act at dopamine receptors.

The physiological factors that control the release of VIP are for the most part speculative and still largely under investigation. Studies in developing rats showed that at puberty an increase in the content of VIP in the brain preceded that of plasma PRL but no sharp increase in plasma VIP concentration occurred (Maletti, Besson, Bataille, Laburthe and Rosselin, 1980). Gonadal steroids which are known to stimulate the release of PRL (Neill, 1980; Section 1.4.3), did not alter the accumulation of cAMP in pituitary cells by VIP (Rotsztejn, 1981) or affect the concentration of VIP in the adenohypophysis (Rotsztejn, Besson, Beraud, Gagnant, Rosselin and Kordon, 1980b). Oestradiol administered to ovariectomised rats modulated the content of VIP in the hypothalamus and pituitary (Maletti *et al.*, 1982). This action of oestradiol was enhanced by high plasma concentrations of PRL (Maletti *et al.*, 1982). In addition, corticosteroids have been reported to modulate the concentration of VIP in the adenohypophysis (Rotsztejn *et al.*, 1980b), the responsiveness of PRL cells to VIP (Rotsztejn *et al.*, 1980a), inhibit the accumulation of cAMP and release of PRL from pituitary cells in culture by VIP (Rotsztejn *et al.*, 1981).

Purpose of the present studies:

The aim of the experiments described in this section was to determine,

1. whether there was an increase in the concentration of VIP in pituitary stalk blood which could be responsible for the spontaneous surge of PRL on pro-oestrus (Gray *et al.*, 1970; Wuttke and Meites, 1970; Neill *et al.*, 1971);

2. the effects of four different anaesthetics (Althesin, Ketalar, Sagatal and urethane) on the concentration of VIP in pituitary stalk plasma, in view of the ability of anaesthetics to alter secretions of hypothalamic and pituitary peptides (Blake and Sawyer, 1972; Smythe and Lazarus, 1973; Lawson and Gala, 1974; Sarkar *et al.*, 1976).

7.2.2 Materials and Methods

(a) Animals:

All experiments were carried out on adult female rats of the Wistar strain, weighing 200-250 g, which were maintained under conditions described in Section 2.1. Only animals which had exhibited at least two successive 4-day oestrous cycles (as assessed by the inspection of daily vaginal smears, described in Section 2.1) were used for experimentation. The injected dose of the selected anaesthetic is given in Section 2.2.1.

Pituitary stalk blood was collected as described in Section 2.2.5 for two consecutive 30 min periods between 15.30 and 18.30 h each day. Peripheral blood was obtained from the external jugular vein before the pituitary stalk was cut. All samples were collected into tubes kept on ice and containing EDTA (5.4 mM/l blood) and Trasylol (5000 KIU/ml). The plasma was stored frozen until assayed.

(b) Radioimmunoassay and calculations:

The concentration of VIP in the plasma samples was determined by a specific radioimmunoassay (Section 2.3, Maletti *et al.*, 1982). The content (concentration x volume per 30 min) was calculated for pituitary stalk blood. The significance of differences between means was determined by the unpaired t-test. The differences between more than two means was determined by analysis of variance followed by the multiple range test (Duncan, 1957; Harter, 1960).

7.2.3 Results

The VIP concentration of peripheral and pituitary stalk plasma was determined using four anaesthetics: alphaxalone-alphadolone acetate (Althesin), ketamine hydrochloride (Ketalar), sodium pentobarbitone (Sagatal) or ethyl carbamate (urethane) and are shown in Table 7.2a and in histogram form in Figure 7.2a.

(a) VIP concentrations in peripheral plasma:

An analysis of variance of the VIP concentrations in peripheral plasma over the four days of the oestrous cycle showed that there was no statistically significant difference between the anaesthetics with respect to the VIP profile. In addition, no statistically significant differences were found on comparison of VIP levels on a particular day of the cycle under different anaesthetics. However, in general, the concentration of VIP in peripheral plasma was significantly ($p < 0.05$) less than in pituitary stalk plasma.

(b) VIP concentrations in pituitary stalk plasma:

Table 7.2a and Figure 7.2a show that the VIP concentration in pituitary stalk plasma was significantly ($p < 0.05$) greater than in peripheral plasma on the same day, under each of the anaesthetics except on dioestrus under Ketalar.

The VIP concentration and content in stalk plasma from animals anaesthetised with Sagatal at metoestrus were significantly greater ($p < 0.05$) than those in metoestrus animals anaesthetised with any of the other anaesthetics. Only under Ketalar was there a significantly ($p < 0.05$) higher concentration of VIP in stalk plasma on pro-oestrus compared to on dioestrus. There were no significant changes in the concentration and content of VIP in stalk plasma on any of the days of the cycle under Althesin and urethane anaesthesia.

TABLE 7.2a: VIP concentration and content in pituitary stalk plasma and peripheral plasma between 13.30 h and 18.30 h during the oestrus cycle. Blood samples were collected under Althesin, Ketalar, Sagatal or urethane. VIP values in pituitary stalk plasma were averaged for the two 30 min collection periods for each individual rat; the value shown is a mean of the values for each rat. VIP content represents the mean value of VIP released into pituitary stalk blood over 30 min. The values are given as the mean ± S.E.M. (n).

Stage of oestrous cycle		Anaesthetics			
		Althesin	Ketalar	Sagatal	Urethane
Oestrus	*Pituitary stalk plasma:*				
	concentration (pg/ml)	176.7 ± 19.0 *(6)*	196.2 ± 44.0 *(6)*	210.6 ± 42.0 *(7)*	156.1 ± 36.0 *(7)*
	content (pg/30 min)	106.5 ± 36.0 *(7)*	107.0 ± 74.0 *(5)*	105.6 ± 28.0 *(7)*	106.9 ± 32.0 *(7)*
	Peripheral plasma	38.0 ± 10.0 *(8)*	47.5 ± 13.0 *(6)*	48.0 ± 8.0 *(7)*	25.0 ± 6.0 *(5)*
Metoestrus	*Pituitary stalk plasma:*				
	concentration (pg/ml)	173.3 ± 39.0 *(5)*	124.1 ± 34.0 *(6)*	243.0 ± 31.0 *(5)*	108.2 ± 15.0 *(5)*
	content (pg/30 min)	69.4 ± 19.0 *(7)*	73.3 ± 6.0 *(4)*	179.8 ± 35.0 *(4)*	90.4 ± 26.0 *(4)*
	Peripheral plasma	43.4 ± 11.0 *(6)*	62.5 ± 9.0 *(7)*	35.0 ± 11.0 *(6)*	37.6 ± 9.0 *(6)*

Dioestrus	*Pituitary stalk plasma*				
	concentration (pg/ml)	219.6 ± 57.0 *(5)*	69.6 ± 12.0 *(4)*	131.3 ± 37.0 *(7)*	148.5 ± 48.0 *(6)*
	content (pg/30 min)	121.3 ± 28.0 *(5)*	69.4 ± 37.0 *(3)*	79.9 ± 32.0 *(7)*	59.8 ± 19.0 *(5)*
	Peripheral plasma	46.1 ± 9.0 *(5)*	24.5 ± 15.0 *(3)*	45.2 ± 9.0 *(7)*	57.4 ± 13.0 *(6)*
Pro-oestrus	*Pituitary stalk plasma*				
	concentration (pg/ml)	182.3 ± 38.0 *(7)*	192.8 ± 35.0 *(7)*	101.8 ± 60.4 *(4)*	156.4 ± 53.0 *(6)*
	content (pg/30 min)	41.7 ± 9.0 *(6)*	138.7 ± 50.0 *(5)*	83.4 ± 50.2 *(5)*	62.3 ± 30.0 *(6)*
	Peripheral plasma	31.3 ± 9.0 *(8)*	58.1 ± 21.0 *(7)*	53.4 ± 9.0 *(5)*	65.9 ± 21.0 *(6)*

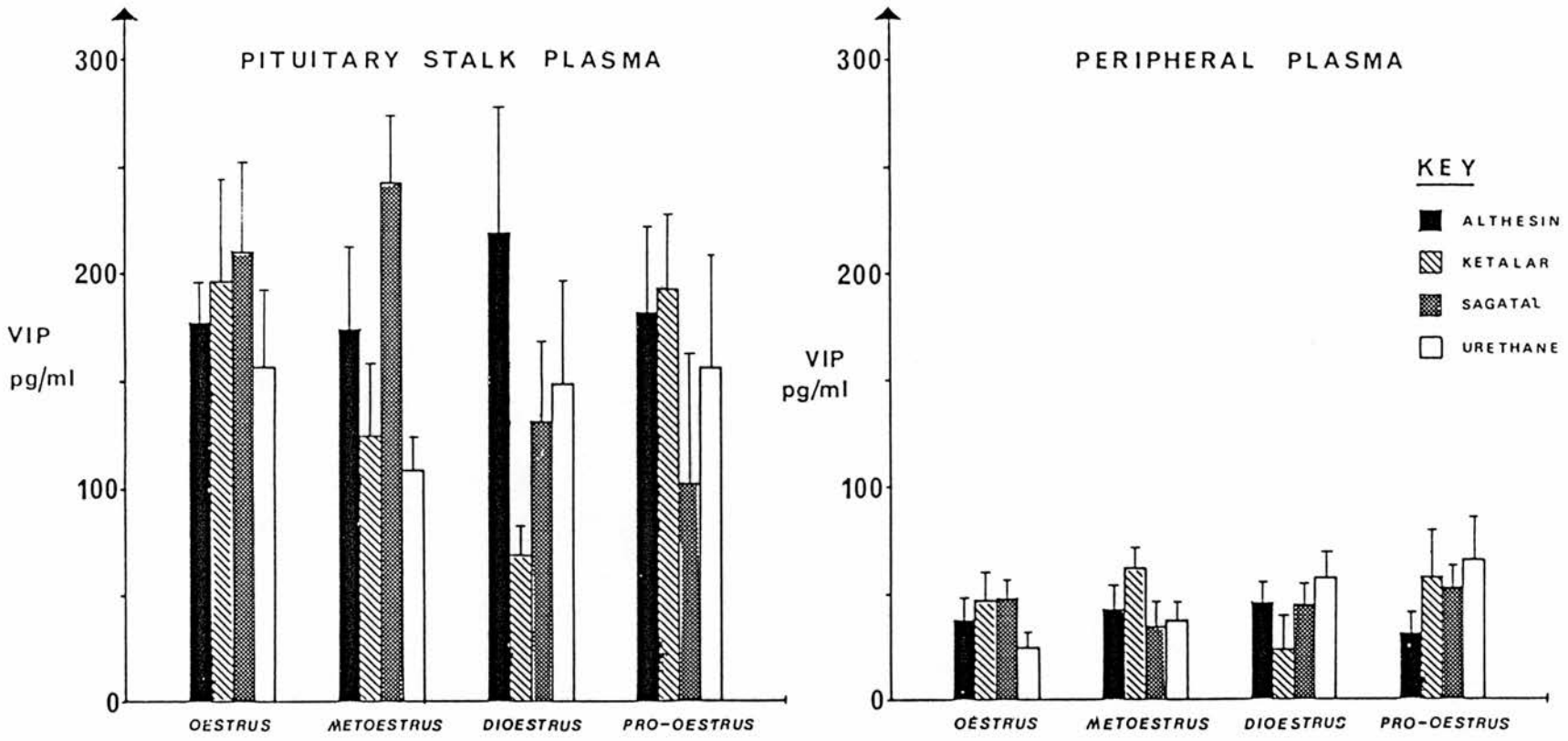


FIGURE 7.2a: VIP concentrations (pg/ml) in peripheral and pituitary stalk plasma collected under Althesin, Ketalar, Sagatal or urethane anaesthesia at various times of the oestrous cycle.

7.2.4 *Discussion and Conclusions*

The VIP concentration in pituitary stalk plasma was overall significantly ($p < 0.05$) greater than in peripheral plasma. However, there is no clear-cut evidence from the VIP concentrations in pituitary stalk plasma that an increase in VIP release precedes the pro-oestrus surge of PRL release. The day of the cycle when maximum VIP concentrations were found in pituitary stalk plasma was dependent on the anaesthetic used. Which of these data represents the physiological VIP concentrations most closely is open to conjecture since pituitary stalk blood samples cannot be obtained without inducing fairly deep anaesthesia and considerable trauma, and the precise influence of the anaesthetics and trauma on VIP and PRL release are not fully understood. Differences in VIP concentrations in pituitary stalk blood collected under urethane and Pentobarbital were also found by Shimatsu *et al.* (1981); the VIP concentration measured under Urethane anaesthesia was 400 pg/ml less than that under Pentobarbital. The prevention of ovulation and the LH surge by neural blocking agents such as urethane, Ketalar and Pentobarbital, administered between 14.00 and 16.00 (the 'critical period') of proestrus (Everett, 1964; Schwartz and Calderelli, 1965; Blake and Sawyer, 1972; Lincoln and Kelly, 1972; Greig and Weisz, 1973) as well as a block of pulsatile LH release in long-term ovariectomised rats (Arendesh and Gallo, 1978; Sarkar and Fink, 1980; Watts and Fink, 1981) is well established. These effects are due primarily to a block of the spontaneous LHRH release (Carter and Dyer, 1979; Sherwood, Chiappa, Sarkar and Fink, 1980) which prevents the subsequent release of gonadotrophins. Althesin, a mixture of the steroids, alphadolone acetate and alphaxalone, on the other hand, has been shown not to block ovulation or the LH

surge, although the magnitude of the LH surge may be reduced (Sarkar *et al.*, 1976). Althesin does not appear to interfere with various forebrain-mediated functions as do anaesthetics such as Ketalar and Sagatal (Timms, 1976), despite evidence obtained using cortical electroencephalography that Althesin is a more potent anaesthetic than either Sagatal or urethane (Fink, Sarkar, Dow, Dick, Borthwick, Malwick and Twine, 1982). Moreover, both urethane (Findell, Larsen, Benson and Blask, 1981) and Sagatal (Carrodi, Fuxe and Hökfelt, 1966; Schanberg, Schildkraut and Kopin, 1967) induce changes in brain monoamine content and turnover. An investigation of the influence of several anaesthetics on the oestrogen-induced afternoon PRL surge showed that ether and Ketamine completely abolished the diurnal PRL surge (Clarke and Gala, 1981); urethane initially suppressed the PRL surge though plasma PRL started rising about 8 h later. This initial inhibitory effect of urethane was also observed with respect to the suckling-induced PRL release (Burnet and Wakerley, 1976; Isherwood and Cross, 1980) and was found to be over in approximately 4 h (Figure 7.2b; Brar, unpublished data) when suckling significantly ($p < 0.05$) increased plasma PRL concentrations. Suckling-induced release of PRL did not occur in rats given Ketalar. These results are opposite to those reported recently by Fink, Koch and Aroya (1983) who were only able to demonstrate an increase in plasma PRL after suckling in rats given Ketalar but not urethane. The reason for this difference is not clear, particularly since the same strain of rats was used in the studies by Fink *et al.* (1983) and those in Figure 7.2b. Fink *et al.* (1983) did, however, find that suckling induced an increase in TRH output into pituitary stalk blood in animals anaesthetised with Ketalar but not in animals given Althesin. On the other hand, the release of Somatostatin into

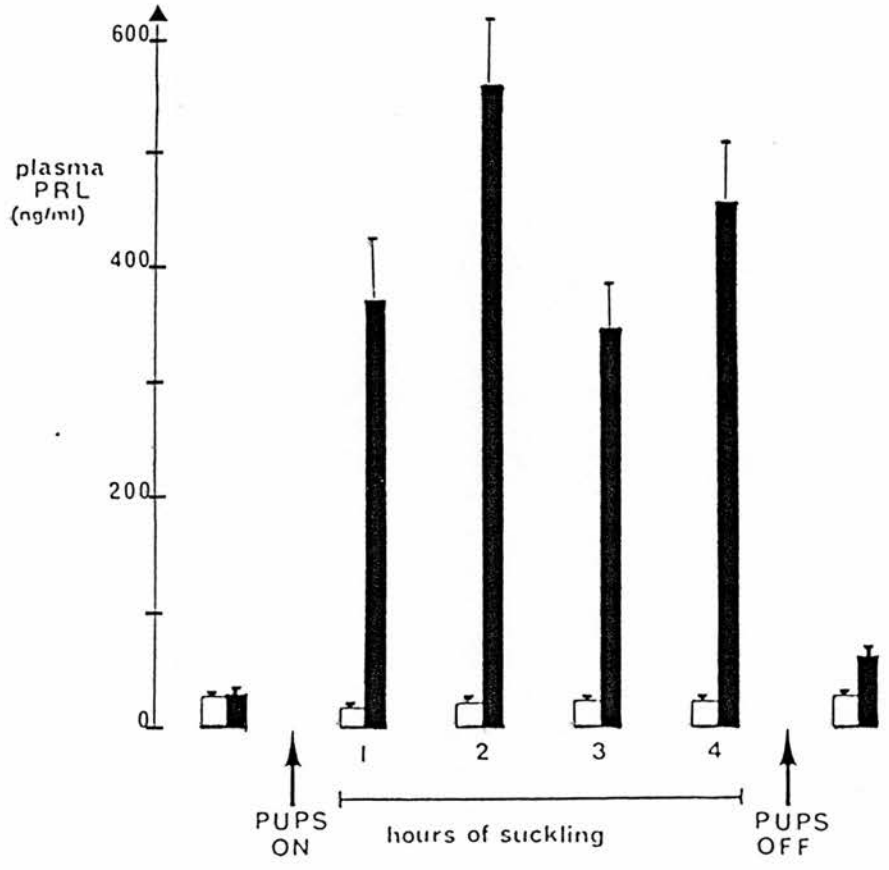


FIGURE 7.2b: Prolactin concentration (mean ± S.E.M.) of peripheral plasma in groups of suckled (■) and non-suckled (□) lactating rats at 10 days post-partum (n=6). Blood samples were collected 4-8 h after the onset of Urethane anaesthesia (1 g/kg, i.p.).

pituitary stalk blood correlated most closely with a decrease in pituitary growth hormone release in animals anaesthetised with urethane as opposed to Althesin or Sagatal (Chihara, Arimura and Schally, 1979). The different effects of anaesthetics on the release of pituitary peptides into systemic blood and hypothalamic peptides into pituitary stalk blood does raise the possibility that the reason that there was no dramatic increase in VIP release into pituitary stalk blood with the surge in PRL release on pro-oestrus was, that all the four anaesthetics used in the experiments described in this chapter masked any changes that may occur in the conscious animal. Perhaps a better model would have been animals made hyperprolactinaemic (e.g. by implantation of pituitary transplants under the kidney capsule, Chapter IV), in which a marked change in the release of any PRF would be expected to occur.

CHAPTER VIII

Summary and Hypotheses

CHAPTER VIII

8.1 Summary

The aim of the experiments in Chapters III and IV was to determine whether testicular steroids exert an inhibitory influence on the secretion of LHRH into pituitary stalk blood. Long-term castration, for 30 days (Chapter III) or 14 days (Chapter IV), did not alter the amount of LHRH released into stalk blood, compared with that in intact control rats. Long-term castration did, however, induce a marked increase in the plasma concentrations of pituitary gonadotrophins (Chapters III and IV), the pituitary content of LH and the number of LHRH receptors in the anterior pituitary gland (Chapter III). Electrical stimulation of the median eminence of castrated rats, using stimulus parameters known to induce the optimum release of LH release in the intact animal, produced significantly lower secretion of LHRH into stalk blood compared with that in intact rats (Chapter IV).

The administration of three major testicular steroids, testosterone (T), oestradiol (OE_2) which is formed by aromatisation of T and 5α-dihydrotestosterone (DHT) which is formed by 5α-reduction of T, by subcutaneous injections for 8 days, all effectively suppressed the post-castration increase in plasma LH concentration. The post-castration increases in FSH concentration was suppressed only by OE_2. Treatment with OE_2 lowered the numbers of pituitary LHRH receptors, although these values were still higher than those in intact control rats. The amount of LHRH in stalk blood was not significantly altered by administration of either OE_2, DHT or T (Chapter III).

In order to determine whether the suppression of gonadotrophin secretion by elevated PRL secretion can be attributed to a suppression of LHRH release or stimulation of DA release into stalk blood, male rats were made hyperprolactinaemic by transplanting two anterior pituitary

glands under the kidney capsule (Chapter IV). Neither the amount of LHRH nor that of DA in stalk blood was altered by increasing plasma PRL concentrations to values found in the lactating rat for a period of 42 days. Electrical stimulation of the median eminence was as effective in hyperprolactinaemic rats as in control rats in increasing LHRH release into stalk blood.

Removal of the testis of hyperprolactinaemic rats resulted in an increase in gonadotrophin secretion which was lower compared with that in control, castrated rats, but the amounts of LHRH in stalk blood were similar in the control and hyperprolactinaemic castrated rats (Chapter IV). Administration of T by subcutaneous implants (10 mm or 30 mm lengths) demonstrated that plasma T concentrations equal to or greater than 4 n.g/ml are required to suppress completely the post-castration increase in the plasma gonadotrophin concentration, both in control rats and hyperprolactinaemic rats. Neither 10 mm nor 30 mm T implants altered the release of LHRH into stalk blood in the control or hyperprolactinaemic castrated rats.

The hyperprolactinaemic rats were more sensitive than the control rats to the anaesthetic effects of the steroid anaesthetic, Althesin.

The release of DA into stalk blood of intact hyperprolactinaemic rats, intact control rats or castrated, control rats with and without T implants, was not significantly different (Chapter IV). Thus, the maintenance of low gonadotrophin release by the testes and elevated plasma PRL concentrations does not appear to be due to a suppression of LHRH secretion into stalk blood.

The enzymatic conversion in the brain and pituitary of oestrogens by 2-hydroxylation, produces the metabolites, CAE_1 and CAE_2. These catechol oestrogens were found to be potent stimulators of LH release

in the immature male rat, at doses (50-100 μg/rat) at which their parent oestrogens, OE_1 and OE_2, were ineffective (Chapter V). CAE_1 had no effect on PRL secretion although CAE_2, OE_1 and OE_2 stimulated PRL secretion in the prepubertal male and female rat. CAE_2 was more potent than OE_2 in stimulating LH release in the prepubertal female rat and had no effect on weight of the ovaries or the uterus. However, CAE_2 suppressed the pre-ovulatory LH surge and uterotrophic effects of PMSG in the immature female rat. OE_1 (2.5 μg/rat) was more potent than OE_2 in stimulating LH release in the immature female rat, although the uterotrophic activity of OE_1 was equal to OE_2. Therefore, the conversion of the oestrogens to catechol oestrogens, CAE_1 and CAE_2, produces metabolites that are capable of stimulating LH and PRL secretion in prepubertal rats but lack the uterotrophic activity demonstrated by their parent oestrogens.

The experiments in Chapters VI and VII were carried out to determine whether there is any basis for considering that the 'gut-peptides' CCK, gastrin and VIP, recently demonstrated to be localised in the brain and pituitary, are physiological hypothalamic-pituitary regulating factors. The release of CCK and VIP, but not gastrin, into pituitary stalk blood was significantly greater than in peripheral blood. Electrical stimulation of the amygdala, dorsal and ventral hippocampus, preoptic area, paraventricular and suprachiasmatic nuclei and the median eminence, using stimulus parameters known to produce the optimum release of LH, did not alter the secretion of either CCK or VIP into stalk blood. The release of gastrin into stalk blood could not be induced by electrical stimulation of the median eminence, preoptic area or the suprachiasmatic nuclei. Removal of the gastric antrum, in order to remove the major peripheral source of gastrin, was found to reduce the

concentrations of both gastrin and CCK in stalk blood. Electrical stimulation of the median eminence of rats with the antrum removed was ineffective in altering the release of CCK or gastrin in stalk blood. The release of VIP into stalk blood, on the other hand, was not altered by the removal of the entire gut which contains large amounts of VIP (Polak *et al.*, 1974; Larsson *et al.*, 1976). An attempt to correlate the release of VIP into stalk blood with the spontaneous surge of PRL on pro-oestrus was complicated by the fact that the four anaesthetics used (Althesin, Ketalar, Sagatal and urethane) may all affect PRL secretion (Section 7.2.4: Burnet and Wakerley, 1976; Clarke and Gala, 1951; Fink *et al.*, 1983). No clear-cut correlation between VIP release into stalk blood on pro-oestrus and an increase in PRL release was found. Therefore, the studies in Chapters VI and VII demonstrated that it is unlikely that CCK and gastrin are physiological hypothalamic regulating factors since the release of these peptides into stalk blood was not greater than in peripheral blood after removal of the gastric antrum, and could not be altered by stimulation of the hypothalamus or other brain areas that contain these. The release of VIP into stalk blood was significantly higher than in peripheral blood, suggesting a putative role for VIP as a releasing factor. However, a physiological role for VIP in stimulating PRL release on pro-oestrus was not conclusively demonstrated, although a suppression of any changes that may have occurred by the anaesthetics used, cannot be ruled out.

8.2 Hypotheses

8.2.1 Modulation of LHRH secretion by testicular steroids and PRL

The demonstration that removal of testicular steroids did not alter the release of LHRH or DA into stalk blood, despite an increase in gonadotrophin secretion (Chapters III and IV; Gay and Midgley, 1969; Yamamoto *et al.*, 1970; Schally *et al.*, 1973) suggests that the negative feedback effects of the testes are exerted mainly at the level of the pituitary gland. This hypothesis is supported by the fact that castration increases pituitary LH content, the number of pituitary LHRH receptors (Chapter III; Gay and Midgley, 1969; Kingsley and Bogdanove, 1973; Clayton *et al.*, 1980; Frager *et al.*, 1980) and the responsiveness of the pituitary gland to exogenous LHRH (Debeljuk *et al*, 1974; Nansel *et al.*, 1979; O'Conner, Allen and Mahesh, 1980).

A pituitary site of action of the testicular steroids, T, DHT and OE_2, is also suggested by the demonstration that these steroids suppress the post-castration rise in gonadotrophin release by a direct negative feedback action on the pituitary gland (McEwen, Pfaff and Zigmond, 1970; Kingsley and Bogdanove, 1971; Debeljuk, Arimura and Schally, 1972). None of these steroids appeared to alter the secretion of LHRH into stalk blood (Chapters III and IV) although they directly inhibit pituitary responsiveness to exogenous LHRH in the castrated rat (Debeljuk *et al.*, 1974; Drouin *et al.*, 1976; Cheung and Davidson, 1977; Henderson and Fink, 1977). However, in the long-term castrated rat, any reduction in the responsiveness of the pituitary gland to LHRH in stalk blood and LH secretion by T, DHT or OE_2 is not accompanied by a complete suppression of the post-castration rises in pituitary LH content and pituitary LHRH receptor numbers (Chapter III; Conne *et al.*, 1980). The post-castration increase in the number of pituitary

LHRH receptors (Chapter III; Clayton and Catt, 1981; Frager *et al.*, 1981; Fraser *et al.*, 1982) may not, therefore, be due to an increased secretion of LHRH into stalk blood. The demonstration that rats immunised against T had elevated numbers of pituitary LHRH receptors (Fraser *et al.*, 1982), indicates the important role of T in the moderating of pituitary LHRH receptors. That androgens can act directly on rat anterior pituitary cells in culture to decrease LHRH binding sites (Giguere *et al.*, 1981) lends further support to the hypothesis that the negative feedback effects of testicular steroids are exerted at the level of the pituitary gland. The requirement of normal LHRH secretion from the hypothalamus to maintain normal numbers of pituitary LHRH receptors, suggested by the facts that administration of an LHRH antagonist, or lesions of the median eminence passive or active immunization of LHRH lowers the number of pituitary LHRH receptors (Clayton *et al.*, 1982a,b; Fraser *et al.*, 1982), is not disputed. The studies in this thesis support the hypothesis that, in the presence of normal LHRH secretion, testicular steroids modulate gonadotrophin secretion, at least in part, by modulating pituitary LHRH receptors.

There are, however, studies suggesting an effect of testicular steroids at the level of the hypothalamus. For example, a decrease in the content of LHRH after castration has been proposed to be indicative of increased LHRH secretion (Moguilevsky *et al.*, 1975; Root *et al.*, 1975; Shin and Howitt, 1976; Gross, 1980; Kalra and Kalra, 1980). There was, however, no significant difference between control and castrated rats in the basal release of LHRH into stalk blood while electrical stimulation of the median eminence of castrated rats induced significantly less release of LHRH into stalk blood than in control rats (Chapters III and IV). Therefore, removal of the testes appears to

decrease the amount of hypothalamic LHRH available for release. Although OE_2, DHT and T are all effective in restoring the hypothalamic LHRH content of long-term castrated rats to values found in control animals, after a period of 3-7 days (Campbell and Ramaley, 1978; Gross, 1980; Kalra and Kalra, 1980), none of these testicular steroids altered the basal release of LHRH or the LHRH release into stalk blood induced by electrical stimulation of the median eminence (Chapters III and IV). Studies in which T, DHT or OE_2 implants have been placed in the hypothalamus (Danguy, Ectors and Pasteels, 1976; Kalra and Kalra, 1978, 1980) indicate that LHRH neurones in both the preoptic area and the arcuate-median eminence complex are sensitive to the feedback from T, DHT and OE_2, in the male rat and OE_2- and androgen-binding sites have been demonstrated by autoradiographic studies to occur in these hypothalamic regions (Pfaff, 1968; Sar and Stumpf, 1973). In addition, T administration restores, while anti-androgen administration prevents, the decrease in hypothalamic LHRH content induced by castration (Kalra and Kalra, 1980). Yet, despite all this evidence supporting an effect of T, DHT and OE_2 at the level of the hypothalamus, these steroids did not alter the secretion of LHRH into stalk blood of long-term castrated rats which does raise the possibility that, in the male rat, Althesin anaesthesia masks any changes that occur in LHRH release into stalk blood.

The ability of elevated plasma PRL concentrations to reduce gonadotrophin secretion and pituitary LHRH receptors in the intact rat and prevent the post-castration increases in plasma gonadotrophin concentration, pituitary LH content and pituitary LHRH receptors (Bartke *et al.*, 1977; McNeilly *et al.*, 1978; Winters and Loriaux, 1978; McNeilly *et al.*, 1980; Fraser *et al.*, 1982; Marchetti and Labrie,

1982), is postulated to be due to modulation of various parameters by PRL at the level of the pituitary gland rather than by suppression of LHRH release. Hyperprolactinaemia reduces the responsiveness of the pituitary to LHRH (Winters and Loriaux, 1978; Greeley and Kizer, 1979; Tresguerres and Esquifino, 1981; Wuchenich and Cheung, 1981), the number of pituitary LHRH receptors (Fraser *et al.*, 1982; Marchetti and Labrie, 1982) and pituitary gonadotrophin concentrations (Bartke *et al.*, 1978; McNeilly *et al.*, 1978). A suppression of the responsiveness of the pituitary to LHRH (Smith, 1980) and a reduction in pituitary LHRH receptors (Clayton *et al.*, 1982) has also been associated with elevated PRL concentrations in the lactating rat. Neither the high (30 mm implant) nor the low (10 mm implant) dose of T altered the release of LHRH into stalk plasma of castrated hyperprolactinaemic rats, but the high dose did suppress the post-castration rise in plasma gonadotrophins. Thus, a pituitary site of action of T in the hyperprolactinaemic rat is also suggested.

The lack of any increase in DA release into stalk blood of hyperprolactinaemic rats, despite elevated plasma PRL concentrations (Chapter IV) and evidence suggesting that TIF DA turnover is increased by elevated plasma PRL concentrations (Hökfelt and Fuxe, 1972; Gudelsky *et al.*, 1976; Moore, Demarest and Johnston, 1980; Morgan and Herbert, 1980), may be due to several reasons. One of these is suggested by Demarest and Moore (1981) who showed that there was a sexual difference in the sensitivity of TIF DA neurones to the stimulatory actions of PRL; the TIF neurones in the female are more sensitive to circulating concentrations of PRL than those in the male. The TIF neurones of the hyperprolactinaemic male rats may therefore not respond to the elevated plasma PRL concentrations.

Another reason why there was no change in the secretion of DA into stalk blood of hyperprolactinaemic rats could be that much higher concentrations of plasma PRL are required to stimulate DA release. This proposal is supported on experiments by Cramer *et al.* (1979) and Weber *et al.* (1983) in rats bearing PRL-secreting tumours (plasma PRL concentrations >1000 ng/ml) compared with rats with pituitary transplants (plasma PRL concentration >200 ng/ml). DA secretion into stalk blood was only increased in rats with PRL-secreting tumours (Cramer *et al.*, 1979). The demonstration that lactating females (day 12 post-partum) which had continuously elevated plasma PRL concentrations (580 ng/ml) did not show increased TIF DA neuronal activity (Demarest, McKay, Riegle and Moore, 1981a) supports the view that the PRL feedback mechanism that activates TIF DA neurones does not always operate in hyperprolactinaemic conditions. Perhaps such a situation is also found in the hyperprolactinaemic male rat. The demonstration that transplanted pituitary tumours that secrete large amounts of PRL reduce the PRL content of the pituitary *in situ* (MacLeod, Smith and De Witt, 1966; Chen, Minaguchi and Meites, 1967) and increase the PIF activity of the hypothalamus (Chen *et al.*, 1967) suggests that an autoregulatory role for PRL should also be considered. Certainly, plasma concentrations of PRL that are induced by implanting 4 pituitary glands (178 ng/ml) that do not alter the release of DA into stalk blood (Cramer *et al.*, 1979) do, however, reduce the PRL content of the pituitary gland (McNeilly *et al.*, 1978; Cramer *et al.*, 1979).

A role for the adrenal steroids in modulating the release of LHRH into stalk blood is likely in view of the fact that hyperprolactinaemia increases the size (McNeilly *et al.*, 1978; Cramer *et al.*, 1979) and function (Schlein, Zarrow and Denenberg, 1974; Vasquez and

Kitay, 1979) of the adrenal gland. In addition, in adrenalectomised male rats bearing PRL and ACTH-secreting tumours, hyperprolactinaemia did not affect gonadotrophin secretion or the androgenic action of T (Weber *et al.*, 1982). Moreover, the suppression of LHRH release but not the increase in DA release into stalk blood induced by PRL- and ACTH-secreting tumours was absent in adrenalectomised tumour-bearing rats (Weber *et al.*, 1983).

8.2.2 Physiological role for catechol oestrogens

The stimulation of LH and PRL secretion in the pre-pubertal male and female rat by acute administrations of CAE_1 or CAE_2 raises the possibility that the conversion of the oestrogens to CAE_1 and CAE_2 is a mechanism by which the effects of the parent oestrogens are prolonged. This hypothesis seems plausible in view of the demonstration that OE_1 and OE_2 were ineffective in stimulating LH release at the low doses of CAE_1 and CAE_2 that stimulated LH release in the male rat. The physiological significance of a stimulation of LH secretion by the catechol oestrogens in the male rat remains to be found, since oestrogens exert a predominantly inhibitory influence of LH secretion in the adult male rat (Chapters III and IV; Gay *et al.*, 1970; Yamamoto *et al.*, 1970).

The mechanism by which the CAEs modulate hormone release from the anterior pituitary gland has been subject to various speculations. These have included the possibility that CAEs prevent the inhibitory effects of DA on LH and PRL release. This attractive hypothesis has been difficult to prove. No conclusive evidence has yet shown that the CAEs provide a direct link between the oestrogenic and adrenergic control of tonic and cyclic release of LH apart from demonstrations that CAEs can inhibit DA synthesis (Lloyd and Weisz, 1978; Foreman and

Porter, 1980) and compete with DA receptors in the rat anterior pituitary gland (Schaeffer and Hseuh, 1979).

The mechanism by which a dose of CAE_2 that stimulates LH secretion and lacked uterotrophic activity, suppresses the release of LH and uterotrophic effect induced by PMSG in the immature female rat (Chapter V) remains unresolved. It is possible that in the PMSG-treated rat, CAE_2 has an anti-oestrogenic effect. This view is supported by the facts that plasma OE_2 concentrations reach a maximum 42-52 h after PMSG treatment (Wilson *et al.*, 1974) and that CAE_2 has a high affinity for receptors in the uterus (Martucci and Fishman, 1976), and the hypothalamus and pituitary gland (Davies *et al.*, 1975). The ability of CAE_2 to block the OE_2-induced accumulation of cAMP in the brain (Paul and Skolnick, 1977) also suggests that an anti-oestrogenic effect of CAE_2 is plausible.

8.2.3 The roles of CCK, gastrin and VIP as releasing factors in the hypothalamo - hypophysial system

The present results (Chapter VI) make it unlikely that gastrin or CCK have a role as releasing factors in the hypothalamo-hypophysial system, as defined by the neurohumoural hypothesis (Section 1.1.1). Gastrin has been reported to be localised primarily in the pituitary (Rehfeld, 1978; Rehfeld *et al.*, 1980), was not secreted in higher concentrations into stalk blood than peripheral blood (Section 6.2.2) and was not released into stalk blood on electrical stimulation of the median eminence. Despite the localization of CCK-immunoreactivity in the external layer of the median eminence (Hökfelt *et al.*, 1978; Loren *et al.*, 1979; Vanderhaeghen *et al.*, 1980; Anhut *et al.*, 1983), electrical stimulation of the median eminence failed to increase the

release of CCK into stalk blood. Although it is possible that the optimal parameters required to induce the release of CCK from the median eminence, were not used, most of the CCK (and gastrin) in the stalk blood appeared to originate from the periphery (Section 6.3.3).

A physiological role for VIP as a releasing factor is proposed on the basis that the release of VIP into stalk blood was significantly greater than in peripheral plasma. The fact that electrical stimulation of the median eminence did not stimulate VIP release into stalk blood can be explained to be due to the fact that very few VIP fibres have been found in the external layer of the median eminence (Larsson *et al.*, 1976; Rostène *et al.*, 1982). In addition, there is no published evidence to suggest that VIP fibres from areas such as the amygdala and supra-chiasmatic nucleus project to the hypophysial portal vessels, or that the optimal parameters required to induce VIP release were not used. The source of VIP in stalk blood has not yet been resolved.

Although a suppression of any release of VIP into stalk blood on pro-oestrus may have been masked by the anaesthetics used, it is possible that the reason that there was no marked changes in VIP release into stalk blood during the oestrous cycle is that the PRL-releasing action of VIP may involve a suppression of the inhibitory effect of DA on PRL release, possibly by an inhibition of DA release (Kato *et al.*, 1978). The proof of this hypothesis and other questions, such as whether the PRL-releasing effects of VIP are enhanced by a brief removal of DA, as found for the action of TRH (Grosvenor and Mena, 1980; Fagin and Neill, 1981), must await further research.

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APPENDICES

APPENDIX A1: Radioimmunoassay for luteinising hormone (LH) and follicle-stimulating hormone (FSH): based on the methods of Greenwood *et al.* (1963); Niswender *et al.* (1968); Daane and Parlow (1971).

A1.1 Stock Solutions

0.01M Phosphate buffered saline (PBS):

8.17 g	NaCl
0.10 g	Na methiolate
0.25 g	$NaH_2PO_4.2H_2O$
1.193 g	Na_2HPO_4 anhydrous

per litre distilled H_2O

pH = 7.6

Antiserum buffer:

400 ml	0.01M PBS
9.306 g	EDTA

dissolved by warming; pH adjusted to 7 with 5N Na OH before adding 1.65 ml normal rabbit serum containing 0.1% Na Azide per 0.5 litre 0.01M PBS.

Assay buffer (0.01M PBS/0.1% BSA):

10.0 g bovine serum albumin (BSA)

per litre 0.01M PBS; filtered before use; stored at 4°C.

0.01M PBS/5% egg white:

5 g egg albumin

per 100 ml 0.01M PBS; centrifuged at 2000 x g for 5 min before use.

A1.2 Iodination

(a) Materials

Columns	12 x 1 cm Biogel P60; coated with 1.5 ml 0.01M PBS/5% egg white.
Hormones	*ovine-LH* (LER-1056-C2; NIADDK) - 500 µg/ml 0.01M PBS; 10 µl aliquots stored at -40°C.
	rat-FSH (FSH-I-1; NIADDK) - 100 µg/ml; 20 µl aliquots stored at -40°C.
$Na^{125}I$	1 mCi/10 µl for LH 2 mCi/20 µl for FSH
Chloramine T	5.0 mg/ml 0.01M PBS; 10 µl used for LH 2.5 mg/ml 0.01M PBS; 25 µl used for FSH made up fresh before use.
Sodium metabisulphate ($Na_2S_2O_5$)	5.0 mg/ml 0.01M PBS; 25 µl used for LH 2.5 mg/ml 0.01M PBS; 25 µl used for FSH made up fresh before use.

(b) Protocol

(i)	Defrost hormone aliquot.
(ii)	Add $Na^{125}I$ and mix gently.
(iii)	Add Chloramine T solution and mix gently.
(iv)	Reaction time: 2 min for LH iodination 45 sec for FSH iodination.
(v)	Add $Na_2S_2O_5$ and mix gently.
(vi)	Apply to column; elute with 0.01M PBS at room temperature.
(vii)	Collect 1 or 0.5 ml fractions into tubes containing 0.5 ml 0.01 PBS/5% egg white.
(viii)	Estimate radioactivity in fractions; retain fractions with peaks of radioactivity.

N.B. *LH and FSH iodination:* two peaks of radioactivity found to elute; the labelled hormone eluted in the first peak and the free ^{125}I in the second peak. The ^{125}I-ovine LH and ^{125}I-rat FSH could be used for up to 10 days after purification after which it could still be used for a further 2 weeks following re-chromatography on a Biogel P60 column.

A1.3 Standards

Ovine LH (NIH-LH-S18; NIADDK) - 0.25, 0.5, 0.75, 1.2, 2.0, 4.0, 8.0 and 16.0 ng/ml 0.01M PBS/1% BSA; 200 µl aliquots stored at -40°C.

Rat FSH (FSH-RP-1; NIADDK) - 62.5, 125, 250, 500, 1000, 1500, 2000 and 4000 ng/ml 0.01M PBS/1% BSA; 200 µl aliquots stored at -40°C.

A1.4 Assay protocol

All incubation at 40°C

Day	LH	FSH
1	200 µl standards/samples	200 µl standards/samples
	200 µl assay buffer	200 µl assay buffer
	200 ul antiserum diluted in antiserum buffer	200 µl antiserum diluted in antiserum buffer
		200 µl ^{125}I-rat FSH (~12,000 cpm) diluted in assay buffer
2	200 µl ^{125}I-ovine LH (~10,000 cpm) diluted in assay buffer	-
4	200 µl anti-rabbit gamma globulin (ARGG) diluted in 0.01M PBS	200 µl ARGG diluted in 0.01M PBS
5	Centrifuge tubes for 30 min at 2000 x g; aspirate supernatant and count pellet	-
7	-	Centrifuge tubes for 45 min at 2000 x g; aspirate supernatant and count pellet

APPENDIX A2: Radioimmunoassay for luteinising hormone-releasing hormone (LHRH) and prolactin (PRL): based on the methods described by Greenwood *et al.* (1963); Nett *et al.* (1973); Pickering (1978)

A2.1 Stock Solutions

0.01M PBS: see A1.1

Antiserum buffer: see A1.1

Assay buffer (for LHRH - 0.01M PBS/0.1% gelatine):

1 g gelatine

dissolve by heating in 0.01M PBS and made up to 1 litre

(for PRL - 0.01M PBS/1% BSA):

see A1.1

0.1M Borate buffer:

0.618 g boric acid
0.1 g Na methiolate
11.8 ml 0.1M Na OH (4 g/100 ml distilled H_2O)

made up to 1 litre with distilled H_2O; pH = 8.6

0.05M Phosphate buffer (PB):

12.5 g $NaH_2PO_4.2H_2O$
59.65 g Na_2HPO_4 anhydrous

per litre distilled H_2O

0.01M PBS/5% egg white: see A1.1

0.01M $NaHCO_3$:

1.14 g $NaHCO_3$

per 100 ml distilled H_2O

Rinse for PRL iodination:

0.1 g KI
0.8 g sucrose

per 10 ml distilled H_2O

A2.2 Iodination

(a) Materials:

Columns	For LHRH - 20 x 0.75 cm Sephadex G25 coated with 0.01M PBS/1% gelatine. For PRL - 25 x 1 cm Sephadex G50 coated with 200 µl human albumen (4.5% in 0.01M PBS).
Hormone	*Synthetic LHRH* (ICI Pharmaceuticals Ltd, Macclesfield) - 1 mg/10 ml 0.01M PBS; diluted to 2 µg/20 µl in distilled H_2O, stored at -40°C. *Rat PRL* (rat-PRL-I-5; NIADDK) - 100 µg/400 µl 0.01M $NaHCO_3$; 5 µg/20 ul aliquots stored at -40°C.
$Na^{125}I$	1 mCi in 10 µl for LHRH and PRL.
Chloramine T	For LHRH: 2 mg/ml 0.01M PBS; 20 µl used. For PRL: 1 mg/ml 0.05M PB; 15 µl used. Made up fresh before use.
Na_2S_2O	For LHRH: 2 mg/ml 0.01M PBS; 20 µl used. For PRL: 2.4 mg/ml 0.05M PB; 50 µl used. Made up fresh before use.

(b) Protocol:

(i)	Defrost hormone.
(ii)	Add 1 mCi ^{125}I and mix gently.
(iii)	Add Chloramine T and mix gently.
(iv)	Reaction times: 15 sec for LHRH iodination 20 sec for PRL iodination.
(v)	Add $Na_2S_2O_5$ solution and mix gently.
(vi)	Transfer to column; elute for LHRH with 0.01M PBS/0.1% gelatine and for PRL with 0.1M borate buffer at pH = 8.6; for PRL iodination, the container holding the mixture of labelled and free hormone, chloramine-T and $Na_2S_2O_5$ is rinsed twice with KI/sucrose and the rinses eluted on the column.
(vii)	Collect fractions for LHRH - collect first 10 ml fraction as a pool then 1 ml fractions until total volume eluted is 22 ml: collect fractions for PRL in 0.5 ml PBS/1% BSA.
(viii)	Estimate radioactivity in fractions; retain fractions with peaks of radioactivity.

N.B. *LHRH iodination:* two peaks of radioactivity found to elute; the free ^{125}I eluted in the first 11-14 ml and ^{125}I-LHRH eluted in the 15-19 ml fractions. The ^{125}I-LHRH could be used without further purification for up to 10 weeks.

PRL iodination: two peaks of radioactivity found to elute; the labelled hormone appeared in the first peak and the free ^{125}I in the second. The ^{125}I PRL was only used in the radioimmunoassay if prepared within 48 h of use.

A2.3 Standards

Synthetic LHRH (ICI Pharmaceuticals Ltd, Macclesfield) - 7.8, 15.5, 31.0, 62.0, 125.0, 250.0 and 500.0 pg/ml 0.01 PBS/ 0.1% gelatine; 200 µl aliquots stored at -40°C.

Rat PRL (rat-PRL-RP-1; NIADDK) - 0.5, 1.2, 2.0, 4.0, 8.0 16.0, 32.0 and 64.0 ng/ml 0.01M PBS/1% BSA; 200 µl stored at -40°C.

A2.4 Assay protocol

All incubations at 4°C.

Day	LHRH	PRL
1	200 µl standards/samples	200 µl standards/samples
	200 µl assay buffer	200 µl assay buffer
	200 µl antiserum diluted in antiserum buffer	200 µl antiserum diluted in antiserum buffer
	200 µl ^{125}I-LHRH (~5000 cpm) diluted in assay buffer	-
2	200 µl ARGG diluted in 0.01M PBS	200 µl ^{125}I-rat PRL (~10,000 cpm) diluted in assay buffer
3	1 ml 0.01M PBS then centrifuge for 45 min at 2000 x g; aspirate supernatant and count pellet	-
4	-	200 µl ARGG diluted in 0.01M PBS
5	-	Centrifuge for 45 min at 2000 x g; aspirate supernatant and count pellet

APPENDIX A3: Protein assay: based on the method by Lowry *et al.* (1951)

A3.1 Reagents

Solution A	-	20.0 g 4.0 g	Na_2CO_3 NaOH
			per 1 litre distilled H_2O
Solution B_1	-	1.0 g 1.56 g	$CuSO_4$, or $CuSO_4.5H_2O$
			per 100 ml distilled H_2O
Solution B_2	-	2.0 g	NaK Tartrate.$4H_2O$
			per 100 ml distilled H_2O
Solution B	-	1:1	v/v of B_1 : B_2
Solution C	-	50:1	v/v of A : B
Solution D	-		Folin Ciocolteu Reagent diluted 1 : 1.5 with distilled H_2O

A3.2 Standards

Stock solution of bovine serum albumin (2.5 mg/ml distilled H_2O) serially diluted to give standards of 50, 100, 150, 200 and 250 µg/ml. A volume of 0.3 ml of these standards was estimated.

A3.3 Protocol

(i)	0.3 ml standards/samples
(ii)	Add 3.0 ml C and mix
(iii)	Stand for 15 min
(iv)	Add 0.3 ml D and mix immediately
(v)	Stand for 30-90 min
(vi)	Read optical density at 750 nm
(vii)	Determine protein concentration by linear regression from a standard curve of log absorbance versus log protein concentration.

APPENDIX A4: Cresyl fast violet stain for frozen sections of formaldehyde-fixed brains: based on the method described by Bancroft and Stevens (1982)

A4.1 Formaldehyde Buffer

10.0	ml	Formaldehyde
0.9	g	NaCl
90.0	ml	0.1M phosphate buffer (pH = 7.4) consisting of

0.296 g $NaH_2PO_4.2H_2O$/100 ml distilled H_2O
1.150 g $Na_2HPO_2.12H_2O$/100 ml distilled H_2O

A4.2 Staining Procedure

1. Leave sections in xylene for about 5 mins.
2. Hydrate through graded alcohols to water.
3. Stain in 1% cresyl fast violet for about 15 mins.
4. Rinse in tap water then pass through 70% alcohol.
5. Leave in acid alochol (1% HCl in 80% alcohol) until the sections are a pale blue colour.
6. Wash in 80% alcohol then in absolute alochol. Arrest the differentiation in xylene.
7. Mount in DPX on sliced which were pre-cleaned in alcohol and coated with Alum Gelatine†.

Results: nuclei-purple/blue; neurones-purple/blue.

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† Alum Gelatine: 1.0 g Gelatine dissolved in 100 ml distilled H_2O by heating and stirring continuously.

0.1 g Chromium potassium sulphate dissolved in 100 ml distilled H_2O.

APPENDIX A5: OFG-Br.AB. method for cells of the anterior pituitary (Slidders, 1961). (Tissue fixed in formaldehyde, wax-embedded sections - 8-10 µm thick were used.)

A5.1 Solutions

Bromine water:

45.0 ml 10% hydrobromic acid (aqueous)
5.0 ml 2.5% potassium permanganate (aqueous)

Alcian blue:

100.0 mg Alcian Blue
1.0 ml sulphuric acid (conc.)
9.0 ml glacial acetic acid
90.0 ml distilled water

Mixed the dye and sulphuric acid, stirred with a glass rod. Slowly added the glacial acetic acid. Stirred again. Made up to 100.0 ml with distilled water and filtered.

Acid alcohol:

99.0 ml 70% alcohol
1.0 ml hydrochloric acid (conc.)

Celestine blue-haematoxylin:

2.5 g Celestine Blue B
25.0 g ferric ammonium sulphate
70.0 ml glycerin
500.0 ml distilled water

The ferric ammonium sulphate was dissolved in the cold distilled H_2O, the celestine blue was added and the mixture boiled for a few minutes. After cooling the stain was filtered and the glycerine added.

Orange G:

500.0 mg Orange G
2.0 g phosphotungstic acid
95.0 ml absolute alcohol
5.0 ml distilled H_2O

Acid fuchsin:

500.0 mg acid fuchsin
0.5 ml glacial acetic acid
99.5 ml distilled H_2O

A5.2 Staining Procedure

Part A:

1. Wash sections in tap water.
2. Treat with bromine water for 5 min.
3. Wash in running tap water for 5 min.
4. Rinse in distilled water.
5. Stain in alcian blue solution for 1 h.
6. Wash well in tap water.

Part B:

1. Stain with celestine blue-haematoxylin.
2. Wash in tap water.
3. Differentiate in acid alcohol.
4. Rinse in 95% alcohol.
5. Stain in orange G for 2 min then rinse in distilled H_2O.
6. Stain in acid fuchsin solution for 2-5 min (until basophil cells are strongly coloured), then rinse in tap water.
7. Treat with 1% phosphotungstic acid for 5 min then rinse in tap water.
8. Stain in 1.5% light green in 1.5% acetic acid for 1 min then rinse in tap water to remove excess stain.
9. Flood with absolute alcohol.
10. Clear in xylene.
11. Mount in DPX on slides coated with alum gelatine (see A4.2).

Acidophils	- orange-yellow
Basophil cells (s)	- dark green-blue
Basophil cells (R)	- magenta-red
Chromophobe cells	- pale grey-green
Nuclei	- grey blue
Red blood cells	- yellow