# *PURIFICATION OF FUNCTIONAL LACTOTROPHS AND SOMATOTROPHS*

**A thesis presented**

**by**

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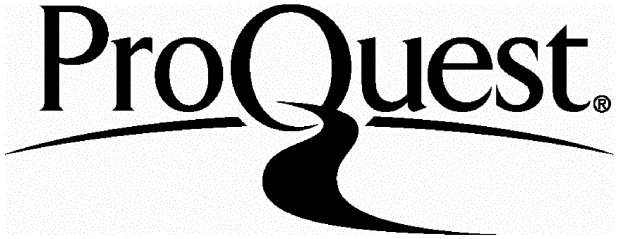

*ABSTRACT*

Data is presented in this thesis demonstrating that using flow cytometry, dispersed, anterior pituitary cells can be analysed and enriched on the basis of their size, granularity and cell surface fluorescence. Two distinct populations of cells, differing in granularity were defined 26 ± 2.2% were more granular and 74 ± 3.5% less granular. Acutely dispersed anterior pituitary cells were labelled with antibodies against four of the anterior pituitary hormones, and cell size and granularity were compared amongst the different hormonal cell types. Somatotrophs were the most granular cell type, gonadotrophs were the largest and corticotrophs the smallest whilst lactotrophs were of intermediate size. Labelling was demonstrated to be dependant upon the secretory state of the cell. Hypothalamic stimulating factors increased cell surface labelling, whilst dopamine and somatostatin decrease labelling. These changes compare favourably with published data obtained by immunocytochemistry.

Fluorescence-activated cell sorting (FACS) was applied to labelled pituitary cells and percentage purity and depletion of other cell types assessed by immunocytochemistry and the reverse haemolytic plaque assay (RHPA). Results demonstrate that fluorescence-activated cell sorting allows almost complete purification of functional lactotrophs and somatotrophs to 96.7 ± 1.7% and 98 ± 1.0% respectively by immunocytochemistry and to 95.8 ± 1.1% and 97 ± 0.8% respectively by RHPA. Depletion of other anterior pituitary cell types to less than 2% was demonstrated by both immunocytochemistry and RHPA. Fluorescence-activated cell sorting to this degree of purity was routinely possible with cell yields of 91 ± 3.4%. To obtain such purity/depletion, it was necessary to use specific antisera of high titre, at concentrations which ensured maximal cell-surface labelling associated with maximal stimulation of hormonal secretion by the appropriate hypothalamic stimulatory factor.

Having established the technique of FACS enrichment this was then compared to magnetic bead separation. Whilst magnetic bead separation is inferior to FACS enrichment it allows the study of newly released hormone and an hypothesis to be advanced as to how the prolactin secretory granule is packaged and exocytosed from the lactotroph.

The secretory characteristics of FACS enriched lactotrophs have been studied using the reverse haemolytic plaque assay and compared to unenriched populations, with particular reference to regulation of prolactin secretion by the 29 amino acid peptide galanin. Using a novel cell blot assay to detect galanin secretion from single cells, a sub-population of

lactotrophs were identified (9% ± 1%) which secrete galanin. These galanin secreting cells were enriched by FACS and their autocrine regulation of the non-galanin secreting lactotrophs studied. Galanin would appear to be cruicial to the regulation of basal and vasoactive intestinal polypeptide stimulated prolactin release. Hyperoestrogenisation increases the number of galanin secreting cells to 39 ± 2% of all lactotrophs and the resulting increase in basal prolactin release is completely abolished by treatment with galanin antiserum. These findings represent direct evidence for paracrine and autocrine regulation of lactotroph function and demonstrate that the effect of oestrogen on prolactin release would appear to be mediated by locally secreted galanin.

Lastly, having demonstrated that a specific galanin antiserum inhibits prolactin release from FACS enriched lactotrophs I was able to show that the same cells do not respond to the galanin antagonist, galantide, allowing the characterisation of a single high affinity anterior pituitary galanin receptor with a $K_d$ of 4.4 ± .34 nM and a $B_{max}$ of 79 ± 8.3 fmol/mg of protein. These data suggest the presence of a novel pituitary galanin receptor, designated GAL-$R_2$, in which the region 3-10 and amino acid 25 are crucial for membrane binding and biological activity, in contrast to the known gut/brain galanin receptor (designated GAL-$R_1$). A number of tissues known to bind galanin were screened. GAL-$R_2$ would only appear to be expressed in the anterior pituitary and hypothalamus.

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# CHAPTER ONE:

# INTRODUCTION

## 1.1 *MORPHOLOGY OF THE PITUITARY: AN HISTORICAL PERSPECTIVE*

Nearly all that is known about the hypothalamo-pituitary axis has been gathered during the twentieth century, building on fragmentary notions from earlier times. Galen regarded the pituitary as a sink for waste products (the Latin for phlegm is pituita) derived in the brain from distillation of animal spirit. That notion was held without question until 1655 when Connor and Schneider concluded on anatomical grounds that the opening in the cribriform plate of the ethmoid bone was for the olfactory nerves and that fluids could not pass from the cranial cavity into the nose. Recognition of the dual embryonic origin of the pituitary from the diencephalon and the buccal epithelium awaited disclosure in 1838 by an embryologist noted for discovering the embryonic gill slits and arches. Rathke described a dorsal out-pocketing from the roof of the stomodeum extending to meet a ventral process from the diencephalic floor. It was not until the latter part of the 19$^{th}$ century when methods for fixing tissues were in use that the concept of the pituitary as a ductless gland was first proposed. Claude Bernard first conceptualised the "milieu interieur" noting that all tissues and organs influence the body as a whole by discharging substances into the blood. The first concept of a hormone, ie a circulating messenger, was proposed by Bayliss and Starling, following their discovery of secretin.

Although the histology of the pituitary clearly shows the glandular features of the pars distalis, the neural lobe did not appear to be a secretory organ. The first real evidence for the true role of the pituitary came from reports by Pierre Marie in 1886 and Benda in 1900 that the condition acromegaly was associated with a tumour of the pituitary gland. However, a full appreciation of the role of the pituitary could only

be determined when surgical manipulation of the gland became possible early this century. By that stage a role for the pituitary in both growth and sexual function was becoming clearer. In 1921 Evans and Long showed that an injection of ox anterior pituitary extract into normal rats could increase their final body weight compared to littermate controls. Indeed later experiments by Smith, who developed a technique for the hypophysectomy of rats, soon demonstrated the dependence of the gonads, thyroid gland, adrenals and growth rate on pituitary secretions. By the late 1920s the effect of hypophysectomy was also shown to be associated with the cessation of lactation and the disturbance of carbohydrate, fat and protein metabolism, water retention and salt balance. These discoveries were followed by the fractionation of the pituitary and the isolation of the hormones responsible for the above effects.

## 1.2 *PITUITARY ANATOMY*

The adenohypophysis is divided into three regions: the pars distalis, pars tuberalis and pars intermedia. The pars distalis is the prominent part of the tissue which consists of cords of secretory cells. The hypothalamic hypophyseal portal system provides a rich blood supply to these cells. The pars distalis lacks a nerve supply, and no fibres can be traced from the hypothalamus to the pars distalis.

The precise role of the pars tuberalis has not been defined, although it is also associated with blood vessels which run from the hypothalamus to the pars distalis. The pars intermedia is the site of release of the hormone alpha melanocyte stimulating hormone ($\alpha$MSH) in many animals. In man it is often fused with the neurohypophysis and this may reflect the fact that $\alpha$ MSH occurs in only small quantities in man. The

pars intermedia and neurohypophysis together are known as the posterior pituitary, the pars distalis and pars tuberalis being known as the anterior pituitary.

The neurohypophysis is less complex in structure than the adenohypophysis and is mainly comprised of swollen nerve endings of axons from cells bodies which originate in the paraventricular and supra optic nuclei of the hypothalamus. The hormones of the neurohypophysis are formed in these cells bodies and then carried down axons which pass through the pituitary stalk and terminate in the infundibular process or pars nervosa (Fig 1.1).

## 1.3 *THE HYPOTHALAMUS AND ITS FUNCTIONAL CONNECTIONS WITH THE ANTERIOR PITUITARY*

The hypothalamus is a region of the brain formed by the diencephalon which lies below the third ventricle. It is continuous with the neurohypophysis via the pituitary stalk. However, as well as producing the hormones of the neurohypophysis, it also produces humoral factors which are carried to the adenohypophysis via the hypophyseal portal vessels (Fig 1.1). The significance of this neurovascular link was first demonstrated by Harris et al in 1948. They showed that both the transplantation of the pituitary to remote sites away from the hypothalamus and the sectioning of the pituitary stalk could lead to a reduction of secretion in hormones from the adenohypophysis. Indeed it appears that blood is carried by this hypothalamic hypophyseal portal system from a capillary plexus in the hypothalamus to another at the pituitary gland providing a means of transferring hypothalamic hormones from the

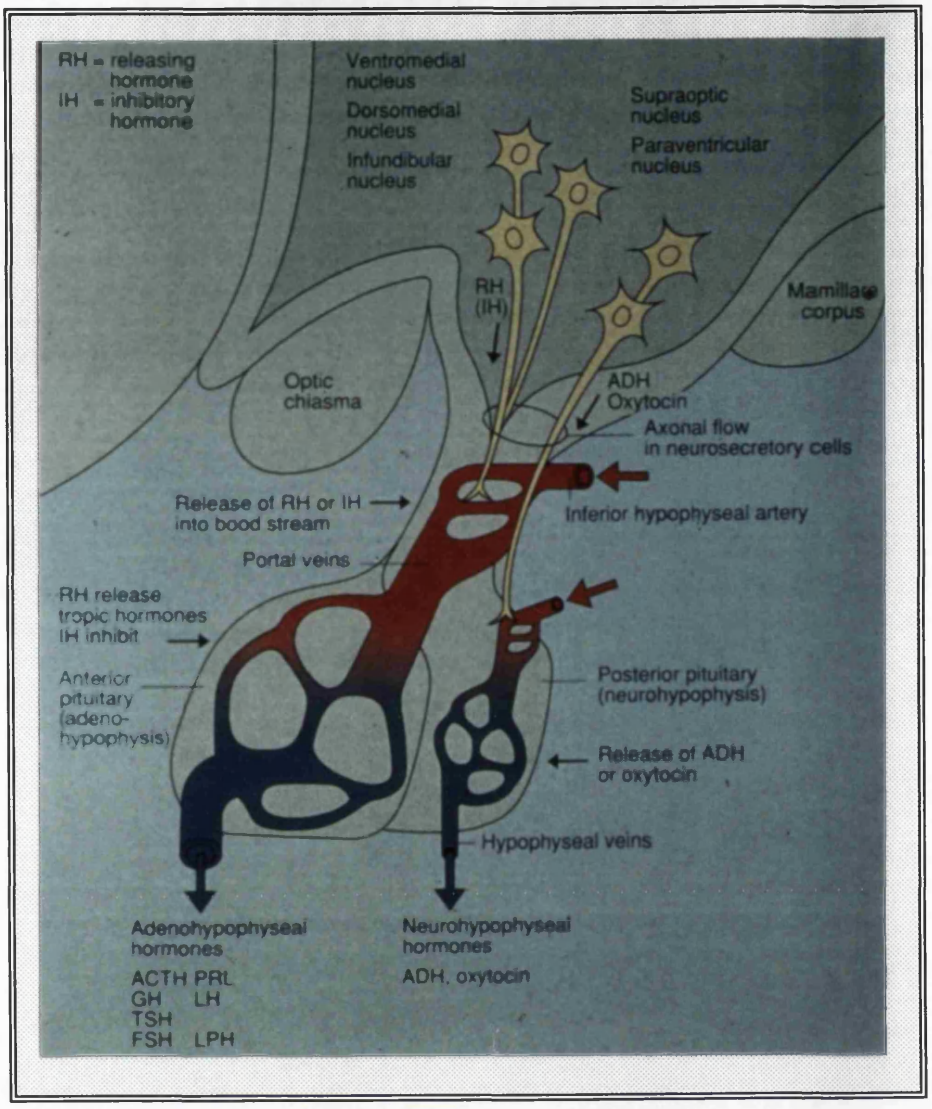



**Figure 1.1**: The hypothalamus and pituitary connected by the hypothalamo-pituitary portal circulation. Releasing and inhibitory hormones (RH and IH) are carried by the portal circulation to reach the adenohypophysis (anterior pituitary) and hence influence hormone release. The hormones of the neurohypophysis (posterior pituitary) are formed in the paraventricular and supra optic nuclei of the hypothalamus and then carried down axons which pass through the pituitary stalk and terminate in the infundibular process or pars nervosa. Reproduction of this figure is with kind permission of Dr H Williams, Sandoz Pharmaceuticals, Frimley Park, UK.

one to the other. As mentioned previously, there appear to be few nervous connections between the hypothalamus and the pars distalis of the adenohypophysis and it is therefore unlikely that pituitary hormone secretion is under the direct regulation of the nervous system.

### 1.3.1 *Hypothalamic hormones*

Several different factors are recognised today as hypothalamic hormones transmitted to the pars distalis via the portal vessels, each factor having more or less selective action on pituitary secretions. The agents of primary importance for pituitary hormone synthesis and release include luteinizing hormone releasing hormone (LHRH or GnRH), which releases luteinizing hormone (LH) and follicle stimulating hormone (FSH); thyrotropin releasing hormone (TRH) the releasing agent for thyroid stimulating hormone (TSH) which has the added capacity for stimulating prolactin secretion; dopamine, the primary inhibitor of prolactin secretion; growth hormone releasing hormone (GRF or GHRH) and somatostatin (SRIF) which at present are thought to be the main regulators of growth hormone secretion, and corticotrophin releasing hormone (CRF) which releases adrenocorticotrophin hormone (ACTH) Fig 1.2. The chemical structures of all of these peptides are known (not just for the human but for most mammals) and commercially available in synthetic form.

Since this thesis will deal predominantly with the control of prolactin and growth hormone secretion, those hypothalamic factors known to alter prolactin and growth hormone release will be considered in greater detail. It is accepted that many of these factors are synthesised and stored in many parts of the body other than the hypothalamus and play many and various roles depending on their location. These other extra-hypothalamo-pituitary functions are beyond the scope of this thesis and are not addressed further.

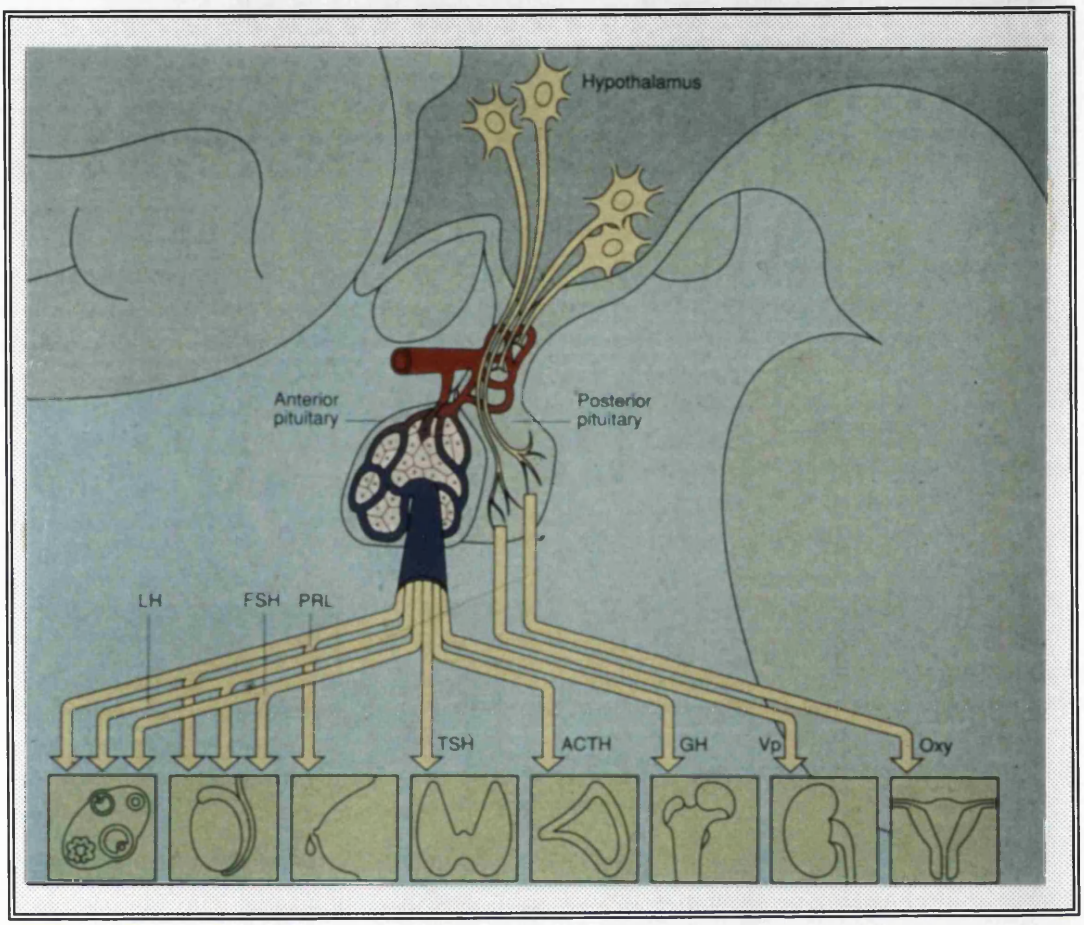



**Figure 1.2**: The anterior and posterior pituitary secrete a range of hormones which in turn act on a number of endocrine target tissues thus regulating their function. Each hormone may act alone or in concert with other factors secreted by the pituitary or even by other glands. Similarly, each secreted hormone may have more than one action at the target gland(s). Reproduction of this figure is with kind permission of Dr H Williams, Sandoz Pharmaceuticals, Frimley Park, UK.

### 1.3.1.1 *Dopamine*

Dopamine is synthesised in the arcuate nucleus and median eminence, but may not be the only inhibitor of prolactin release since there is now increasing evidence that the posterior pituitary produces one or more prolactin inhibiting factors[1].

Dopamine suppresses both the synthesis and release of prolactin by lactotrophs. Although intra-cellular calcium may participate in its action, the primary mechanism of dopamine action appears to be via cyclic adenosine mono-phosphate (cAMP) production[1-6]. Dopamine and its agonists inhibit pituitary adenylate cyclase and in lactotroph enriched cell fractions will suppress cAMP levels within minutes[7]. Accumulation of cAMP causes large increases in prolactin release and in prolactin gene transcription[6,8]. Receptors for dopamine are located in the plasma membrane of lactotrophs where they are negatively coupled to adenylate cyclase[3,6,9]. These are called D-2 dopamine receptors. Haloperidol and Domperidone are examples of D-2 receptor antagonists[3,6,9-12].

### 1.3.1.2 *Somatostatin*

The main function of somatostatin (somatotropin-release inhibitory factor) is a cyclic 14 amino acid peptide (tetradecapeptide), bridged by a sulphur-sulphur bond, was believed to be the regulation of growth hormone secretion. Somatostatin related peptides are now known to constitute a family that includes the original identified peptide (designated somatostatin 14), an N-terminal extended somatostatin (somatostatin 28), several species specific variants and larger prohormone forms[13,14].

Somatostatin and many of its related peptides are now known to be potent inhibitors of the release of many hormones from various organs and in 1987 Bloom coined the term "endocrine cyanide" to encompass its many inhibitory actions[15].

Immunocytochemical studies have revealed that somatostatin, originally identified in the hypothalamus, is also widely distributed in the gut, pancreas, CNS and peripheral nervous system. Recent evidence has demonstrated that somatostatin neurones in the paraventricular nucleus project directly into the median eminence of the hypothalamus[9,16,17]. Indeed, the highest concentration of somatostatin in the brain has been detected in the median eminence. From the median eminence the peptide is transported via the hypothalamic hypophyseal portal system to the adenohypophysis. Specific binding sites for somatostatin have been described in rat pituitary plasma membranes[18,19] and a good correlation observed between the binding affinities of several somatostatin analogues and their potencies as inhibitors of growth hormone release from rat pituitary cells[20-23].

### 1.3.1.3 *Thyrotropin releasing hormone*

TRH is the smallest of the hypothalamic releasing factors. It is a tri-peptide whose sequence is well conserved amongst all mammalian species. Larger molecular forms of 6 - 10 amino acids are now thought to represent pro-hormones. TRH receptors are well characterised on thyrotrophs and lactotrophs[19,24-26]. $IP_3$ hydrolysis acts as the predominant second messenger[27-29]. Whilst cAMP may also be involved, its role remains unclear[30-32]. The prolactin stimulating effect of TRH is very rapid, occurring within seconds and increases prolactin gene transcription in about 2 minutes[33-35].

Terminals containing TRH immunoreactivity are distributed in the external layer of the median eminence and several other areas of the hypothalamus. A large amount of the total TRH immunoreactivity of the CNS is found outside the hypothalamus, particularly in the olfactory bulb and spinal cord[16,36,37].

### 1.3.1.4 *Growth hormone releasing factor*

The relationship between the presence of certain ectopic tumours and the condition acromegaly was first noted in the early 1960s, and it was later shown that an extract of such a tumour released growth hormone both *in-vivo* and *in-vitro*. Further tumours were examined and the substance responsible for such GRF activity was finally found to be a peptide[38,39]. The highest level of GRF activity was found in a pancreatic islet cell tumour associated with secondary acromegaly and this discovery led to the final isolation and sequencing of GRF. In 1982 Guillemin et al published the sequence of GRF, a 44 amino acid amide[40]. The structure of GRF from several different species has now been determined, including that of the rat, pig, ox, sheep and horse[41-43]. All are structurally very similar, although rat GRF does exhibit a 30% non-homology compared to human GRF.

Many investigators have used in-situ hybridization and immunocytochemistry to localise human GRF synthesis and storage. Specific staining has been localised in the hypothalamus, particularly in the arcuate and ventromedial nuclei with nerve fibres projecting into the median eminence ending in contact with the hypothalamic hypophyseal portal vessels[41,44-46]. Similar observations have been reported in the rat. Specific binding sites for GRF on pituitary cells and plasma membranes have been demonstrated and it appears that only partial receptor occupancy is required for a full

biological effect[47]. As well as stimulating growth hormone release, GRF has also been shown to stimulate growth hormone synthesis. Exposure of pituitary cells to GRF for 24 hours results in increased pituitary growth hormone content and a significant increase in growth hormone mRNA levels, which occurs after 6 hours exposure[48,49].

## 1.4 *CELL TYPES OF THE ANTERIOR PITUITARY*

Original studies to determine the different cell types present in the anterior pituitary used a variety of staining techniques. Pituitary cell types could be divided into those that took up stain, the chromophils and those that did not, the chromophobes. The stain was associated with the secretory granules. The chromophils could be divided into those cells which stained with basic dyes, the basophils, and those which stained with acidic dyes, the acidophils. The acidophils are made up of the somatotrophs and lactotrophs which stain with orange G and erythrosin respectively. The basophils are cells containing TSH, FSH or LH (glycoprotein hormones) which stain using a periodic acid Schiff procedure. The chromophobes have been identified as corticotrophs, but possibly include other cell types (eg folliculo-stellate cells).

Such staining techniques have been used in conjunction with endocrine ablation studies, such as adrenalectomy, thyroidectomy and castration. These procedures remove the negative feedback of the hormone secreted by the target glands, and pituitary cell types secreting the trophic hormones are rendered hyperactive. Such changes in the activity of the cells could be detected with staining procedures such as those described above. Another technique used to study pituitary cytology is that of

electron microscopy. The variable size, shape and electron opacity of the prominent secretory granules provides a further basis for the identification of different cell types. In 1973 Hopkins and Farquahar[50] described a new procedure, the production of single pituitary cells by trypsin digestion. After dispersion, the function and morphological integrity of the cells was maintained, as shown using electron microscopy, and the different cell types could be more easily identified. Thus the cellular site of synthesis, transport and release of peptide hormones could now be identified and studied[51].

Immunochemical and immunofluorescent staining techniques have provided evidence that a different cell type exists for each of the six hormones produced by the anterior pituitary with the probable exception of the gonadotroph. However, more recently the reverse haemolytic plaque assay method of detecting hormone release from single cells, first described for pituitary cells by Neill and Frawley[52] and double gold immuno-electron microscopy[53] have shown that normal rat pituitary cells exist which appear to contain and release both growth hormone and prolactin, namely mammosomatotroph. These observations cast some doubt on the one cell, one hormone theory.

## 1.5 *EVIDENCE FOR PITUITARY PARACRINE/AUTOCRINE CONTROL*

Paracrine and autocrine effects have recently been found to be of importance in the regulation of haematological and immunological responses[54-58]. It has been hypothesised that endocrine cells are arranged in glands to facilitate cell to cell communication. It has now been found that each endocrine gland synthesises and

secretes certain peptides whose function is, as yet, unknown. Evidence for local control mechanisms in the pituitary include the topographical arrangement of the different cell types, the local synthesis and storage of these bioactive peptides, changes in the synthesis and content of these peptides as a result of endocrine manipulations and the effect of these substances on pituitary hormone release.

It should be emphasised at this point that some confusion in the literature exists as to the precise definitions of the terms paracrine and autocrine. I have used both words in this thesis to describe the influences of a cell **type** on its neighbour, thus paracrine implies the effects of one cell type on another whilst relationships between sub-populations within a specific cell type (see chapter 5) are referred to as autocrine.

### 1.5.1 *Topographical arrangement of cell types in the anterior pituitary*

It is clear that the topographical distribution of cell types in the pituitary is not random. Gonadotrophs and lactotrophs are frequently found in close association with each other[59,60]. Many of these lactotrophs are cup shaped, embracing and completely surrounding gonadotrophs[61,62]. It has been suggested that gonadotrophs and lactotrophs form specialised junctional complexes between each other[63-65].The length of attachment of these junctions varies between 50 and 300 nm. It should be noted however, that not all lactotrophs have affinity for gonadotrophs and indeed there would appear to be subpopulations of gonadotrophs which either do or do not adhere to lactotrophs[66]. The hypothesis that the topographical affinity of lactotrophs for gonadotrophs may have a functional significance is supported by several observations. There include dramatic developmental changes, sex differences and alterations during the oestrous cycle in the affinity of lactotrophs for the gonadotrophs[59]. Cup shaped cells do not seem to occur before puberty and in adult life they are more numerous in the female than in the male[59].

Corticotrophs have a stellate shape and form cytoplasmic extensions which may be very long[67-69]. Cytoplasmic extensions often engulf or completely encircle a somatotroph[66].

Thyrotrophs are the least dispersed throughout the pituitary, predominantly forming clusters, particularly in the centre of the gland[66]. Although cell extensions and associations suggest inter-cellular communication, functional morphological correlates have not been reported.

Recent evidence has demonstrated variability in the distribution of somatotrophs in the pituitary using sections in the sagittal and dorsoventral planes. Sections were stained for growth hormone and pronounced differences in percentage staining were noted. Following perifusion of these slices a marked increase in GRF induced growth hormone release was seen only in the most ventral sections. This response did not correlate with the percentage of growth hormone cells in that region[70]. A similar study has demonstrated variation in prolactin secretion and TRH responsiveness from different sagittal and dorso-ventral sections using the reverse haemolytic plaque assay[71].

### 1.5.2 *Neuropeptide synthesis and storage*

The number of peptides described in the literature that are synthesised and/or stored in the anterior pituitary seems to increase on an almost weekly basis. A recent review[72] estimated that over 200 small proteins or peptides had been described that might play a paracrine regulatory role in anterior pituitary function. Techniques used

in such studies include immunocytochemistry or radioimmunoassay (RIA) of whole pituitaries to assess peptide content and to demonstrate local synthesis, *in-situ* hybridization or northern blotting were used[73-76].

### 1.5.3 *Endocrine manipulations*

Thyroidectomy dramatically decreases pituitary content and mRNA quantity of neurotensin and galanin but increases synthesis and content of substance P, Neuropeptide Y (NPY), neuromedin U and VIP[73-78]. The above effects are not seen in the hypothalamus nor are they mimicked by adrenalectomy or gonadectomy, suggesting a specific functional significance of these peptides in the pituitary.

In contrast, oestrogen manipulation (in either normal male or female animals) produces huge changes in the pituitary synthesis and content of both VIP and galanin as well as the expected increase in prolactin synthesis and content but has little or no effect on NPY or neurotensin[79-86].

Other studies include treatment with dexamethasone contrasted with adrenalectomy[86-89] and changes in prolactin secretion using dopamine agonists and antagonists[90-92]. These studies have noted differing, but specific, patterns of change in the neuropeptide levels with each of the endocrine manipulations, reflecting in part, alterations in the numbers of the pituitary cell types.

Recent studies have advanced these findings by attempting to demonstrate colocalisation of these peptides with the various classical pituitary hormonal cell types both in the normal and in the endocrine manipulated state. Immunocytochemistry has revealed very weak NPY immunoreactivity in scattered cells in control rat pituitaries, but in hypothyroidism a greater number of positive cells were seen and the relative staining intensity was increased. NPY colocalised to the thyrotroph both in the control and in the hypothyroid state[73].

The increase in galanin staining in control and hyperoestrogenised state has been attributed to an increase in the frequency and amounts of galanin expression in the lactotroph. In contrast to the above, the increase in VIP synthesis and content induced by hyperoestrogenisation or thyroidectomy is not attributable to an increase in lactotroph VIP content. Indeed VIP appears to co-localise to some other cell type, possibly the folliculo-stellate cell[78,93].

### 1.5.4 *Regulation of pituitary hormone release by locally secreted factors*

Despite the demonstration of large numbers of peptides in the anterior pituitary, little or no data exists to indicate that they are released from their cells of synthesis/storage and a surprisingly few number of peptides have been shown to have a direct effect on pituitary hormone release (rather than, as is more common, a modulatory role in the secretion and action of hypothalamic releasing or inhibitory factors). Some of the earlier studies, using perifusion systems or in static culture, which demonstrated direct effects of peptides on anterior pituitary hormone release could be criticised because: (a) pharmacological rather than physiological doses of peptides were used: (b) changes in hormone release were often very slow, occurring between 10 - 60

minutes after the addition of the peptide: (c) stimulation, when it did occur was often less than 50% of basal value and not dose dependent. These studies were therefore of doubtful physiological significance.

The pioneering work of Denef and colleagues[94-97] using aggregates of enriched pituitary cells has significantly advanced the field of cell to cell communication. It was these studies that provided the first evidence that paracrine regulation does occur and that the presence or absence of locally secreted factors may greatly influence anterior pituitary hormone release. Denef's work has centred around those factors which may regulate prolactin, growth hormone and luteinizing hormone release. With respect to gonadotrophs, the intimate contact occurring with lactotrophs *in-vivo* does not seem necessary. The probable agent mediating the gonadotroph/lactotroph interaction appears to be angiotensin II (AII) released from gonadotrophs in response to GnRH[98-101]. AII stimulates prolactin release. The effect of AII can be blocked with specific AII antagonists. AII receptors have been found on pituitary cells, presumably lactotrophs.

In a separate study Denef and co-workers demonstrated that the folliculo-stellate cells (previously thought to be non-secretory) inhibited the secretion of prolactin when co-cultured with lactotrophs as cell aggregates. The prolactin secretory response to AII and TRH was inhibited by such co-aggregates[72,102-104]. Thus, an inhibitory paracrine factor of folliculo-stellate cell origin seems indicated, but its identity is unknown.

More recently his work has centred on trying to mimic the changes induced by various *in-vivo* endocrine manipulations in his culture system, thus allowing the study of neuropeptide secretion and their influence on hormone release[89,105].

Following on from Denef's lead, other groups have used immunoneutralisation of various peptides to further study their role in pituitary function. Nagy et al demonstrated, using the reverse haemolytic plaque assay (RHPA), that locally produced pituitary VIP acts in an autocrine fashion in both GH3 cells (a clonal mammosomatotroph cell line) and primary pituitary cell cultures and that VIP antisera and antagonists decreased basal prolactin but not growth hormone secretion[106]. More recently, Lam et al[107] showed that VIP secretion from cultured dispersed cells from hypothyroid animals (but not from euthyroid controls) could be measured and was stimulated by TRH and GRF, but not by CRF or GnRH. The addition of VIP antisera significantly decreased the basal prolactin secretion indicating that, in the hypothyroid state, VIP exerts a tonic stimulatory effect on prolactin secretion. Exposure to anti-VIP had no effect on basal growth hormone secretion, nor did it have any effect on GRF induced growth hormone release.

## 1.6 *THE ISOLATION OF SPECIFIC PITUITARY CELL TYPES*

The heterogeneity of the anterior pituitary, the six hormones being secreted by at least five different cell types, presents a problem in interpreting hormonal responses to hypothalamic releasing hormones, the putative paracrine factors and their mechanism of action. Thus it is important to purify the individual cell types and several methods

for such purification have been developed. These methods have exploited the differences in density and size of the individual cell types after enzymic dispersion to provide a means for their separation. Such methods have included centrifugation or sedimentation at unit gravity of pituitary cells through gradients of dextran, bovine serum albumin (BSA), Percoll, and sucrose.

### 1.6.1 *Velocity sedimentation at unit gravity*

The technique of velocity sedimentation at unit gravity is the most widely used method for pituitary cell separation. The technique was first applied to the mammalian pituitary by Hymer et al[108]. When pituitary cells are enzymatically dissociated from intact tissue they are for the most part spherical particles and thus sedimentation rates are predominantly dependent on the density and size of the cells being separated. For their sedimentation behaviour in linear gradients of BSA it has been shown that the density of the different rat pituitary cell types covers a range of 1.05 to 1.08 g/cm. Since 1 and 3% BSA have densities of 1.010 and 1.016 respectively, the difference between pituitary cell density and surrounding medium may be as much as 0.06 g/cm depending on the specific cell type and its sedimentation rate in the chamber. The sedimentation behaviour of pituitary cells in shallow BSA gradients maintained at unit gravity is at least partially dependent upon cell density differences. However, there appears to be universal agreement that differences in cell size constitute the primary factor in determining sedimentation rate.

Methods other than unit gravity sedimentation include:-

### 1.6.2 *Density gradient centrifugation*

When used in combination with the unit gravity method this has proved useful for obtaining populations of somatotrophs of greater than 90% purity[108] and corticotroph enrichment by five fold[109]. Continuous density gradients of BSA in the former case and Percoll in the latter were used in these experiments. More recently discontinuous gradients of BSA or Percoll have been used to separate pituitary cells[110-112]. However use of discontinuous gradients are associated with decreased cell recovery and a considerable loss of viability.

### 1.6.3 *Centrifugal elutriation*

Recently a new unit gravity cell separating device has become available using the technique of counter-streaming centrifugation (centrifugal elutriation). This procedure also separates cells on the basis of cell size. It is well suited to those situations where large numbers of pituitary cells are being separated. Childs et al have recently shown[68,113] that using the results of morphometric studies, the surface area of corticotrophs increased after stimulation by adrenalectomy and decreased after inhibition of secretion by ion channel blockers. The increased size may be correlated with enhanced secretion of storage granules (ie increased fusion of secretory granules with the cell surface) or be due to osmotic effects. They then showed that CRF stimulation also increased the size of corticotrophs, GRF increased the size of somatotrophs and similarly GnRH increased the size of gonadotrophs. Thus separation by re-elutriation after 2-4 hours of treatment with the stimulatory hormone, allowed further purification on the basis of the increased cell size. However the yield from

centrifugal elutriation, particularly when performed twice, was no more than 50-60% and the viability was less than 80%.

1.6.4 *Separation on the basis of cell surface receptors*

In an effort to obtain enrichment of cell types by means other than variations in cell size and density, neither of which directly correlate with hormonal content, a number of groups have attempted to separate cells on the basis of their cell surface receptors for the various specific hypothalamic releasing hormones.

Separation of pituitary cells by affinity chromatographic methods have been reported in the case of TRH receptor positive cells[114]. This was accomplished by coupling TRH to a sephadex column. The TRH bound to the receptors on the thyrotrophs and lactotrophs thus slowing them down. The bound cells were then recovered in later fractions or eluted off the column at a later stage. Like centrifugal elutriation, however, the cell yield and viability was sub-optimal and separation of lactotrophs from thyrotrophs impossible.

In contrast to solid phase separation Thorner et al reported[115] that by conjugating a GnRH analogue to fluorescent microspheres, cells which possessed GnRH receptors (gonadotrophs) could be enriched by fluorescence activated cell sorting (FACS). Results demonstrated a cell recovery of 30% but with 99% viability. Enrichment of gonadotrophs from 7.4% to 52% was possible but the speed of separation was a limiting factor, since it took 12 - 16 hours of continuous sorting to produce half a million cells!

Similar work by Schwarz and Vale[116] using a fluorescent CRF analogue demonstrated excellent corticotroph enrichment, but as with the work of Thorner, FACS sorting was extremely slow since the starting sample contained only 5 - 6% labelled cells.

## 1.7 *AIMS OF THESIS*

The purpose of this thesis was to:- (1) apply modern-day fluorescence-activated cell sorting (FACS) to dispersed anterior pituitary cells. (2) Study the secretory characteristics of FACS enriched cells compared to unenriched populations, using the reverse haemolytic plaque assay with particular reference to regulation of prolactin secretion by the peptide galanin. (3) Investigate the changes in the secretory states of such enriched populations of lactotrophs induced by oestrogen manipulation.

# CHAPTER TWO:

# FLOW CYTOMETRIC ANALYSIS OF FUNCTIONAL ANTERIOR PITUITARY CELLS

## 2.1 *INTRODUCTION*

Flow cytometry is routinely used to analyze and separate living cells. It measures both cell size and granularity. Forward or narrow-angle light scatter (FALS) is a good indicator of cell size, whilst ninety degree or perpendicular light scatter (PLS) is related to cytoplasmic granulation and nuclear shape[117].

By measuring angles of light scatter, a fluorescence-activated cell sorter (FACS) is capable of analyzing and sorting cells on the basis of their size and granularity as well as fluorescence. Use of fluorescent antibodies or ligands allows sorting of cells on the basis of their surface antigens[118]. Little work, however, has been published on the application of the FACS to dispersed pituitary cells.

As described in Chapter 1 purification of gonadotrophs and corticotrophs had been possible using fluorescent analogues of GnRH and CRF to label pituitary cells which were then be enriched using early FACS machines[115,116]. The major limitations of these machines were the low speed of sorting, which was partly a function of the low percentage positivity in both studies (7 - 10% for gonadotrophs and 5 - 6% for corticotrophs) and the poor cell recovery and viability after sorting due to cell damage by turbulence, excessive sheath fluid pressure and inadequate smoothing of the FACS flow cell orifice.

Many of the above "design faults" have been eradicated in the modern generation of FACS machines and the first application, using such a machine, to dispersed pituitary cells was by Hadfield et al in 1986[117,119]. These studies demonstrated that partial enrichment of the differing cell types was possible by sorting on the basis of FALS

(cell size) and PLS (cell granularity). It was not surprising, however, that the results were no better than the existing gradient enrichment methodologies since they too separated cells using the same parameters! It should be emphasised, that Hatfield's studies demonstrated that the newer FACS machines were capable of accurately separating cells on the basis of two simultaneous parameters (FALS and PLS) which was not previously possible, without lysing or damaging the cells and at a rate of 500-800 cells/second.

Later in 1986 St John et al[120] demonstrated that lactotrophs might be identified using an antibody against the anterior pituitary hormone prolactin. However cell-surface prolactin labelling was found to be present on only 40-50% of lactotrophs (as judged by intracellular staining). Subsequent work[121] also failed to demonstrate greater than 56% cell surface labelling on diethylstilboestrol-induced prolactinoma cells. St. John et al[120] also found that when using a final dilution of 1/1000 (the same dilution used for prolactin labelling) for antibodies against each of the other anterior pituitary hormones, much lower labelling was obtained than would be expected from published histochemical values.

Neither group could explain the nature of the cell-surface prolactin labelling. St. John et al[120] stimulated and inhibited prolactin secretion with a number of factors (including thyrotropin releasing hormone, vasoactive intestinal polypeptide and dopamine) to assess the effects on the amount and intensity of surface labelling, but failed to demonstrate consistent effects.

The objectives of the studies reported here were, first to disperse anterior pituitaries into single, viable cells and analyze them on the basis of their laser light scatter characteristics. Secondly, to label live acutely dispersed anterior pituitary cells with antibodies against anterior pituitary hormones and compare cell size and granularity amongst the different hormonal cell types. Thirdly, to demonstrate that labelling is dependent upon the secretory state of the cell and to show that factors which are known to alter secretion change the intensity and percentage of cell-surface labelling accordingly. Finally, to use dual-colour fluorescence labelling to assess the percentage of cells which simultaneously secrete both prolactin and growth hormone on their cell-surface, and to investigate the effects of the appropriate hypothalamic releasing factor on the labelling of each hormone.

## 2.2 *MATERIALS AND METHODS*

### 2.2.1 *Buffers*

Buffer A was calcium- and magnesium free Hank's balanced salt solution (Gibco, Paisley. UK) containing HEPES (25 mmol/l, Sigma, Dorset, UK); pH 7.4, and bovine serum albumin (BSA); fraction V, (4 mg/ml, Sigma).

Buffer B was Earles balanced salt solution (Gibco, Paisley, UK) containing 25 mmol HEPES/l (Sigma, Dorset, UK), pH 7.4, and 4 mg BSA/ml (fraction V).

Culture medium was made using Eagle's minimal essential medium (Gibco, Paisley, UK), 10% (v/v) Fetal Calf Serum (Gibco, Paisley, UK), 25 mmol HEPES/l, 10 mmol bicarbonate/l (Gibco), 400 units penicillin/ml, 400 $\mu$g streptomycin/ml and 2.5 $\mu$g amphotericin/ml solution (Gibco) and 20 mmol L-Glutamine/l (Gibco) at pH 7.4

### 2.2.2 *Reagents*

Stimulation/inhibition of anterior pituitary hormone secretion was performed using human thyrotropin releasing hormone (TRH; Peninsula, Liverpool, UK), rat growth hormone releasing hormone (GRF; Sigma), human gonadotrophin releasing hormone (GnRH; Peninsula), rat corticotrophin releasing hormone (CRF; Sigma), dopamine (Sigma) and somatostatin (Peninsula).

### 2.2.3 *Preparation of cell suspensions*

Suspensions of cells were prepared from the anterior pituitary using modifications of standard methods[122]. All experiments were performed with cells from dispersed pituitaries of non-pregnant randomly cyclic Wistar rats (Interfauna, Huntingdon, UK), weighing 200-250g.

Pituitaries were removed from rats immediately after death (by decapitation) and transferred to buffer A. Pituitary lobes were dissected free of intermediate and posterior lobes and gently minced. The tissue was washed twice with phosphate buffered saline (PBS) (Oxoid, Hampshire, UK) and then incubated whilst shaking at 37°C for 1 h in buffer A containing 0.75 mg collagenase/ml (Boehringer, East

Sussex, UK), followed by incubation for 30 min whilst shaking at 37°C in buffer A containing 0.125% (v/v) trypsin (Sigma) and 0.1mg DNAase/ml (Type IN; Sigma), and then incubation for 15 min whilst shaking at 37°C in buffer A containing 0.5% Trypsin and 0.2mg DNAase/ml (type I).

The resulting cell suspension was rinsed by at least three cycles of centrifugation and resuspension in fresh culture medium. The number of cells in the suspension was determined with a haematocytometer and viability checked using the methods of trypan blue (Sigma) and propidium iodide (Sigma) exclusion. The final yield was normally 2.0 - 2.6x$10^6$ cells/pituitary, with a viability of >98%.

### 2.2.4 *Antibodies*

Cell-surface labelling was performed using antibodies kindly donated by Dr A F Parlow and the National Hormone and Pituitary Programme (NHPP; formerly NIADDK) raised against the ß subunit of human luteinizing hormone (LH; NHPP anti-hLH IC-1), the ß subunit of human thyrotropin (TSH; NHPP anti-hTSH IC-1) and human adrenocorticotrophin (ACTH; NHPP anti-hACTH IC-1). Surface labelling for rat prolactin and rat growth hormone was performed using antibodies raised in sheep, kindly donated and previously validated by Dr D.J. Flint, Hannah Research Institute, Ayr[123].

All antibodies were used at a final dilution of 1:100-1:200/ml in buffer B. All experiments were performed at a final concentration of 2x$10^6$ cells/ml antibody solution.

**Addendum to 2.2.6**

For all stimulatory and inhibitory factors used (TRH, GRF, GnRH, CRF, dopamine and somatostatin) dose response experiments were perfomed (data not shown). Maximal surface labelling was obtained using a concentration of 100 nM for each stimulatory peptide and maximal inhibition obtained with 1mM dopamine and 100 nM somatostatin.

Fluorescence labelling was performed using fluorescein isothiocyanate-conjugated (FITC) goat anti-rabbit and anti-sheep immunoglobulin G (Sigma) diluted 1:25 in culture medium, FITC goat anti-monkey immunoglobulin G (Nordic, Feltham, Middx) diluted 1:25 in culture medium and phycoerythrin-conjugated (PE) goat anti-rabbit immunoglobulin G (Seralabs, Sussex, UK) diluted 1:10 in culture medium.

### 2.2.5 *Immunological labelling*

Cell suspensions to be used for immunological labelling of cell-surface antigens were incubated at 37°C in culture medium for 2 h after dissociation and before further processing. Cells were then washed twice and the pellet then resuspended in buffer B containing the primary antibody and incubated for 1 hour at 4°C. The cells were rinsed three times by centrifugation in culture medium to remove excess primary antibody and then incubated in FITC or PE secondary antibody for a further 1 hour at 4°C.

Dual colour fluorescence labelling was performed using sequential labelling with three rounds of washing between the addition of each layer of antibody.

After labelling, cells were rinsed in culture medium and maintained at 4°C in culture medium whilst awaiting sorting.

### 2.2.6 *Stimulation and inhibition experiments*

To assess the effects of known hypothalamic stimulatory and inhibitory factors on cell-surface labelling, maximal stimulatory/inhibitory doses of these factors were, at different times, added to the dispersed cells at the time of incubation with primary

antibody. Thus the cells were exposed to maximal stimulatory/inhibitory doses (100 nmol/l in all cases) for 1h.

### 2.2.7 *FACS analysis*

Cell analysis was performed on an electronically programmable individual cell sorter (EPICS CS, Coulter Electronics, Luton, Bedford, UK). Exciting light came from a 5 W argon laser emitting 200 mW at 488nm.

For analysis the EPICS was operated at rates between 2000-5000 particles/s. In all experiments, windows and gates were defined on the basis of FALS, PLS and log green and log red fluorescence. All experiments were performed using a 76 $\mu$m nozzle tip and sterile PBS as sheath fluid. The FACS was standardized before and during each experiment with 10.3 $\mu$m Fluorospheres (Fullbright grade II, Coulter Electronics).

Results are displayed as dual parameter histograms (64x64 channels) and represent 10-15x$10^4$ cells/histogram.

### 2.2.8 *Immunocytochemistry*

Acutely dispersed cells were fixed in 2% (v/v) paraformaldehyde in PBS at room temperature for 2 h ($10^5$ cells/500$\mu$l 2% paraformaldehyde). Fixed cells were permeablised by treatment for 10 min with 0.4% (v/v) Triton X-100 (Sigma) in PBS at room temperature ($10^5$ cells/100 $\mu$l of Triton X-100). The cells were then rinsed three times in culture medium before labelling with primary and secondary antibodies as described above for unfixed cell suspensions. Final dilutions for each hormone

antibody used for intracellular labelling were as follows: rat prolactin (using the NHPP IC-1 antibody), 1/10,000; rat growth hormone (using a previously characterized antibody supplied by Dr Grindeland, NASA, Ames, California, USA)[124] 1/20,000; human ACTH (NHPP IC-1 antibody), 1/5000; and rat LH (NHPP antibody supplied for radioimmunoassay), 1/2,000. The resulting labelled cell suspensions were analyzed on the FACS as described above.

### 2.2.9 *Antisera validation*

Quenching was performed for both intracellular and cell-surface labelling for all antibodies, using iodination grade pituitary hormones supplied by the NHPP (rat prolactin, rat growth hormone, human ACTH, rat TSH, and rat LH). Each hormone was used at a final concentration of 25-50$\mu$g/ml antiserum. Absorption of antibody with purified peptide was performed at 4°C for 24 h before labelling in buffer B (as above).

## 2.3 *RESULTS*

### 2.3.1 *Cell size and granularity*

Using the FALS and PLS properties of dissociated anterior pituitary cells a number of different populations could be defined (Fig 2.1). In addition to the area representing debris and red blood cells (labelled A), two distinct populations of granular and less granular cells (labelled B and C) were characterized. The more granular population represented 26 $\pm$ 2.2% of the total pituitary cell population with the remaining 74 $\pm$ 3.5% being less granular. In all subsequent experiments the debris and red cells (ie population A, Fig 2.1) were electronically gated out.

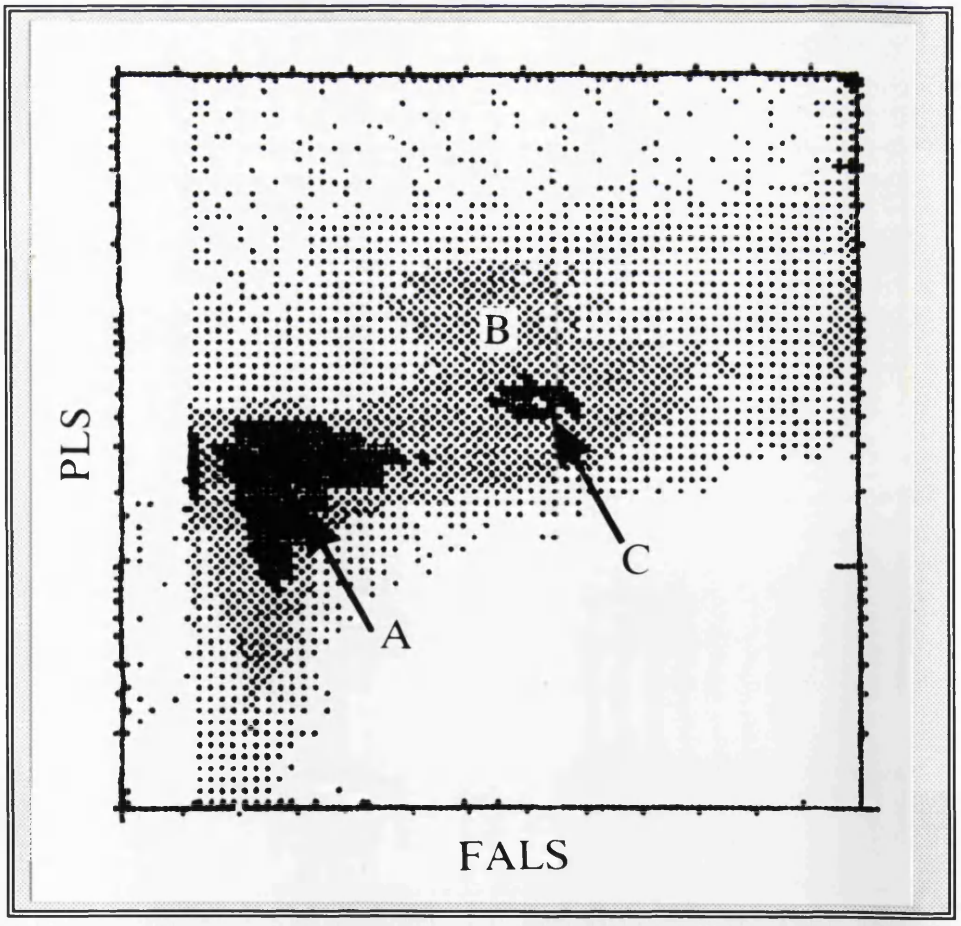



**Figure 2.1**

**Figure 2.2**

Size (FALS) on the X axis versus granularity (PLS) on the Y axis, gated on fluorescence for each of the antibodies raised against four of the anterior pituitary hormones. Somatotrophs (Panel A) tended to fall into two distinct populations, 78 $\pm$ 2.9% with a higher PLS signal (cellular granularity) and the remaining 22 $\pm$ 1.5% less granular. The other cell types had lower PLS signals and were thus less granular. Gonadotrophs had the highest FALS signal, i.e. were the largest cell type (Panel B), whilst corticotrophs were the smallest (Panel C) and lactotrophs were of intermediate size (Panel D).

### 2.3.2 *Cell size and granularity of labelled cells*

Figure 2.2 shows the size (FALS) versus the granularity (PLS) gated on fluorescence for each of the antibodies raised against four of the anterior pituitary hormones. Growth hormone cells tended to fall into two distinct populations, 78 $\pm$ 2.9% with a higher PLS signal (cellular granularity) and the remaining 22 $\pm$ 1.5% less granular. The other cell types had lower PLS signals and were thus less granular. Gonadotrophs had the highest FALS signal, i.e. were the largest cell type, whilst corticotrophs were the smallest and lactotrophs were of intermediate size. To date specific cell-surface labelling for thyrotrophs has not been obtained, using the NHPP antisera.

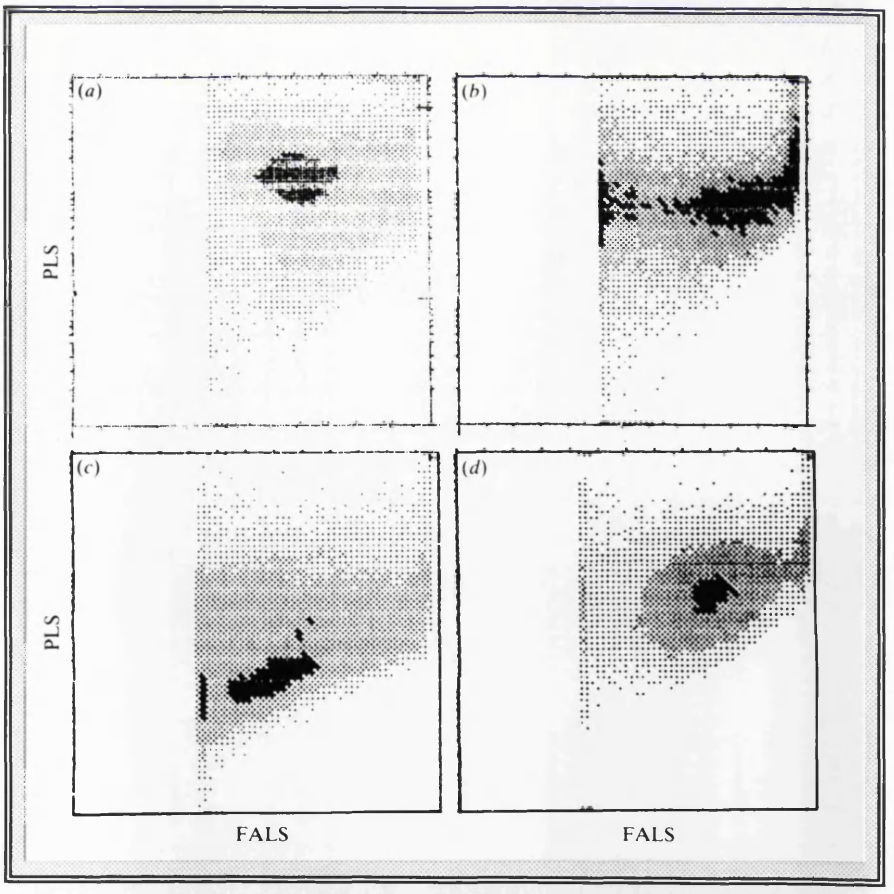



**Figure 2.2**

**Addendum to 2.3.3**

For all primary and secondary antisera used in these studies serial dilutions were performed. Optimum labelling, (ie highest intensity labelling with a minimum of non-specific "background" labelling) was determined for both primary and secondary antisera (data not shown). Optimal labelling was obtained using a dilution of 1/100 for all primary antisera and dilutions of 1/25 for FITC conjugated secondary antisera and 1/10 for PE conjugated antisera.

### 2.3.3 *Cell-surface labelling of live cells*

Preincubation of all the antisera used for cell-surface labelling (see materials and methods) with 50$\mu$g of the homologous peptide completely abolished cell surface labelling whilst incubation with other anterior pituitary hormones did not affect labelling. In particular the anti-rat prolactin and anti-rat growth hormone antisera used (supplied by Dr D Flint) demonstrated less than 1% crossreactivity with rat growth hormone and rat prolactin respectively even at the final dilution of 1/100.

When primary antibody dilutions of 1/1000 were used the percentage of all pituitary cells labelling for each hormone were similar to those found by St. John et al[120]. However, when the primary antibody dilutions were decreased to 1/100, significantly higher, yet still specific, labelling for lactotrophs, somatotrophs and gonadotrophs was obtained (Table 2.1). These higher values for percentage labelling agree with previously published findings based upon immunocytochemistry of dissociated and undissociated pituitary tissue[125,126] and these latter concentrations of primary antibody were used thereafter. Maximum labelling with primary antibody occurred after an incubation time of 1 h, and was therefore used in all further experiments. Incubation times for longer periods, up to 4 h, did not significantly increase surface labelling for any of the hormone antisera used.

*Table 2.1*

Basal percentage of surface labelling of rat pituitary cells with various hormone antibodies (diluted 1/100 and 1/1000) and surface labelling (using 1/100 dilution of antibody) after addition of stimulatory (100nM TRH during prolactin labelling, 100 nM GRF during GH labelling, 100nM GnRH during LH labelling and 100nM CRF during ACTH labelling) and inhibitory factors (1mM dopamine in the case of prolactin labelling and 100 nM somatostatin in the case of all other hormones) added to the cell suspension at the time of primary antibody labelling. Values are means ± S.E.M., n=6 separate experiments in all cases.

**Surface labelling (%)**

| Antibody | Basal 1/1000 | Basal 1/100 | Stimulated 1/100 | Inhibited 1/100 |
|---|---|---|---|---|
| **rPRL (IC)** | **23 ± 1.3*** | **44 ± 1.8** | **49 ± 1.7*** | **16 ± 1.0*** |
| **rGH (Ha)** | **2 ± 0.4*** | **7 ± 1.1** | **29 ± 1.1*** | **2 ± 0.4*** |
| **hLH (IC)** | **3 ± 0.6*** | **6 ± 0.8** | **10 ± 0.9*** | **5 ± 0.8$^{NS}$** |
| **hACTH (IC)** | **1 ± 0.8$^{NS}$** | **2 ± 1.3** | **6 ± 0.6*** | **1 ± 0.4$^{NS}$** |

IC denotes antisera donated by the NHPP and Ha denotes the antibody donated by Dr D.J. Flint, Hannah Research Institute.

* = $p<0.01$ compared to basal 1/100

NS = not significant

When viewed by fluorescence microscopy, labelling was confined to the surface of each labelled cell. Fig 2.3 demonstrates surface labelling of lactotrophs and somatotrophs. Surface labelling for gonadotrophs and corticotrophs was identical, but considerably less numerous and thus are not shown. The cell-surface labelling was patchy. Those cells which had uniform intracellular labelling could be shown to be non-viable by counter staining with propidium iodide. More than 98% of cells with discrete, patchy cell-surface labelling excluded propidium iodide. Results of antibody binding by fluorescence microscopy of 400-600 cells from eight to ten randomly chosen high-power fields, correlated extremely well with results obtained by FACS analysis. Controls, using either no primary antibody or the appropriate non-immune serum at a final dilution of 1:100, yielded non specific fluorescent labelling of less than 1% for both FITC and PE.

### 2.3.4 *Intracellular labelling of fixed cells*

FACS analysis confirmed that fixation with paraformaldehyde and treatment with Triton X-100 did not change the FALS/PLS patterns observed with unfixed cells (Fig 2.1). The percentage of labelled cells was very similar to that obtained from the maximally stimulated, cell-surface labelling (50 $\pm$ 2.1% for prolactin, 31 $\pm$ 1.9% for growth hormone, 9.7 $\pm$ 0.8% for luteinizing hormone and 6.3 $\pm$ 0.6% for ACTH) whilst the FALS/PLS patterns, gated on fluorescence (i.e. the positive staining cells), were identical to those obtained using live cells (Fig 2.2). Validation of antisera binding was performed for all hormones. There was complete abolition of intracellular labelling using 25$\mu$g of homologous peptide.

A

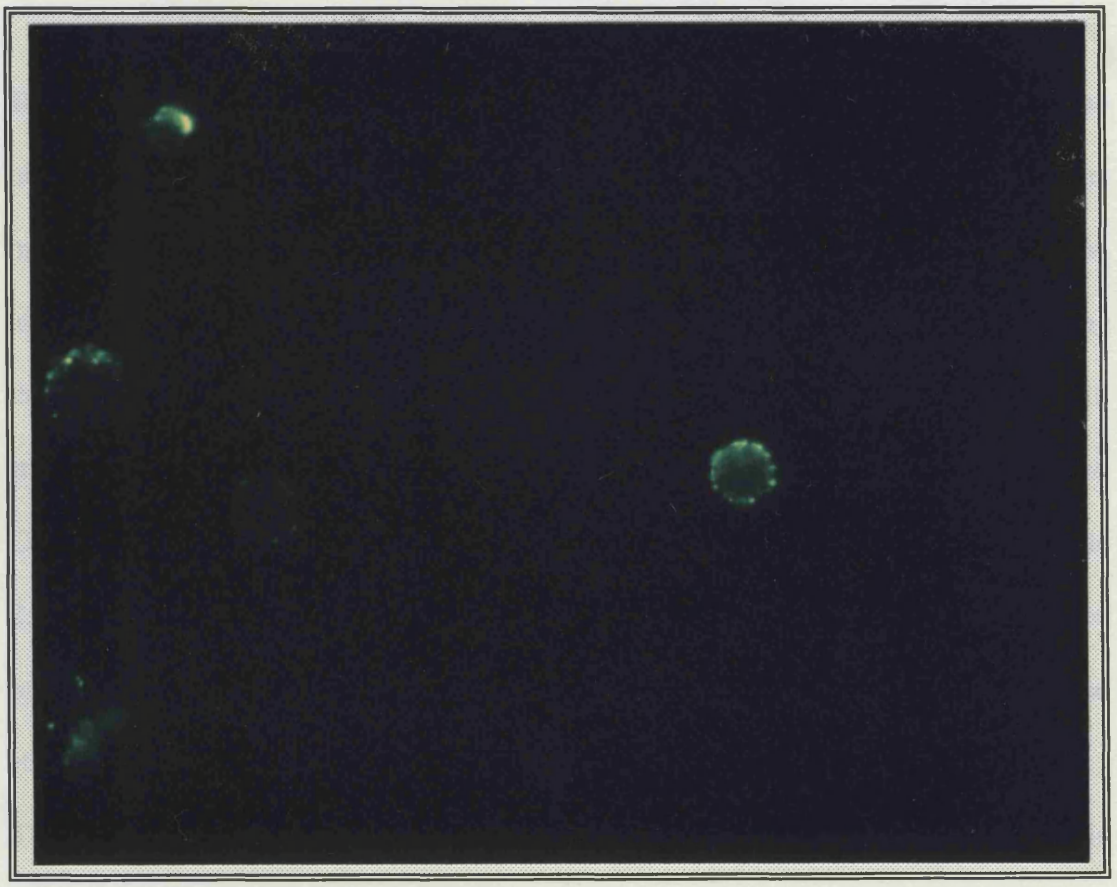
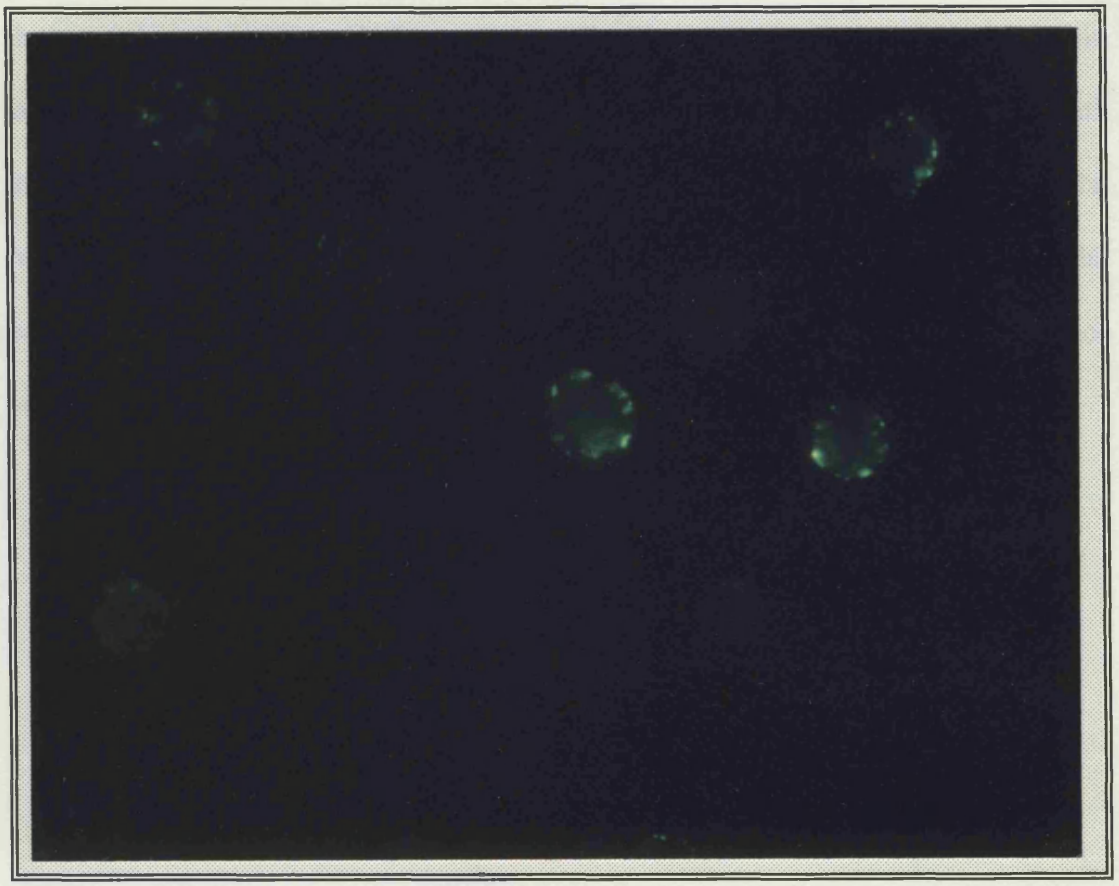


**Figure 2.3:** High power (x400) photographs of cell-surface labelling using an anti-prolactin antibody (A) and anti-growth hormone antibody (B). Note in both cases that the labelling is confined to the cell surface and is discontinuous.



B

### 2.3.5 *Changes in secretory state on cell-surface labelling*

To investigate the hypothesis that the patchy cell-surface labelling represents the phase of secretory granule fusion with the cell-surface (exocytosis), when hormone is thus present on the cell-surface, experiments were performed to study the effects on the intensity and percentage of cell-surface labelling when the cells were incubated during the addition of primary antibody with maximal doses (100 nmol/l in all cases) of both stimulatory agents (the hypothalamic releasing hormones TRH, GRF, GnRH and CRF) and inhibitory agents (dopamine and somatostatin). Results confirmed (Table 2.1) that the cell-surface labelling was significantly affected by agents which increased or decreased cellular secretion in the case of lactotrophs, somatotrophs and stimulation (but not inhibition) of gonadotrophs and corticotrophs.

There was a marked variation in intensity of cell-surface fluorescence for prolactin (and to a lesser extent for growth hormone, but not for the other cell types), such that when analyzing fluorescence a three decade log scale was used in order to visualize the complete spectrum of fluorescence. To analyze this variation in prolactin labelling, the FALS scatter (cell size) of the brightest lactotrophs was compared with the FALS scatter for the dimmest. No significant difference was noted between the two populations (mean channel numbers 76 $\pm$ 1.2 vs 75 $\pm$ 1.7), thus obviating the possibility that the largest cells were the brightest since they have a greater surface area.

### 2.3.6 *Dual-Colour Labelling*

Dual-colour fluorescence labelling was performed to assess the percentage of cells simultaneously expressing prolactin and growth hormone on their cell-surface. Two parameter histograms of red and green fluorescence were generated to allow analysis of those cell with red only, green only and both red and green labelling (Table 2.2). A further two parameter histogram was generated of FALS against PLS gated both upon red and green fluorescence thus allowing comparisons of size and granularity of those cells simultaneously expressing both hormones on their cell-surface as compared with those expressing only one. Using the NHPP anti-rat prolactin IC-1 and the anti-rat growth hormone donated by Dr D.J. Flint (to allow simultaneous incubation of both primary and secondary antisera since the NHPP antibody was raised in rabbit and the growth hormone antibody in monkey) dual prolactin and growth hormone secretors (mammosomatotrophs) could not be defined either in the basal state or after maximal stimulation with TRH (100nmol/l) and/or GRF (100nmol/l).

*Table 2.2*

Dual-colour cell-surface immunofluorescence of rat pituitary cells analyzed using flow cytometry. Prolactin was labelled with the red dye phycoerythrin and growth hormone with the green dye fluorescein isothiocyanate. Results are a mean of three separate experiments.

| | Green and/or red surface labelling (%) | | |
|---|---|---|---|
| **Antibody** | **red only** | **green only** | **Both red and green** |
| **rPRL(IC) red** | **45** | **0** | **0** |
| **rGH(Ha) green** | **0** | **6** | **0** |
| **Both antisera** | **44** | **5** | **0** |
| **Both antisera + TRH** | **49** | **5** | **0** |
| **Both antisera + GRF** | **42** | **28** | **0** |
| **Both antisera + TRH and GRF** | **48** | **27** | **0.8** |

IC denotes antisera donated by the National Hormone and Pituitary Programme and Ha denotes the antibody donated by Dr D.J. Flint, Hannah Research Institute. The fluorescence-activated cell sorter is able to detect both green and red dyes simultaneously thus allowing differentiation between cells labelled with red only, green only and both dyes on their surfaces.

## 2.4 *DISCUSSION*

These findings demonstrate that populations of live cells dissociated from the anterior pituitary of the rat and from human pituitary adenomas (data not shown), can be labelled by antibodies against the pituitary hormones prolactin, growth hormone, ACTH and LH. Unlike the previously published work[120,121], these studies achieve considerably higher percentage labelling by using a higher ratio of antibody to cell number. Comparing size and granularity (which in turn is proportional to cellular density) amongst the pituitary cell types, the results concur with previously reported data obtained by centrifugal elutriation[68,127], gravity sedimentation[110-112] and immunocytochemical analysis of unlabelled FACS sorted pituitary cells[117,119]. The cell-surface labelling obtained compares well with the many published reports using classical immunocytochemistry[69,128-132]. It is worthy of note that this higher concentration of primary antibody used in surface labelling was very similar to that used in a reverse haemolytic plaque assay[133,134].

The variation in intensity of prolactin and growth hormone cell-surface fluorescence correlates well with previous studies[133,134], which demonstrated, by plaque assay, a considerable variation in prolactin and growth hormone plaque area under both basal and stimulated conditions. In contrast, plaque area is uniform under basal conditions in the few studies published using reverse haemolytic plaque assays of corticotrophs[68] and gonadotrophs[113,135].

The changes seen in cell-surface labelling in response to stimulation and inhibition is in accord with the many published *in-vivo* and *in-vitro* models. The failure of St. John et al[121] to demonstrate reproducible changes in labelling when stimulatory and inhibitory factors were added may have been due to the addition of the factors 1 h

before primary antibody labelling, i.e. the effect on secretion is short lived, which is born out by superfusion experiments using pulsed stimulatory and inhibitory factors[136,137] and therefore was no longer evident at the time of labelling.

On the basis of this work, the most likely explanation for the phenomenon of cell-surface labelling is that hormones are being labelled as the exocytotic secretory granules fuse with the cell-surface. This is supported by the observations-

(1) A suspension of dispersed anterior pituitary cells may be labelled using hormone antibodies in amounts which compare favourably with published values from histochemical and reverse haemolytic plaque assay studies.

(2) With stimulation, all cells that contain a particular hormone may be labelled if appropriate concentrations of antibody and releasing factors are used.

(3) The intensity and percentage of cell-surface labelling achieved varied according to the secretory state of the cell.

The inability to detect mammosomatotrophs using dual colour analysis, or by growth hormone content of lactotrophs enriched by fluorescence-activated cell sorting (FACS) and prolactin content of growth hormone FACS enriched cells (see chapter 3) is at odds with the sequential plaque data of Frawley et al[52] and the double immuno-gold labelling studies of Nikitovitch-Winer et al[53]. Possible explanations include sex and strain differences in the animals used (male vs female and Sprague Dawley vs Wistar rats), differing specificities of the anti-prolactin and growth hormone antibodies used and differences in times of culture. Acutely dispersed cells were used in these studies, whilst Frawley's group cultured cells for 24 - 48 h prior to sequential plaque assay.

# CHAPTER THREE:

# PURIFICATION OF FUNCTIONAL LACTOTROPHS AND SOMATOTROPHS USING FLUORESCENCE-ACTIVATED CELL SORTING

## 3.1 *INTRODUCTION*

The use of dispersed anterior pituitary cell preparations to study the mechanisms controlling hormone synthesis and secretion circumvents the problems of limited diffusion and associated cell death at the centre of the tissue which are often encountered when using intact pituitaries or hemipituitaries[50,51,138].

The problem of cellular heterogeneity, the six anterior pituitary hormones being produced by at least five different cell types, still exists even when using fully dissociated pituitary cell preparations. Consequently, when the actions of hypothalamic releasing hormones on such cells are studied, one cannot be sure that overall changes in the secretion of various hormones reflect those occurring from a single cell type. For example growth hormone releasing factor (GRF)[139] may stimulate prolactin as well as growth hormone secretion. Similarly thyrotropin releasing hormone (TRH) stimulates thyroid stimulating hormone (TSH) and prolactin[140] secretion and may also influence growth hormone release[141].

In order to study the specific inter- and intracellular pathways involved in the regulation of any one of the anterior pituitary hormones (paracrine and autocrine effects), the preparation of a functionally pure, homogeneous cell population is required.

Work by Barker and colleagues[120,142,143] demonstrated that lactotrophs could be labelled using an antibody against the anterior pituitary hormone prolactin and thereby enriched by fluorescence activated cell sorting (FACS) as judged by cell-surface and intracellular immunofluorescence. Cell-surface prolactin labelling, however, was present on only 40-50% of prolactin cells (as judged by intracellular staining); nearly

half the cells that contained prolactin could not be sorted into the enriched population and thus the preparation of a lactotroph depleted population was not possible. Whilst these lactotrophs enriched by FACS were relatively pure (80-90%), contamination with other cell types necessitated verification of hormonal content by intracellular histochemistry before subsequent use.

The purpose of this study was to produce enriched and depleted viable populations of anterior pituitary cell types, using antibodies raised against the anterior pituitary hormones. These cells were cultured and percentage purity/depletion of other cell types assessed using reverse haemolytic plaque assays (RHPA) and immunocytochemistry.

## 3.2 *MATERIALS AND METHODS*

For buffers, reagents, preparation of cell suspension, immunological labelling and immunocytochemistry, please see Chapter 2.

### 3.2.1 *Antisera*

Antisera raised in sheep against rat prolactin and rat growth hormone, kindly donated by Dr D. J. Flint, Hannah Research Institute, Ayr, Scotland were used at all times for sorting. Aliquots of two million cells were labelled at a final dilution of 1/100/ ml of antisera in the presence of 100nmol TRH/l or 100 nmol GRF/l as appropriate.

The antibody for prolactin RHPA was generously donated and characterized by Drs L.S. Frawley and F.R. Bookfor, University of South Carolina, Charleston, USA[71,144] and was used at a final dilution of 1/100. The antibody used for growth hormone RHPA was raised by Dr Grindeland, NASA Ames, California, USA in monkey (see methodology for immunocytochemistry, see chapter 2) and was used at a final dilution of 1/50.

### 3.2.2 *Fluorescence activated cell sorting*

Cell sorting was performed on an electronically programmable individual cell sorter (EPICS CS, Coulter Electronics, Luton, Bedford, UK). Exciting light came from a 5-W argon laser emitting 200 mW at 488 nm. When sorting, the machine was operated at rates up to 800 cells/s. In all experiments windows and gates were defined on the basis of forward angle light scatter, log perpendicular light scatter and log green fluorescence. All experiments were performed using a 76 $\mu$m nozzle tip and sterile PBS as sheath fluid, cells were sorted directly onto the surface of culture medium (5 mls).

The EPICS CS sorts two populations at one time, the left channel was always termed the "enriched" ie highly fluorescent cell population and the right channel the "depleted" ie the non-fluorescent cell population. The remaining cells (those not fulfilling the pre-defined criteria for either the right or left sorting channels) and debris were sent to waste (Fig 3.1).

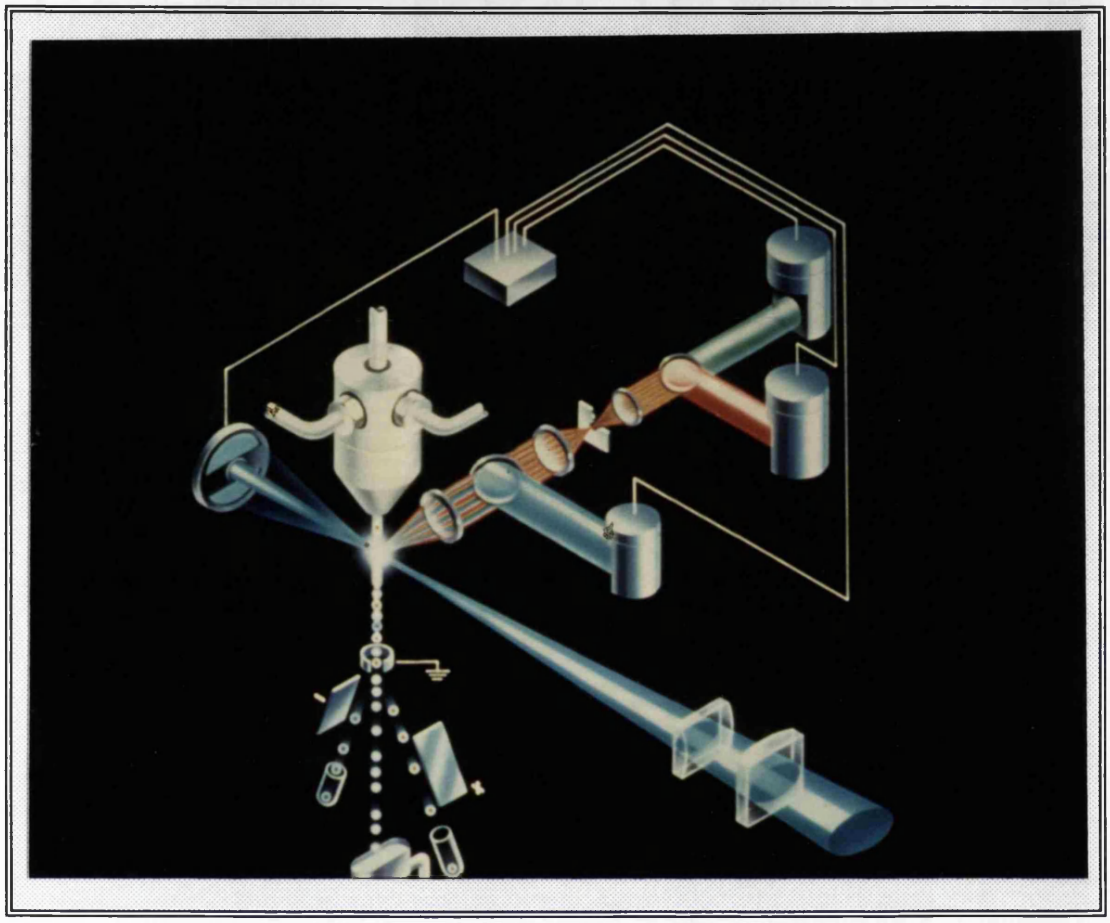


**Figure 3.1**: Schematic diagram of the fluorescence-activated cell sorting process. Cells are injected, under pressure into a flow cell and mixed with sheath fluid, Relative pressures are adjusted between the two fluids, such that when they leave the flow cell, one droplet contains one cell. Cells then meet the in-coming laser beam, focused by a number of lenses, and the resulting light scatter is detected by photomultipliers and analysed accordingly. Drops, containing cells, which meet pre-set criteria of size, granularity and intensity of fluorescence are charged either positively, negatively or not at all. The charged droplets then fall between the high voltage deflection plates and are consequently sorted either to the right or left. Unwanted cells and debris are not deflected and hence are sent to waste.

In a separate set of experiments cells were separated on the basis of the intensity of prolactin cell-surface labelling. The 20% most intense prolactin surface labelling cells were sorted to the left whilst the least bright 20% were sorted to the right. Similarly the "negative" cells (ie with little or no prolactin cell-surface labelling) were separated into those cells with a low degree of cell-surface labelling (sorted left) and those with no cell-surface labelling (sorted right).

The sorter was standardized before and during each experiment with 10.3 $\mu$m Fluorospheres (Fullbright grade II, Coulter Electronics). Results are displayed as dual parameter histograms (64x64 channels) and represent 10-15x$10^4$ cells/histogram.

### 3.2.3 *Reverse haemolytic plaque assay*

The method of Neill & Frawley[134] was used for prolactin and growth hormone plaque assays. In brief, sorted cells were cultured for 48 hours on polylysine-coated (0.5mg/ml, Sigma) petri dishes to prevent cell clumping and were briefly trypsinized (0.0125% v/v) for 5 min the next morning to remove the cells from the plates. The cells were allowed to recover in culture medium for a further 2 h before use. Cunningham chambers were constructed using polylysine coated microscope slides, double-sided adhesive tape and coverslips. A mixture of $10^5$ sorted pituitary cells and 1 ml 18% (v/v) sheep red blood cells (Tissue Culture Services, Buckingham, Kent) conjugated to Protein A (Sigma) by chromium chloride was infused into the chambers and allowed to attach to the slide for 1 h. Excess cells were then removed by washing with buffer B before the addition of antibody and secretagogue. On average one chamber contained 750-1000 pituitary cells. Maximal plaque formation occurred after

3 and 4 h for prolactin and growth hormone respectively, in the presence of 100 nmol TRH/l or 100 nmol GRF/l. Plaque formation was induced by the addition of 1/25 (v/v) guinea-pig complement (Gibco) for 45 min. Cells were fixed in 2% (v/v) glutaraldehyde (Sigma) and counterstained with 0.1% (v/v) tolidine blue (Sigma) to facilitate counting of cells not forming plaques.

## 3.3 *RESULTS*

### 3.3.1 *Sorting of unlabelled cells*

Unlabelled pituitary cells were separated on the basis of size and granularity. Cell recovery after sorting was 91 $\pm$ 3.4%. Light microscopy after sorting confirmed that all pituitary cells fell into the more and less granular populations (marked B and C respectively, in Figure 2.1, page 47) and that population A comprises only red blood cells and debris. No cell-surface prolactin or growth hormone labelling could be detected using FACS analysis after sorting or fluorescence microscopy within population A other than non-specific labelling of debris (which could be proven to be debris by counterstaining with propidium iodide).

### 3.3.2 *Sorting of surface labelled lactotrophs and somatotrophs*

Pituitary cells were, at different times, labelled with antisera against either prolactin or growth hormone and separated from unlabelled cells by sorting. Debris and red blood cells were electronically gated out and not sorted (ie allowed to go to waste).

FACS reanalysis of the sorted cells revealed almost total depletion of the more granular population from purified lactotrophs and the converse to be true for purified somatotrophs, compared with controls (Fig 3.2).

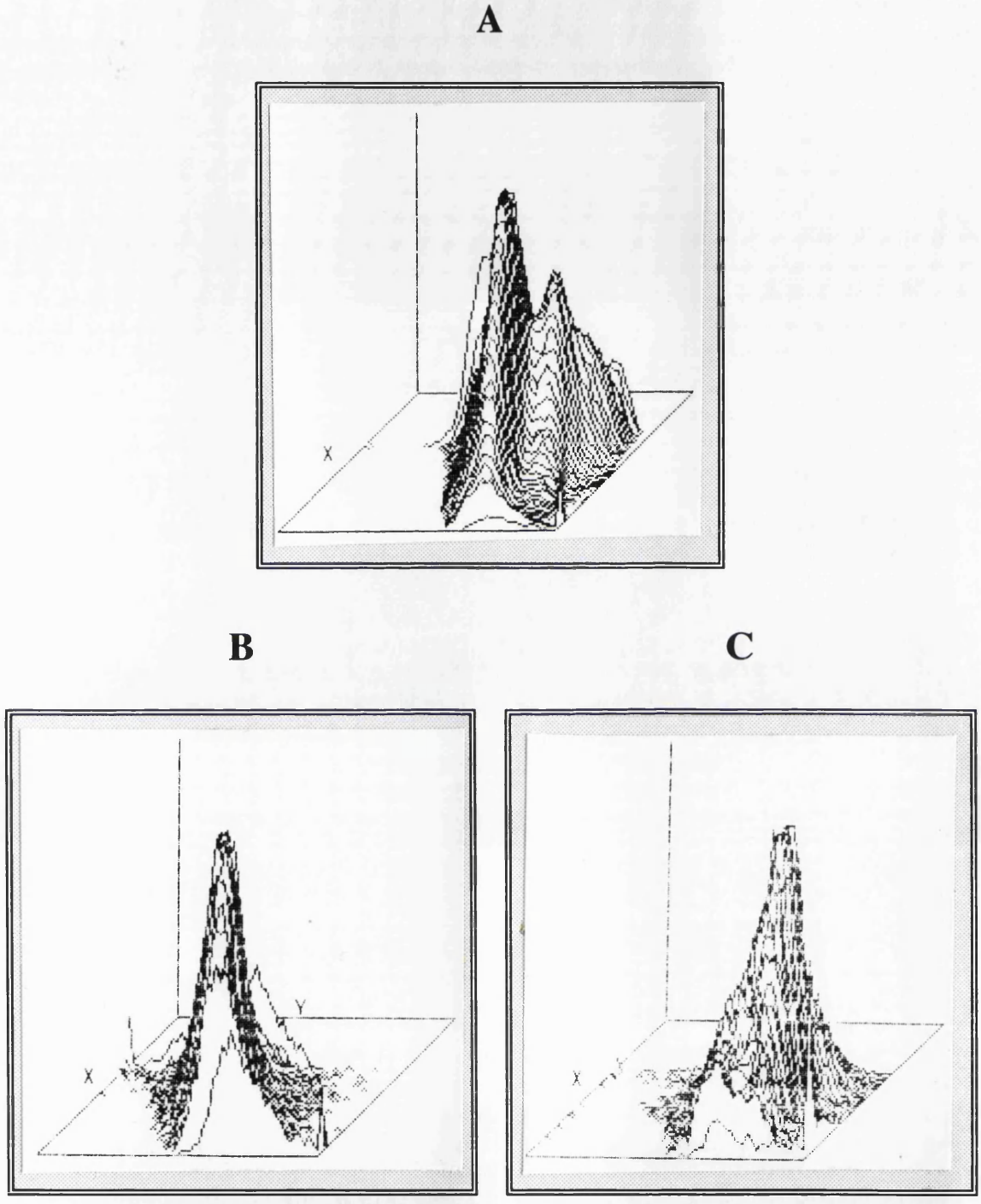



**Figure 3.2:** Three dimensional histograms of PLS (cell granularity) on the X axis, FALS (cell size) on the Y axis and cell number on the Z axis for (A) Control unenriched cells, (B) FACS enriched lactotrophs and (C) FACS enriched somatotrophs. This demonstrates that somatotrophs are the most granular, whilst lactotrophs are bigger but less granular. Debris and red cells have been electronically gated out.

**Legend to Table 3.1**

Purity of sorting of FACS enriched and depleted populations of lactotrophs and somatotrophs, judged by cell content (immunocytochemistry) and secretion (RHPA). Results are expressed as mean $\pm$ SEM, N=5 separate experiments in all cases.

The purity of both the enriched lactotrophs and somatotrophs was 97.2 ± 1.8% as judged by cell-surface staining after sorting and was less than 2 ± 0.9% in the depleted populations. Purity as judged by immunocytochemistry and RHPA was always >95% for each of the cell types in the enriched populations and <2% in the depleted population (Table 3.1). There was little or no loss of cellular viability amongst the sorted cells by trypan blue and propidium iodide exclusion.

*Table 3.1*

| Hormone | Control | PRL enriched | PRL depleted | GH enriched | GH depleted |
|---|---|---|---|---|---|
| | **Cell content (%) assessed by Immunocytochemistry** | | | | |
| PRL | 49 ± 1.4 | 96.7 ± 1.7 | 1.8 ± 0.9 | 1.2 ± 0.5 | 66 ± 1.4 |
| GH | 29 ± 1.7 | 1.3 ± 0.7 | 57 ± 2.3 | 98 ± 1.0 | 0.7 ± 0.2 |
| LH | 7.1 ± 1.0 | 0.8 ± 0.7 | 18.6 ± 1.2 | 0.4 ± 0.1 | 11.1 ± 2.6 |
| | **Cell secretion (%) assessed by RHPA** | | | | |
| PRL | 46 ± 2.3 | 95.8 ± 1.1 | 1.0 ± 0.5 | 1.0 ± 0.5 | 69 ± 1.4 |
| GH | 27 ± 2.0 | 1 ± 0.5 | 60 ± 1.5 | 97 ± 0.8 | 1 ± 0.5 |

### 3.3.3 *Sorting differing intensities of prolactin cell-surface labelling*

Instead of sorting all prolactin surface labelled cells to the left and those with no surface labelling to the right (see above), lactotrophs were sorted on the basis of the intensity of the prolactin cell-surface labelling. Those lactotrophs with the most intense cell-surface prolactin staining (brightest 20%) were sorted to the left and those lactotrophs with a low level of surface staining (20% least bright) were sorted to the right. Sorted cells were cultured overnight and then fixed for immunocytochemical analysis (Table 3.2). Whilst both populations of cells were >95% enriched for intracellular prolactin and depleted of growth hormone and LH, the intensity of intracellular prolactin fluorescence was not significantly different in the brighter cells compared with the less bright population (mean channel number 128 $\pm$ 2 vs 130 $\pm$ 3, $p>0.05$).

A further experiment was then performed to study the content of those cells with little or no prolactin cell surface labelling (ie the "depleted population"). Those cells demonstrating no cell surface labelling, were greatly enriched in growth hormone containing cells, compared to those of intermediate brightness (Table 3.2) despite the fact that both populations were depleted of prolactin. The converse was true for intracellular luteinizing hormone, those prolactin depleted cells with no cell surface labelling contained considerably less luteinizing hormone than those with a low degree of fluorescence.

*Table 3.2*

Comparison of cell content of control (unpurified) lactotrophs compared to those enriched by fluorescence-activated cell sorting (FACS). Cell content of the sorted cells was assessed by immunocytochemistry (IC) for prolactin (PRL), growth hormone (GH) and luteinizing hormone (LH) and analyzed by FACS. Values are means ± S.E.M., n=6 separate experiments in all cases.

| | Intracellular staining (%) | | | | |
|---|---|---|---|---|---|
| **IC** | **Control** | **20% brightest PRL enriched** | **20% least bright PRL enriched** | **20% brightest PRL depleted** | **20% least bright PRL depleted** |
| **PRL** | **49 ± 1.6** | **97.9 ± 1.7** | **95.1 ± 1.6** | **1.9 ± 0.7** | **1.2 ± 0.6** |
| **GH** | **30 ± 1.5** | **1.3 ± 0.7** | **3.9 ± 1.3** | **45 ± 1.8** | **89 ± 2.8** |
| **LH** | **6.8 ± 1.4** | **0.9 ± 0.7** | **1.1 ± 0.6** | **24 ± 1.9** | **4.8 ± 1.0** |

## 3.4 *DISCUSSION*

These findings demonstrate that using prolactin or growth hormone labelled cells, highly enriched and depleted populations of functional lactotrophs and somatotrophs may be obtained using fluorescence activated cell sorting (FACS). Purity was judged not only by cell surface and intra-cellular staining but also by secretion using the reverse haemolytic plaque assay.

The work of St John et al[120] suggested that whilst purification of a sub-population of lactotrophs was possible, the preparation of a lactotroph depleted population was not, since only 50% of lactotrophs had cell surface staining for prolactin. However when the final concentration of prolactin antibody was increased from 1:1000 (which is the concentration used by St John et al) to 1:100 and when used in combination with maximal secretory stimulation using TRH, all lactotrophs demonstrated cell surface staining thus allowing almost complete FACS purification and depletion. When concentrations of 1:100 were used for the antibody directed against growth hormone, with maximal GRF stimulation, then similar purification and depletion was possible (Table 3.1). When comparing the content of cells with differing intensities of prolactin surface labelling (maximally stimulated with TRH for 1 hour during the primary antibody labelling), there was no difference in prolactin content between those cells secreting more prolactin (and therefore having a greater intensity of prolactin cell surface labelling) and those with less surface labelling. This emphasises that hormonal secretion is not directly related to intra-cellular stores of that hormone, indeed newly synthesised prolactin may, under certain circumstances, be released preferentially over older, stored prolactin granules[145,146].

Since lactotrophs represent nearly half of all cells in the adult female rat pituitary, it would have been expected that the percentage of growth hormone containing cells would double in the prolactin depleted population from 28% to 56% and indeed this was the case (Table 3.1). However when stratifying the prolactin depleted population on the basis of the low level of cell surface labelling it became obvious that whilst all the cells were depleted of prolactin the somatotrophs and gonadotrophs were not evenly distributed amongst the remaining cells (Table 3.2). Explanations for this might include a low degree of crossreactivity of the anti-prolactin antibody with cell surface luteinizing hormone but not growth hormone or, perhaps more likely, that gonadotrophs have surface receptors for prolactin. These receptors may bind a low level of secreted prolactin and be labelled by the anti-prolactin antibody, thus having a low intensity of cell surface labelling. Low level prolactin labelling would distinguish the gonadotrophs from the somatotrophs but would not be sufficiently intense to allow them to fall into the "true" prolactin positive cell population. Somatotrophs have no prolactin receptors and thus are non-fluorescent. Support for this theory comes from work by Denef's group[102] who have demonstrated functional communication between gonadotrophs and lactotrophs thus raising the possibility of prolactin surface receptors on gonadotrophs.

The somatotrophs in the prolactin depleted population with no cell surface labelling represent a sub-population of all somatotrophs despite being enriched to 89%, thus necessitating sequential sorting of cells labelled with either anti-prolactin or growth hormone antibodies if complete purity for both cell types is required.

The finding that enriched lactotrophs neither contain, nor secrete growth hormone and similarly that somatotrophs do not contain or secrete prolactin adds weight to the dual colour work (see Chapter 2) that in these preparations the mammosomatotroph (MS) cannot be delineated. However Nikitovitch-Winer et al[53] noted that MS cells were intensely granular and smaller than the mono-hormonal lactotrophs and somatotrophs. It was thus possible that MS cells may have been excluded by the FACS analysis and sorting if they were small enough to fall into population A (see Fig 2.1) and were thus gated out and allowed to go to waste. To this end population A was separated from populations B and C by sorting on the basis of size and granularity alone. Pituitary cells were not demonstrated within population A nor was cell surface labelling detectable for prolactin or growth hormone within this population.

In conclusion almost complete purification and depletion of functional anterior pituitary cell types is possible, using FACS. However to obtain complete purity one needs specific antisera of high titre, used at concentrations which ensure maximal cell surface labelling.

# CHAPTER FOUR:

# MAGNETIC BEAD SEPARATION OF ANTERIOR PITUITARY CELLS

## 4.1 *INTRODUCTION*

Application of the technique of flow cytometry to dispersed live anterior pituitary cells allows analysis and sorting on the basis of cell surface labelling to almost complete purity and depletion. However the capital cost and running expenses of a fluorescence activated cell sorter (FACS) are high and to enrich $5x10^5$ cells takes 2-6 hours of continuous sorting, depending on the percentage cell surface labelling. Thus FACS sorting is costly, labour intensive and slow to generate large numbers of enriched or depleted cells. To overcome the problem 1-5 micron diameter magnetic beads have been manufactured which have high stability, high uniformity, unique paramagnetic properties, low particle-particle interaction, and high dispersability[147]. Different reactive groups: hydroxyl, carboxyl and amino groups allow attachment of anti-IgG antibodies or proteins A or G[147]. These beads are incubated with primary antibody coated cells and then separated from the unbound cells by a strong magnetic field. This technique has the advantages of being fast and large numbers of cells may be treated at once. The viability of the cells does not seem to be affected by the process and the beads drop off the cell surface after 24 hours in culture without harming the cells. This technique is in routine use in immunology and haematology[148-150].

Chapters 2 and 3 propose, that as the secretory granules of anterior pituitary cells fuse with the cell surface there would appear to be sufficient hormone on the cell surface at that time to be labelled by polyclonal anti-hormone antibodies and thus analyzed and separated by flow cytometry. Magnetic bead separation has therefore been applied to these labelled pituitary cells and compared to the purity and depletion obtained by FACS enrichment.

## 4.2 *MATERIALS AND METHODS*

For buffers, reagents, preparation of cell suspension, immunological labelling, FACS analysis and immunocytochemistry, please see chapter 2.

### 4.2.1 *Bead Separation*

After primary antibody labelling with either the NHPP rabbit anti-rat prolactin, the sheep anti-rat prolactin or the sheep anti-rat growth hormone, cells were washed by centrifugation, three times in Buffer B (pre-equilibrated to 4°C). Two million labelled cells (at different times experiments were performed using 0.5 - 5 million cells) were then incubated for 30 minutes at 4°C with 100$\mu$l of the beads using either Dynal (DY) beads (Dynal - Oslo, Norway) or Advanced Magnetics (AM) beads (Advanced Magnetics - Cambridge, MA, USA) coated with anti-rabbit or sheep IgG (conjugated by the manufacturers) in 100$\mu$l of Buffer B to allow binding of the beads to the cells.

The suspension of cells and beads were then gently layered onto 10 mls of Buffer B in a 15 ml conical tube whilst in the magnetic separator (Dynal Oslo, Norway) and allowed to stand for 5 minutes at room temperature. During this time cells bound to the magnetic beads were attracted to the side of the tube, whilst unbound cells remained in suspension. The unbound cells were then gently removed by pipetting and a further 10 mls of Buffer B added to the cells adherent to the side of the tube. These purified cells ie those with cell surface primary antibody, which had bound the IgG coated beads and hence been attracted in the magnetic field to the side of the conical tube, were at that point, either agitated, using a mechanical vortex machine

for 2-3 minutes to remove the beads from the cells, or placed in a petri-dish overnight (in 10 mls of culture medium at 37°C) to allow the beads to drop off as the secreted hormone/antibody/bead complex became detached from the cells.

## 4.3 *RESULTS*

### 4.3.1 *Controls*

Pre-incubation of all the antisera used for cell surface labelling (see methods) with 50$\mu$g of the homologous hormone completely abolished cell surface labelling and thus abolished binding of the beads to the cells. Incubation with other anterior pituitary hormones did not affect labelling. In particular the rPRL and rGH antisera used (supplied by Dr D Flint and the NHPP) demonstrated less than 1% cross-reactivity with rGH and rPRL respectively even at the final dilution of 1/100.

Similarly, absence of primary antibody or substitution with 1/100 non-immune rabbit or sheep serum (as appropriate) completely abolished cell surface labelling and subsequent binding of the beads, as assessed by light microscopy of at least 10 high power fields.

### 4.3.2 *Analysis of cell purity and depletion*

Cell recovery after antibody labelling and separation using the magnetic beads demonstrated a yield of 94 $\pm$ 4.4% within the range of 0.5 - 5 million cells separated within one conical tube.

*Table 4.1*

Purity and depletion of other cell types of magnetic bead purified lactotrophs and somatotrophs as assessed by immunocytochemistry (IC) and analyzed by FACS. DY denotes Dynal beads and AM those manufactured by Advanced Magnetics. N=5 separate experiments in all cases.

| IC | Control | PRL: DY enriched | PRL:DY depleted | GH: DY enriched | GH:DY depleted |
|---|---|---|---|---|---|
| PRL | 49 ± 1.6% | 88 ± 2.2% | 31 ± 1.7% | 2.8 ± 0.7% | 67 ± 2.6% |
| GH | 30 ± 1.5% | 3 ± 0.5% | 38 ± 1.3% | 87 ± 1.9% | 20 ± 1.9% |
| | | PRL: AM enriched | PRL:AM depleted | GH: AM enriched | GH: AM depleted |
| PRL | | 86 ± 2.3% | 35 ± 1.5% | 4.2 ± 1.1% | 65 ± 2.4% |
| GH | | 7 ± 0.9% | 39 ± 1.5% | 88 ± 1.9% | 21 ± 1.6% |

Table 4.1 shows that both DY and AM beads are equally effective at separation, giving a purity of 86-88%, but neither bead type gave good depletion, the DY bead being slightly better than the AM bead. Using higher bead:cell ratios (up to a maximum of 500:1) did not improve the purity or depletion for either bead type. Immunocytochemistry results are expressed as the percentage of positive cells in each sample, analyzed on the FACS, for either prolactin or growth hormone. Cells labelled with primary antibody and incubated with the magnetic beads (see methods) but not separated in the magnetic field (ie unpurified) were used as controls.

To check the viability of magnetic bead separated cells, prolactin or growth hormone labelled cells were mixed with either bead type, separated and then plated in petri-dishes over night. By the next morning no beads were adherent to the cell surface by examination under light microscopy of at least 10 high power fields. There was no decrease in viability (>96%) in the purified cells compared to controls as assessed by trypan blue exclusion. Cells which were purified and then vigorously mixed to remove the beads also showed no loss of viability when cultured overnight.

### 4.3.3 *Removal of beads*

Enriched cells were vigorously mixed to remove the beads from the cell surface to attempt to characterise where the "break" occurred. After mixing, cells were washed and then labelled with appropriate FITC conjugated antibody. No labelling was visible under fluorescence microscopy or using FACS analysis implying the break was not at the primary antibody/anti-IgG complex. To test whether the hormone was pulled from the cell surface or whether the anti-hormone antibody was pulled off the

hormone, the purified cells, after mixing, were immediately reincubated in fresh primary antibody in the presence of TRH or GRF or in the absence of the appropriate stimulatory hormone but in the presence of 100 nmol dopamine or 100 nmol somatostatin and then incubated with FITC conjugated second antibody. Results (Table 4.2) show that reincubation in the presence of the inhibitory factors, to stop the release and hence labelling of further hormone, almost completely abolishes cell surface labelling. Reincubation without inhibitory factors (but in the presence of TRH or GRF) demonstrates that approximately half the lactotrophs and somatotrophs re-release prolactin or growth hormone during the one hour addition of the anti-hormone antibody and thus once again demonstrate cell surface labelling.

*Table 4.2*

Cells separated by magnetic bead separation, vigorously mixed to remove the beads and then reincubated with anti-prolactin or anti-growth hormone antibodies in the presence or absence of 100 nmol TRH (thyrotropin releasing hormone), 100 nmol GRF (growth hormone releasing factor) 1 $\mu$mol DA (dopamine) or 100 nmol SRIF (somatostatin). N=5 separate experiments in all cases and figures in brackets represent SEMs.

| **Cell surface labelling** | **Control** | **Incubation + TRH or GRF** | **Incubation + DA or SRIF** |
|---|---|---|---|
| **PRL** | **49 ± 1.3%** | **41 ± 1.2%** | **4 ± 0.9%** |
| **GH** | **29 ± 1.8%** | **45 ± 1.9%** | **2.5 ± 0.4%** |

### 4.3.4 *Validation of the two prolactin antibodies*

Simultaneously labelled cells with both the rabbit and sheep anti-prolactin antibodies (in the presence of 100 nmol TRH) which were subsequently labelled with appropriate FITC conjugated second antibodies, showed that both antibodies labelled approximately 48 ± 1.3% of all pituitary cells and that these populations of cells were identical in size and granularity when gating on fluorescence was performed. When both second antibodies were simultaneously added, 50 ± 1.4% labelling was obtained. To confirm that all prolactin cells were being simultaneously labelled with both antibodies the rabbit anti-prolactin antibody was labelled with second antibody conjugated to phycoerythrin and the sheep anti-prolactin with the FITC conjugated second antibody. As expected 50 ± 1.8% of all cells (i.e. all lactotrophs) simultaneously labelled with both fluorescent dyes when dual colour FACS analysis was performed. Cross reactivity between the two antisera was judged to be greater than 95%.

### 4.3.5 *Bead purification removes prolactin cell surface labelling*

Cells were simultaneously labelled with both prolactin antibodies (in the presence of 100 nmol TRH), as above and were then separated using the DY anti-rabbit IgG beads. The depleted and purified cells were then vigorously mixed to remove the beads, washed and then incubated with FITC conjugated anti-sheep IgG antibody (see Figure 4.1). No labelling was obtained in the purified population and 32 ± 1.0% in the depleted population whilst labelling of control cells i.e. as above but without the addition of the beads, was 49 ± 1.2%. If these purified cells with no surface

labelling were allowed to recover in culture medium for one hour at 37°C and then relabelled with sheep anti-prolactin, in the presence of 100 nmol TRH (see methods) and then FITC conjugated anti-sheep IgG antibody, 78 ± 2.3% labelling was obtained, indicating that most lactotrophs had secreted further prolactin within the one hour recovery period (since the maximum labelling expected would be 88% - see Table 4.2).

To confirm that the removal of all cell surface prolactin was specific rather than some shearing effect of the beads on all surface determinants, a further experiment was undertaken in a similar manner to the above. Instead of simultaneously labelling with both prolactin antibodies, cells were co-incubated with the rabbit anti-prolactin and sheep anti-growth hormone antibodies (in the presence of 100 nmol TRH and GRF). After beading with the DY anti-rabbit (to remove cell surface prolactin) FITC conjugated anti-sheep IgG was added to label the growth hormone. As expected labelling was reduced from 28 ± 0.8% in the control to 3 ± 0.3% in the prolactin purified cells and increased in the depleted population, but only to 38 ± 1.1%, confirming the lack of complete prolactin depletion by beading. Thus removal of the prolactin labelled with the rabbit anti-prolactin antibody abolishes all prolactin labelling but does not affect growth hormone labelling.

## 4.4 *DISCUSSION*

Flow cytometric analysis and sorting allows simultaneous purification and depletion of differing anterior pituitary cell types (see chapters 2 and 3). This is a slow and labour intensive process but has the advantage of providing high purity of the entire cell type, and much additional information on the morphological characteristics of the separated cells. Whilst the degree of purity obtained by sedimentation at unit gravity or centrifugal elutriation is similar to that obtained by FACS sorting; in contrast neither centrifugal nor unit gravity sedimentation purify the complete population and thus only separate a sub-population of cells.

It was expected that the use of magnetic beads would not only be quick and cheap but give very similar results to that obtained by FACS sorting[151,152]. Whilst the degree of purification was similar for beads and FACS (88% vs 96-97% for prolactin and 87% vs 97-98% for growth hormone) the results for depletion using magnetic bead separation were disappointing (Table 4.1), only 30-40% of lactotrophs or somatotrophs may be purified and thus only a sub-population of cells may be obtained by this method. Such results are explicable on the basis of the sensitivity of the two techniques. At a low level of cell surface antigen expression and therefore low primary antibody labelling, the voltage and therefore the sensitivity of the green fluorescence photomultiplier tube in the FACS may be increased but with the beads no such compensation is possible. Thus those cells expressing low levels of prolactin and growth hormone (there is a large heterogeneity in the amount of expressed cell surface prolactin and growth hormone, see chapter 2) may be detected and sorted into the purified population using the FACS whilst the bead technique "misses" those cells

with a low level of surface hormone expression hence explaining the poor depletion results. The two prolactin antibodies available, even when used at a final concentration of 1/50 (data not shown) were probably not of sufficient titre to label all the cell surface hormone to a sufficiently high level to be detected by the beads and thus ensure complete depletion.

After incubation overnight of FACS sorted cells less than 2% cell surface labelling was detected, in what was a >97% pure population. The same finding appeared to be true for beading, since no beads remain attached to the cell surface after overnight incubation. If however the cells were vigorously mixed immediately after separation in the magnetic field the beads were pulled off the cell surface and a number of possibilities existed as to where the "break" occurred. These results suggest that the weakest point of the cell/hormone/antibody/bead complex may be the bond between the hormone and the cell. Yet tearing the hormone from the cell surface did not seem to affect cellular viability either in the short term or after 24 hours in culture.

Removal of those prolactin molecules bound to the rabbit anti-prolactin antibody and hence to the bead also removed those molecules bound to the sheep anti-prolactin. To explain this one must postulate some intimate structural relationship between the two groups of prolactin molecules. Whilst it is possible that the two anti-prolactin antisera used bind to two separate epitopes on the same prolactin molecules (which the cross-reactivity studies indicate is not the case), an alternative hypothesis is that during exocytosis, the core of the hormone granule is transiently trapped in the lipid bi-layer of the surface membrane and it is at this point that the intact hormone core is labelled with the anti-hormone antisera. Removal of the trapped prolactin core by the bead

separation technique would simultaneously remove the prolactin bound to the sheep anti-prolactin antibody, thus explaining the complete abolition of surface labelling after magnetic bead separation. It is not possible at present, to state whether this phenomenon represents *in vivo* conditions or may be due to the primary antibody incubation conditions at 4°C causing trapping of the partially secreted granule in the cell membrane.

In conclusion, magnetic beads may be used to purify pituitary cell types but gives inferior results to FACS sorting. The purification process takes about 30 minutes (compared to 2-6 hours for FACS sorting) to perform, once primary antibody labelling has been performed and allows at least 5 million cells to be treated in one tube. In contrast to FACS sorting, depletion by bead separation was incomplete, thus only a sub-population of cells appeared to be purified.

# CHAPTER FIVE:

# GALANIN IS A PITUITARY AUTOCRINE REGULATOR OF PROLACTIN RELEASE

## *5.1 INTRODUCTION*

Having achieved the first objective of this thesis in chapters 2 - 4, that of enriching functional lactotrophs from dispersed rat anterior pituitary cells, the secretory characteristics of these cells were investigated. I wished to answer one of the fundamental question relating to the pituitary - "do paracrine interactions regulate lactotroph function and are they of importance in patho-physiological states?"

The mechanisms that regulate prolactin secretion from the anterior pituitary are complex. TRH and dopamine are the only factors that have clearly been demonstrated to have a physiological role in the regulation of prolactin release. Vasoactive intestinal polypeptide (VIP) and more recently, galanin, a 29 amino acid peptide, have been shown to be synthesised and stored by the anterior pituitary[79,80,153,154] and to release prolactin in a number of *in-vivo* and *in-vitro* systems[155-157]. The cellular localisation of VIP is still unclear but present data would suggest it is not a lactotroph derived peptide[78,93,158]. Galanin, is localised to the lactotroph, somatotroph and thyrotroph in female rats[158] and by double immuno-gold labelling to be co-stored with prolactin in the same secretory granule[85]. Galanin release from dispersed, hyperoestrogenised anterior pituitary cells is stimulated by TRH and inhibited by dopamine and somatostatin[159], these results parallel prolactin release.

Both VIP and galanin are exquisitely sensitive to the oestrogen status of the animal, exposure to 17$\beta$ oestradiol for 14 days causes up to a 4000 fold increase in message levels[73,79] whilst peptide content in the anterior pituitary rises by up to 500 fold[79,160]. *In-situ* hybridisation and immunocytochemistry have shown that these changes occur

almost exclusively in the lactotroph for galanin[158] but in another cell type, possibly the folliculo-stellate cell, for VIP[158]. Ovariectomy markedly decreases pituitary galanin and VIP synthesis and content[86,158] and abolishes galanin staining in the lactotroph[85]. Significant changes in pituitary galanin synthesis and content are noted throughout the oestrous cycle and parallel changes in plasma oestrogen levels[161]. Most recently, wide fluctuations in pituitary galanin gene expression have been noted during pregnancy, with an 8-fold stimulation of expression induced by suckling[162].

In this chapter a quantitative galanin cell blot assay is described, thus allowing the study of galanin release from single cells. In the intact female rat, galanin is secreted by a small minority (8%) of lactotrophs and by no other cell type. Galanin secreting lactotrophs have been greatly enriched, by fluorescence activated cell sorting, allowing the study of their role in prolactin release. These data demonstrate that the galanin secretors regulate the prolactin secretion of the remaining lactotrophs in an autocrine fashion. VIP stimulation of prolactin is a paracrine event and acts via galanin release. The actions of TRH are mediated in part by galanin and thus may be termed galanin dependent.

## *5.2 MATERIALS AND METHODS*

For pituitary dispersion, FACS analysis and enrichment and prolactin RHPA please see chapters 2 and 3.

### *5.2.1 Endocrine manipulation of oestrogen status*

Surgical ovariectomy was carried out 3 weeks prior to sacrifice; sham operated litter mates were used as controls.

In a separate set of experiments, hyperoestrogenisation was induced for a 3 week period, as previously described[86], by the subcutaneous injection of 2mg of 17 $\beta$ oestradiol (Sigma) in 100 $\mu$l linseed oil on day 1 followed by an identical injection 10 days later; litter mates injected on both occasions with linseed oil alone were used as controls. The animals were sacrificed at 21 days ie 11 days after the second injection.

Once the 2 experimental groups (with their appropriate controls) were sacrificed, the pituitaries were dispersed and lactotrophs enriched by FACS. In order to maintain the *in-vivo* endocrine environment in culture, FACS enriched lactotrophs were maintained in homologous serum culture medium at 37°C for the 48 hours prior to prolactin RHPA. Homologous serum culture media was prepared using the method described by Lam et al[107]: serum from each animal was heat inactivated for 60 minutes at 57°C, passed through a 1 $\mu$ filter and then diluted to a 10% solution in the identical reagents to the culture medium described in chapter 2. Foetal calf serum was still added to the media but at the reduced amount of 2.5%

*5.2.3 Peptides*

Porcine galanin and porcine VIP was used throughout these experiments, TRH as described in Chapter 2.

*5.2.4 Satistics*

All results presented in this chapter are expressed as mean $\pm$ SEM, N=5 separate experiments in all cases.

*5.2.2 Measurement of galanin release by cell blot assay*

The method of Kendall and Hymer was used[163]. Dispersed cells were briefly trypsinized (0.0125% v/v) for 5 minutes on the day of use to remove the cells from the culture plates. The cells were allowed to recover in culture medium for a further 2 h before use. Chambers were constructed using microscope slides, a 2x2 cm squares of polyvinylidene difluoride transfer membrane (Imobilon PVDF, Millipore), double-sided adhesive tape and coverslips. Membrane was pre-wetted in methanol for 10 seconds and then rinsed with distilled water for 5 minutes and then equilibrated with DMEM for 1 hour before use. 100 $\mu$l of DMEM containing $10^4$ dispersed cells were added to the chambers and then incubated for 6 hours at 37°C. The transfer membranes were then incubated for 30 minutes at room temperature with 0.15% phenylhydrazine hydrochloride (Sigma) in PBS (to block endogenous peroxidase activity), followed by 1 hour at room temperature in PBS containing 1% BSA to block any unoccupied binding sites. After the blocking procedure, galanin bound to the membrane was immunostained with: 1) an antiserum raised in rabbits against rat galanin used at a final dilution of 1:1,000[158] for 18 hours at 4°C; 2) goat anti-rabbit immunoglobulin conjugated to horse-radish peroxidase (HRP, Sigma) final dilution 1:1,000 for 1 hour at room temperature; 3) donkey peroxidase-antiperoxidase (PAP, Sigma) 1:2,000 for 1 hour at room temperature; 4) Substrate, 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) 0.05% in 0.01M citrate buffer, pH 5.2, initiated with 3% hydrogen peroxide for 30 mins at room temperature. The resulting area and density of the colour reaction was quantified with a light microscope (Olympus) and the Cue-2 image analysis system. Results are expressed as mean $\pm$ SEM, n=5 in all cases.

*5.3 RESULTS*

*5.3.1. Dispersed, unenriched cells*

Using the cell blot assay 4.6 ± 0.3% of all dispersed anterior pituitary cells were shown to be galanin secretors. TRH and VIP significantly stimulated galanin release ($p < 0.01$), paralleling prolactin secretion (Fig 5.1).

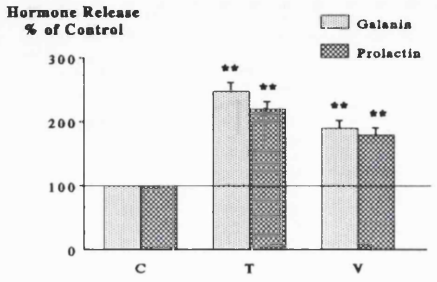



**Figure 5.1**

Using antisera raised against VIP ($V_{as}$) and galanin ($G_{as}$), immunoneutralisation studies were performed. Results demonstrate that both antisera inhibit basal prolactin secretion, $G_{as}$ being more potent than $V_{as}$, 88 ± 5.1% vs 55 ± 2.7% inhibition, (fig 5.2a). Controls included the substitution of the antisera with (a) 1/100 non-immune rabbit serum and (b) eight other antisera at the same dilution raised in rabbits against glucagon or insulin and (c) antisera were pre-incubated for 24 hours at 4°C with 25 $\mu$g of VIP or porcine galanin. All controls failed to demonstrate an inhibitory effect on prolactin release.

Both exogenously added VIP and galanin stimulated prolactin secretion to the same extent (Fig 5.2b). Galanin and VIP were not additive in stimulating prolactin release, whilst the response to galanin was additive with that of TRH (Fig 5.2b) at the concentrations used, whilst VIP did not enhance the effect of TRH on prolactin release. The $G_{as}$ completely abolished VIP stimulated prolactin release, but $V_{as}$ did not modulate galanin stimulated prolactin release. Given the decrease in basal prolactin release caused by $V_{as}$ or $G_{as}$ the subsequent response to the addition of TRH in combination with either antisera remained robust, implying a direct effect of TRH on prolactin secretion (Fig 5.2a).

A

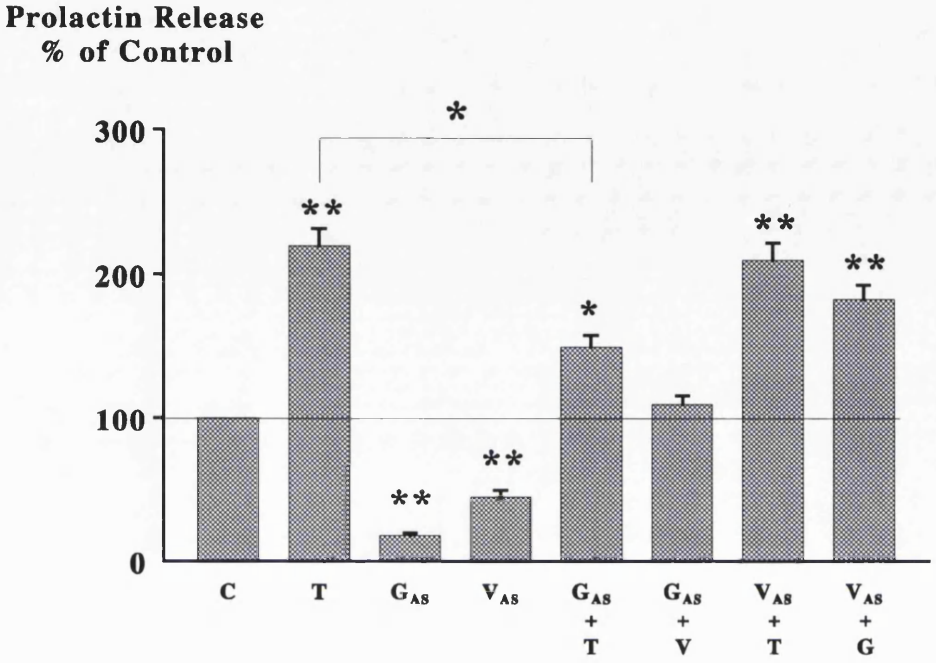
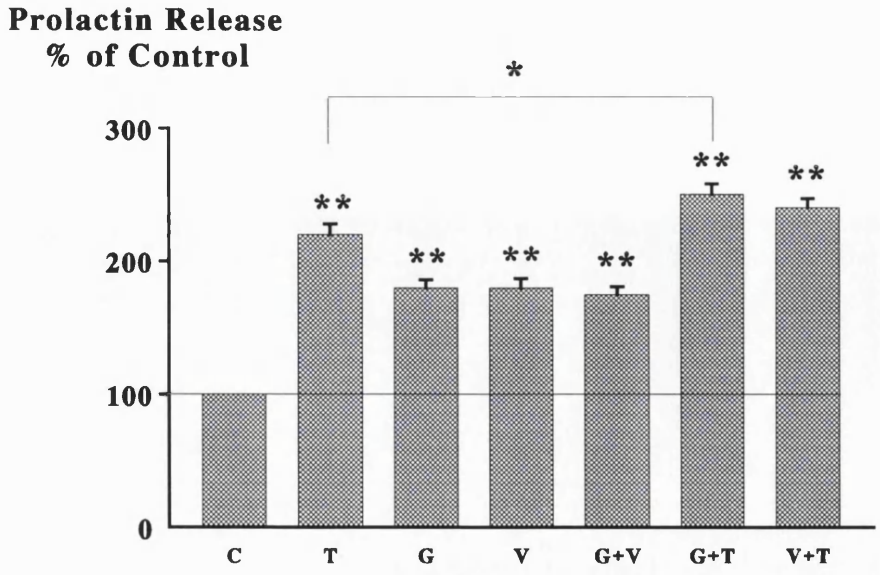



**Figure 5.2:** Prolactin secretion expressed as a percentage of basal secretion in response to thyroliberin (T), vasoactive intestinal polypeptide (V), galanin (G), the VIP antiserum ($V_{as}$), the galanin antiserum ($G_{as}$) or in combination. All peptides were used at a concentration of 1 nM. **, $P<0.01$; and *, $P<0.05$.




B

**Addendum to 5.3.2**

Basal prolactin release as assessed by mean plaque was lower in FACS enriched lactotrophs than in the unenriched mixed population (as indeed is the case for the basal release of the non-galanin secreting lactotrophs compared to the galanin secreting lactotrophs - see page 93). In both cases data is presented graphically, normalising all secretions to the base line for that particular group of FACS enriched cells, rather than try and compare all groups to the starting (ie mixed) population, which makes the graphical representation difficult to interpret. The way the data is presented however, does not affect the subsequent statistical analysis or interpretation of the results.

*5.3.2 FACS enriched and depleted lactotrophs*

Galanin secreting cells represented 8.8 ± 0.9% of all lactotrophs, no galanin secretors were identified in the lactotroph depleted population. Lactotroph sensitivity to TRH, galanin or $G_{as}$ was unchanged in the enriched lactotrophs. In contrast, the lactotrophs were supersensitive to the addition of VIP and inhibition of basal prolactin release by $V_{as}$ was now abolished (Fig 5.3). Basal prolactin plaque area was significantly reduced by FACS enrichment when compared to the unenriched dispersed cell population (0.049 ± 0.006$\mu m^2$ vs 0.07 ± 0.007$\mu m^2$, $p<0.05$).

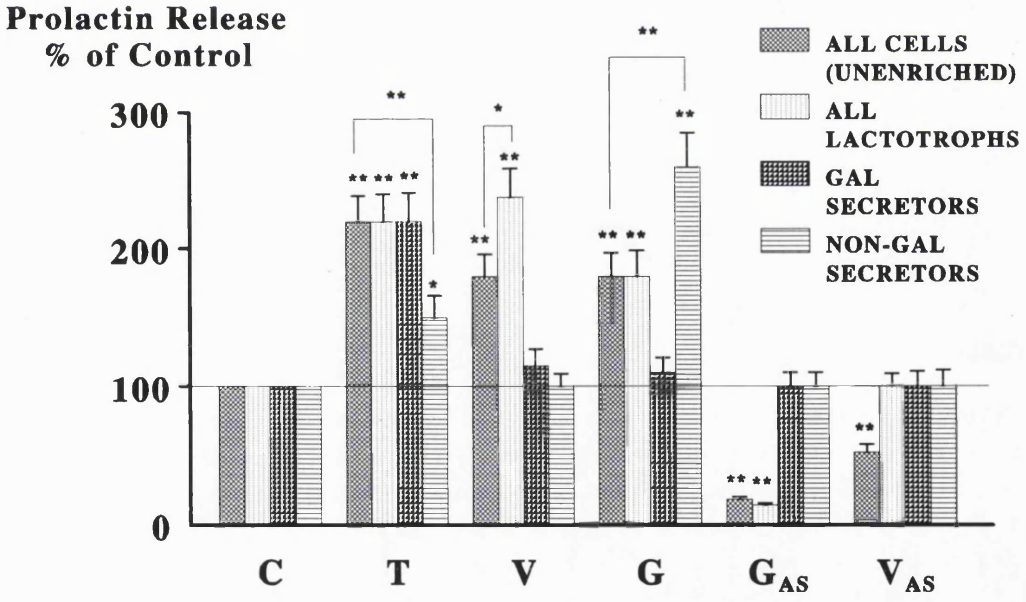



**Figure 5.3:** Prolactin secretion from unenriched cells, FACS enriched lactotrophs and sub-sets of lactotrophs (see text for details) was measured and expressed as a percentage of basal secretion in response to thyroliberin (T), vasoactive intestinal polypeptide (V), galanin (G), the vasoactive intestinal polypeptide antiserum ($V_{as}$) or the galanin antiserum ($G_{as}$). All peptides were used at a concentration of 1 nM. **, $P<0.01$; and *, $P<0.05$.

*5.3.3 FACS enrichment of galanin secretors*

Lactotrophs were stratified by the amounts of cell surface prolactin expressed and sorted in aggregates of 10%. The 10% of lactotrophs expressing the least surface prolactin were 88 $\pm$ 7.4% galanin secretors. Basal galanin blot area was significantly lower than in the unenriched cells (0.007 $\pm$ 0.001$\mu m^2$ vs 0.011 $\pm$ 0.008$\mu m^2$, $p>0.05$), mirroring the decrease in prolactin plaque area. The remaining 90% of lactotrophs, expressing greater amounts of surface prolactin, were galanin non-secretors. Galanin and non-galanin secreting lactotrophs were separated by FACS. The galanin secretors retained a normal response to TRH but were unresponsive to the addition of galanin, $G_{as}$, $V_{as}$ or VIP. VIP stimulates galanin secretion from the galanin secreting lactotrophs, causing an increase in galanin blot area by 1.95 $\pm$ 0.23 fold compared to unstimulated galanin secretors ($p<0.01$), but does not directly increase prolactin release.

Basal prolactin release from the non-galanin secretors was significantly lower than the galanin secretors, 0.028 $\pm$ 0.003$\mu m^2$ vs 0.049 $\pm$ 0.006$\mu m^2$, $p<0.01$. The non-galanin secreting lactotrophs were supersensitive to the addition of galanin and unresponsive to VIP, $V_{as}$ and $G_{as}$. Response to TRH stimulation was diminished to the same extent as that observed in the unenriched dispersed cells, when stimulated with TRH in the presence of $V_{as}$ (Fig 5.2a).

*5.3.4 Oestrogen manipulation*

Dispersion of individual pituitaries yielded 2.6 $\pm$ 0.32x$10^6$ cells/pituitary for both control groups, 9.8 $\pm$ 0.79x$10^6$ cells/pituitary for the hyperoestrogenised animals and 2.1 $\pm$ 0.21x$10^6$ cells/pituitary for the ovariectomised animals. All dispersed cells had a viability of >98%.

FACS analysis demonstrated that hyperoestrogenisation decreased growth hormone labelling with a concurrent decrease in PLS signal (indicative of cell granularity) mean channel number of 132 $\pm$ 14 vs 148 $\pm$ 15 $p<0.05$, whilst prolactin labelling was significantly increased as compared to controls (68 $\pm$ 3.2% vs 49 $\pm$ 2.8% $p<0.01$). It was also noted that the FALS (indicative of cell size) of the lactotrophs was 40% greater in the hyperoestrogenised animals when compared to controls (FALS mean channel number 200 $\pm$ 25 vs 149 $\pm$ 22 $p<0.01$). Ovariectomy did not change growth hormone surface labelling or cell size, however prolactin surface labelling was significantly reduced to 37 $\pm$ 2.9% compared to controls ($P<0.01$)

Ovariectomy reduced the number of galanin secreting cells to below the detectable limit of the blot assay, whilst hyperoestrogenisation for the same period of time increased the number of secretors to 39 $\pm$ 2.4% of all lactotrophs. Mean galanin blot area was unchanged in the hyperoestrogenised state compared to controls (0.011 $\pm$ 0.002$\mu m^2$ vs 0.013 $\pm$ 0.003$\mu m^2$, $p>0.05$). Ovariectomy reduced both the number of lactotrophs compared to the control group (37 $\pm$ 2.8% vs 49 $\pm$ 3.7%, $p<0.01$) and basal prolactin secretion to 28 $\pm$ 3.2% of control cells, $p<0.01$. The decrease in basal prolactin release is similar to that noted in the eugonadal non-galanin

secreting lactotrophs when compared to unenriched cells. Ovariectomised lactotrophs demonstrated a decreased responsiveness to the addition of VIP, TRH, $G_{as}$ and $V_{as}$ (Fig 5.4). Sensitivity to the addition of exogenous galanin was increased, as with the non-galanin secretors in the control group of animals. Hyperoestrogenisation significantly increased the number of lactotrophs compared to the control group (68 $\pm$ 5.9% vs 49 $\pm$ 3.7%, $p<0.01$) and basal prolactin release by 2.4 $\pm$ 0.18 fold ($p<0.01$). Oestrogen treatment decreased the response to the addition of TRH, VIP and galanin. Hyperoestrogenised lactotrophs were unresponsive to the addition of $V_{as}$ but exquisitely sensitive to $G_{as}$ decreasing prolactin release to a significantly greater extent ($p<0.01$) than that observed in eugonadal lactotrophs (Fig 4) indicating that in states of high oestrogen exposure basal prolactin secretion is almost completely galanin dependent.

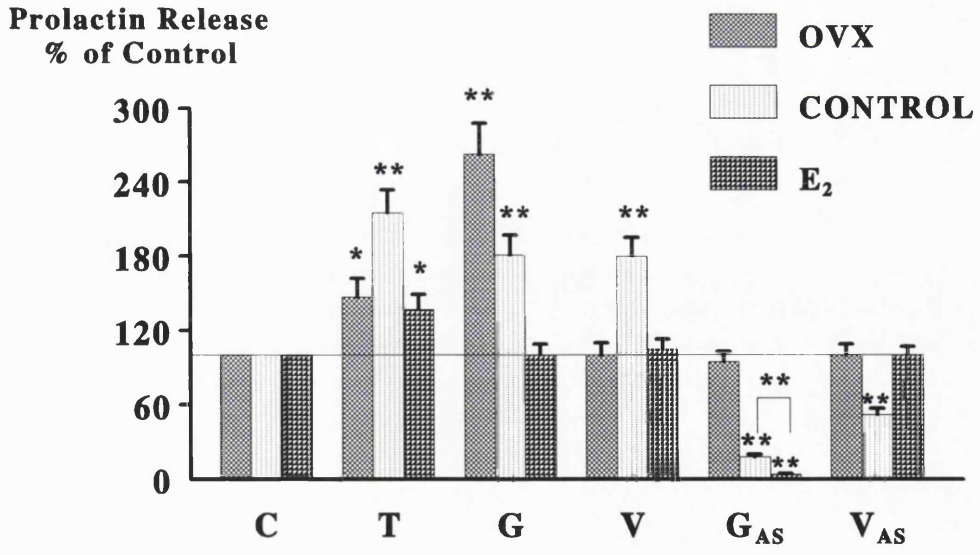



**Figure 5.4:** Effects of ovariectomy (OVX) or hyperoestrogenisation ($E_2$). Prolactin secretion was measured and expressed as a percentage of basal secretion in response to thyroliberin (T), vasoactive intestinal polypeptide (V), galanin (G), the VIP antiserum ($V_{as}$) or the galanin antiserum ($G_{as}$). All peptides were used at a concentration of 1 nM. **, $P<0.01$; and *, $P<0.05$.

## 5.4 *DISCUSSION*

Galanin is a 29-amino acid peptide originally isolated from porcine intestine[164] and subsequently reported to be widely distributed in many tissues[165,166]. Galanin immunoreactivity is abundant in the central and peripheral nervous systems, highest levels of both mRNA and peptide content being found in the hypothalamus and anterior pituitary[79,167]. Galanin has been shown by both immunocytochemistry and *in-situ* hybridisation to colocalise to the lactotroph and to a lesser extent to the somatotroph in male and female rats[73,79,160] and has been demonstrated, by electron microscopy, to be stored in the prolactin secretory granule[85].

These studies demonstrate that galanin stimulates prolactin release and that immunoneutralisation of locally secreted galanin inhibits prolactin release. These effects are unchanged when FACS enriched lactotrophs are studied thus indicating that galanin is acting in an autocrine rather than paracrine manner. The newly raised galanin cell blot assay allows the study of galanin release from single cells and confirms that galanin is secreted by a minority (some 9%) of lactotrophs and by no other cell type. Galanin secretion is stimulated by TRH and VIP and inhibited by dopamine (data not shown), release is paralleled by prolactin secretion. The ability to enrich and separate galanin and non-galanin secreting lactotrophs by FACS has greatly aided understanding of the interplay between the two sub-populations of lactotrophs. The galanin secreting lactotrophs are, as might be expected, unresponsive to the addition of exogenous galanin with no additional increase in prolactin release. Similarly, prolactin secretion from the galanin secretors is not inhibited by the $G_{as}$, demonstrating that whilst these cells produce galanin they are not dependent upon it

for their basal prolactin release. In contrast, the non-galanin secreting lactotrophs are almost totally dependent on galanin for their basal prolactin release and deprivation of galanin for the 48 hours between FACS enrichment and the RHPA causes an upregulation of the response to the addition of galanin such that the incremental prolactin release rises from 1.8 fold in the unenriched dispersed cells and FACS enriched lactotrophs (all lactotrophs ie a mixture of the galanin and non-galanin secretors) to over 3 fold in the galanin non-secretors.

As with galanin, VIP stimulated prolactin release by 1.8 fold. There was no additive effects of the simultaneous addition of both peptides. $V_{as}$ also inhibits basal prolactin release as previously noted by a number of groups[106,107,168] but is less potent than the $G_{as}$ when used at the same dilution. The demonstration that the $V_{as}$ has no effect on basal prolactin release in FACS enriched lactotrophs (either the entire population or the two sub-populations studied) further strengthens the immunocytochemistry data[78,93,158,169] that VIP is not a lactotroph derived peptide and is thus acting in a paracrine fashion. Similar to the upregulation of galanin response in the non-galanin secretors, the entire lactotroph population when deprived of VIP for 48 hours is hyper-responsive in its prolactin release. Neither the galanin or non-galanin secreting enriched lactotrophs responded by increasing prolactin release when stimulated with VIP. From these data VIP would appear not to be directly stimulating prolactin release but to be acting solely on the galanin secreting lactotrophs to increase their release of galanin which in turn increases prolactin release from the non-galanin secreting lactotrophs. This proposed mechanism would explain the delay observed by a number of groups in the action of VIP (45 - 60 secs) when compared to the first phase of TRH (4 - 6 secs) stimulation of prolactin release[170-172]. Since basal prolactin

and galanin release is decreased in the FACS enriched lactotrophs, VIP would appear to be acting as a tonic primer of prolactin release, and whilst not essential to the function of the lactotroph (since responsiveness to galanin and TRH is maintained in the enriched lactotrophs) may be necessary as a modulator of prolactin responsiveness. VIP may be part of a "cascade" process which augments small incremental changes in peptide levels (eg to interleukins and cytokines), coordinating prolactin release in times of infection and stress, when serum prolactin levels are elevated[173-176].

Galanin would also seem to mediate, at least in part, the actions of TRH. TRH stimulation of prolactin is maintained in FACS enriched lactotrophs, but was diminished in non-galanin secretors, reflecting the absence of galanin for the 48 hours between FACS enrichment and RHPA. TRH stimulation of prolactin has been demonstrated, in a number of different experimental paradigms, to have a biphasic response[177,178]. It is possible therefore, that the late or delayed phase represents stimulation of galanin secretion which acts on the non-galanin secreting lactotrophs to stimulate prolactin release.

In order to study the effects of oestrogen status on galanin regulation of lactotroph function, endocrine manipulated animals were studied. Ovariectomy decreased basal prolactin release presumably due to the absence of galanin secretors, thus also explaining the decrease in responsiveness to the addition of VIP, TRH, $V_{as}$ and $G_{as}$. Hyperoestrogenisation increased both the number of galanin secretors and the total number of lactotrophs. The observed increases in the number and size of the lactotrophs in the hyperoestrogenised state concur with published data[179,180]. It is of interest that the observed blot area around each galanin secreting cell did not increase in the hyperoestrogenised state, implying that the effect of oestrogen is to recruit new galanin secreting lactotrophs rather than to increase the galanin release/cell. Basal prolactin release in the hyperoestrogenised state was high and responsiveness to VIP and TRH decreased implying that the high endogenous galanin secretion is stimulating prolactin secretion to near maximal levels in the basal state. Hyperoestrogenised lactotrophs were unresponsive to the addition of $V_{as}$ but exquisitely sensitive to $G_{as}$ decreasing prolactin release to a significantly greater extent than that observed in unenriched cells (Fig 4) indicating that in states of high oestrogen exposure basal prolactin secretion is almost completely galanin dependent.

Chronic hyperoestrogenisation in rats first induces lactotroph hyperplasia, followed by adenoma formation and finally the development of prolactinomas[181-183]. Since the hyperoestrogenised lactotroph would appear, from these studies, to be so dependent on galanin for its function, a specific pituitary galanin antagonist might be of value in the treatment of prolactinomas.

# CHAPTER SIX:

# CHARACTERIZATION OF A NOVEL GALANIN RECEPTOR IN THE RAT ANTERIOR PITUITARY

## 6.1 *INTRODUCTION*

In the previous chapter, galanin, a 29 amino-acid peptide originally isolated from porcine intestine[164] was demonstrated to be an autocrine regulator of prolactin release. Galanin is also widely distributed in gut, pancreas, muscle and the peripheral and central nervous systems[165,166]. Highest levels of galanin synthesis and storage occur within the hypothalamus in the median eminence[167].

Various studies previously published, as well as those described in this thesis, have demonstrated the effects of galanin on basal and stimulated release of prolactin[156,184], growth hormone[185-188] and luteinizing hormone (LH)[189,190] either from dispersed pituitary cells or at the hypothalamic level affecting dopamine, somatostatin (SRIF) and gonadotropin releasing hormone (GnRH) release into the portal circulation. Recently[189] galanin has been shown to be episodically released into the hypothalamo-pituitary portal circulation at concentrations in the nanomolar range, providing further evidence for its putative role as a modulator of pituitary function.

In view of the high concentrations of galanin reaching or secreted by the anterior pituitary, it is paradoxical, therefore, that a number of studies have failed to demonstrate anterior pituitary binding of $^{125}$I-Tyr$^{26}$ labelled galanin by membrane assay or by autoradiography in the rat or pig[191,192] whilst both studies demonstrated good thalamic and/or hypothalamic specific galanin binding.

Bartfai et al have recently synthesised a high affinity galanin antagonist by fusing galanin 1-13 with substance P (5 - 11) which they termed M-15 or galantide[193]. This antagonist has been shown to be a potent inhibitor (at doses of 1 nM or less with an $IC_{50}$ of 0.1 nM) of the actions of galanin on the pancreas, smooth muscle and hippocampus.

This chapter demonstrates that the galanin antagonist, galantide, has no effect on basal or galanin stimulated prolactin release. Using galanin labelled with the $^{125}$I-Bolton and Hunter reagent at the N-terminus a single high affinity galanin receptor in the pituitary is characterised. Unlike the brain/gut galanin receptor, the C terminal part of the peptide is crucial for pituitary membrane binding.

## *6.2 MATERIALS AND METHODS*

For pituitary dispersion, FACS enrichment and prolactin RHPA please see chapters 2 and 3.

### *6.2.1 Peptide synthesis*

Porcine galanin and its fragments 2-29, 3-29, 5-29, 8-29, 10-29 and 20-29 were synthesised by Dr P Byfield (CRC, Harrow) on Rink's amide-type resin (Novabiochem, Nottingham, UK) using Fluorenemethyloxycarbonyl (FMOC)-protected amino acids and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexaflurophosphate (HBTU) activation chemistry in an automated peptide synthesiser

(Model 431A, Applied Biosystems, Warrington, UK). Peptides were cleaved from the resin and side chain protecting groups removed. Peptide material was precipitated from the resulting solution and purified to homogeneity by HPLC on C8 columns (Aquapore RP300, Anachem, Luton, UK). Fidelity of synthesis was confirmed by amino acid analysis and mass spectroscopy. The galanin 1-15 used in these studies was synthesised as previously described[194] and kindly donated by Dr N Yanaihara (University of Shizuoka, Japan).

### *6.2.2 Preparation of porcine and rat mono$^{125}$I-Tyr$^{26}$ galanin and porcine $^{125}$I-Bolton and Hunter-N terminus galanin*

Synthetic porcine and rat galanin 1-29 were iodinated by the iodogen method to yield mono $^{125}$I-Tyr$^{26}$ galanin as previously described[195].

Synthetic porcine galanin (5 nMol in 20$\mu$l of 0.2 M borate buffer, pH 8.2) was incubated at 4°C for 2 hours with 1 nMol of $^{125}$I-Bolton and Hunter reagent and separated from the free reagent on a C18 reverse phase HPLC column. All iodinations were performed by Dr M Ghatei, in our laboratory.

### *6.2.3 Preparation of membranes*

Fifty female rats/experiment were killed by decapitation and tissues quickly removed. Tissues were homogenised as previously described[196] for 2 minutes in 50 mM HEPES buffer, pH 7.4, containing 0.25M sucrose and protease inhibitors, using an ultra-turrax homogeniser. The homogenate was spun at 1500g at 4°C for 20 minutes in ice cold buffer, the supernatant was then spun at 4°C for 1 hour at 100,000g. The

resulting pellet was resuspended in homogenisation buffer without sucrose and spun for a further 1 hour at 4°C at 100,000g. The final pellet was then resuspended in buffer (without sucrose) at a concentration of 2.5 mg/ml and frozen at -70°C in 500$\mu$l aliquots for future use in equilibrium binding experiments.

### *6.2.4 $^{125}$I-Galanin binding to tissue membranes*

Experiments were performed in 20 mM Hepes buffer pH 7.4 containing 5 mM magnesium chloride (Sigma), 0.1% (w/v) bacitracin, 40 KIU/ml aprotinin, 1 mM EDTA (Sigma) and 1% (w/v) BSA fraction V in the presence of 0.1 nM of $^{125}$I-galanin, 50 $\mu$g of the membrane preparation and increasing concentrations of unlabelled porcine galanin or of other galanin receptor ligands. Non-specific binding was assessed in the presence of 200 nM galanin. All assays were carried out in siliconised polypropylene tubes (final volume 500 $\mu$l) with an incubation period of 45 minutes at 22°C. Bound and free label were separated by centrifugation at 15,600g for 2 mins at 4°C. Specific binding was calculated from the non-specific binding subtracted from the total binding expressed as absolute counts or as a percentage of total binding.

### *6.2.5 Peptides*

Porcine galanin was used throughout these experiments unless otherwise stated.

### *6.2.6 Satistics*

Results are expressed as mean $\pm$ SEM, n=3 separate experiments in all cases.

## *6.3 RESULTS*

### *6.3.1 Effects of galanin on prolactin release in the reverse haemolytic plaque assay*

FACS enriched lactotrophs were purified from dispersed anterior pituitary cells, obtained from randomly cycling female rats, as described in chapter 3. Both rat and porcine galanin caused an equipotent dose-dependent stimulation of prolactin release up to a maximum of 180 $\pm$ 12% of control secretion at a dose of 100 nM with an $ED_{50}$ of 6 nM for both peptides. This compares to a 220 $\pm$ 19% increase in release by 100 nM TRH (Fig 6.1).

The addition of a specific galanin antiserum, raised in rabbits caused a profound and dose dependent inhibition of basal prolactin release, to a minimum of 12 $\pm$ 0.5% of control secretion using an addition of 1/100 of the antiserum (Fig 6.1). Addition of galantide at concentrations up to 1 $\mu$M had no effect on basal prolactin release. Similarly, when the antagonist was used at a concentration of 1 $\mu$M, concurrently with 100 nM galanin no attenuation of stimulation was observed (Fig 6.1).

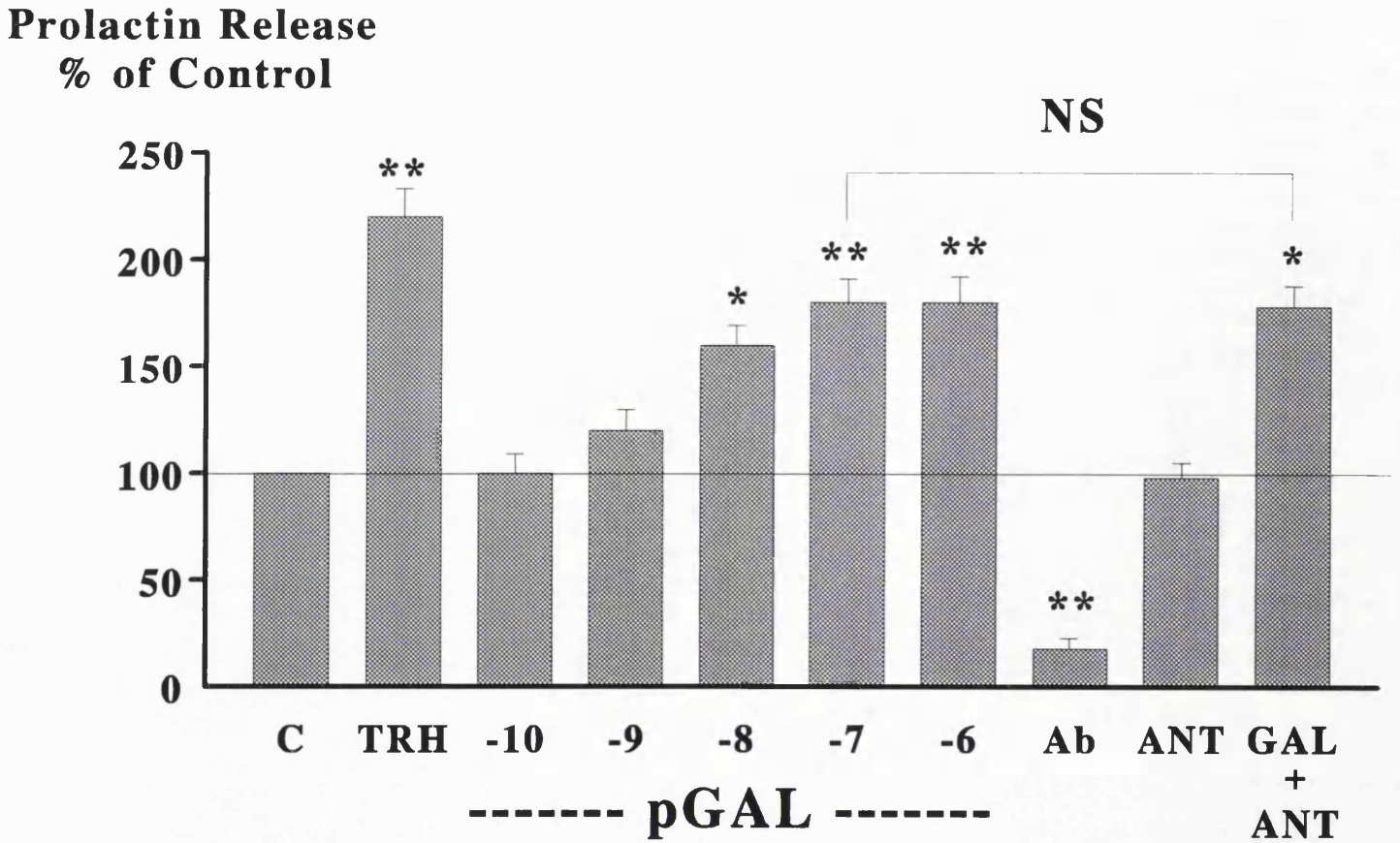



**Figure 6.1:** Prolactin secretion, assessed by reverse hemolytic plaque assay, as a percentage of control. Effects of thyrotropin releasing hormone (TRH, 100 nM), increasing doses of porcine galanin (denoted as pGAL), galanin antisera (denoted as Ab, 1/100 dilution), the galanin antagonist galantide (denoted as ANT, 1 $\mu$M) and a combination of galanin 100 nM and galantide 1 $\mu$M . **, $P<0.01$; *, $P<0.05$ and NS, not significantly different from control.

Various N- and C-terminal galanin fragments were also tested in the RHPA. Galanin 2-29 and 3-29 were fully active at concentrations of 1 $\mu$M when compared to galanin 1-29. There was a diminishing stimulation of prolactin release using galanin 5-29 and 8-29 (Fig 6.2). Galanin 1-15, 10-29 and 20-29 had no significant prolactin releasing activity when tested at a concentration of 1 $\mu$M.

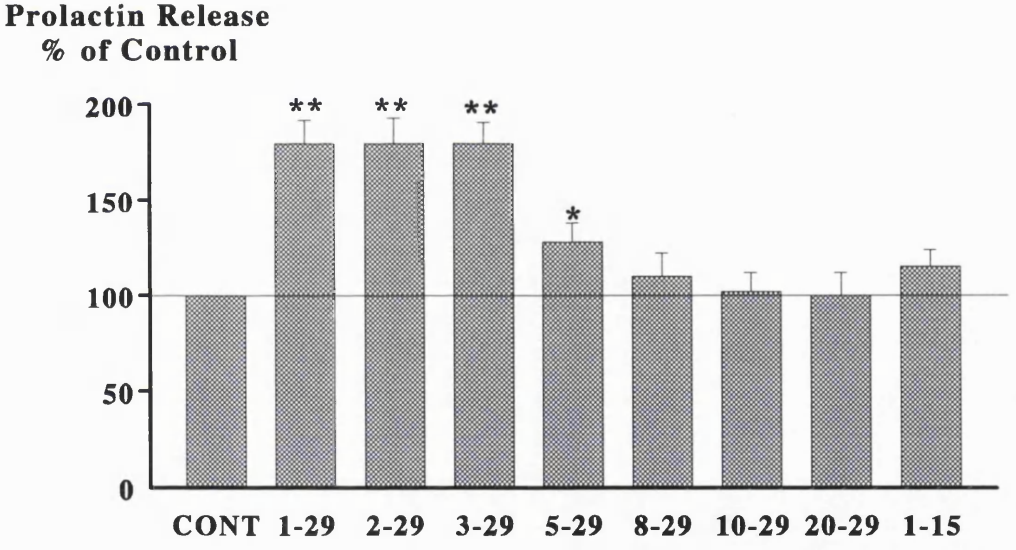


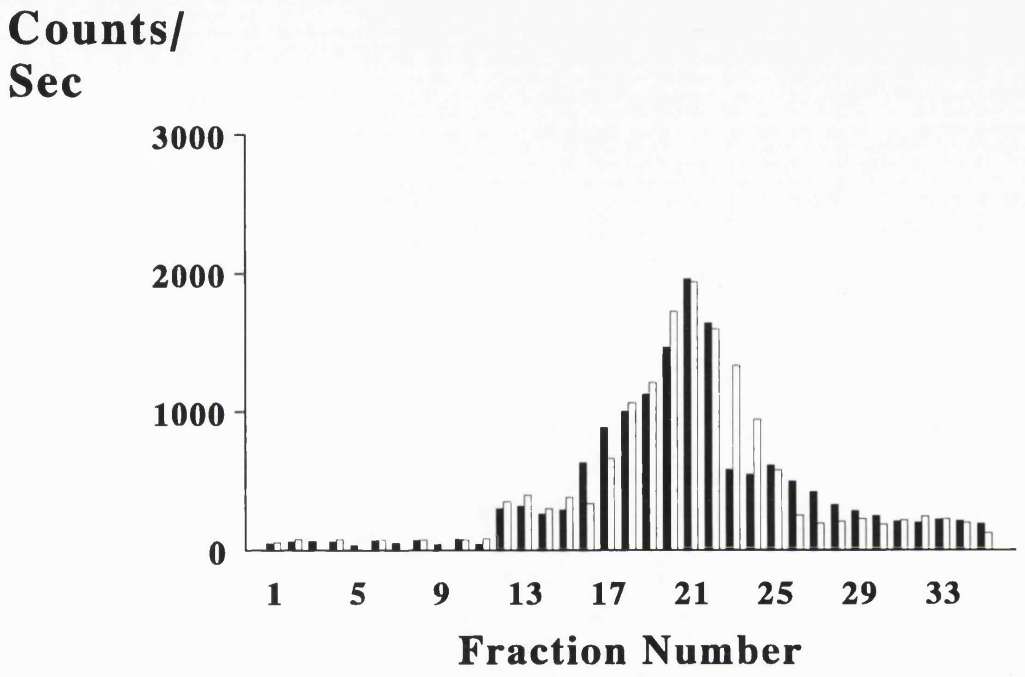

**Figure 6.2**

*6.3.2 Binding studies*

Specific binding to the thalamic membrane preparation (at a single ligand concentration) was 94.5 ± 6.1 fmol/mg of protein for both porcine and rat $^{125}$I-Tyr$^{26}$ galanin, whilst pituitary membrane specific binding was insignificant at 2.3 ± 0.14 fmol/mg of protein.

Iodination of porcine galanin using $^{125}$I-Bolton and Hunter (BH) reagent produced 2 peaks when purified on HPLC (designated fractions 46 and 48). With thalamic membranes, both fractions gave identical specific binding to that obtained with the $^{125}$I-Tyr$^{26}$ galanin. Fraction 46 also bound the pituitary membranes with a specific binding of 50.2 ± 2.2 fmol/mg of protein whilst fraction 48 specific binding was insignificant. Since the BH reagent could label only the free N amino terminus or the side chain of the $^{24}$Lys of porcine galanin, further studies were undertaken to determine which of these fraction 46 represented. Both fractions were run on a G-25 Sephadex column (using a 0.01 M phosphate buffer containing 0.5% BSA w/v) after incubation for 6 hours at 37°C with either a 50 mM HEPES buffer pH 7.4 containing 1 mM cobalt chloride (Sigma) or the above buffer containing 0.5 units of aminopeptidase M (Boehringer). Aminopeptidase M sequentially cleaves amino acids from the N-terminus if the peptide is not blocked. Thus if the BH reagent had labelled the N-terminus, and blocked the free amino group, the peptide would not be cleaved. In contrast, if the BH reagent labelled at the $^{24}$Lys then the peptide could be cleaved up to the $^{13}$Pro (Aminopeptidase M is unable to cleave prolyl peptide bonds) leaving a residual labelled 16 amino acid peptide.

The results suggest that Fraction 46 (top) is N-terminally BH labelled whilst fraction 48 (bottom) is labelled at the $^{24}$Lys. Closed bars represent incubation with buffer and open bars after incubation with Aminopeptidase M. All further studies were performed using fraction 46, $^{125}$I-Bolton and Hunter N-terminal labelled galanin.



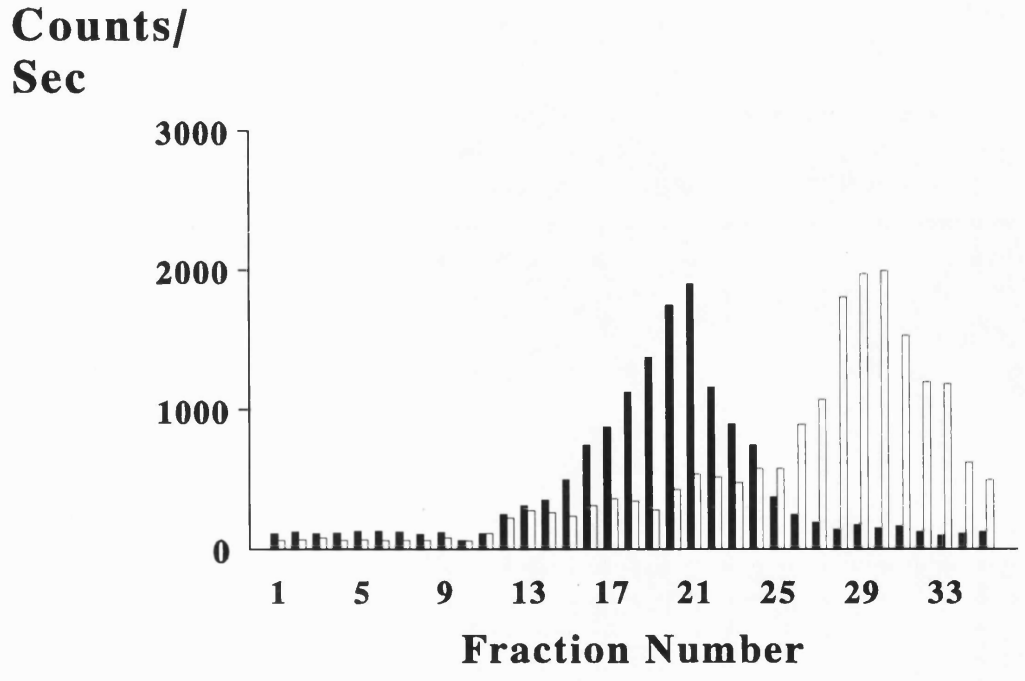




**Figure 6.3**

Scatchard analysis of saturation binding data, demonstrated a $K_d$ of 4.4 ± .34 nM for the anterior pituitary membrane (top) and 1.3 ± .12 nM for the thalamic membrane (bottom), whilst the $B_{max}$ for the pituitary and thalamus were 79 ± 8.3 and 222 ± 17 fmol/mg of protein respectively and were thus in good agreement with existing published brain and hippocampal values[197,198] (Fig 6.4).

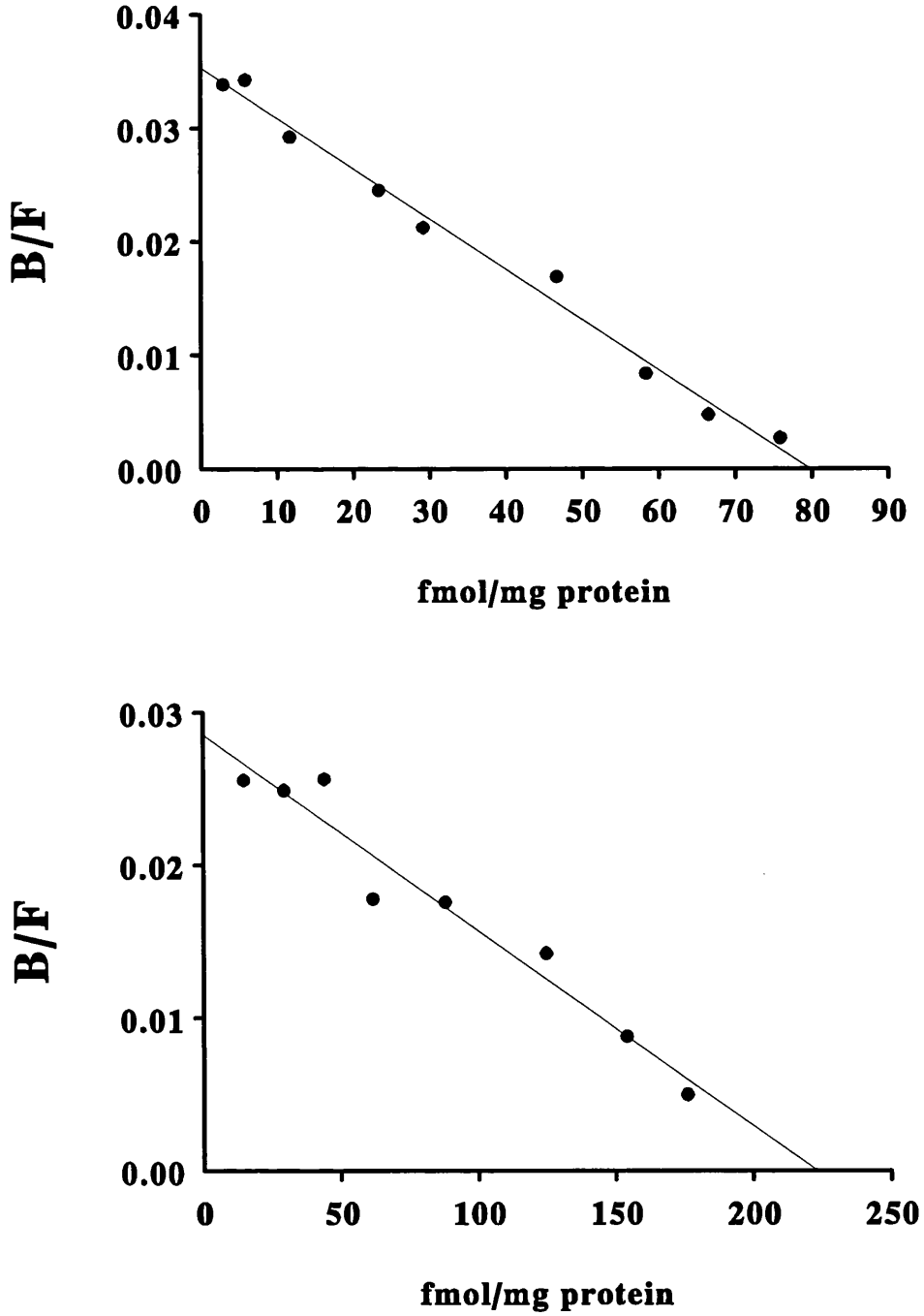



**Figure 6.4**

The $IC_{50}$ for unlabelled porcine galanin (closed circles) in the anterior pituitary (top) and thalamus (bottom) were 0.51 ± .04 and 0.58 ± .06 nM respectively. The $IC_{50}$ for galantide (open circles), however differed greatly, the thalamic membranes giving a value of 0.5 ± .05 nM, identical to the published value[193], whilst the pituitary yielded a value of >10 μM (Fig 6.5).

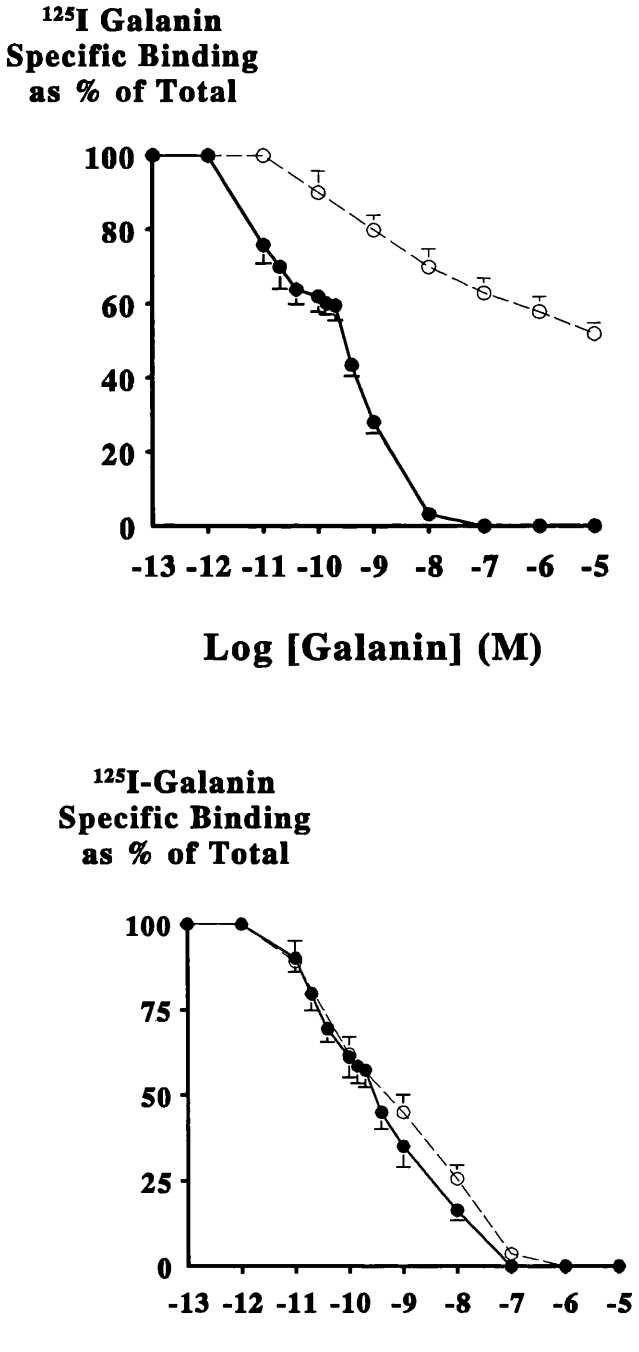



**Figure 6.5**

Displacement of the labelled galanin with the various galanin fragments was tested and the results parallelled those obtained in the RHPA. Galanin 2-29 and 3-29 fully displaced the label, having an $IC_{50}$ of 0.6 $\pm$ .04 and 0.96 $\pm$ 0.7 nM respectively. The $IC_{50}$ for galanin 5-29 was 200 $\pm$ 13 nM, whilst the $IC_{50}$ for 8-29 and 1-15 were greater than 10 $\mu$M. Galanin 10-29 and 20-29 demonstrated no displacement of receptor binding (Fig 6.6).

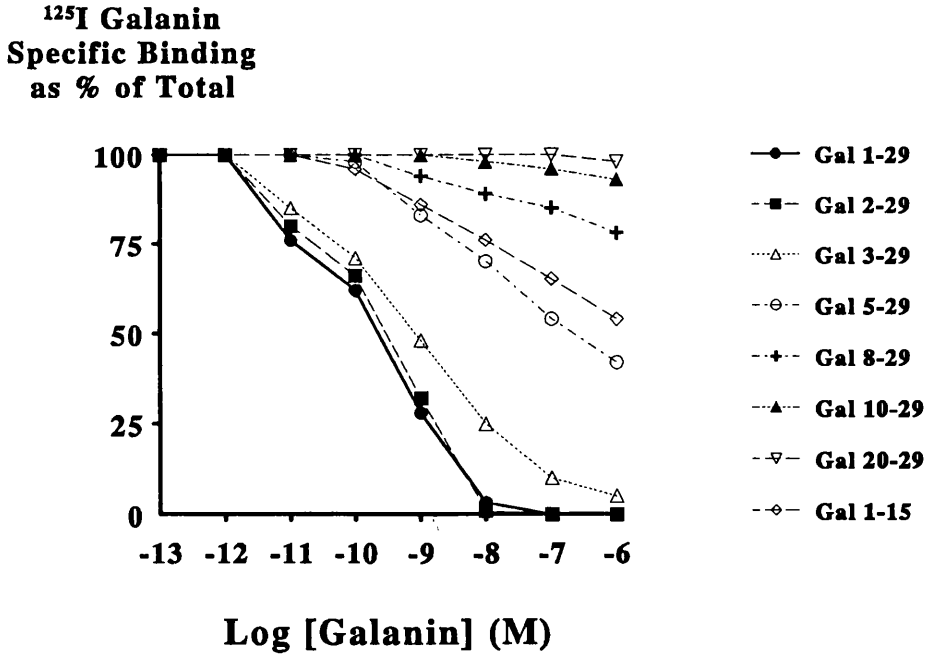



**Figure 6.6**

Displacement of the N-terminal labelled $^{125}$I-Bolton and Hunter porcine galanin from pituitary membranes with unlabelled galanin fragments. Standard error bars are not shown for the sake of clarity.

Binding of the $^{125}$I-Bolton and Hunter N-terminal labelled porcine galanin was assessed in a number of tissues, known to express galanin receptors, in the absence or presence of 200 nM unlabelled porcine galanin, the galanin 3-29 fragment or galantide. Hippocampus, thalamus, cortex, stomach, duodenum, ileum, and pancreas demonstrated equal displacement of the label with porcine galanin and galantide and no displacement with the galanin 3-29 fragment. In contrast, galantide and galanin 3-29 displacement of the label using the hypothalamic membranes was 83 $\pm$ 4.7% and 32 $\pm$ 1.7% respectively, of that observed with unlabelled galanin.

No displacement of the labelled porcine galanin from pituitary membranes was observed with the following peptides, all at 1 $\mu$M,: thyrotropin-releasing hormone (TRH), growth hormone releasing hormone (GHRH), corticotropin releasing factor, gonadotropin releasing hormone (GnRH), somatostatin (SRIF), dopamine, vasoactive intestinal polypeptide, neuromedins U and B, calcitonin gene related peptide, substance P, neuropeptide Y, neurotensin and pituitary adenylate cyclase activating peptide.

*6.4 DISCUSSION*

Modulation of the release of the anterior pituitary hormones prolactin, growth hormone and luteinizing hormone by the peptide galanin is now well documented[156,184-190] and may well represent a summation of a number of different interactions. These include (a) changes in the release of hypothalamic stimulating and inhibiting factors eg TRH, GHRH, GnRH, dopamine and SRIF, (b) effects of hypothalamic galanin secreted into the portal circulation and acting directly on basal and/or stimulated hormone release from the lactotroph, somatotroph or gonadotroph and (c) locally synthesised and secreted galanin acting in a paracrine and/or autocrine manner.

Chapter 5 described the stimulation of prolactin release by galanin and inhibition of basal release using a specific galanin antiserum. It was expected, therefore, that the recent characterisation[193] of a new, highly potent, galanin antagonist galantide would inhibit prolactin release to a similar extent to that obtained with the antiserum and thus allow further study of the role galanin plays in mediating pituitary function.

Failure to demonstrate inhibition of both basal and galanin stimulated prolactin release with galantide and the surprising absence of $^{125}I$ labelled galanin specific binding in the anterior pituitary by either autoradiography or membrane displacement, demonstrated by a number of groups[191,192] was inexplicable and cast doubt as to the role that galanin may play in pituitary physiology.

To explain these inconsistencies a separate pituitary galanin receptor is postulated. The studies reported here suggest a single high affinity galanin receptor in the anterior pituitary does exist (designated GAL-$R_2$) and is different from the previously characterised receptor (designated GAL-$R_1$) found in the brain, gut, pancreas and smooth muscle.

The many published structure-activity studies on the effects of galanin fragments and analogues on the GAL-$R_1$[199-204] have demonstrated that the active receptor binding part of the peptide resides at the N-terminal portion of the peptide. The presence of the unmodified, first two N-terminal amino acid residues is particularly important for retention of high biological potency, since galanin 2-29 and 1-15 are nearly as potent biologically and at receptor displacement as the full 1-29, whilst 3-29, 10-29 and 20-29 are inactive.

In contrast to the above, the first two N-terminal amino acids are not crucial for binding to the pituitary GAL-$R_2$ since the 3-29 fragment retains full biological activity. Receptor binding and release of prolactin decrease with increasingly shorter C terminal fragments such that the 10-29 and 20-29 fragments have no significant activity. These data indicate that the 3-10 region of galanin is crucial for binding to the GAL-$R_2$. However, the addition of the $^{125}$I-Bolton and Hunter reagent to the $^{24}$Lys and $^{125}$I to the Tyr$^{26}$ of galanin both modify the peptide in some way thus inhibiting binding to the GAL-$R_2$ (whilst both bind the GAL-$R_1$). Thus the three amino acids in positions 24-26 may also be required for pituitary receptor binding in accord with the inactivity of the 1-15 fragment.

Given the above data it is not surprising that galantide (a chimera of galanin 1-13 and substance P 5-11) is not a pituitary galanin antagonist and only weakly displaces labelled GAL, to a similar degree to the 1-15 fragment. These data would suggest that since two regions of the peptide appear to be required for GAL-$R_2$ binding, the preparation of a specific pituitary galanin antagonist is likely to require both the 3-10 and 24-26 moieties of the peptide.

In order to assess whether the GAL-$R_2$ was unique to the anterior pituitary a number of regions of the brain and gut, known to bind or respond to galanin$^{204-210}$ were screened. In all but one of the tissues tested, galantide and unlabelled galanin equally displaced the label, whilst the 3-29 fragment caused no displacement of the label at all, implying that they only possess GAL-$R_1$ receptors. In the hypothalamus, however, 17% lower displacement was observed with galantide than with unlabelled galanin, whilst the galanin 3-29 fragment was able to displace 32% of the label. Since the 3-29 fragment is able to fully displace the label from the GAL-$R_2$ receptor, these data would support the hypothesis that the hypothalamus expresses both GAL-$R_1$ and GAL-$R_2$ whilst the other tissues examined exclusively express GAL-$R_1$.

In summary, these data demonstrate a novel pituitary galanin receptor, designated GAL-$R_2$ in which region 3-10 and the amino acids 24 and 26 are crucial for biological and membrane binding activity, in contrast to the known gut/brain galanin receptor (designated GAL-$R_1$) in which activity is dependent only on the first fifteen N-terminal amino acids. The previously characterised GAL-$R_1$ antagonist, galantide, does not bind to the GAL-$R_2$ and has no direct effect on the pituitary.

# CHAPTER SEVEN:

# SUMMARY AND FUTURE WORK

Paracrine and autocrine effects have recently been found to be of importance in the regulation of haematological and immunological responses. It has been hypothesised that endocrine cells are arranged in glands to facilitate cell to cell communication. It has been found that each endocrine gland synthesises and secretes certain peptides whose role and function is, as yet, unknown. I have used the expression of one of these peptides, galanin in the anterior pituitary, as a model of autocrine and paracrine regulatory systems in endocrine glands. Evidence for local control mechanisms in the pituitary include the topographical arrangement of the different cell types, the local synthesis and storage of these bioactive peptides, changes in the synthesis and content of these peptides by endocrine manipulations and the effect of these substances on pituitary hormone release.

I have established a novel method of isolating fully functional lactotrophs and somatotrophs and, using the newly defined techniques of reverse haemolytic plaque assay and cell blotting, have demonstrated that FACS enriched lactotrophs are regulated by galanin, an oestrogen dependent peptide. It is hypothesised that galanin, despite being widely distributed throughout the body, is of crucial importance to pituitary function and the demonstration of a unique hypothalamo-pituitary galanin receptor strengthens this hypothesis.

Having completed my thesis, I am attempting to further characterise the role galanin plays in the control of lactotroph growth and function, I am applying the techniques of molecular biology in order to study how galanin regulates the activity of these cells. To identify the interactions between the roles that hypothalamic and locally derived pituitary galanin play in controlling lactotroph function, I will generate

transgenic animals that specifically lack galanin expression in the lactotroph and somatotroph as well as mice deficient in galanin gene expression.

To focus on the local pituitary effects of galanin I will use the previously cloned cis-acting regulatory DNA sequences of the *pit-1*/GHF1 gene, to drive the expression of the coding portion of the galanin gene, or its 5' untranslated region, in reverse orientation. The targeted expression of anti-sense galanin sequences should destabilize and/or markedly reduce the translation of the endogenous galanin gene in the adenohypophysis, thus allowing the effects of galanin deficiency in lactotrophs and somatotrophs to be examined.

The role of the galanin gene in the development of the entire organism will also be studied in a mouse strain carrying a loss-of-function mutation at the endogenous galanin locus. This mouse strain will be generated by first targeting the galanin gene by homologous recombination in embryonic stem (ES) cells. The chimeric mice thus produced will be bred and their progeny tested for germ line transmission of the disruption of the galanin locus. Heterozygous animals will be bred to homozygosity, and the effects of the lack of normal function of the galanin gene will be examined.

These studies in conjunction with the data presented in this thesis should increase our knowledge of the role galanin plays in lactotroph function and allow a greater understanding of the patho-physiology of the prolactinoma.

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