# SOMATOTROPH REGULATION IN DWARF RATS

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by

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## ABBRIEVIATIONS

| | |
|---|---|
| ABC | avidin biotin complex |
| ACTH | corticotrophin |
| AP-1(2) | activating protein 1 (2) |
| AS | albino Swiss |
| BSA | bovine serum albumen |
| BTF | basic transcription factor |
| cAMP | cyclic adenosine 3',5'-monophoshate |
| CRE(B) | cAMP response element (binding protein) |
| CRF | corticotrophin releasing factor |
| CPI | Complete™ peptidase inhibitor cocktail |
| cpm | counts per minute |
| DA | dopamine |
| DAB | diaminobenzidine tetrahydrochloride |
| $dH_2O$ | distilled water |
| D-MEM | Dulbecco's minimal essential medium |
| DNase | deoxyribonuclease |
| d1 | postnatal day 1 |
| e1 | embryonic day 1 |
| $E_2$ | 17β-oestradiol |
| ER(E) | oestrogen receptor (response element) |
| FACS | fluorescence activated cell sorting |
| FGF | fibroblast growth factor |
| FIAU | 1-(2-deoxy-2-fluoro-β-δ-arabinofuranosyl)-5-iodouricil |
| FITC | fluorescein-isothionate |
| FS | folliculo-stellate |
| FSC | forward scatter |
| FSH | follicle stimulating hormone |
| GABA | gamma aminobutyric acid |
| GFAP | glial fibrillary acidic protein |
| GH | growth hormone |
| hGH | human growth hormone |
| rGH | rat growth hormone |

| | |
|---|---|
| bGH | bovine growth hormone |
| GHRP | growth hormone releasing peptide |
| GHS | growth hormone secretagogue |
| GHS-R | growth hormone secretagogue receptor |
| GRF | growth hormone releasing factor |
| GRF-R | growth hormone releasing factor receptor |
| GnRH | gonadotrophin releasing factor |
| GSUα | glycoprotein subunit α |
| HBSS | Hank's balanced salt solution |
| HPLC | reverse phase high performance liquid chromatography |
| HSV1-TK | herpes virus 1 thymidine kinase |
| iv | intravenous |
| icv | intracerebroventricular |
| IGF-I | insulin-like growth factor-1 |
| IL-1 | interleukin-1 |
| IL-1R | interleukin-1 receptor |
| IL-6 | interleukin-6 |
| kDa | kilodalton |
| LH | luteinizing hormone |
| LI | like immunoreactivity |
| α-MSH | α-melanocyte stimulating hormone |
| mRNA | messenger ribonucleic acid |
| NIDDK | National Institute of Diabetes & Digestive & Kidney Diseases |
| NMS | normal monkey serum |
| NRS | normal rabbit serum |
| PACAP | pituitary adenylate cyclase activating polypeptide |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PE(-SA) | phycoerythrin (-streptavidin) |
| PEG | polyethylene glycol |
| Pit-1 | pituitary specific transcription factor |
| PI | phosphatydylinositol |
| PKA | protein kinase A |
| PL | placental lactogen |

| | |
|---|---|
| POMC | pro-opiomelanocortin |
| PRL | prolactin |
| Prop-1 | Prophet of Pit-1 |
| PVDF | polyvinylidene difluoride |
| RHPA | reverse haemolytic plaque assay |
| RIA | radioimmunoassay |
| RIPA | radioimmunoprecipitation buffer |
| SDR | spontaneous dwarf rat |
| SDS | sodium lauryl sulphate |
| SDS-PAGE | sodium lauryl sulphate-polyacrylamide gel electrophoresis |
| S.E.M. | standard error of the mean |
| SS | somatostatin |
| SSTR /sst | somatostatin receptor |
| TBS | tris buffered saline |
| TGR | transgenic growth retarded |
| TH | tyrosine hydroxylase |
| TRH | thyrotrophin releasing hormone |
| $T_3$(R) | thyroid hormone (receptor) |
| TSH | thyroid stimulating hormone |
| V | volts |
| VIP | vasoactive intestinal peptide |

## ABSTRACT

Growth hormone (GH) and prolactin (PRL) are synthesized in specialized secretory cells in the anterior pituitary gland. The cells producing these hormones are thought to develop from a common precursor cell which co-expresses both GH and PRL. Somatotrophs are under stimulatory and inhibitory control from hypothalamic factors, influencing GH release, GH gene expression and somatotroph proliferation. The autosomal recessive dwarf rat (*dw/dw*) exhibits a 40% reduced growth rate compared to normal rats of the same (AS) strain. While the secretory function of *dw/dw* somatotrophs is relatively intact, somatotroph proliferation and GH gene expression is markedly reduced, resulting in a 75-80% reduction in somatotroph number and a 95% reduction in pituitary GH. This specific deficiency is a result of impaired cAMP accumulation in response to growth hormone releasing factor (GRF) due to the inability of Gsα to activate adenylate cyclase (Downs and Frohman, 1991).

*Dw/dw* anterior pituitary cultures, in my hands, showed no detectable increase in cAMP, while cultures from AS rats showed an increase in cAMP in a dose dependant manner. Both AS and *dw/dw* pituitary cultures responded to GRF with GH release in a dose dependent manner, illustrating the distinction between the cAMP dependant responses (GH gene expression and somatotroph proliferation) and the secretory response.

I further investigated the cAMP response to GRF in *dw/dw* anterior pituitary cultures by manipulating the somatotroph number in the culture to control for the reduced proportion of GH cells in the *dw/dw*. This thesis provides the first description of fluorescence activated cells sorting (FACS) to purify somatotrophs from *dw/dw* pituitary cells. *Dw/dw* somatotrophs enriched to over 40% by fluorescence (equivalent to an AS pituitary culture) did not show cAMP accumulation in response to GRF. In addition, cultures of AS and *dw/dw* pituitary cells mixed to contain just 2% normal somatotrophs (about 6 fold less than a *dw/dw* pituitary) were still able to exhibit a significant cAMP response to GRF. These data support the existence of an inherent defect in the GRF signalling pathway in these cells.

While my data support the existence of the GRF signalling defect in the *dw/dw* somatotrophs, I also present data that the phenotype is not restricted to this cell type. SDS-PAGE of anterior pituitary extracts suggested that the PRL content in the *dw/dw* pituitary was increased compared to the AS. Radioimmunoassay of pituitary extracts confirmed a 2-3 fold higher PRL content in both male and female adult *dw/dw* rats, despite profound pituitary hypoplasia. As

somatotrophs and lactotrophs belong to the same lineage, I therefore decided to investigate the proportions of both cell types during development. This was carried out using two colour FACS analysis, which also allowed the quantification of the mammosomatotroph precursor and intermediate cells. At 9 days, the *dw/dw* pituitary contained 4 fold higher percentage of PRL staining cells, suggesting that the *dw/dw* defect also affects lactotroph development. Allowing for a 40% reduced cell number in the *dw/dw* pituitary, the total lactotroph number remained higher in the *dw/dw* than the AS pituitary until at least 23 days of age. In the adult, while the percentage of lactotrophs was higher in the *dw/dw*, this probably did not translate to an actual increase in lactotroph number due to the reduced pituitary size. However, as the PRL content was still higher at this time, this would mean that each lactotroph would contain more PRL.

Since interconversion of GH and PRL cell types is sensitive to oestrogen, I also analyzed cells from AS and *dw/dw* male rats treated with 17β-oestradiol ($E_2$: 25μg/day for 10days). The proportions of GH, PRL and double positive cells in AS males was unchanged by $E_2$. The total percentage of GH positive cells in the *dw/dw* rats did not change, but the number also staining for PRL increased after $E_2$ treatment. The number of GH positive/PRL negative cells in the estrogen treated *dw/dw* male was similar that in the female *dw/dw*.

I therefore propose that the observed signalling defect is not the cause of the phenotype, as previously suggested, but a symptom of an earlier developmental defect affecting the equilibrium of the GH and PRL cell lineage. The equilibrium between GH and PRL, which already rests closer to the lactotroph phenotype in the *dw/dw* can then be further displaced by $E_2$.

Another aim of the work in this thesis was to screen for somatotroph specific proteins, which could be used as marker proteins in the analysis of somatotrophs. Attempts were made to identify proteins up-regulated by GRF, by GRF treating cultures in the presence of [$^{35}$S]methionine and separating the cell extracts by SDS-PAGE for autoradiography. In addition, proteins were screened for differing in abundance in AS and *dw/dw* pituitary extracts and between extracts of FACS sorted somatotrophs and lactotrophs. Several candidate bands were detected, which may be potential somatotroph marker proteins. In addition, a potential lactotroph marker was identified which, in light of the lactotroph phenotype described in this thesis, may also be valuable in the analysis of the *dw/dw* rat.

# GENERAL INTRODUCTION

In this introduction I shall first provide a general background to my field of interest, followed by a specific introduction to my project in section 1.5.

## 1.1 THE PITUITARY GLAND

### 1.1.1 Anatomy of the Pituitary Gland

The pituitary gland consists of an anterior, a posterior and an intermediate lobe. Although the intermediate lobe has the same embryonic origins as the anterior lobe they are separated by the hypophyseal cleft in many species, including the rat. Instead, the intermediate lobe adheres to the posterior lobe, forming the neuro-intermediate lobe, and during dissection can be easily separated from the anterior lobe. The pituitary gland lies below the hypothalamus, which contains clusters of neurones making up hypothalamic nuclei. The hypothalamus plays a role in both anterior and posterior pituitary hormone axes, however, the mechanisms differ.

The posterior pituitary is made up of terminals of magnocellular neurones whose cell bodies are located in the paraventricular and supraoptic nuclei of the hypothalamus. Oxytocin and vasopressin are synthesized within the cell bodies and then are transported to the terminals in secretory granules for storage, awaiting release into the circulation.

The intermediate lobe contains cells which synthesize α-melanocyte stimulating hormone (α-MSH), cleaved from Pro-opiomelanocortin (POMC) precursor protein.

The focus of this thesis is the anterior lobe, which is comprised of endocrine cells making their peptide, protein or glycoprotein hormones products, under the influence of hypothalamic factors. The hypothalamus supplies the anterior pituitary with stimulatory and inhibitory factors, thus controlling the hormone output. For instance, growth hormone (GH) releasing factor (GRF) is synthesized mainly in the arcuate nucleus, while somatostatin (SS) involved in GH inhibition is mainly synthesized in the periventricular nucleus. These nuclei contain clusters of cell bodies whose axons project to the median eminence,

where they release their products into a specialized vascular link to the anterior pituitary known as the hypophyseal portal system. GRF and SS have opposing effects on the somatotrophs of the anterior pituitary, influencing GH release, GH gene expression and somatotroph proliferation by mechanisms which will be described in detail later. Anterior pituitary hormone axes are subject to feedback mechanisms acting at the level of the pituitary and/or hypothalamus. Feedback is mediated by the hormone itself or by factors produced by their target organs.

### 1.1.2 Anterior Pituitary Cells Types

#### 1.1.2.1 Endocrine Cells

Early classification of anterior pituitary cells was based on simple histological methods, characterizing cells as acidophilic or basophilic. The cell types could be further sub-classified by modifications of the methods. For instance Herlant's tetrachrome stains acidophils either orange or red, dividing them into two cell types. Using combinations of such staining techniques, Purves *et al* (1966) devised a sub-classification of acidophils and basophils. This classification was based on the hormonal products of the cell types determined by the physiological changes in the pituitary gland after the removal of peripheral endocrine organs.

The availability of specific antibody staining techniques such, as immunocytochemistry, allowed cell types to be identified directly by their products (Nakane, 1970). In addition, electron-microscopy has been used to visualize ultrastructural characteristics of the cell types, again revealing differences between the two groups of acidophils. Somatotrophs have round granules of 200-400μm, whereas lactotrophs have irregular shaped granules of 400-600μm. Nikitovitch-Winer *et al* (1987) described a cell type smaller than either somatotrophs or lactotrophs with granules of 50-100μm which stained for both GH and prolactin (PRL) by double immunogold labelling.

The current nomenclature for anterior pituitary hormone producing cells is based on their hormonal products. Acidophils are divided into somatotrophs (GH) and lactotrophs (PRL), and basophils into corticotrophs (corticotrophin: ACTH), gonadotrophs (luteinizing hormone: LH and follicle stimulating hormone: FSH) and thyrotrophs (thyroid stimulating hormone: TSH). Where hormones are co-

expressed, the names are modified to indicate the products, for instance cells expressing both GH and PRL are known as mammosomatotrophs or somatomammotrophs. The former will be used in this thesis, although the terms are synonymous.

1.1.2.2 Folliculo-stellate cells

In addition to the endocrine cells described above, non-granular folliculo-stellate (FS) cells are also present in the anterior pituitary. Rinehart and Farquhar (1953) described star-shaped cells forming long processes which surround the endocrine cells of the anterior pituitary. FS cells can be identified by immunoreactivity to the neuronal marker S-100 and glial fibrillary acidic protein (GFAP). Many functions have been attributed to these cells, including the ability to modulate hormone release from endocrine cells. FS cells can be purified to 65% on a bovine serum albumen (BSA) gradient (Allaerts and Denef, 1989; Baes *et al*, 1987). When this cell fraction was allowed to re-aggregate with other fractions, depleted of FS cells, the response of somatotrophs and lactotrophs to stimulatory (GRF, thyrotrophin releasing hormone (TRH) and Angiotensin II) and inhibitory (SS and dopamine (DA)) factors was attenuated. These cell aggregates formed structures such that very few GH or PRL cells were not in contact with an FS cell, although modulation still occurs if the aggregates are re-dispersed and plated as single cells (Baes, *et al*, 1987). FS cells release substances implicated in the modulation of various endocrine cells (reviewed by Allaerts *et al*, 1990), eg follistatin inhibits FSH release; fibroblast growth factor (FGF) modulates the responsiveness of lactotrophs and thyrotrophs and may be involved in oestrogen-induced tumour formation; S-100 can stimulate release of PRL from cultured anterior pituitary cells. The presence of FS cells was found to correlate with IL-6 bioactivity in fractions of BSA gradient fractions of separated pituitary cells (Vankelecom *et al*, 1989). Pituitary adenylate cyclase activating polypeptide (PACAP) treatment of the FS-like cell line TtT/GF caused increased cAMP levels and IL-6 release (Matsumoto *et al*, 1993). Interleukin-6 (IL-6) can release GH, PRL and LH from anterior pituitary cells *in vitro* (Spangelo *et al* 1989). FS cells may also be involved in proliferation of pituitary cells as the tumourogenesis of the somatotrophic cell line MtT/S was enhanced when co-transplanted with MtT/GF cells (Koyama *et al*, 1995). Roles of FS cells in uptake and degradation of waste materials or debris are suggested by the presence of

phagocytotic vesicles and lysosomes, and the ionic composition of the interstitial fluid may also be modulated by these cells (reviewed by Allaerts *et al*, 1990).

### 1.1.3 Anterior Pituitary Development

The anterior pituitary (adenohypophysis) develops from Rathke's pouch protruding from the roof of the embryonic mouth. As it develops, it migrates towards the posterior pituitary, developing from neural ectoderm, to form the mature pituitary. The hypothalamus develops in parallel with the pituitary gland. Members of the POU (Pit-1, Oct-1, *unc-86*) family of transcription factors are involved in the development of the nervous system (Rosenfeld *et al*, 1996). Brn-1, 2 and 4 are involved in the differentiation of specific neuronal phenotypes. An example of their involvement in the establishment in the hypathalamo-pituitary axis is illustrated by the deletion *Brn-2*. This mouse fails to differentiate mature neurosecretory neurones of the paraventricular and supraoptic nucleus and lacks a posterior pituitary (Schonemann *et al*, 1995; Nakai *et al*, 1995).

The development of the endocrine cells in the rat anterior pituitary can be traced by the appearance of specific cell type markers. The first hormone marker to be detected is the glycoprotein subunit α (GSUα), common to thyrotrophs and gonadotrophs (Simmons *et al*, 1990). In the rat, GSUα mRNA is detected on embryonic day (e)11. GSUα expression is activated by P-Lim, a member of the LIM (Lin-11, Isl-1, Mec-3) homeodomain transcription factors (Bach *et al*, 1995). On e14, mRNAs for POMC and TSHβ are first detected (Simmons *et al*, 1990), while the first committed cell type to contain detectable hormone is the corticotroph on e16 (Chatelain *et al*, 1979; Setalo and Nakane, 1976).

The pituitary specific transcription factor, Pit-1 (Ingraham *et al*, 1988; Nelson *et al*, 1988), is another member of the POU family of transcription factors. It is a 31 or 33 kilodalton (kDa) protein (from alternative transcriptional start sites) consisting of a 60 amino acid carboxy terminal homeodomain joined *via* a 15 amino acid linking peptide to a 75 amino acid POU specific domain. The homeodomain includes a helix-turn-helix motif involved in DNA binding and the POU specific domain is involved in the sequence specificity. Pit-1 mRNA and protein are first detected in the rat anterior pituitary on e15 in the caudomedial

region (Simmons *et al*, 1990). Pit-1 protein is restricted to somatotrophs, lactotrophs and thyrotrophs where it has roles in proliferation, maintenance and cell specific gene expression. The Snell mouse (*dw/dw*: Snell, 1929) possesses a point mutation, and the Jackson mouse ($dw^J/dw^J$), a gross rearrangement of *Pit-1* (Li *et al*, 1990). Both these models lack somatotrophs, lactotrophs and mature thyrotrophs, illustrating the importance of Pit-1 in pituitary development. (A summary of mouse and rat dwarf models is presented in table 1.1). In addition, cases of combined pituitary hormone deficiency affecting GH, PRL and TSH in humans have been shown to be caused by mutations in *PIT-1* (Cohen *et al*, 1996; Haugen and Ridgeway, 1995). Conversely, pituitary adenomas secreting combinations of GH, PRL and TSH have been shown to contain PIT-1 protein (Sanno *et al*, 1994).

Although the maintenance of thyrotrophs in the adult is dependant on Pit-1, the appearance of a population of cells expressing TSHβ at the rostral tip of the pituitary precedes Pit-1. A second population of thyrotrophs is then detected in the caudal region after the appearance of Pit-1. In Snell mice, these caudal cells lack TSHβ, although they still express GSUα. The early rostral tip thyrotrophs are still found in Snell mice, although these cells disappear from both mutant and wild-type animals after birth (Lin *et al*, 1994).

The Ames mouse (*df/df*) has a similar phenotype to the Snell and Jackson, however the defective gene has been mapped to chromosome 11, whereas *Pit-1* is located on chromosome 6. In contrast to the Snell and Jackson mice, the Ames pituitary contains occasional foci of somatotrophs, lactotrophs and thyrotrophs, although they are completely insensitive to excess GRF (Gage *et al*, 1995). Analysis of the pituitary development of Ames compared to Snell mice suggested that the gene product acted earlier in the cell lineage (Anderson *et al*, 1995; Gage *et al*, 1996). For instance, the earliest genetic marker for Rathke's pouch is a homeobox gene product *Rpx* or *Hesx1*, expressed between e7.5-14.5 in the mouse. Due to the spatial and temporal pattern, it has been suggested that the down regulation of *Rpx* is mediated by Pit-1 (Hermesz *et al*, 1996). The extinction of *Rpx*, which occurs normally in the Snell mouse, fails to occur in the Ames mouse (Gage *et al*, 1995). The Ames gene has been recently cloned and found to code for a novel *paired*-like homeodomain factor, termed Prophet of Pit-1 or *Prop-1*

(Sornson *et al*, 1996). It is likely that Prop-1 is involved, either directly or indirectly, in the initial expression of *Pit-1*. Wild-type, but not mutant, Prop-1 was found to bind the early enhancer region of *Pit-1*, although it was not sufficient alone to cause *Pit-1* expression (Sornson *et al*, 1996). Cases of combined pituitary hormone deficiency in man have been linked with mutations in *PROP-1* (Wu *et al*, 1998). In addition to GH, PRL and TSH, the affected individuals also exhibited deficiencies in FSH and LH, implying a role of PROP-1 in gonadotroph development.

Once Pit-1 is activated, there is a lag before GH is detected. A cell line (GHFT1 cells) has been developed that may represent an early progenitor cell at this stage, by targeting SV40 T-antigen tumorogenisis by the *Pit-1* promoter. This cell line, despite containing Pit-1 protein, is unable to activate GH or PRL genes (Lew *et al*, 1993). Once the expression of Pit-1 is established, it's levels are maintained by autoregulation. It is conceivable that the level of Pit-1 must reach a threshold level before it is able to transactivate GH. The Pit-1 level in the GHFT1 cell line is lower than in GC or $GH_3$ cells, and may be too low to transactivate GH (Lew *et al*, 1993).

GH is detected a few days after the appearance of Pit-1, with GH mRNA present at e18 (Simmons *et al*,1990) and GH protein detectable by immunocytochemistry on e19 (Chatelain *et al*, 1979). The somatotroph population increases rapidly, reaching proportions similar to adult by birth (Setalo and Nakane, 1976). The Little mouse (*lit/lit*) possesses a point mutation in the GRF receptor and adults show pituitary hypoplasia, reduced GH protein and mRNA (Lin *et al*, 1993). These mice show normal embryonic somatotroph development, but severe somatotroph hypoplasia in adulthood, showing that GRF signalling is not absolutely necessary for early somatotroph development, but is required for post-natal proliferation. Although GRF signalling is not required for fetal somatotroph proliferation, hypothalamic extracts can stimulate GH secretion from e18 somatotrophs *in vitro* (Khorram *et al*,1983). GRF-R mRNA is present on e19.5 (the earliest time point investigated) in the rat (Korytko *et al*, 1996) and on e14.5 in the mouse (Lin *et al*, 1992). The lack of GRF-R in the Snell mouse suggests the involvement of Pit-1 in it's expression (Lin *et al*, 1992).

Although there are reports of occasional PRL positive cell in fetal pituitaries (Gash *et al*, 1982; Selato and Nakane, 1976), large numbers of lactotrophs are not present until a few days after birth. Hoeffler *et al* (1985) found no lactotrophs in 24 hour cultures of e18-21 rats and negligible PRL release from pituitary cells until post-natal day (d)4 by reverse haemolytic plaque assay (RHPA) when the percentage of PRL secretors expanded rapidly to about 9%. On d5, roughly 2/3 of the GH or PRL secreting cells secreted GH only, less than 2% secreted PRL only and the remaining third secreted both hormones. Similarly, Chatelain *et al* (1979) detected cells containing both GH and PRL (mammosomatotrophs) by immunocytochemistry of sequential sections of e21 rats.

Further evidence that lactotrophs are derived from GH containing precursors came from elegant transgenic cell ablation studies. Behringer *et al* (1988) fused the structural gene for diphtheria toxin A chain to the GH promoter, leading to death of any cells expressing the product. In addition to the absence of GH cells, PRL cells were also dramatically reduced. This suggested that the majority of lactotrophs at some stage express the GH gene, or that cells that express the gene are necessary for the survival of lactotrophs. The presence of a small remaining population of PRL positive cells (several hundred per pituitary) in these mice suggested that a subset of lactotrophs are derived from precursors that do not express GH. In addition, a few GH staining cells were also detected (about 10 per pituitary), however these were attributed to the rare loss of ability to activate the transgene due to gene deletion, mutation or epigenetic events such as DNA methylation.

In a refinement of this approach, Borrelli *et al* (1989) created transgenic mice expressing the herpes virus 1 thymidine kinase (HSV1-TK) gene under the control of the GH or PRL promoter. This rendered the expressing cells sensitive to 1-(2-deoxy-2-fluoro-β-δ-arabinofuranosyl)-5-iodouricil (FIAU) added to their drinking water, which is converted to a lethal metabolite in dividing cells. Mice bearing the GH promoter driven transgene, and treated with FIAU, were dwarfed and had virtually no somatotrophs and lactotrophs. If the FIAU was removed from the drinking water, the somatotroph population returned within three weeks, followed by recovery of lactotrophs at 6 weeks. Therefore, not only are lactotrophs derived from the same precursor as somatotrophs, but these stem

cells are also present in the adult pituitary with the potential to differentiate into somatotrophs and lactotrophs. The stem cell expansion may have been driven by the high level of GRF that would have presumably arise as a result of lack of feedback by GH. This experiment shows that the GH/PRL lineage is replaceable, even in the adult. Conversely, when the transgene was PRL promoter driven, there was no reduction in either somatotrophs or lactotrophs, indicating lactotroph differentiation occurs post-mitotically.

Frawley and Miller (1989) reported that although appreciable PRL secretion is not detected before d3, the PRL mRNA is detected in rats at least 3 days earlier. They suggest that this translational blockage is due to the lack of association of PRL mRNA with ribosomes. A milk-borne signal appears to be necessary to induce the increase in PRL secretion in neonates, as aqueous milk extracts can increase the percentage of cells secreting PRL (Porter and Frawley, 1991). Milk from early lactation (d2-4) was more effective than late (d15-16). This effect was mimicked by basic FGF, which increased the percentage of PRL secretors from <0.5% to 4% *in vitro* (Porter *et al*, 1994). In addition, FGF binding sites have been detected on the rat mammosomatotroph cell line, $GH_3$ (Yamashita *et al*, 1995; Cheifetz *et al*, 1988). However, it is unlikely that the milk-borne factor is basic FGF, as gel filtration chromatography revealed the fraction of the milk extract of 2-8kDa (Porter and Frawley, 1991; Porter *et al*, 1993), whereas FGF has a molecular weight of 16kDa. It may, however be a fragment of FGF or another factor that acts to increase FGF indirectly.

## 1.2 REGULATION OF SOMATOTROPHS AND LACTOTROPHS

### 1.2.1 History of Growth Hormone

The first solid evidence of a link between growth and the pituitary gland in mammals came in 1921, when Evans and Long produced gigantism in rats by repeated intraperitoneal injection of bovine anterior pituitary emulsion. Although early attempts at hypophysectomy in dogs had proved unsuccessful due to a high mortality (reviewed in Evans *et al*, 1966), it was successfully performed on rats (Smith, 1930) leading to dwarfism, reversible by treatment with pituitary extracts. The introduction of a sensitive bioassay based on the effects of GH on the thickness of uncalcified epiphysial cartilage of the tibia (Evans *et al*, 1943)

allowed the purification of bovine (b)GH (Li and Evans, 1944). Human (h)GH was later purified (Li and Papkoff, 1956) and shown to treat hypopituitary dwarfism (Raben, 1958) whereas bGH had no effect in humans (Bennett *et al*, 1950). Sequencing of hGH revealed it to be a 191 amino acid protein with two disulphide bridges (Li *et al*, 1969). Subsequently, rat (r)GH was found to be 190 amino acids long with 66% homology to hGH (Seeberg *et al*, 1977). In addition to the species specificity of hGH and bGH on growth, hGH was shown to have lactogenic activity in the pigeon crop sac bioassay, indicating it's action on pigeon PRL receptors (Lyons *et al*, 1960). This cross-reactivity of GH and PRL between certain species is due to structural similarities between the proteins and their receptors (Russel and Nicoll, 1990). GH and PRL along with placental lactogen (PL) are members of a family of hormones which arose from a common ancestral molecule by gene duplication (Russel and Nicoll, 1990; Moore *et al*, 1982; Barta *et al*, 1981). The rat possesses a single GH gene, a single PRL gene and several PL genes, whereas humans have a single PRL gene and a cluster of GH and PL genes (Davis *et al*, 1988). An important aspect of this cross-specificity is the action of hGH on mouse and rat PRL receptors, and this must be borne in mind when used in animal studies and as a reporter gene in transgenic models.

### 1.2.2 Actions of Growth Hormone

GH promotes growth of tissues both directly and indirectly *via* insulin-like growth factor 1 (IGF-I). IGF-I production occurs mainly in the liver, although it may be produced locally in many tissues and have autocrine or paracrine actions. GH receptors are present on bone and cartilage, so the effects of GH are probably a combination of direct and indirect effects. However, GH has little activity on cartilage or muscle *in vitro* (Isaksson *et al*, 1985) leading to the endocrine theory that IGF-1 action mediates GH effects. This theory was tested by Skottner *et al* (1989) who compared hGH and IGF-I replacement in the GH deficient *dw/dw* rat. IGF-I alone had less effect on longitudinal bone growth than hGH, suggesting that IGF-I can only stimulate bone growth after direct effects of GH on chondrocytes or prechondrocytes. IGF-I treatment caused similar increases in body weight to hGH, although some organ weights differed between hGH and IGF-I treated animals, again differentiating between direct and indirect GH actions.

GH also has metabolic effects, paradoxically acting in both insulin-like and insulin-antagonistic ways (Isaksson *et al*, 1985). The early (1 hour) insulin-like effects are shown by lowering of blood glucose and free fatty acid levels in fasted, hypothysectomized rats. The late (5 hour) effects of GH show a rise in free fatty acid levels in the blood. The hGH 20KDa variant was reported to lack these metabolic effects, although it's growth promoting activity was intact (Lewis, 1984) but this may have been due to impure preparation, as recombinant hGH 20kDa was later shown to induce an early insulin-like effect (Tinsley *et al*, 1986). hGH fragments have been isolated that have been claimed to have metabolic effects without growth promoting activity. Insulin-like activity is elicited by hGH 1-43, whereas hGH 44-191 shows diabetogenic activity. However, neither of these naturally occurring fragments binds to the GH receptor, raising the possibility that metabolic effects of GH are mediated by a separate receptor (Rowlinson *et al*, 1996), and that the effects of GH were artefacts.

### 1.2.3 Somatostatin

Somatostatin (SS) or Somatotrophin Release Inhibitory Factor (SRIF) was first isolated from ovine hypothalamus (Brazeau *et al*, 1973) and found to be cyclic tetradecapeptide (SS-14). The N-terminally extended SS-28 was found to be biologically active and secreted into the portal blood, in addition to the previously described SS-14 (Meyers *et al*, 1980; Millar *et al*, 1983). SS is found mainly in the brain (amygdala and hypothalamus) and gastrointestinal tract (stomach, pancreas and colon) (Benoit *et al*, 1982). Hypothalamic SS immunoreactive neurones are found mainly in the periventricular nucleus, projecting to the median eminence (Alpert *et al*, 1976), although they are also detected in the arcuate and ventromedial nuclei and the cortex (Finley *et al*, 1978).

The actions of SS are mediated by a family of G-protein coupled receptors known as SSTR1-5 (sst1-5) (reviewed in Reisine and Bell, 1995). SSTR1 was cloned from human pancreatic islet RNA using a polymerase chain reaction (PCR)-based strategy for identifying new G-protein coupled receptors (Yamada *et al*, 1992). SSTR2 was isolated by the use of [$^{125}$I-Tyr$^{11}$]SS and [$^{125}$I-Tyr$^{3}$]SMS-201-955 (a SS analogue) to screen a rat brain library expressed in mammalian cells (Kluxen *et al*, 1992). Due to the sequence similarity, and the absence of introns, the other subtypes could then be isolated from cDNA or genomic libraries by low stringency

hybridization using SSTR1 or SSTR2 as probes (Reisine and Bell, 1995). A cryptic intron is present in the SSTR2 gene, resulting in 2 splice variants of this receptor, known as SSTR2A and SSTR2B (Vanetti *et al*, 1994). It is thought that SSTR2B, 3 and 5 are coupled to $G_{ia1}$ and inhibit adenylate cyclase, while SSTR1, 2A and 4 signal *via* other mechanisms. Raynor *et al* (1993) proposed that inhibition of GH release by SS was mediated only by SSTR2, although SSTR5 (the only subtype that has a higher affinity for SS-28 than SS-14) has also been suggested to have a role (Brazeau *et al*, 1981). Kimura *et al* (1989, 1998) have shown that 17β-oestradiol ($E_2$) treatment down-regulates SSTR2 and up-regulates SSTR5, allowing the inhibition of PRL by SS (see section 1.2.8).

### 1.2.4 Growth Hormone Releasing Factor

Although SS was isolated first, the main influence of the hypothalamus on the growth axis was known to be stimulatory from early stalk section and pituitary transplantation studies. Thus, the existence of a GH releasing factor (GRF) was hypothesized long before it's isolation. Between 1969 an 1981 the field of neuroendocrinology saw the characterization of thyrotrophin releasing factor (TRH), gonadotrophin releasing factor (GnRH) and corticotrophin releasing factor (CRF) (reviewed in Guillemin, 1986), but GRF remained elusive. Unlike the other releasing factors, GRF was not initially isolated from hypothalamic tissue but from two pancreatic tumours removed from acromegalic patients (Rivier *et al*, 1982; Guillemin *et al*, 1982). Both tumours contained large quantities of a 40 amino acid peptide (hGRF-40) although Guillemin's group also isolated a C-terminally extended amidated hGRF-44 and a shorter hGRF-37. The major form in human hypothalamus is a 44 amino acid amidated peptide (Mayo *et al*, 1983), whereas rGRF is a 43 amino acid peptide with a free C-terminus (Spiess *et al*, 1983). GRF neurones are found mainly in the arcuate nucleus (Ibata *et al*, 1986), and like SS neurones, project to the median eminence where their products can be released into the portal blood. A few GRF neurones are also found in the ventromedial nucleus, although these are not involved in the control of the GH axis (Li *et al*, 1996). GRF acts by binding specific receptors on the somatotroph. Cloning of the human and rat GRF receptors (GRF-R) revealed them to be 7-trans-membrane G-protein coupled receptors (Mayo, 1992). The rat GRF-R was shown to have a possible alternative isoform, possessing an insertion of 41 amino acids in the predicted third intracellular loop.

On reaching the anterior pituitary, GRF and SS act in opposite ways on GH release, GH gene expression and somatotroph proliferation. The mechanisms involved in these processes, described in sections 1.2.5 and 1.2.6, are summarized in figure 1.1.

### 1.2.5 Secretion of Growth Hormone

The rat shows a sexually dimorphic pattern of GH secretion, regulated by the amounts of GRF and SS arriving at the pituitary in the portal blood. Unlike the female rat which shows a relatively continuous irregular GH output (Clark *et al*, 1987), the male rat releases GH in a regular pulsatile manner with a period of about 3.3 hours. Exogenous administration of GRF lead to variable responses, however the use of trains of GRF demonstrated that there was a regular pattern to the responsiveness, also with a period of about 3 hours (Clark *et al*, 1985). SS immunoneutralization leads to a more uniform response to exogenous GRF in male rats (Tannenbaum and Ling, 1984). Thus, SS is also thought to be released in a variable manner, reducing the sensitivity of somatotrophs to exogenous GRF when at high levels. A male-type pattern of GH secretion was shown to be induced by intermittent infusions of SS (150 minutes on, 30 minutes off) to female rats (Clark and Robinson, 1988). This also increased the growth rate in these animals, supporting the observation that a pulsatile pattern of GH is more effective at promoting growth.

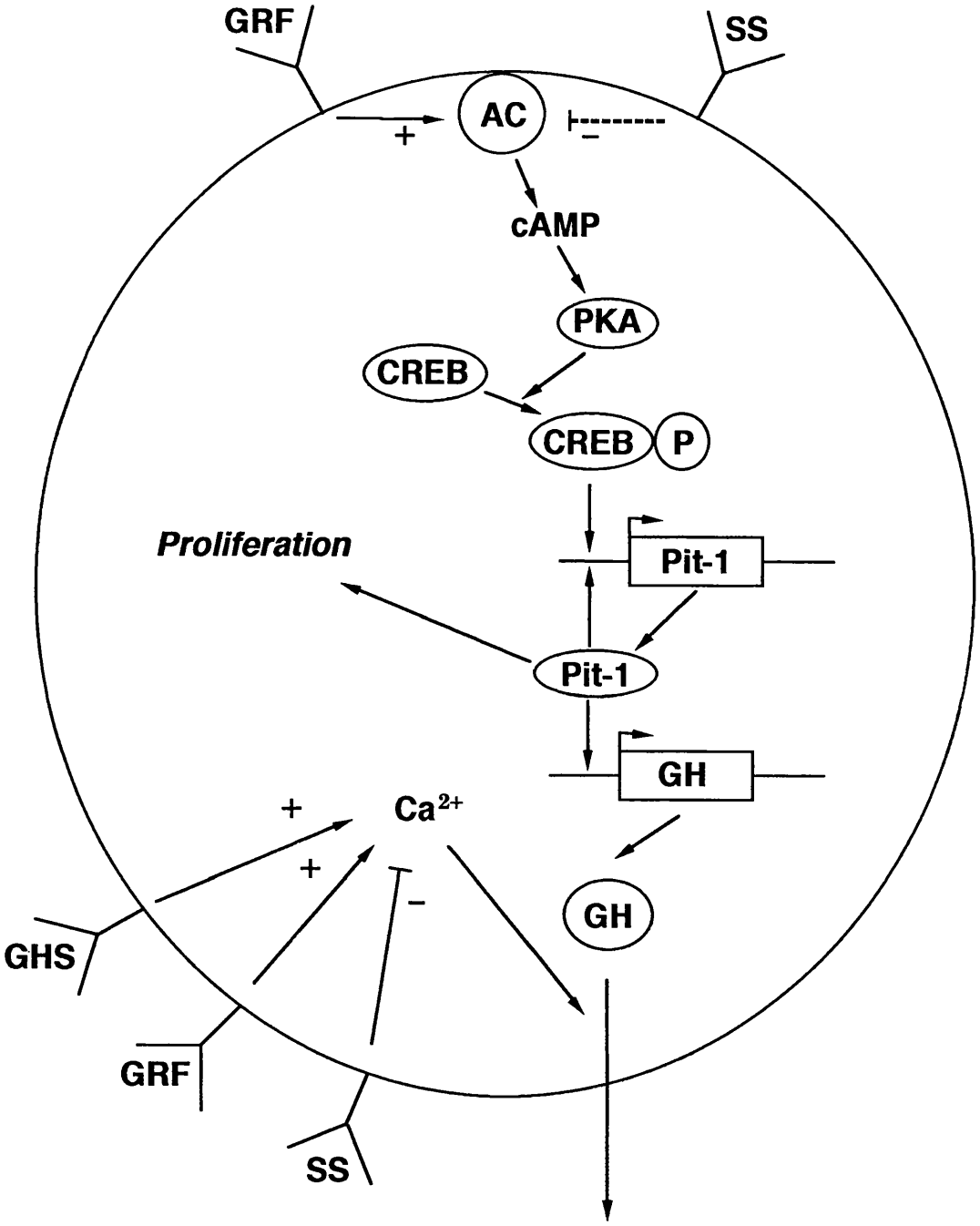
GH gene expression is not inhibited to the same extent as GH secretion (indicated by a dotted line in figure 1.1), thus during a GH trough, GH is still synthesized (Barinaga *et al*, 1985). Pre-treatment of rats (Turner and Tannenbaum, 1995) and short children (Dickerman *et al*, 1993) with SS increased responsiveness to subsequent GRF challenges. Furthermore, fasted rats (as a model of high SS) also showed a greater GH release in response to GRF *in vivo* and *in vitro* (Tannenbaum *et al*, 1989). In contrast, anterolateral hypothalamic deafferentation, periventricular nucleus lesions or SS antiserum treatment of rats reduced the pulsatile responsiveness to GRF (Soya and Suzuki, 1990) and chronic exposure of anterior pituitary cultures to GRF caused a desensitization (Bilezikjian and Vale, 1984). SS may restore responsiveness to GRF by allowing GRF to replenish stored GH without causing its release.

**Figure 1.1: Schematic of the mechanisms involved in GH release, GH gene expression and somatotroph proliferation.**

GRF occupation of the GRF receptor causes an activation of adenylate cyclase (AC) *via* a $G_s$ protein, which causes an increase in cAMP levels. This activates protein kinase A (PKA), which in turn phosphorylates the cAMP response element binding protein (CREB). One of the target promoters of this transcription factor is *Pit-1.* Pit-1 protein acts as a transcription factor, binding to sites on the GH promoter to stimulate GH gene expression. Pit-1 also binds it s own promoter, thus the levels are controlled by autoregulation. Pit-1 is also necessary for the proliferation and maintenance of somatotrophs.

The control of GH secretion by GRF is distinct from the cAMP dependant pathway. Increased intracellular $Ca^{2+}$ levels stimulate the secretion of GH from the releasable stores. SS is more inhibitory to the release of GH than to the cAMP dependant pathway, thus the inhibition of AC by SS is illustrated by a dotted line.

GH secretagogues also cause GH secretion by stimulating an increase in intracellular $Ca^{2+}$ levels *via* PI turnover. GH secretagogues do not affect cAMP levels alone, but do potentiate the effects of GRF.




*GH Secretion*

On withdrawal of SS, there is a large rebound release of GH (Clark and Robinson, 1988). This is partly mediated at the pituitary level, as removal of SS from cells in culture will elicit a rebound (Cowan *et al*, 1983), although the effect is much larger *in vivo* suggesting interaction within the hypothalamus (Clark *et al*, 1988). Intracerebroventricular (icv) injection of SS caused an increase in plasma GH levels in conscious rats, which could be blunted by GRF antiserum (Murakami *et al*, 1987). Dickson *et al* (1994) describe a population of neurones in the arcuate nucleus (putative GRF neurones) whose rate of firing is inhibited during stimulation of the periventricular nucleus. A subpopulation of these arcuate neurones exhibited a rebound hyperactivation, suggesting that increased GRF neuronal activity may be involved in the rebound GH secretion response. There is also immunocytochemical evidence that SS neurones in the periventricular nucleus project to the arcuate nucleus (Ishikawa *et al*, 1987) and for SS binding sites on GRF neurones (Epelbaum *et al*, 1989). In addition, dual chromagenic and autoradiographic *in situ* hybridization revealed that, of the GRF neurones in the arcuate nucleus, 15% expressed the SSTR1 and 15% expressed the SSTR2 genes (Tannenbaum *et al*, 1998). SSTR3 mRNA has also been localized to the arcuate nucleus (Senaris *et al*, 1994).

In addition to innervating the arcuate nucleus to regulate GRF neurones, SS is also thought to influence it's own production by an ultrashort feedback loop. SS added to the culture medium inhibited the release of SS from dispersed hypothalamic cultures (Richardson and Twente, 1986). In addition, SSTR1 mRNA shows the same distribution in the hypothalamus as SS (Beaudet *et al*, 1995). While this loop may be mediated *via* autocrine stimulation of autoreceptors, SS has been shown to stimulate gamma aminobutyric acid (GABA) secretion from hypothalamic cultures (Gillies, 1997,). GABA antagonists, in turn, stimulated SS release from hypothalamic cultures (Gillies and Davidson, 1992). It is therefore possible that inhibition of SS release by SS is mediated *via* additional neuronal pathways.

Feedback mechanisms occur at the level of the pituitary and hypothalamus in order to regulate the GH axis. For instance, the GH pulse released as a result of the rebound response may act in turn to stimulate SS. If GH bursts are prevented by GRF antiserum, the pulses of SS no longer occur. If a bolus of hGH

is then administered, the SS increase occurred as usual (Sato *et al*, 1989). Furthermore, administration of exogenous pulses of hGH resulted in the endogenous rGH pulses becoming entrained, thus supporting the involvement of the GH pulse in modulating GRF and SS patterns (Carlsson and Jansson, 1990). In addition, GH increased SS, and reduced GRF in conditioned medium and cell extracts of hypothalamic cultures (Vazquez *et al*, 1993). In the same study, IGF-I increased SS levels in the medium, but had no effect on GRF. Furthermore, IGF-I was found to feed back at the level of the pituitary, reducing GRF stimulated GH secretion from perfused anterior pituitary cells (Ceda *et al*, 1987) and pituitary explant cultures (Goodyer *et al*, 1984). In addition to GH secretion, IGF-I also reduces GH mRNA levels in anterior pituitary cultures (Yamashita and Melmed, 1986).

The complicated interplay between GRF, SS and GH has recently become even more complex with the development of a group of peptide and non-peptide GH secretagogues. Growth hormone releasing peptide-6 (GHRP-6) was the first potent secretagogue to be described and was found to release GH *in vivo* and *in vitro* (Bowers *et al*, 1984; Sartor *et al*, 1985). Slight modification of GHRP-6 lead to the creation of GHRP-2 and hexarelin. Non-peptide secretagogues, such as benzolactam derivatives (L-692,429 and L-692,585) and spiroindanes (MK-0677) are orally active, thus have considerable potential as therapeutic agents (Smith *et al*, 1997). Although GH secretagogues were thought to release GH specifically, there are reports of cortisol and PRL responses in rats (Thomas *et al*, 1997) and humans (Massoud *et al*, 1996).

The difference in time course of GH secretagogues compared to GRF, and the fact that pre-treatment with GRF reduced subsequent response to itself, but not GHRP-6, raised the possibility that a different mechanism of action was involved (Sartor *et al*, 1985). However, since the Little mouse does not respond to GHRP-6, this indicates that an intact GRF receptor system is apparently needed (Jansson *et al*, 1986), a paradox that has yet to be resolved. In rat pituitary cultures, GRF causes activation of protein kinase A (PKA) and accumulation of cAMP. GHRP-6 alone had no effect on cAMP, but potentiated the effects of GRF (Wu *et al*, 1996). In rat anterior pituitary cultures, GHRP-6 did not increase Pit-1 mRNA, unlike GRF or forskolin (Soto *et al*, 1995). Pit-1 is involved in GH gene

expression and somatotroph proliferation (see section 1.2.5), thus GHRP-6 probably acts only on release of stored GH. GH secretagogues have been shown, by patch clamp studies and indo-1 $Ca2^{+}$, to elevate intracellular $Ca^{2+}$ levels (Herrington and Hille, 1994). Phosphatydylinositol (PI) turnover is also increased in response to GH secretagogues (Adams *et al*, 1998).

Since GH secretagogues are more potent *in vivo* than *in vitro,* and more potent in conscious than anaesthetized animals, it was thought likely that in addition to pituitary effects, these compounds may act centrally (Clark *et al*, 1989; Dickson *et al*, 1993). GHRP-6, L-629,585 and L-629,429 induced *c-fos* expression in a population of arcuate nucleus neurones (Dickson *et al*, 1995b). 25% of GHRP-6 stimulated Fos positive cells also contained GRF (Dickson and Luckman, 1997). The possibility of *c-fos* induction being mediated by feedback from the GH axis was ruled out as Fos staining was also present in the little mouse, although this model does not respond to GHRP at the level of the pituitary. Furthermore, neither GH or IGF-I administered icv to *dw/dw* rats caused an increase in the number of Fos positive cells (Dickson *et al*, 1995a). In the anaesthetized guinea pig, much smaller doses of secretagogues icv, compared to intravenously (iv), were needed to elicit GH release, suggesting that the primary action of GHRP-6 is hypothalamic in this model (Fairhall *et al*, 1995).

The GH secretagogue receptor (GHS-R) was recently cloned (Howard *et al*, 1996; Pong *et al*, 1996) and found to be a 7 trans-membrane domain G-protein coupled receptor. *In situ* hybridization revealed the receptor to be located in the arcuate and ventromedial nuclei (Bennett *et al*, 1997) and, at much lower levels, in the anterior pituitary (Guan *et al*, 1997). GHS-R has been localized to certain human tumours expressing GH or PRL, where increased PI turnover was stimulated by GHRP-2 (Adams *et al*, 1998). GHS-R mRNA was also detected by PCR in $GH_3$ cells (Adams *et al*, 1998). $E_2$ treatment of male rats increased the level of expression in both the arcuate and periventricular nuclei (Carmignac *et al*, 1998). In addition, in the same study untreated female and $E_2$ treated male *dw/dw* rats showed substantial PRL release in response to GHRP-6. PRL release from pituitary cells derived from lactating rats has also been reported (Cheng *et al*, 1993). As the GHS-R is only found on cells containing GH (Hreniuk

*et al*, 1996; Smith *et al*, 1997), the release of PRL in both of these models suggests that GH secretagogues may be acting on mammosomatotrophs.
Interestingly, GH secretagogues and GRF have been shown to target different sub-populations of somatotrophs in rats by RHPA (Mitani *et al*, 1996).
The GH secretagogue story is an example of reverse endocrinology. In most systems, the ligand is discovered and used as a tool to locate the receptor. In this case, the receptor has been discovered, along with a selection of agonists, however no endogenous ligand has been discovered as yet. The existence of a functional receptor, conserved between species, suggests that it has an endogenous role, but probably in secretion rather than in GH synthesis or somatotroph proliferation.

### 1.2.6 Proliferation and Cell Specific Gene Expression

Although the development of the somatotroph population in the fetal pituitary is not dependant on GRF, proliferation of somatotrophs in the mature pituitary gland is driven by GRF. The little mouse shows normal levels of GH and Pit-1 in the embryonic gland but severe pituitary hypoplasia in the adult, reflecting a preferential loss of somatotrophs (Lin *et al*, 1993). Although the mutation is in the GRF receptor, specific to the somatotroph, this mouse also shows decreased PRL pituitary content. As previously described, the lactotroph population is thought to be derived post-mitotically from mammosomatotroph precursors. Mice transgenic for hGRF were found to have mild hyperplasia of mammosomatotrophs (Stefaneanu *et al*, 1989), indicating that these cells also proliferate in response to excess GRF. In addition, human patients with ectopic GRF also show hyperplastic anterior pituitaries (Sano *et al*, 1988), thus even the adult pituitary has enormous proliferative potential.

*In vitro*, somatotrophs showed a 20 fold increase in the percentage of $^{3}$H-thymidine labelled nuclei and a 60% increase in GH positive cells when treated with 5nM GRF for 18 days (Billestrup *et al*, 1986). The effect was reproduced by forskolin, suggesting that adenylate cyclase mediated cAMP accumulation was involved. 40% of GH secreting tumours have mutations which lead to a constituitively active $G_s\alpha$ proteins (Landis *et al*, 1989) and targeting cholera toxin (an irreversible $G_s\alpha$ activator) to somatotrophs by the GH promoter leads to somatotroph hyperplasia and elevated serum GH levels (Burton *et al*, 1991).

Signalling *via* cAMP occurs by phosphorylation of a cAMP Response Element Binding Protein (CREB) and it s subsequent binding to a cAMP Response Element (CRE) in the promoter of the genes. A transgenic mouse with a non-phosphorylatable CREB, which presumably competes with endogenous CREB, has a pituitary selectively deficient in somatotrophs, indicating the involvement of this pathway in somatotroph proliferation (Struthers *et al*, 1991). The expression of proto-oncogenes is often associated with proliferation and differentiation in response to growth factors. The proto-oncogene *c-fos* is one gene with a CRE in it's promoter, and GRF has been shown to stimulate fos expression in pituitary cultures (Billestrup *et al*, 1987).

Another target gene of CREB is *Pit-1*, which possesses two CREs in it s promoter. In addition to activation by GRF, Pit-1 up-regulates it s own gene expression due to the presence of a Pit-1 response element in it's own promoter. Pit-1 is detected in Snell mice until birth, indicating that autoregulation is not needed in early development, although the levels are reduced by e18.5 (Rhodes *et al*, 1993). The mutant form of Pit-1 produced in embryonic Snell mice is unable to transactivate the *Pit-1* gene. Mutant Pit-1 is not found after birth, confirming that autoregulation is essential in the mature pituitary. A second form of positive autoregulation of the pathway is also present, as cAMP dependant pathways also up-regulates the expression of GRF receptors (Horikawa *et al*, 1996; Lin *et al*, 1992).

Pit-1 response elements on GH and PRL genes were identified by DNase footprinting (Bodner and Karin, 1987; Ingraham *et al*, 1988; Nelson *et al*, 1988). The GH promoter has two Pit-1 sites and the PRL promoter has 4 proximal and 4 distal sites. The TSHβ promoter also has a site capable of binding Pit-1 (Kim *et al*, 1995). Expression of Pit-1 in HeLa cells leads to expression of both GH and PRL (Mangalam *et al*, 1988) however Pit-1 is not sufficient alone or all GH or PRL cells would co-express both hormones. Cell specific expression is achieved by co-ordination of Pit-1 with other transcription factors, some of which are cell specific and others ubiquitous. Somatic cell hybridization of non-GH expressing L-cells with pituitary derived $GH_3$ cells was found to result in extinction of GH production (McCormick *et al*, 1988) suggesting that repressor elements are also involved in the cell specific expression.

The rGH gene possesses a thyroid hormone ($T_3$) response element (TRE), to which the $T_3R$-$T_3$ receptor-ligand complex binds specifically. Activation of this TRE in co-operation with cAMP mediated Pit-1 binding have been shown to increase GH gene expression (Copp and Samuels, 1989). This is supported by *in vitro* studies showing that thyroid hormone deprivation reduced the GH content of the cells (Dieguez *et al*, 1985). A zinc-finger protein, Zn-15, also synergizes with Pit-1 in GH expression by binding the "GH Z box" between the two Pit-1 response elements (Lipkin *et al*, 1993). Mutation of the GH Z box reduces GH expression by more than 100 fold. Glucocorticoids induce GH synthesis in $GH_3$ and GC cell lines (Yu *et al*, 1977) and anterior pituitary primary culture (Evans *et al*, 1982). They also appear to modulate the sensitivity of the somatotroph to GRF, as dexamethasone restores pulsatile GH secretion caused by adrenalectomy (Krempels *et al*, 1994) and increases GRF receptor mRNA in rat pituitary cultures (Tamaki *et al*, 1996).

The PRL promoter possesses an oestrogen response element, ERE, which binds ligand associated oestrogen receptor (ER). Co-operation of ER and Pit-1 are involved in targeting cell specific gene expression of PRL (Nowakowski *et al*, 1994). In addition, the rPRL promoter has sequences for ubiquitous transcription factors including activating protein 1 (AP-1), AP-2 and basic transcription factor (BTF) (Gourdji and Laverriere, 1994). $T_3R$ has been shown to negatively regulate the PRL gene by either interfering with AP-1 in the hPRL promoter (Pernasetti *et al*, 1997) or competing with ER for the ERE in the rPRL promoter (Glass, 1988).

Various splice variants of Pit-1 have been reported, which may possess differential specificity for their target genes. A 36 kDa variant known as Pit-1β, GHF-2 or Pit-2 is present in rat (Vila *et al*, 1993; Theill *et al*, 1992; Diamond and Gutierrez-Hartmann, 1996) and human (Delhase *et al*, 1995). This variant has a 26 amino acid insert at the end of exon 1 and has reduced ability to transactivate the PRL, TSHβ or *Pit-1* promoter. Δ4Pit-1 is another naturally occurring variant, resulting form the splicing out of the POU specific domain coded for by exon 4 (Voss *et al*, 1993). This variant can neither bind nor transactivate the PRL promoter. Transplantation of $GH_3$ cells, which express both GH and PRL, into Wistar-Furth rats results in an increase in the levels of Δ4Pit-1 and

repression of PRL gene expression (Day and Day, 1994 a,b). After returning the tumours to culture, the levels of Δ4Pit-1 fell coupled with the return of PRL expression. Pit-1T (Haugen *et al*, 1994) has a 14 amino acid insert at the end of exon 1, similar to Pit-1β. In combination with Pit-1, Pit-1T synergistically activated TSHβ but not GH or PRL in $GH_3$ cells and a TSH cell line lacking other Pit-1 variants.

### 1.2.7 Mammosomatotrophs

In addition to the roles of GH and PRL co-expressing cells in pituitary development, mammosomatotrophs are also present in the mature pituitary gland. In the cow, all acidophils are reported to be mammosomatotrophs, with the proportion of each hormone varying from cell to cell (Kineman *et al*, 1990). Mammosomatotrophs were identified in the rat by double immunogold staining of pituitary sections (Nikitovitch-Winer *et al*, 1987) and RHPA (Frawley *et al*, 1985; Leong *et al*, 1985), identifying cells containing and releasing both hormones respectively.

In addition, evidence that these cell types can reversibly interconvert, or transdifferentiate (Beresford, 1990), came from two conceptually similar studies. The relative proportions of GH, PRL and double secreting cells were measured by RHPA in virgin, pregnant and lactating rats. The proportion of PRL secretors increased and the proportion of GH secretors decreased during this time without altering the total cell number. The mammosomatotroph appeared to serve as a functional intermediate (Porter *et al*, 1990). Support for this theory comes from the fact that the cell populations returned to pre-gestational proportions after weaning, thus showing that the changes were reversible (Porter *et al*, 1991). The second study showed that the proportion of GH only- and double- secreting cells fluctuated throughout the bovine estrous cycle, without affecting the number of PRL only secreting cells (Kineman *et al*, 1991). Both of these experiments show that changes in the steroidal status of the host lead to shifts in cell specific expression of GH and PRL. Cortisol and progesterone caused an increase in both GH only- and double- secreting cells and a decrease in PRL secreting cells in bovine pituitary cultures (Kineman *et al*, 1992). In the same study, $E_2$ had no effect on the cell populations. However, Boockfor *et al* (1986) found that $E_2$ treatment of rat pituitary cultures caused an increase in the proportion of double

secretors and a decrease in cells secreting GH only. The effects of $E_2$ treatment of rat pituitary cells will be covered in more detail in section 1.2.8.

An alternative mechanism for the alteration of the distribution of cell types in the pituitary is apoptosis and mitosis (Nolan *et al*, 1998). The basal rate of apoptosis and mitosis results in 1.58% turnover of the anterior pituitary cell population per day, thus the impact of this mechanism cannot be dismissed. Adrenalectomy, followed by dexamethasone replacement resulted in a burst of apoptotic activity and reduced mitotic figures (Nolan *et al*, 1998). In a separate study, Fischer 344 rats were $E_2$ treated, resulting in lactotroph hyperplasia. bromocriptine treatment, after the withdrawal of $E_2$, resulted in increased rate of apoptosis in the hyperplastic lactotrophs. Apoptotic bodies were subsequently phagocytosed by FS cells (Drewett *et al*, 1993).

In addition to steroids, hypothalamic peptides can affect the proportion of the cell types. For instance, GnRH treatment of rat pituitary cultures caused an increase in PRL secretors and a decease in GH secretors. This pattern was not changed if cytosine arabinoside was included, suggesting that the effects were due to transdifferentiation, and not proliferation, in this case (Hoeffler and Frawley, 1987; Frawley and Hoeffler, 1988). GRF also caused an increase in the number of PRL cells, but only in the absence of cytosine arabinoside. If all lactotroph differentiation is post-mitotic, GRF may be expanding the mammosomatotroph or somatotroph population and the cells then interconvert to become lactotrophs.

Frawley and Boockfor (1991) proposed that, as somatotrophs and lactotrophs are thought to both be derived from the same precursor cells and are able to functionally interconvert in the mature pituitary, they may represent a single cell type. It is conceivable that all acidophils have the potential to express GH and PRL and that the somatotroph and lactotroph phenotypes represent the two extremes, while mammosomatotrophs have an intermediate phenotype. If this is the case, then cells referred to as somatotrophs or lactotrophs would have to be redefined as cells showing somatotroph or lactotroph phenotype at a particular time. This raises the question of by what criteria are somatotrophs and lactotrophs defined. For instance, is any cell that contains or secretes GH a

somatotroph? Are the hormone products the only markers of somatotroph or lactotroph phenotype, or are other proteins differentially expressed that could define the cell types? (See section 1.5.2)

Cell lines derived from rat pituitary tumours (eg $GH_3$, $GH_4C_1$) and cultures (eg $P_0$) frequently secrete both GH and PRL (Kineman and Frawley, 1994). Acromegalic patients have been reported to release GH in response to TRH (Irie and Tsushima, 1972) and PRL in response to GRF (Losa *et al*, 1985) which is typical of mammosomatotrophs. In addition, mice transgenic for GRF developed hyperplasia of mammosomatotrophs (Stefaneanu *et al*, 1989). It would appear that either mammosomatotrophs are more prone to proliferate, or that hyperplasia or tumour formation reduces the cell specific constraints of gene expression. Although cell lines are often regarded as clonal populations, variation in the secretory characteristics is common. For instance, $GH_3$ cells consist of a mixture of GH only-, PRL only- and double secreting cells (Kineman and Frawley, 1994). Furthermore, the proportions of the subpopulations can also be influenced by exogenous factors. For instance, $E_2$ treatment of $P_0$ cells increased the proportion of PRL secreting cells without affecting the GH secreting cells. In addition the rate of proliferation was reduced to 20% of control (Kineman and Frawley, 1994).

### 1.2.8 Oestrogen Treatment

$E_2$ treatment has been shown to recruit "GH-only" cells to produce PRL, although the mechanism differs depending on the time course. Acute $E_2$ treatment both increases the rate of PRL gene transcription in pre-existing lactotrophs and recruits PRL production in somatotrophs to increase the total PRL positive population. This recruitment occurs exclusively in the inner region of the anterior pituitary (Porter and Frawley, 1992) and is abolished by posterior lobectomy (Murai and Ben-Jonathon, 1990). The effect has been studied *in vitro* by Ellerkmann *et al* (1991, 1992) by RHPA. Anterior pituitaries were co-cultured with neuro-intermediate cultures on a separate, removable coverslip. The increase in PRL secretors was abolished if the neuro-intermediate culture was removed before the 3 hour $E_2$ treatment, and could be mimicked by α-MSH. The effect was prevented by the protein synthesis inhibitor cyclohexamide but not the

RNA synthesis inhibitor actinomycin D, thus the recruitment is regulated posttranscriptionally (Porter *et al*, 1992).

The long term effects of $E_2$ are not mediated by α-MSH, as the increase in PRL positive cells anterior pituitary cultures treated for 5-6 days did not require the presence of the neuro-intermediate lobe in the culture (Leiberman *et al*, 1982; Boockfor *et al*, 1986). Sequential RHPA for GH and PRL was used on $E_2$ treated cultures to show that the increase in total PRL secretors was due to recruitment of somatotrophs to produce PRL without affecting the total percentage of GH secretors (Boockfor *et al*, 1986). This study did not, however, exclude the possible contribution of lactotroph mitosis, suggested by Corenblum *et al* (1980). Lieberman *et al* (1982) found that cytosine arabinoside, a DNA synthesis inhibitor, had only a minor effect on the $E_2$ induced PRL synthesis, suggesting that mitosis only has a small contribution to the total increase. Long term treatment of Fischer 344 rats with diethylstilbestrol however, induces large prolactinomas (Hymer and Motter, 1988; Phelps and Hymer, 1983), supporting the contribution of mitosis in these particularly estrogen sensitive rats (Wiklund *et al*, 1981). The presence of ERs in lactotrophs and EREs on the PRL promoter suggests that the actions are direct, however there is also evidence for the mediation of $E_2$ effects by galanin. Galanin, a 29 amino acid peptide, is localized throughout the hypothalamus and pituitary. It is found in lactotrophs of female and $E_2$ treated, but not lactotrophs of male rats. Galanin release is regulated by the same factors as PRL (Hyde and Keller, 1991) and co-localized in the same secretory granules as PRL (Hyde *et al*, 1991) in $E_2$-treated Fischer 344 rats. The effects of long term $E_2$ are abolished by galanin antiserum (Wynick *et al*, 1993), and homozygous galanin mutant mice show no proliferative response to $E_2$ in addition to reduced basal prolactin pituitary content and secretion (Small *et al*, 1998).

Kimura *et al* (1986) reported SS inhibition of PRL, coincident with an increase in SS binding sites in the anterior pituitary, was dependant on the presence of $E_2$, later confirmed by RHPA by Goth *et al* (1996). $E_2$ treatment for 4-6 weeks of ovarectomized rats caused up-regulation of the SSTR2 subtype and down regulation of SSTR5 subtype (Kimura *et al* 1989, 1998). The two subtypes differ in affinity to various SS agonists and in signalling mechanisms. SS decreases

basal and forskolin stimulated cAMP in pituitary membrane preparations from $E_2$ treated rats, however control ovarectomized rats showed no decrease in cAMP levels, suggesting that SS action on decreasing GH secretion are mediated by other pathways (Kimura *et al*, 1989). The SSTR2 has been shown mediate SS effects by inhibition of voltage gated $Ca^{2+}$ channels and reducing the intracellular $Ca^{2+}$ concentration (Fujii *et al*, 1994).

### 1.2.9 Somatotroph and Lactotroph Heterogeneity

It is simplistic to describe somatotrophs and lactotrophs as populations of identical cells. Both have been shown to exhibit heterogeneity structurally and functionally. Somatotrophs have been separated into type I and type II by BSA (Snyder *et al*, 1977) and Percoll (Lindstrom and Savendhal, 1996) density gradients, where type I were less dense than type II. Type II cells were found to release more GH under basal conditions, whereas type I showed a higher rate of GH synthesis than type II (Snyder *et al*, 1977). Type II cells were more responsive and sensitive to GRF although a higher proportion of type I cells responded to SS (63% *vs.* 17%) (Lindstrom and Savendhal, 1996). Functional heterogeneity of somatotrophs was also shown by RHPA (Frawley and Neill, 1984) where GRF treatment (100nM) of anterior pituitary cells produced a bimodal distribution of plaque size. Furthermore, Hoefler *et al* (1987) found that IGF-1 acts only on a subpopulation of somatotrophs, and this population is, at least partly, distinct from the SS responsive cells. Similarly, differential responses of lactotrophs to TRH (Hu and Lawson, 1994), and to PRL and DA (Frawley and Clark, 1986) have been reported. Analysis of the calcium currents in somatotrophs and lactotrophs have revealed silent and spontaneously active subpopulations of these cells, which may also relate to secretory function of these cells (Lewis *et al*, 1988).

These subpopulations are only grouped by virtue of their hormone contents and secretory function and it is not known whether these subtypes represent different developmental stages. Again, this raises the question of by what criteria are cell types classified.

## 1.3 DWARF MODELS

Much has been learned about the mechanisms involved in the GH axis by investigating animals models defective in part of it. It is only recently that artificially created models have been available, however nature has provided us with spontaneous dwarfs which have been invaluable in the study of the GH axis. The characteristics of these dwarf mice and rats are summarized in table 1.1.

In addition to the Snell, Jackson, Ames and Little mice described in section 1.1.3, three spontaneously dwarfed rats have been described all of which show autosomal recessive inheritance. The SDR rat possesses a single base substitution at the 3' splicing site of the third intron of the GH gene. This results in a deletion in the GH mRNA and a premature translational termination (Takeuchi *et al*, 1990). Unlike the Snell mouse (Wilson and Christiensen, 1981), electron microscopy reveals that presumptive somatotrophs are present, although these cells have few or no secretory granules (Nogami *et al*, 1989a). Immunocytochemistry of the SDR pituitary, which is of similar weight as controls, reveals a high percentage of cells which do not stain for ACTH, PRL, TSH, LH or S-100 (Nogami *et al*, 1993). The relative increase in these non-staining cells appears to correlate with the somatotroph population in controls, suggesting that the SDR contains a normal number of presumptive somatotrophs that do not contain GH protein. Although immunocytochemistry did not detect GH staining cells, GH mRNA was shown to be present in the anterior pituitary by *in situ* hybridization (Nogami *et al*, 1993).

The lack of GH feedback would be expected to influence the levels of hypothalamic factors. This would result in an increased stimulation of the pituitary, conceivably causing hyperplasia of presumptive somatotrophs. Indeed, GRF mRNA has been shown to be increased (Kamegai *et al*, 1997), but somatotroph hyperplasia is not apparent. It is likely that GH is not the only product of the somatotroph and other products may be involved in the feedback mechanisms controlling the proliferative signals.

## Table 1.1: Spontaneous rat and mouse dwarfs.

| Model | Defect | Phenotype | Reference |
|---|---|---|---|
| Snell Mouse (*dw/dw*) | Point mutation in *Pit-1* | Hypoplastic pituitary lacking somatotrophs, thyrotrophs and lactotrophs. Sterile. | Snell, 1929; Li *et al*, 1990 |
| Jackson mouse ($dw^J/dw^J$) | Gross rearrangement of *Pit-1* | Hypoplastic pituitary lacking somatotrophs, thyrotrophs and lactotrophs. Sterile. | Li *et al*, 1990 |
| Ames mouse (*df/df*) | Point mutation in *Prop-1* | Hypoplastic pituitary lacking somatotrophs, thyrotrophs and lactotrophs. Sterile. | Anderson *et al*, 1995 Gage *et al*, 1995; Sornson *et al*, 1996 |
| Little mouse (*lit/lit*) | Point mutation in GRF-R | Hypoplastic pituitary with severely reduced somatotroph number. No cAMP or GH secretion in response to GRF. Mild PRL deficiency. Mildly reduced fertility (males take longer to sire young) | Lin *et al*, 1993 |
| Spontaneous dwarf rat (SDR) | Point mutation in GH | Pituitary normal weight. Increased population of non-hormone producing cells. Reduction in lactotroph number. | Takeuchi *et al*, 1990; Nogami *et al*, 1993 |
| Hypothyroid rat (*rdw*) | Secretory defect of the thyroid | Reduced GH and PRL protein and mRNA. Increased TSHβ mRNA. Many proteins expressed at different level in thyroid. Sterile. | Koto *et al*, 1988; Oh-Ishi *et al*, 1998 |
| Dwarf rat (*dw/dw*) | Unknown | Reduced somatotroph number. Reduced GH content per somatotroph. Reduced cAMP response to GRF, although secretory response relatively intact. PRL and lactotroph number normal or increased. | Charlton *et al*, 1988); Downs and Frohman, 1991; Carmignac, 1991 |

In addition to the GH phenotype, the PRL levels in serum and pituitary of the SDR are 50% and 65% of controls respectively and PRL mRNA is about 30-55% of controls. The PRL gene structure and mRNA size are not different to controls (Nogami *et al*, 1989b). By immunocytochemistry, the lactotroph population is reduced by 40% in the male and 30% in the female compared to controls (Nogami *et al*, 1993). This reduction does not fully account for the PRL mRNA and protein contents of the pituitary, thus the content per cell must also be reduced.

The *rdw* rat (Koto *et al*, 1988) has pituitary hypoplasia and reduced GH and PRL protein (1/15 and 1/30 of control) and mRNA (1/30 and 1/100 of control) (Oh-Ishi *et al*, 1998). Due to the similarity with the Snell mouse, also showing pituitary hypoplasia and multiple hormone deficiency, *Pit-1* was an obvious candidate for the defect. However, similar levels of Pit-1 mRNA and protein were found in *rdw* and control rats. Furthermore, the distribution of Pit-1 was similar to controls and the amino acid sequence was also identical (Ono *et al*, 1994). In contrast to the Snell mouse, the level of TSHβ mRNA was about 7 fold higher in the *rdw* than controls. Analysis of protein extracts of various organs by 2D-gel electrophoresis revealed that in addition to differences in hormone levels in the pituitary, the thyroid gland had much more severe abnormalities (Oh-Ishi *et al*, 1998). At least 18 proteins were present at higher concentration than controls, and 17 proteins were at reduced concentrations. Three of the proteins present at high levels were identified, and found to belong to the chaperone protein family. Accumulation of these proteins may be caused by a disorder affecting protein folding and secretion. The same study provided confirmation that the thyroid gland is responsible for the phenotype, as transplantation of thyroid glands from control animals into *rdw* infants allowed these animals to mature to normal size and fertility, facilitating breeding of homozygotes. Thus the pituitary phenotype in this model is secondary to the thyroid defect.

The *dw/dw* rat (Charlton *et al*, 1988) has reduced pituitary GH content (5-10% of normal) and reduced somatotroph cell number (15-20% of normal). Pituitary size and body weight are about 40% reduced. Downs and Frohman (1991) calculated the GH content per somatotroph to be 40% of normal somatotrophs. The releases of GH per cell is reduced compared to normal somatotrophs, measured by plaque area in RHPA (Kineman *et al*, 1989), but when expressed as

percentage of content, the basal GH secretion in culture is not significantly less than normal (Downs and Frohman, 1991). GRF treatment caused a 4 fold increase in plaque size in both *dw/dw* and control cells, indicating that the *dw/dw* somatotrophs still respond to GRF with GH release (Kineman *et al*, 1989). Plaque size is only semi-quantitative, thus measurements of GH release in culture give a better indication of secretion. *In vivo*, the reduced basal and GRF stimulated GH peaks were largely attributed to the reduced pituitary content (Carmignac and Robinson, 1990). Shortly after, investigation of the GRF dose response *in vitro* revealed *dw/dw* cultures be 2.5 fold less sensitive to GRF than control, and at maximal stimulation the *dw/dw* cultures responded 25% less with respect to GH content (Downs and Frohman, 1991).

In contrast to this slight impairment of secretory function, the ability of GRF to stimulate cAMP accumulation is severely diminished. 10nM GRF caused a 63 fold increase in cAMP levels in cultured normal pituitaries, compared to 1.9 fold in *dw/dw* cultures, extrapolating to a 6-10 fold deficiency per somatotroph (Downs and Frohman, 1991). They also investigated the effects of other factors on cAMP accumulation and GH release. The GH release was included due to the fact that the compounds used were non-specific and would act on other cell types as well as somatotrophs. The cAMP and GH secretion response to forskolin was not different, however the GH secretion and cAMP responses and sensitivity to cholera toxin were impaired in the *dw/dw*. This lead to the hypothesis that the defect was due to the inability of $G_s\alpha$ to stimulate adenylate cyclase in the *dw/dw*. However, other $G_s\alpha$ mediated responses, such as the increase in ACTH release in response to cholera toxin, were still intact in the *dw/dw*, thus any defect in $G_s\alpha$ would have to be restricted to the somatotrophs. In a later study, no differences in size, quantity or distribution of $G_s\alpha$ were found, and sequencing did not reveal any mutations (Zeitler *et al*, 1993). They also found no differences in the content or size of the Pit-1 mRNA. This is in contrast to Houston *et al*, (1991), who reported significantly lower Pit-1 mRNA levels in *dw/dw* pituitaries. The most important point raised by these studies on signal transduction, is that the defect in the *dw/dw* distinguishes between the actions of GRF. While GH secretion is relatively intact, the main deficiency appears to be the reduced number of somatotrophs and the inability to synthesize GH. This was also shown *in vivo* by Turner and Tannembaum (1995). As described earlier,

somatotroph proliferation and GH gene expression are regulated through cAMP mediated mechanisms. There is evidence, however that GRF also activates other signalling pathways, for instance Canonico *et al* (1983) proposed that GRF also stimulates PI turnover, although this is disputed by others (Kraicer and Sims, 1994). The model proposed by Kraicer and Sims (1994) is that GRF signalling also causes the opening of sodium channels, resulting in depolarization of the cell and thus opening of voltage gated $Ca^{2+}$ channels. It is of interest that the GRF receptor possesses two splice variants, one of which is predicted to have an extra 41 amino acids in the third intracellular loop (Mayo, 1992). An analogous alternative splice site in the PACAP receptor alters it's coupling to cAMP and phospholipase C second messenger systems (Spengler *et al*, 1993). A difference in the relative levels of these splice variants could explain the selective deficiency in *dw/dw* cAMP dependant signalling, however both variants have been detected in *dw/dw* pituitaries by RT-PCR (Hoeh and Zeitler, 1993).

The *dw/dw* differs from the Little mouse which is unable to respond to GRF. Other, differences between these two models suggest that the defect in GRF signalling may not be the cause of the phenotype in the *dw/dw*. The somatotroph population in the Little mouse develops normally in the embryonic pituitary, thus GRF signalling is not required at this stage. The *dw/dw* however, already has severe deficiencies in GH content and somatotroph number before birth (Carmignac *et al*, 1993b; Zeitler *et al*, 1994).

The Snell, Jackson and Ames mice all lack somatotrophs, lactotrophs and thyrotrophs due to mutations in *Pit-1* (Snell and Jackson) and *Prop-1* (Ames) and the *rdw* hypothyroid rat also shows severe deficiency in prolactin. In addition, although the GRF receptor mutation in the little mouse and the GH mutation in the SDR rat would not be expected to affect the lactotroph phenotype, both also show a reduction in PRL. The *dw/dw* rat is unique in that it does not show a deficiency in PRL content (Charlton *et al*, 1988; Bartlett *et al*, 1990) or cell number (Kineman *et al*, 1989). The percentage of lactotrophs were even found to be higher than the control, although this may compensate for reduced pituitary size (Kineman *et al*, 1989). In addition basal and TSH stimulated plaque size were similar to control lactotrophs suggesting that the *dw/dw* lactotrophs secretory function is normal. PRL deficiency has been linked to infertility

(Barkley *et al*, 1982), and PRL treatment of Snell and Ames dwarves promotes testicular growth and fertility (Bartke *et al*, 1977) and increases testicular PRL and LH binding sites (Bohnet and Friesen, 1976). Even the milder PRL deficiency in the little mouse leads to the males taking longer to sire young (Chubb, 1987). In contrast, exogenous PRL given to normal rats results in precocious puberty (Clemens *et al*, 1969). *Dw/dw* rats show normal testicular function and fertility, although litter sizes tend to be lower possibly due to maternal size (Bartlett *et al*, 1990). However, a fertility defect has been reported in one sub-colony of *dw/dw* rats, provided by our lab (Breier *et al*, 1996).

## 1.4 THE STUDY OF PITUITARY CELL TYPES

### 1.4.1 Detection of Mammosomatotrophs

Many studies investigating mammosomatotroph populations employ the use of RHPA. The standard RHPA is performed in a Cunningham incubation chamber, constructed by attaching a coverslip to a poly-L-lysine coated microscope slide with two pieces of double sided tape. Protein-A-coated ovine red blood cells are allowed to attach to the slide, and then dispersed pituitary cells are added in medium containing primary antibody. After the incubation period, the plaques are developed using guinea pig complement, which causes the red blood cells to lyse around cells releasing antigens specific to the antibody used. The cells can then be fixed and counterstained for subsequent quantification of plaque forming cells.

The method can be modified in two ways to detect mammosomatotrophs. The simultaneous plaque assay is performed exactly as described, and plaque forming cells in the presence of GH, PRL or a mixture of both antisera quantified. The mammosomatotroph population is calculated by subtracting the proportion of plaque forming cells in response to both antisera from the sum of those formed by individual antisera. Alternatively, the sequential plaque assay can be used, where the same cell population is tested with one antiserum and then the other. The main difference with this method is that the ovine red blood cells are not allowed to attach as the method has to be performed twice. The pituitary cells are allowed to attach to the slide and then the medium, antibody and protein-A-coated red blood cells infused. As with the previous method, the plaques are

developed with guinea pig complement. The plaques formed are recorded photographically and then the cells are washed and the method repeated with the other antibody and fresh protein-A-coated red blood cells. By looking at the photographs, the cells which form plaques in the presence of both antibodies are the mammosomatotroph population.

It is important to consider the criteria by which a method identifies a cell type. RHPA detects cells which are releasing hormone. Methods that rely on the staining of cells, such as immunocytochemistry or immunofluorescence, identify cells by their hormonal content. Both methods may miss mammosomatotrophs as a cell may be storing but not releasing a hormone, or they may be making a hormone for immediate release and thus no stores will be detectable.

When using immunocytochemistry to detect mammosomatotrophs, the method used for visualization needs to be considered. While peroxidase- or alkaline phosphatase- labelled methods can give beautiful two colour staining, if the two antigens are within the same cell or granule they can be difficult to visualize. There are several reports of the use of double immunogold labelling to visualize mammosomatotrophs at the electron microscope level (Nikitovitch-Winer *et al*, 1987; Papka *et al*, 1986). In this method, the second antibodies are conjugated to two different sizes of gold particles (eg 10nM and 20nM), such that cells containing both antigens can be detected. The advantage of this method is that the distribution of staining within the cell can also be seen. Staining for GH and PRL can be seen mainly within the secretory granules, with some staining in the rough endoplasmic reticulum (Papka *et al*, 1986).

Another way of visualizing double stained cells is by immunofluorescence. The fluorescence microscope can filter out light of different wavelengths, thus the sample can be stained simultaneously for both antigens using second antibodies conjugated to fluorescent markers of different colours such as fluorescein-isothionate (FITC: green) and phycoerythrin (PE: red). By superimposing images taken with different filters, double fluorescent cells can be detected.

Fluorescence activated cell sorting (FACS) has also been used to detect mammosomatotrophs (Shinkai *et al*, 1995). Instead of double fluorescently

labelling tissue sections, the cells are dispersed and then stained. Single and double stained cells are quantified as the cells suspension is passed through the FACS machine. The advantage of using FACS analysis as method to quantify cell types is that 10000 cells can be counted from a sample in about a minute, and the data file automatically stored. In addition, many samples can be analysed in a single run (>100) and the method can be completed within a day.

### 1.4.2 Purification of Pituitary Cell Types

Cell types within the anterior pituitary can be separated according to characteristics such as size, density and antigenic specificity.

The size and density of anterior pituitary cells differs between cell types, eg somatotrophs are generally of medium size and high density; lactotrophs are small cells with low density (Scheikl-Lenz *et al*, 1985). These differences in characteristics can be used to separate different sizes and densities of cells in the hope of enriching different cell types. Density gradient centrifugation using BSA (Snyder *et al*, 1977) and Percoll (Lindstrom and Savendhal, 1996) gradients have been used to separate anterior pituitary cells. The cells equilibrate with the area of the gradient at the same density as themselves, and fractions can be collected containing the various cells types. For instance, Allaerts and Denef (1989) collected 9 fractions from a 0.3-2.4% BSA gradient, fraction 1 being the least dense. FS cells were enriched to 65% in fraction 2, lactotrophs to 55% in fraction 4, and somatotrophs to 48% in fractions 7-9. As mentioned earlier, however, pituitary cells exhibit heterogeneity in structure as well as function. The distribution of somatotrophs (Snyder *et al*, 1977; Lindstrom and Savendhal, 1996) and lactotrophs (Hu and Lawson, 1994) on the gradient can be bimodal. This is useful for separating the subpopulations of a single cell type, but could interfere with the isolation of a single cell type from the others.

Separation by size can be achieved by counterflow centrifugation or centrifugal elutriation. The cells are centrifuged in a specially designed rotor and are prevented from pelleting by a continuous counterflow of media (Hymer and Hatfield, 1983) and is well suited to the purification of minority cell types. For instance, corticotrophs have been enriched from 9-10% to over 90% (Childs *et al*, 1988). This paper also illustrates another method to aid cell purification. The

size and density of cells can be manipulated to make them more distinct from the rest of the cell types, in this case by adrenalectomy.

Scheikl-Lenz *et al* (1986) combined centrifugal elutriation with density gradient sedimentation to separate cells firstly by size and then by density. Using these methods, they separated 18 fractions containing various proportions of different cells types.

Flow cytometry can also separate cells according to their physical characteristics, as measured by scatter of laser beams. Forward scatter (FSC) is a measure of cell size, determined by the refraction of the laser beam. Side scatter (SSC) measured by reflection and diffraction of the laser beam by structural components of the cell, thus is a measure of complexity or granularity. The characteristics of the cell types analysed by flow cytometry reflect the observations from density gradient and counterflow centrifugation. For instance, somatotrophs have high SSC and medium FSC. Cells sorted for high SSC were 2.4 fold enriched for somatotrophs, whereas cells sorted for medium SSC were 2.5 fold enriched for lactotrophs (Hatfield and Hymer, 1986b). There is a technical difficulty with sorting by side scatter, due to the vibration of the nozzle (to separate the stream into droplets) interfering with the detection of scatter characteristics. Physiological manipulations can also be used to alter the scatter characteristics of the cells; $E_2$ treatment increased the FSC and SSC of lactotrophs, thus making them easier to purify (Hatfield and Hymer, 1986a).

As pituitary cell types are defined by the hormone or hormones that they produce, the most definitive way to select for a cell type is by it's product. FACS can be utilized for this purpose, separating cells by fluorescent markers as well as the scatter characteristics previously described. Although pituitary hormones are stored intracellularly, there appears to be sufficient antigen on the surface of live cells to specifically bind primary antibodies (St. John *et al*, 1986; Wynick *et al*, 1990b). These are subsequently labelled with fluorescent molecules *via* secondary antibodies.

Other markers can be used to label specific cell types, such as fluorescent ligands eg CRF for corticotrophs, (Schwartz *et al*, 1986), rhodamine conjugated GnRH

antagonist for gonadotrophs (Edwards, 1983) and FITC-conjugated β-subunit of cholera toxin for somatotrophs (Lewis *et al*, 1988).

Another method of separating cells by antigenic specificity is by magnetic beads, which has been utilized for the purification of gonadotrophs (Valenti *et al*, 1995) and lactotrophs and somatotrophs (Wynick and Bloom, 1990). Although this method is quicker than FACS sorting (30 minutes after labelling as opposed to several hours) it only selected 30-40% of the immunoreactive cells possibly due to cleavage of the cell surface hormone off the cell (Wynick and Bloom, 1990).

## 1.5 SPECIFIC AIMS OF THIS THESIS

### 1.5.1 Investigation of Somatotrophs and Lactotrophs in Dwarf Models

The current literature viewpoint concerning the *dw/dw* is that the defect lies in the inability of $G_s\alpha$ to stimulate adenylate cyclase in the somatotroph. This does not, however, fully explain the phenotype of this model as reduced GH is apparent as early as e18, before GRF signalling is necessary to maintain the somatotroph population. If the signalling defect was the cause, this model would resemble the little mouse more closely, showing no reduction in somatotrophs until after birth. The GRF-R mutation in the little mouse and the GH mutation in the SDR rat would be expected to only affect the somatotroph population, however both of these models also show a mild deficiency in PRL. A similar somatotroph specific signalling mutation in the *dw/dw* would be expected to yield a similar phenotype, however there was apparently no deficiency in PRL reported in this model (Charlton *et al*, 1988; Bartlett *et al*, 1990). Thus, the PRL system in *dw/dw* rats is anomalous compared with other somatotroph hypoplasia models, which given the theory of PRL cells being derived from GH cells, is curious. While the GH phenotype of the *dw/dw* has been extensively studied, the PRL axis in this model has been largely ignored. **One of the aims of the work in this thesis, therefore, was to re-evaluate the somatotroph and lactotroph phenotype in the *dw/dw*, of which NIMR houses the original colony.** In addition, *in vitro* studies were employed to further investigate the responses to GRF.

The existence of mammosomatotrophs in the normal adult pituitary is well established, but their presence in *dw/dw* pituitaries has not been previously reported. The absence of PRL deficiency despite the early onset of GH deficiency suggests that precursor cells are likely to be present. In addition, mammosomatotrophs serve as intermediate cells during transdifferentiation of somatotrophs and lactotrophs, thus their continued presence in adults is also interesting. **In order to quantify the various pituitary cell populations, FACS analysis was employed.** FACS analysis has been previously used to investigate pituitary cell populations (eg Shinkai *et al*, 1995; Hatfield and Hymer, 1985), although it has not previously been used to investigate dwarf rats.

Although the *dw/dw* phenotype shows autosomal recessive inheritance, there is some evidence of a gene dosage effect (Houston *et al*, 1991; Houston, 1992; P.A. Bennett, personal communication), as the heterozygote was found to exhibit an intermediate phenotype with respect to Pit-1 and GH mRNA. If there is a gene dosage effect, then it has some bearing on the earlier characterization of this model, which used heterozygotes as controls. In this thesis, **the pituitary cell types of heterozygotes were also quantified by immunocytochemistry, and the behaviour of the cells *in vitro* was examined.**

In addition to the *dw/dw* rat, another dwarf model is available (and was created) in our lab. The transgenic growth retarded rat (Flavell *et al*, 1996) targets hGH to the GRF neurones, inducing local feedback within the hypothalamus. This rat shows reduced growth rate, pituitary GH content and mRNA, pituitary size and somatotroph number. In addition, the PRL content of the pituitary is reduced, although the lactotroph number is normal (Thomas *et al*, 1996). This PRL deficiency is likely to be due to the lactogenic activity of hGH. **The somatotroph and lactotroph populations of TGR rats were also investigated by FACS analysis, as well as the effects of tissue culture,** in order to provide a useful control in which somatotroph deficiency is secondary to a hypothalamic effect of a transgene. It should be stressed that, while the TGR is an interesting model in itself, it's role in this thesis is primarily as a control for comparison with the *dw/dw* rat.

### 1.5.2 Somatotroph Specific Protein Markers

It is often useful to use marker proteins to identify cell types, other than their major product, for instance tyrosine hydroxylase (TH) is used as a marker protein for dopamine neurones. The difficulty in defining somatotrophs, lactotrophs and intermediates has been eluded to earlier, and stems from the use of their hormone products as markers. **The aim of the work in chapter 6 is to attempt to identify marker proteins for the somatotroph.** The advantage of a marker protein over GH itself are two-fold;

1. The GH content of a somatotroph is not constant. Stores are built up and then released, altering the GH content with time. Also, the GH content in a *dw/dw* somatotroph is greatly reduced compared to normal rats, while the SDR rat lacks GH protein completely. Thus, the use of an alternative protein

to mark and define somatotrophs may be more reliable. A non-releasable marker protein is more likely to reflect cell numbers. If so, it may be possible simply to measure the amount of such a protein in a pituitary extract to quantify the cell numbers

2. If the marker protein identified is involved in the processes of GH gene expression or somatotroph proliferation, it may be reduced or defective in the *dw/dw*. If this is the case, it may bring us closer to understanding the mechanisms of the defect in this model.

The ability of somatotrophs and lactotrophs to transdifferentiate demonstrates the need for markers other than GH for somatotroph function. If the somatotroph is not a committed cell type, somatotroph specific proteins should be transient markers for somatotroph function. These markers may be expressed in mammosomatotrophs as well as cells expressing GH alone. Alternatively, a somatotroph specific protein involved in the repression of PRL expression may only be expressed in pure GH secretors. A candidate protein would be expressed at a higher level in the Albino Swiss AS than the *dw/dw* rat due to the reduced somatotroph number in the latter. It may also be induced by GRF treatment if it is involved in somatotroph function.

### 1.5.3 Purification of Pituitary Cells from AS and *dw/dw* Rats

Comparisons of pituitary GH and PRL cells would be of would be of greater value if they were performed on pure cells instead of the mixture of cell types found in the pituitary. In particular, comparing the responses of pituitary cultures from AS and *dw/dw* rats, where the somatotroph number differs greatly, can be difficult. A deficiency in the *dw/dw* (reduced GH or cAMP, for example) may be due to low GH cell number, or a real deficiency per somatotroph. In addition, the investigation of proteins specific to somatotrophs could be aided by using pure cells. Somatotrophs from AS and *dw/dw* rats could be compared directly to screen for missing or mutated proteins in the *dw/dw*. Somatotrophs could be compared to lactotrophs to further investigate the specificity of GH and PRL expression. FACS sorting has previously been applied successfully to purify cells from pituitary cell dispersions (Hatfield and Hymer, 1985; St. John *et al*, 1986; Wynick *et al*, 1990b) and **the final aim of this thesis is apply FACS sorting to purify somatotrophs and lactotrophs from *dw/dw* as well as AS rats.**

# MATERIALS AND METHODS

## 2.1 ANIMALS

Animals were housed in plastic cages with up to 6 animals per cage in a dedicated animal facility (Laidlaw, blue floor). Albino Swiss (AS) and *dw/dw* (on an AS background) rats were bred in the NIMR specific pathogen free facility and transferred to Laidlaw after weaning. TGR rats were bred in Laidlaw as TGR male/AS female crosses. Hemizygous pups were tailed at 3 weeks and DNA extraction for PCR to identify positive transgenics. AS/*dw* heterozygotes were bred in Laidlaw as *dw/dw* male/ AS female crosses.

Animals were allowed free access to water and standard laboratory rat chow, under a 12:12 hour light/dark regime, at constant temperature.

## 2.2 PREPARATION OF *IN VITRO* SYSTEMS

### 2.2.1 Preparation of Dispersed Anterior Pituitary Cells

The dispersion of pituitary cells is fundamentally similar for cell culture, FACS sorting and FACS analysis. All reagents were obtained from Sigma (Poole).

Anterior pituitaries were removed from rats (usually 8-10 weeks old) and placed in a petri dish of Hank's balanced salt solution (HBSS) for transport from the animal facility. If the cells were destined for culture or sterile sort followed by culture, the following procedures were carried out in a BoiMAT Class II microbiological safety cabinet (Medical Air Technology Ltd, Manchester). If no cell culture was involved, the method was carried out on the bench.

The cells were enzymically dispersed using a method modified from Weiner *et al* (1983) and Ceda *et al* (1987). The pituitaries were chopped into small pieces and dispersed in HBSS containing enzymes. In the case of cell culture, 0.3mg/ml collagenase type 1A and 0.25% trypsin was used. In the case of FACS sorting or analysis, trypsin was omitted to avoid digestion of antigens. For cell culture and cell sorting, the pituitaries were pooled in 20-40ml and maintained at 37°C in an incubator. Pituitaries for FACS analysis were dispersed individually in round

bottom FACS tubes (Falcon, London) in a shaking waterbath at 37°C. After 1/2 hour, DNase 1 was added to the dispersion to a final concentration of 0.05mg/ml. The cells were mechanically sheared (using a syringe with a sterile filling tube for large volumes or a 1ml pipette for single pituitaries) at intervals of about 10 minutes until no visible clumps remained (usually about 2 hours). The cell suspension was passed though a 40μm cell strainer (Falcon) and centrifuged at 130g (900rpm: IEC Centra-4R centrifuge, International Equipment Company, Dunstable) for 10 minutes. The resulting pellet was processed for culture (section 2.2.2), FACS sorting (section 2.2.3) or FACS analysis (section 2.4.2).

The viability of the cells was tested by dye exclusion tests. An aliquot of cells for culture were tested by trypan blue exclusion and visualized and counted in a haemocytometer chamber. An aliquot of cells for FACS analysis were resuspended in 50μg/ml propidium iodide in 0.1% sodium citrate and analysed by FACS. Both methods revealed the dispersed cells to consistently >98% viable.

### 2.2.2 Primary Culture of Anterior Pituitary Cells

Cells were cultured using a method modified from McNicoll *et al* (1990) and Leiberman *et al* (1982) using reagents were obtained from Sigma, unless otherwise stated. The suspension was centrifuged at 130g for 10 minutes, the pellet resuspended in 20ml HBSS and re-centrifuged. The HBSS was aspirated and the cells resuspended in full medium (D-MEM, 15% Horse serum, 2.5% Fetal calf serum (PAA, Weiner Strasse, Austria), 2mM Glutamine and Penicillin / Streptomycin / Amphotericin) and plated in either 24- or 6-well plates (Falcon). 24-well plates were seeded at either 1/4 pituitary or 500,000 cells in 1ml per well, and 6-well plates were seeded at 1 pituitary in 2ml per well. Cultures for immunocytochemistry were seeded in 24-well plates containing poly-L-lysine coated coverslips. The yield of cells was usually 3-4 million cells per AS pituitary, 2-2.5 million cells per *dw/dw* pituitary. The medium was changed on day 2 or 3 of culture and cells were routinely treated on day 5.

### 2.2.3 Fluorescence Activated Cell Sorting (FACS)

Dispersed anterior pituitary cells were prepared from 18 rats, as described in section 2.1.1. The pellet of dispersed pituitary cells was resuspended in 1ml FACS Buffer (10g/l NaCl, 0.25g/l KCl, 1.37g/l $Na_2HPO_4$, 0.25g/l $KH_2PO_4$, 1g/l

BSA, natural pH) and carefully layered onto about 15ml FACS Buffer + 4% BSA. This was centrifuged for 5 minutes at 100g (600rpm: Beckman Model J-6B Centrifuge) to remove blood cells and cell debris. Most of the cell population was incubated on ice for 30 minutes in primary antibody made up in FACS Buffer (monkey-anti-rat GH (1:2000) or rabbit-anti-rat PRL (1:8000)). Both primary antibodies were obtained from The National Institute of Diabetes, Digestive & Kidney Diseases (NIDDK). A small aliquot of the cells (about 1 million) was removed and incubated in normal (non-immune) monkey (NMS) or normal rabbit serum (NRS) as a control (at the same concentration as the antibody).

After 30 minutes, the cell suspension was diluted with FACS buffer (1ml in the normal serum tube and 20ml in the staining tube). The cells were centrifuged for 5 minutes at 300g (1000rpm: Beckman Model J-6B Centrifuge), the supernatant removed and the cells resuspended in secondary antibody for rGH (PE conjugated goat-anti-human-IgG; Sigma) or for rPRL (FITC conjugated swine-anti-rabbit-IgG: High Wycombe, Bucks.). Both secondary antibodies were diluted 1:20 in FACS Buffer. The tubes were incubated on ice for 30 minutes. The cells were again diluted in FACS buffer and centrifuged for 5 minutes at 300g. The cells were resuspended in D-MEM at about 1 million per ml.

An aliquot of unsorted cells was removed to serve as a control, either after the 4% BSA cleaning step or after the second antibody step. The remainder of the cells were sorted for fluorescence, with respect to the normal serum control on a "FACS Vantage" (Becton-Dickinson, Mountain View, CA). Positive and negative cell populations were collected into tubes containing a small volume of D-MEM.

When the cells were to be cultured after sorting, the following modifications to the method were made to maintain sterility;

1. all buffers and reagents were filter sterilized,
2. the method was carried out in the flow hood,
3. the cells were resuspended in full medium prior to sorting and sorted into full medium. The medium was filtered as, although it was already sterile, small particles from the sera registered on the FACS machine.
4. the FACS machine lines were flushed with 70% ethanol for 30 minutes prior to sorting.

After the sorted cells were collected, they were centrifuged at 300g for 5 minutes and the supernatant aspirated. The cells were plated in full medium for culture, or extracted for protein analysis (section 2.5.1) together with the unsorted control cells.

## 2.3 TREATMENT OF PRIMARY CULTURES

### 2.3.1 Treatment with Hypothalamic Factors

On day 5, the culture medium was aspirated and the cells washed twice and incubated with warmed treatment medium (D-MEM with or without 0.1% BSA) with or without various factors added.

Cultures were incubated for three hours to study hormone release or 30 mins to study cAMP accumulation, due to differences in the time courses of these parameters.

At the end of the treatment period, the medium was aspirated into microfuge tubes and stored at -20°C until analysed. The cells from each treatment group were washed 3 times in ice cold 50mM Phosphate Buffered Saline, pH7.4 (PBS) or D-MEM and extracted for protein analysis (section 2.5.1) or cAMP radioimmunoassay (section 2.5.2).

### 2.3.2 Radiolabelled Amino Acid Incorporation

Differential protein synthesis was investigated by radiolabelled amino acid incorporation. For radiolabelled methionine incorporation experiments, methionine free D-MEM (Gibco, Paisley) was used to maximize the specific activity of radiolabelled supplement and thus the level of radiolabel incorporation. [$^{35}$S]methionine or [3H]methionine (Amersham International, Little Chalfont, Bucks.) was used at 0.74MBq/ml. The treated cells were extracted (section 2.5.1) and were analysed by sodium lauryl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE: section 2.6.5) or High Performance Liquid Chromotography (HPLC: section 2.5.7).

## 2.4 IMMUNO-STAINING METHODS

### 2.4.1 Harvesting of Cultured Cells for FACS Analysis

The medium was removed and the cells washed twice in HBSS. 1ml HBSS + 0.25% trypsin + 0.05mg/ml DNase 1 per well (6-well plate) was added and the plate incubated at 37°C. The cells were gently agitated by pipetting at 10 minute intervals until the they were in suspension. They were passed through a 40µm cell strainer into round bottom FACS tubes (1 well per tube). The tubes were centrifuged at 65g (500rpm: Beckman Model J-6B Centrifuge) and the supernatant aspirated.

### 2.4.2 FACS Analysis

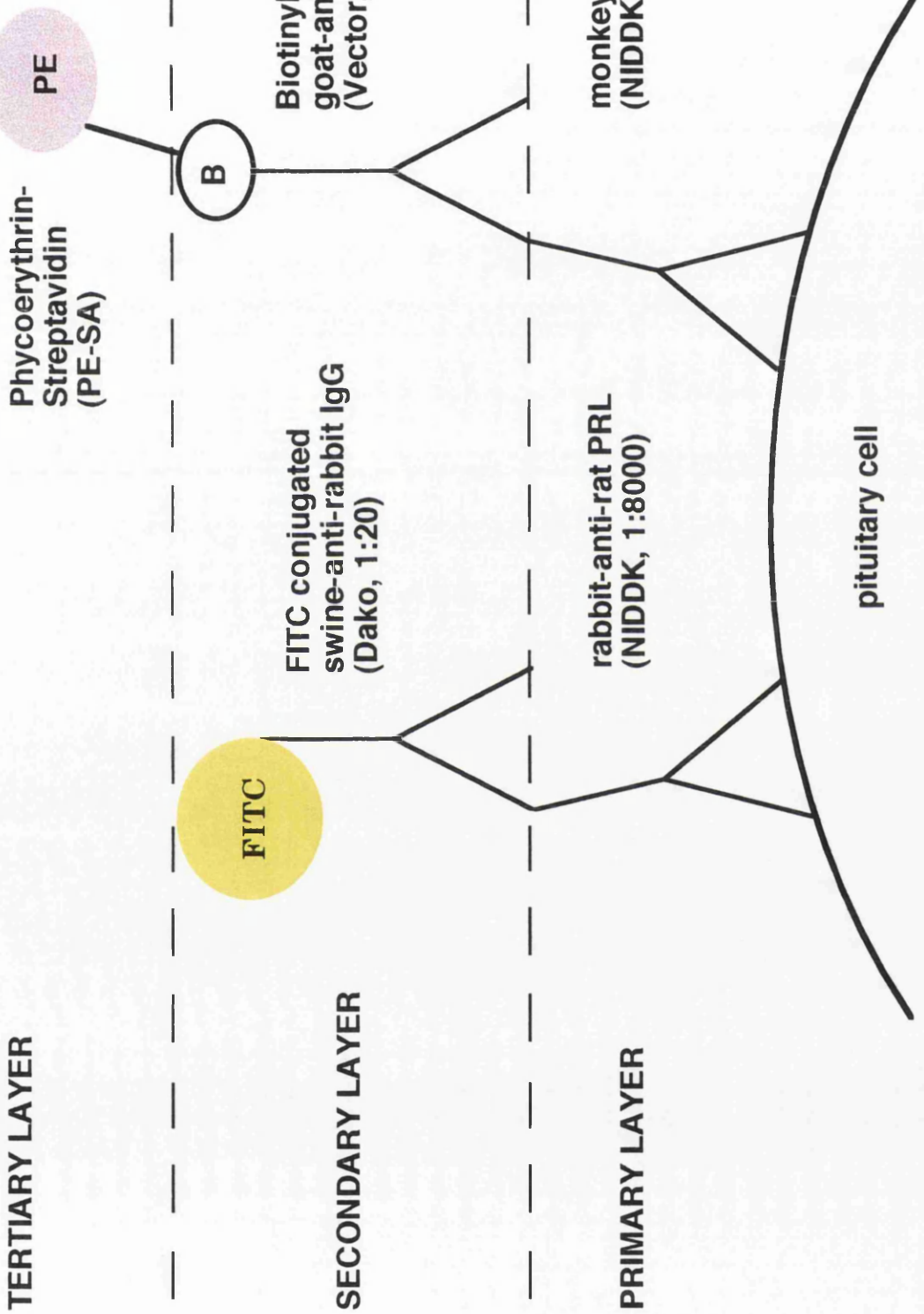
The pellet of about 1 million cells (section 2.2.1 or section 2.4.1) was resuspended in FACS buffer + 0.1% sodium azide and centrifuged at 300g. The tubes were aspirated and the cells fixed in 50µl 4% paraformaldehyde for 5 minutes on ice. The cells were washed with 1ml FACS buffer + azide and centrifuged at 300g for 5 minutes. (At this stage the cells could be stored at 4°C in 50µl FACS buffer + azide for several weeks). The tubes were aspirated and the cells permeablized in FACS buffer + 0.2% saponin (Sigma). The tubes were centrifuged at 130g (700rpm: Beckman Model J-6B Centrifuge) for 5 minutes. (The centrifugation was slower because saponin treated cells are more fragile. For the same reason the tubes were flicked by hand rather than vortexed to mix while in saponin).

The tubes were aspirated and 50µl primary antibody added (monkey-anti-rGH and/or rabbit-anti-rPRL in FACS buffer + saponin). A normal serum control tube was also included in each experiment to act as a background. The tubes were incubated on ice for 30 minutes. The primary antibody was washed out with 1ml FACS buffer + saponin, and the tubes centrifuged at 130g for 5 minutes. 50µl of secondary antibody was added diluted 1:20 in FACS buffer + saponin. The tubes were incubated on ice for 30 minutes. Again, the antibody was washed out with 1ml FACS buffer + saponin, spun at 130g and aspirated. In the case of double staining, the PE conjugated secondary antibody was replaced with a biotinylated secondary antibody (biotinylated goat-anti-human-IgG; 1:200: Vector Laboratories, Burlingame, CA) and a tertiary layer used;

**Figure 2.1: Schematic of two colour FACS antibody systems.**

Fixed, saponized cells were incubated in the two primary antibodies for 30 minutes. They were washed and incubated in the two secondary antibodies for 30 minutes. The swine-anti-rabbit, which binds the PRL primary antibody is directly conjugated to FITC. Biotinylated goat-anti-human IgG binds the GH primary antibody. This was subsequently fluorescently labelled by the use of a tertiary PE-SA layer.

Single PRL staining was carried out using the system shown. Single GH staining also used a two layer system with a PE conjugated goat-anti-human IgG secondary antibody.


TERTIARY LAYER
SECONDARY LAYER
PRIMARY LAYER
Phycoerythrin-Streptavidin (PE-SA)
PE
B
Biotinylated goat-anti-human IgG (Vector, 1:20)
FITC conjugated swine-anti-rabbit IgG (Dako, 1:20)
FITC
rabbit-anti-rat PRL (NIDDK, 1:8000)
monkey-anti-rat GH (NIDDK, 1:82000)
pituitary cell

50µl of phycoerythrin-streptavidin (PE-SA) was added to the pellet. The tubes were incubated for 30 minutes on ice and again washed in 1ml FACS buffer + saponin, centrifuged at 130g and aspirated. A schematic of the antibody systems used is shown in figure 2.1.

Once the staining was complete, the saponin was washed out with 1ml FACS buffer + azide. The cells were spun again at 130g and resuspended in 250µl FACS buffer + azide.

The cells were analysed on either "FACS Vantage" or "FACS Star Plus" (both Becton-Dickinson) and data from 10000-20000 cells collected. Where two colour analysis was performed, single colour control samples were used to correct for fluorescence cross-over between channels. Data was analysed using WinMDI. Briefly, the population of cells was electronically gated to exclude cell debris and red blood cells. The remaining endocrine cells were plotted with FITC fluorescence (PRL) on the X-axis and PE fluorescence (GH) on the Y-axis. The quadrant was placed to separate positive and negative stained cells by estimating the boundaries between the populations and using the normal serum control as a guide. The program then automatically displayed the relative proportions of cells.

### 2.4.3 Immunocytochemistry

On day 5 of culture some dishes of cells grown on poly-L-lysine coated coverslips were washed twice in ice cold PBS and fixed for immunocytochemistry for 30 minutes in 4% paraformaldehyde in PBS. After fixation the cells were washed for 1 minute in distilled (d) $H_2O$

Whole pituitaries were fixed overnight in 4% paraformaldehyde in PBS and then transferred into 70% ethanol. Paraffin embedding, tissue sectioning 4µm and mounting onto microscope slides was carried out by the NIMR histology department. Sections were then stored at 4°C until stained. Prior to immunocytochemistry, the sections were de-waxed in Histoclear (National Diagnostics, Georgia, USA) and re-hydrated for 20 seconds in each 100%, 70%, 30% ethanol and then washed in $dH_20$ for 1 min.

The sections or coverslips were then incubated for 30 minutes in 0.9% $H_2O_2$ in methanol to inactivate endogenous peroxidase. They were subjected to 15 minute 0.1% trypsin (at 37°C) and 0.5% triton washes. They were washed in Tris Buffered Saline (TBS: 0.05M Tris-HCl pH 7.6-7.8, XM NaCl), blocked in NS/BSA (5% BSA, 20% normal serum of the same origin as the secondary antibody in TBS) for 30 minutes and then incubated with primary antibody in NS/BSA overnight at 4°C or for 1 hour on the bench. After extensive washing in TBS, the cells were incubated for 30 minutes in biotinylated second antibody and washed again.

Positive cells were stained brown using diaminobenzidine tetrahydrochloride (DAB: Sigma) after incubation with peroxidase conjugated avidin-biotin complex (Dako) or red using Fast Red (Dako) after incubation with alkaline phosphatase conjugated avidin-biotin complex.

The nuclei were then counterstained with Gill's haematoxylin (BDH, Lutterworth, Leic.). Coverslips were mounted inverted on slides using water based mountant (Glycergel: Dako). The sections were mounted in Glycergel and covered with a coverslip.

Immunocytochemistry for GH was carried out using monkey-anti-rGH at 1:2000 (NIDDK) followed by biotinylated goat-anti-human-IgG at 1:200 (Vector Laboratories).

Immunocytochemistry for PRL was carried out using rabbit-anti-rPRL at 1:8000 (NIDDK) followed by biotinylated swine-anti-rabbit-IgG at 1:200 (Dako).

Immunocytochemistry for the interleukin-1 receptor (IL-1R) was carried out using rabbit-anti-IL-1RI (M-20: Autogen Bioclear, Calne, Wiltshire) at 0.1-5μg/ml followed by biotinylated swine-anti-rabbit-IgG at 1:200 (Dako). Various modifications of the above method were used to optimize the staining. These are described in chapter 6.

## 2.5 PREPARATION OF EXTRACTS

### 2.5.1 Preparation of Extracts for Protein Analysis

Extracts were made from anterior pituitaries, form sorted cells and from cultures. Various extraction buffers were used: PBS, D-MEM + Complete™ peptidase inhibitor cocktail (CPI: Boerhinger Manheim, Lewes, East Sussex), radioimmunoprecipitation (RIPA) buffer (9.1mM $Na_2HPO_4$, 1.7mM $NaH_2PO_4$, 150mM NaCl, pH 7.4 + 1% NP40 + 0.5% Na deoxycholate + 0.1% SDS) + CPI. The tissue / cells were placed in the buffer (a rubber policeman was used to harvest the cells in culture) and homogenized in a glass homogenizer on ice. The homogenate was transferred to a microfuge tube, frozen and thawed and the insoluble debris removed by centrifugation for 5 minutes at 14000rpm (ALC Micro Centrifugette 4212, Camlab, Cambridge). The supernatant was aliquoted and stored at -20°C until analysis.

### 2.5.2 Preparation of Extracts for cAMP Analysis

After removing the culture medium from the cells, they were washed twice in ice cold PBS. 500µl of ice cold acid alcohol (95% ethanol, 0.1M HCl) was then added to each well and scraped with a rubber policeman. The plate was then taped shut and kept at -20°C overnight. The homogenate was then transferred to a screw top microfuge tube. The well was washed with a further 500µl of acid alcohol and this was added to the microfuge tube. The homogenate was microfuged at 14000rpm for 5 minutes and the supernatant transferred to a new tube. The extract was stored at -20°C until assayed for RIA. 500µl of the extract was transferred to a new tube and evaporated to dryness (Speed Vac Concentrator, Savant). The extract was resuspended in 500µl PBS and 100µl and 25µl assayed in duplicate as described in section 2.6.4.

## 2.6 ANALYSIS OF EXTRACTS

### 2.6.1 Total Protein Assay

Total protein of the protein extracts (section 2.5.1) was determined by modification of the method of Lowry, *et al* (1951). Briefly, samples were compared to a standard curve of 0-100µg BSA. 49ml of buffer A (20g/l $Na_2CO_3$, 4g/l NaOH, 0.2g/l K/Na Tartrate) was mixed with 1ml of buffer B (5g/l $CuSO_4$).

Samples (in duplicate) and standards (in triplicate) were made up to 100µl in $dH_2O$ and 3ml of the buffer mixture added to each tube. After 10 mins, 300µl of buffer C was added (1:1 Folin-Ciocalteu phenol reagent (BDH) in $dH_2O$) and the tubes incubated on the bench for 1 hour. For measurement of the absorbance at 740nm in this colorimetric assay, a Beckman DU-64 Spectrophotometer was used. When the samples had been extracted in D-MEM, a blank of D-MEM was also assayed, due to the amino acid content. Due to the fact that SDS interferes with the Lowry assay, RIPA buffer extracts were not be analysed for protein content.

### 2.6.2 Radioimmunoassay for rGH

rGH was assayed by radioimmunoassay (RIA) using reagents supplied by NIDDK (Carmignac and Robinson, 1990). The results were expressed as ng in terms of the standard GH-RP-2. The standards (0.016-16ng/tube) were prepared by double dilution in a volume of 100µl in assay buffer (50mM PBS, pH 7.4 + 0.3% BSA). A typical standard curve gave a 50% $B_0$ of about 0.25ng.

The conditioned medium samples (assayed to determine GH release) were usually assayed at 1:5, 1:25 and 1:125 dilutions in assay buffer to a volume of 100µl. Pituitary extracts and primary culture extracts were diluted further to allow measurement within the assay range.

The tracer was iodinated by the iodogen method (Salacinski *et al*, 1981), purified on a G75 Sephadex column in assay buffer and used at 4000-6000 cpm/100µl. The antibody (anti-rat Growth Hormone-RIA-5) was used at a working concentration of 1:10,000, thus when added at 100µl the final concentration in the 300µl assay was 1:30,000.

After incubation overnight at room temperature, or up to 72 hours at 4°C, antibody bound from free tracer was separated by polyethylene glycol (PEG) (Desbuquois & Aurbach, 1971). 600µl of PEG buffer (18% PEG, 1.5% γ-globulins, 0.01% Triton X-100 in 0.05M Tris buffer, pH 8.4) was added to each tube, incubated for 30 minutes at room temperature and then centrifuged at 2400g for 10 minutes at 4°C (3000rpm: Beckman Model J-68 Centrifuge). The supernatant

was aspirated and the bound fraction (pellet) counted for 3-5 minutes per tube (LKB 1277 Gammamaster automatic gamma counter).

When increased sensitivity was needed, the addition of tracer was delayed by 24 hours. The assay was then incubated for a further day before separating the bound and free fractions. In this delayed addition assay, the standard curve was 0.002-2ng and a typical standard curve gave a 50% $B_0$ of 0.05ng.

**Inter assay variation was 7.1%**

### 2.6.3 Radioimmunoassay for rPRL

rPRL was assayed by the same method as rGH using reagents supplied by NIADDK (Clark *et al*, 1987). The results were expressed as ng in terms of the standard PRL-RP-3. The antibody (anti-rat Prolactin-RIA-9) was used at a final concentration of 1:45,000 and the standards (0.016-16ng/tube) prepared by double dilution. A typical standard curve gave a 50% $B_0$ of about 0.25ng. The samples were prepared in the same way as for rGH RIA

The tracer was iodinated by the lactoperoxidase method (Morrison and Bayse, 1970), purified on a G75 Sephadex column in assay buffer and used at 4000-6000 cpm/100µl. Antibody and tracer were added to the tubes to a final volume of 300µl and the assays incubated overnight at room temperature, or up to 72 hours at 4°C.

Antibody bound from free tracer was separated in the same way as for rGH and the bound fraction (pellet) counted for 3-5 minutes per tube.

**Inter-assay variation was 3.9%**

### 2.6.4 Radioimmunoassay for cAMP

cAMP was assayed by radioimmunoassay (RIA) using reagents supplied by NIADDK and Sigma. The results were expressed as fmol in terms of cAMP standard (Sigma). The antibody (CV-27) was used at a final concentration of 1:400,000 in acetate buffer (50mM sodium acetate buffer, pH6.2 + 0.5% BSA).

The samples, prepared in section 2.5.2, and the standard were acetylated by addition of 2% v/v triethylamine and 1% v/v acetic anhydride (Sigma). Usually, 100µl and 25µl of the acetylated extracts (made up to 100µl with PBS when necessary) were assayed in duplicate. The standards (0.1-800 fmol/tube)

prepared by double dilution of acetylated standard in 100µl PBS. A typical standard curve gave a 50% $B_0$ of about 20 fmol.

2.0'-mono succinyl cAMP tyrosine methyl ester (Sigma) was iodinated by the lactoperoxidase method, purified by a methanol gradient in 0.1% trifluoroacetic (TFA) acid on Sep-Pak C-18 cartridges (Waters, Milford, Massachusetts). It was used at 4000-6000 cpm/50µl acetate buffer. Antibody and tracer were added to the tubes to a final volume of 250µl and the assays incubated overnight at 4°C.

Antibody bound from free tracer was separated in the same way as for rGH and rPRL (although only 500µl PEG was used) and the bound fraction (pellet) counted for 3-5 minutes per tube. **Inter-assay variation was 11.3%**

### 2.6.5 SDS-PAGE

Protein extracts were separated on miniature (Mini-Protean II Electrophoresis Cell: Biorad, Hemel Hempstead, Herts.) and larger (V15.17 Vertical Gel Electrophoresis Apparatus: Life Technologies Inc., Paisley) acrylamide gels (Laemmli, 1970). All reagents were obtained from Biorad unless otherwise stated. Briefly, 0.75mm thick 12% acrylamide gels were poured with a 6% acrylamide staking gel. Where the samples were dilute, they was either evaporated to a smaller volume (Speed Vac Concentrator, Savant) or concentrated using a Microcon-3 (Amicon, Beverly, MA). Samples containing 5-20µg protein were mixed with 5-15µl sample buffer (0.0625M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.002% bromophenol blue) such that the total volume was <15µl (miniature gel) or <30µl (large gel). The tubes were boiled for 5 mins, cooled on ice and loaded onto the gel. Broad range (for staining) or Kaleidoscope pre-stained (for $^{35}S$ gels or Western analysis) SDS-PAGE standards were included for reference. The gels were run in electrode buffer (1.5g/l Tris base, 7.2g/l glycine, 0.5g/l SDS) at 200 volts (V) for about 45 mins (small) or 4 hours (large) until the dye front reached the bottom of the gel.

Some gels were stained in 0.1% Coomassie brilliant blue in 40% methanol, 10% acetic acid overnight and then destained in 15% methanol, 10% acetic acid. Others were silver stained according to the manufacturer's instructions obtained

with the silver stain plus kit (Biorad). After staining the gel was dried between two sheets of cellophane.

When $^{35}S$ labeled extracts had been separated using SDS-PAGE, the gel was soaked in 1M Sodium Salicylate (BDH) for 15 minutes, dried and exposed to film (Biomax MR single emulsion film, Kodak, Rochester, NY).

### 2.6.6 Western Analysis

After separating the proteins by SDS-PAGE, they were transferred to a polyvinyldene difluoride (PVDF) membrane (Biorad). Briefly, the membrane was pre-wetted in methanol, washed in $dH_2O$ and then soaked in transfer buffer (25mM Tris-HCl pH 8.3, 150mM glycine, 20% methanol) along with sheets of filter paper cut to size. The gel (also pre-soaked in transfer buffer) and the membrane were sandwiched together between the filter paper (2 thicknesses on either side) and the proteins transferred at 40V for 1 hour in the Mini-Protean II Electrophoresis Cell. The membrane was washed with TBS (20mM Tris-HCL pH 7.6, 137mM NaCl) and then blocked for 1 hour in TBS + 5% milk + 0.1% Tween 20. The membrane was then incubated with primary antibody in TBS + 5% milk + 0.1% Tween 20 overnight at 4°C or on the bench for 1 hour. After extensive washing in TBS + 0.1% Tween 20, the membrane was incubated in biotinylated second antibody for 1 hour. The membrane was again washed extensively and incubated in peroxidase conjugated avidin-biotin complex (Dako) and then visualized with DAB.

Primary and secondary antibody systems were the same as for immunocytochemistry (see section 2.4.3).

### 2.6.7 Extraction of Protein from Acrylamide Gel

Attempts were made to extract protein bands from acrylamide gel slices, using the method obtained from Amicon. The gel was stained overnight in 0.1% Coomassie blue in 50% methanol, 10% acetic acid. It was then destained for 2 hours in 7% acetic acid, 12% methanol. The band was cut out of the gel and placed in wash buffer (50% methanol) in a microfuge tube. The tube was sonicated for 20 mins. The was buffer was removed, and 100µl extraction buffer added (100mM $NaHCO_3$, 8M Urea, 3% SDS, 0.5% Triton X-100, 25mM

dithiothreitol). The tube was incubated for 20 mins at 60°C and then homogenized in a glass homogenizor. The homogenate was transferred back to the tube, and the homogenizor rinsed with another 100µl of extraction buffer. The homogenate was incubated for a further 2 hours at 60°C. The homogenate was placed into a micropure-0.22 separator (a pre-filter to remove gel slurry) in a Microcon-3 concentrator (Amicon). The tube was rinsed with a further 200µl extraction buffer. The assembly was spun in a microfuge at 1400rpm for 10 mins. The concentrate was washed in 400µl D-MEM and re-concentrated. The concentrate was spun into a new tube and stored at -20°C until used.

### 2.6.8 HPLC

Extracts containing 100µg of protein or 500µl conditioned medium were injected onto a Nucleosil5C8 column (HPLC Technology Ltd., Macclesfield) equilibrated with 80% solvent A (MQ H20 + 0.1% TFA (BDH)) and 20% solvent B (80% Acetonitrile (Romil Ltd., Cambridge) + 0.06% TFA). The samples were eluted with a gradient of increasing solvent B for 1 hour at a flow rate of 1ml/min. 1 minute fractions were collected and stored at -20°C until analysis. The gradient was as follows;

| Time (mins) | 0 | 5 | 20 | 50 | 55 | 60 |
|---|---|---|---|---|---|---|
| % B | 20 | 20 | 50 | 80 | 100 | 100 |

## 2.7 STATISTICAL ANALYSIS

Results were presented as mean ± S.E.M. unless otherwise stated and statistical analysis was carried out in Instat 2.01. Where two groups were compared, Students t-test or Welch Alternate t-test (which assumes Guassian populations, but does not require the SD to be equal). Where multiple groups were compared, Analysis of variance (ANOVA) was used initially. The data were then post-tested using Bonferroni's comparison of selected pairs to determine the groups showing significant differences. Dose response curves were post-tested for linear trend and by Dunnet's multiple comparison to compare each point to the control group.

# ANALYSIS OF CELL TYPES IN ADULT AS, *DW/DW* AND HETEROZYGOTE RATS

## 3.1 INTRODUCTION

The initial characterization of the *dw/dw* (Charlton *et al*, 1988) reported that the PRL content of the pituitary was not significantly different to heterozygote controls. However, this was based on relatively few observations, and has since received relatively little attention. In addition, the use of heterozygote controls in early studies introduced another variable. The aim of the work in this chapter was to re-evaluate the GH and PRL pituitary contents and cell numbers in AS, *dw/dw* and AS/*dw* heterozygotes. By comparing AS and heterozygote rats directly it was possible to show if a slight phenotype exists and thus previous reports using AS or heterozygotes as controls could be compared.

Both immunocytochemistry and FACS analysis were used to quantify the cell types and the results compared. Immunocytochemistry is the standard method for studies of this nature, whereas FACS analysis has been used to a lesser extent. Due to the common origin of somatotrophs and lactotrophs I wanted to investigate the presence of mammosomatotrophs in the *dw/dw* pituitary, and FACS analysis is a method by which this is possible. By comparing the results with those of immunocytochemistry, it was possible to validate FACS analysis as a viable method for quantification of cell types in the *dw/dw* and AS rats.

## 3.2 EXPERIMENTAL RESULTS

### 3.2.1 Experiment 1: GH and PRL Contents of Adult AS, AS/*dw* and *dw/dw* Pituitaries

9-10 week old rats (AS, AS/*dw* and *dw/dw*; males and females; n=5-7) were weighed and then stunned and decapitated. The anterior pituitaries were removed and extracted in 1ml PBS + CPI. The extracts were diluted and assayed for GH and PRL. Results were expressed per pituitary and per mg protein (as determined by Lowry protein assay). Statistical analysis was carried out by ANOVA followed by Bonferroni's post test for selected pairs.

Fig 3.1a shows the body weights of the AS, *dw/dw* and heterozygotes at the time of sacrifice. As expected, the *dw/dw* rats were significantly lighter than the AS controls. However, both male and female heterozygotes were slightly, but significantly, heavier than the controls. In all cases, the female rats were significantly lighter than the males.

The amount of soluble protein per pituitary (Figure 3.1b) reflected the smaller pituitary size in the *dw/dw*. The protein level in the heterozygote was not significantly different to that of the AS and there were also no significant sex differences in any of the groups.

Figure 3.2a shows the amount of GH-like immunoreactivity (LI) expressed as a percentage of the soluble protein. The GH in the *dw/dw* was greatly reduced in males and females, as expected. The GH in the heterozygote was not significantly different from the AS. The mean percentage of GH was lower in female animals, although this only reached significance in the AS/*dw*. The GH deficiency in the *dw/dw* was even more dramatic when expressed per pituitary (Fig 3.2b), as not only was the concentration greatly reduced but the pituitaries were much smaller. The heterozygote still showed no significant differences in GH or PRL compared to the AS.

Surprisingly however, the PRL concentration in both male and female *dw/dw* pituitaries was substantially higher than the AS. Even when the PRL levels were expressed as PRL per pituitary (Figure 3.3b), the levels of PRL in the *dw/dw* were still significantly higher than the AS. The mean PRL levels were higher in female animals, although this only reached significance in the *dw/dw* when expressed as percentage protein, and only in the AS and *dw/dw* when expressed as ng/pituitary.

**Figure 3.1a: Body weights of AS, AS/*dw* and *dw/dw* rats.**

The facing graph shows the body weights of the rats before sacrifice at 9-10 weeks old.

**P<0.01, ***P<0.001 *vs.* AS of the same sex. $^{+++}$P<.001 male *vs.* female. ANOVA followed by Bonferroni's post-hoc test (n=5-7).

**Figure 3.1b: Soluble Protein Contents of Anterior Pituitaries of AS, AS/*dw* and *dw/dw* rats.**

Pituitaries were extracted in 1ml PBS + CPI and assayed for total protein by Lowry protein assay.

***P<0.001 *vs.* AS of the same sex. ANOVA followed by Bonferroni's post-hoc test (n=5-7).

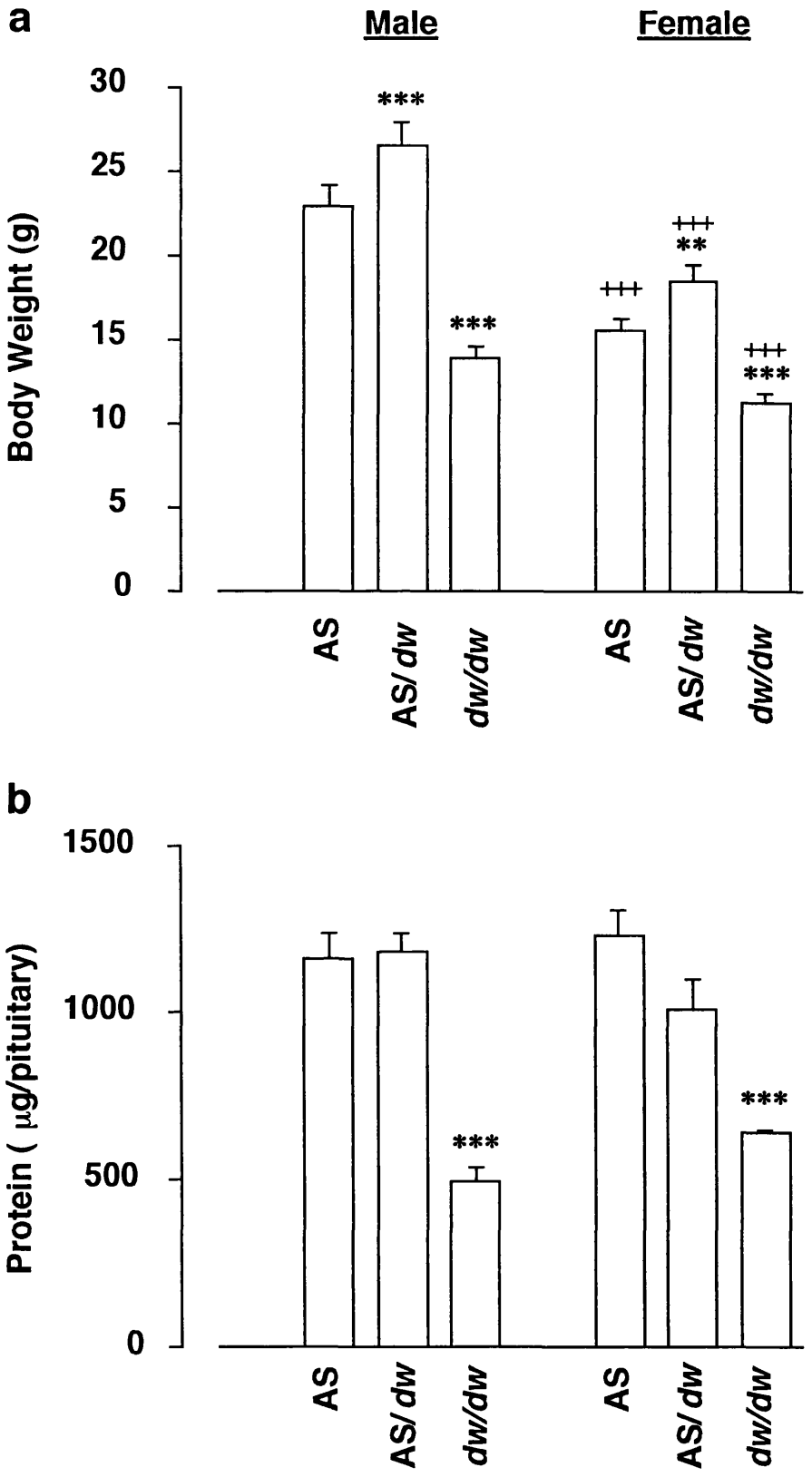

a
Male
Female
Body Weight (g)
30
25
20
15
10
5
0
***
***
+++
**
+++
***
AS
AS/dw
dw/dw
AS
AS/dw
dw/dw
b
Protein ( μg/pituitary)
1500
1000
500
0
***
***
AS
AS/dw
dw/dw
AS
AS/dw
dw/dw

**Figure 3.2: GH contents of anterior pituitaries of AS, AS/*dw* and *dw/dw* rats.**

Pituitary extracts were assayed for GH and expressed as percentage of soluble protein (a) and μg/pituitary (b).

***P<0.001 *vs.* AS of the same sex. $^{+}$P<0.05, $^{++}$P<.001 male *vs.* female. ANOVA followed by Bonferroni's post-hoc test (n=5-7).

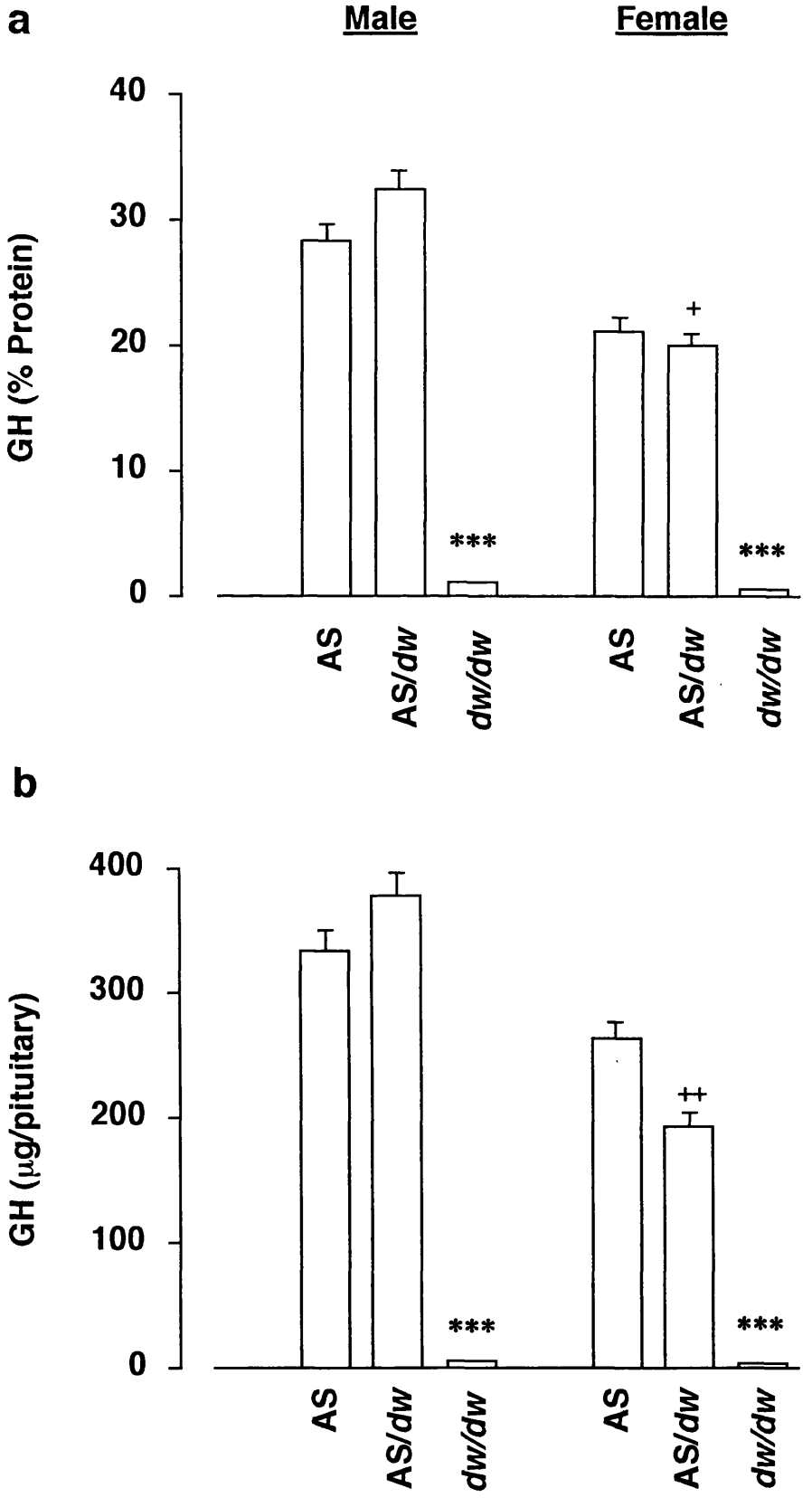
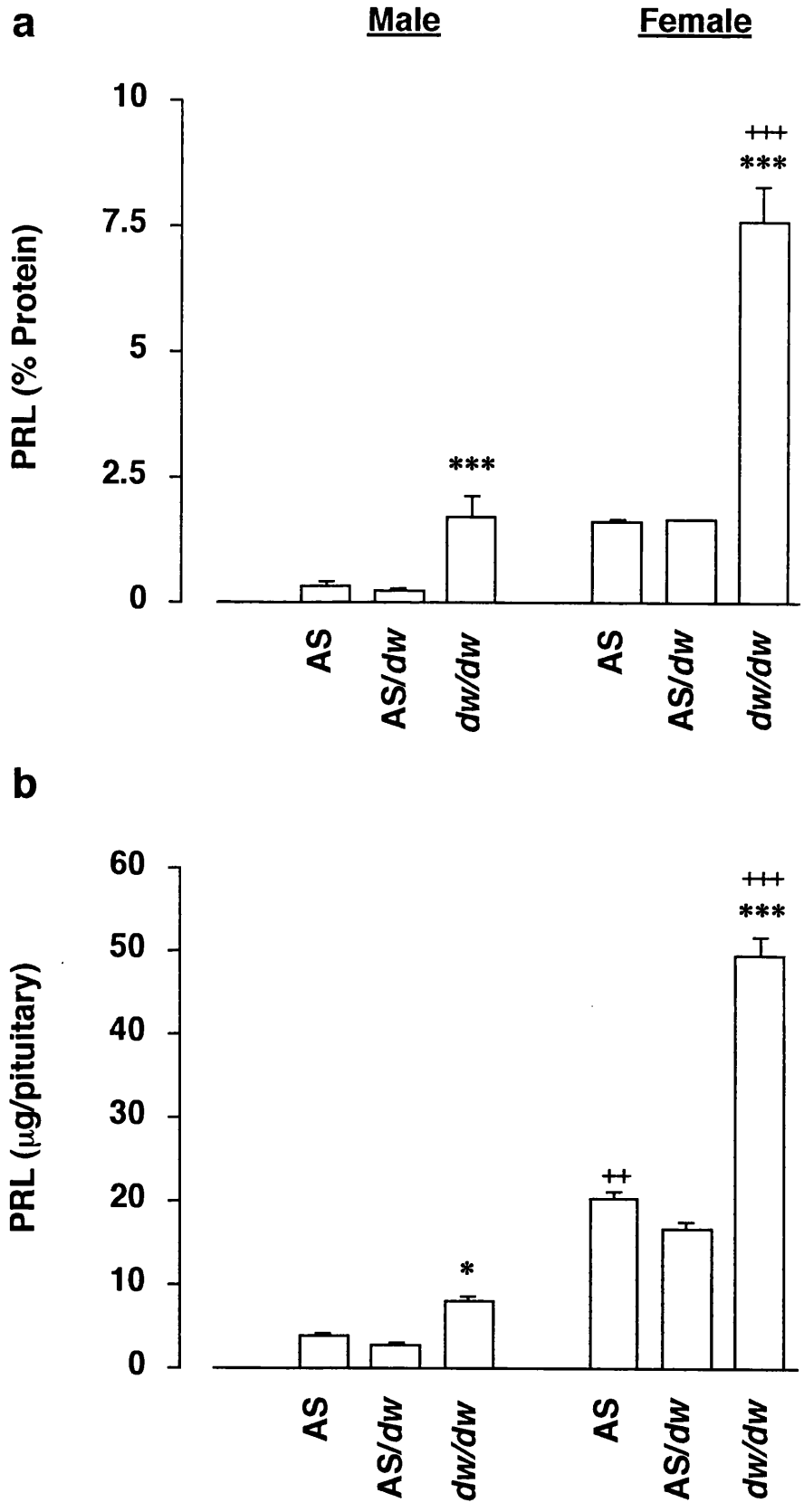

a
Male
Female
GH (% Protein)
40
30
20
10
0
***
+
***
AS
AS/dw
dw/dw
AS
AS/dw
dw/dw
b
GH (μg/pituitary)
400
300
200
100
0
***
++
***
AS
AS/dw
dw/dw
AS
AS/dw
dw/dw

**Figure 3.3: PRL contents of anterior pituitaries of AS, AS/*dw* and *dw/dw* rats.**

Pituitary extracts were assayed for PRL and expressed as percentage of soluble protein (a) and μg/pituitary (b).

*P<0.05, ***P<0.001 *vs.* AS of the same sex. $^{++}$P<0.01, $^{+++}$P<.001 male *vs.* female. ANOVA followed by Bonferroni's post-hoc test (n=5-7).


a
Male
Female
PRL (% Protein)
10
7.5
5
2.5
0
***
+++
***
AS
AS/dw
dw/dw
AS
AS/dw
dw/dw
b
PRL (µg/pituitary)
60
50
40
30
20
10
0
*
++
+++
***
AS
AS/dw
dw/dw
AS
AS/dw
dw/dw

### 3.2.2 Experiment 2: Immunocytochemistry of AS, AS/*dw* and *dw/dw* Pituitaries

9-10 week old rats (AS and *dw/dw*; males and females; n=3) were stunned and decapitated. The pituitaries were removed and fixed in 4% paraformaldehyde at 4°C overnight and then placed into 70% ethanol. They were wax embedded and 4μm sections cut by the in-house histology department. The sections were stored at 4°C until GH and PRL immunocytochemistry was performed. Antibody specificity had previously been shown by controls using normal serum or serum pre-adsorbed overnight with 10μg/ml GH or PRL (Flavell *et al*, 1996).

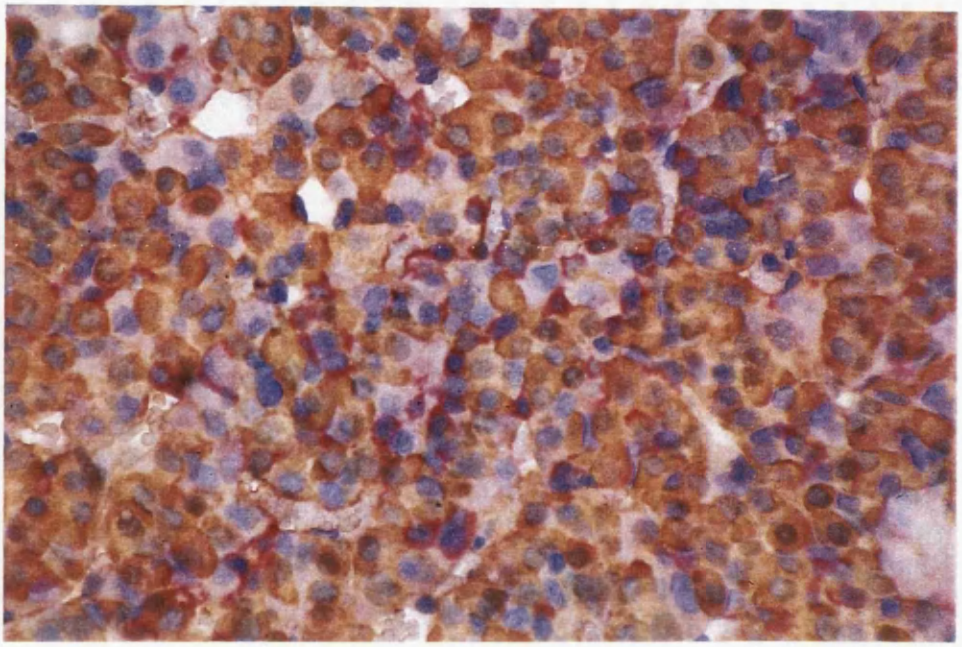
Fig 3.4 shows an example of double immunocytochemistry for GH (stained brown by DAB), and PRL (stained red by fast red). The pictures give an immediate visual impression of the differences in the proportion of cell types in male AS and *dw/dw* pituitaries. The *dw/dw* appears to have less GH staining and more PRL staining than the AS. The cell numbers were quantified by cell counting. For each animal a total of 1000-2000 cells were counted, using an eyepiece graticule, over 3 different areas to accurately estimate the proportion of the total cell number in each animal. The results are expressed as mean percentage of total ± S.E.M. of the three animals and statistical analysis was carried out by ANOVA followed by Bonferroni's post test for selected pairs. The cell numbers, estimated from immunocytochemistry of sections, are shown in figure 3.5. The proportion of cells staining for GH in male and female *dw/dw* rats was dramatically less than in AS rats. Conversely, the proportion of cells staining for PRL was significantly higher in male and female *dw/dw* rats than in AS rats. However, allowing for the reduction in pituitary size, this level of increase could be compensatory such that the total number of lactotrophs is the same in AS and *dw/dw*. This was addressed in a later experiment in this chapter.

AS, AS/*dw* and *dw/dw* females all had a significantly higher percentage of PRL staining cells, although there were no significant sex differences in somatotroph number. The heterozygote showed no significant differences to the AS in these cell numbers, indicating that there is not an intermediate phenotype of these animals for GH and PRL cell numbers.

**Figure 3.4: Immunocytochemistry for GH and PRL of AS and *dw/dw* pituitary sections.**

The photographs show double, sequential, immunocytochemistry for GH and PRL on pituitary sections from (a) AS and (b) *dw/dw* adult male rats. GH is stained using DAB (brown) and PRL is stained with fast red. The nuclei were counterstained with haematoxylin.

a



b

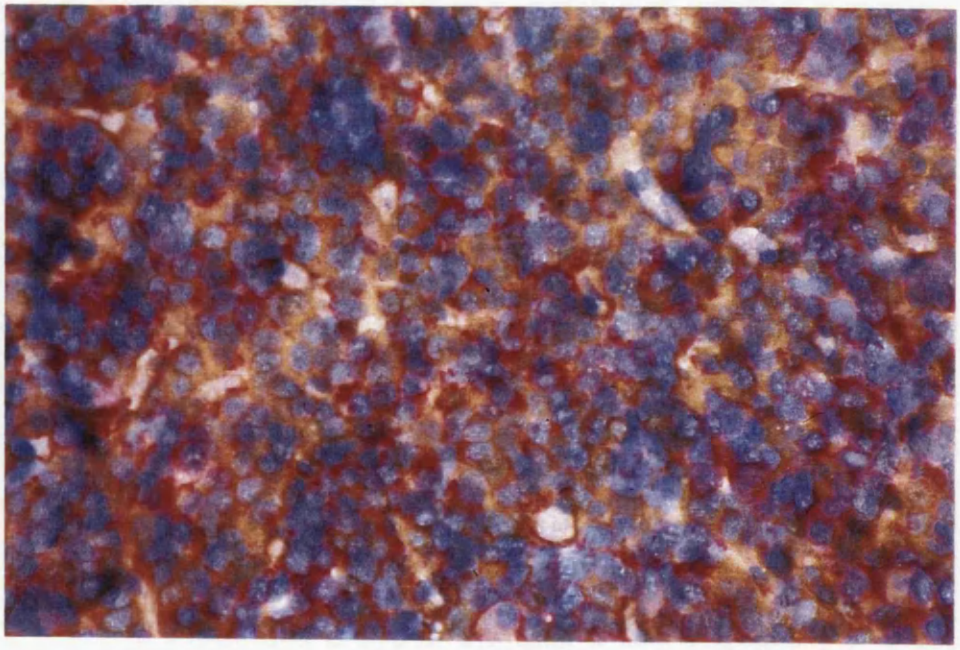
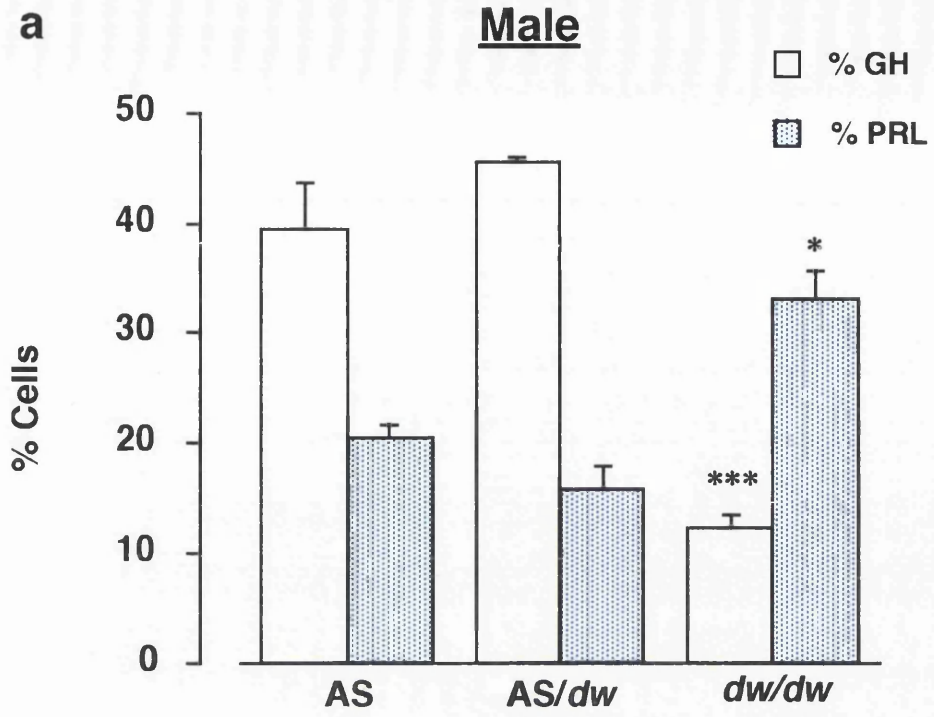
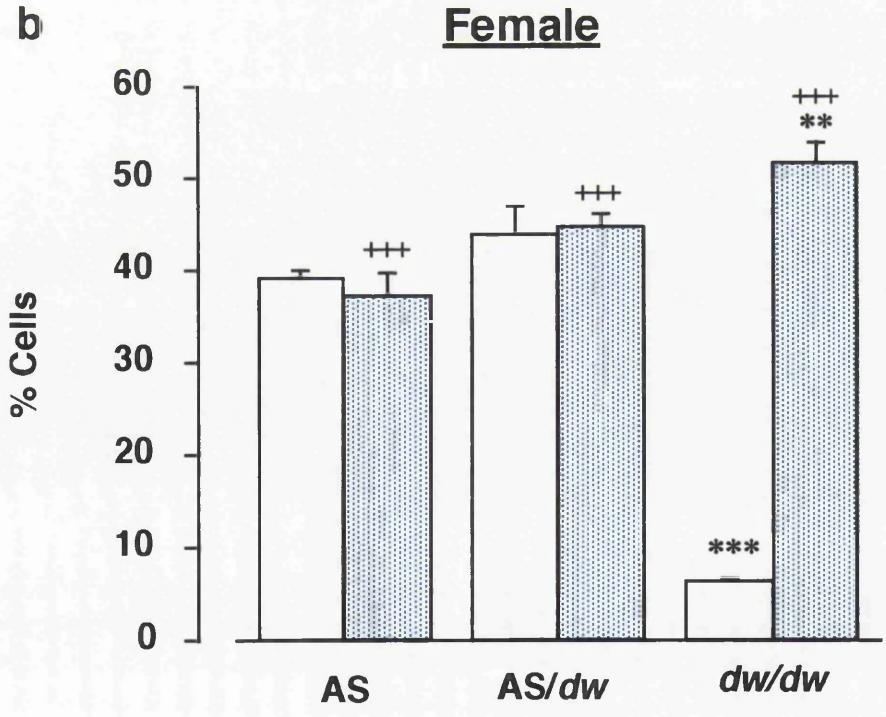


**Figure 3.5: Cell counts of GH and PRL immunocytochemistry on AS, AS/*dw* and *dw/dw* pituitary sections.**

Pituitary sections were stained for GH or PRL and counterstained with haematoxylin. 1000-2000 cells were counted, using an eyepiece graticule, over three different areas to accurately estimate the GH or PRL staining population in each animal.

*P<0.05, **P<0.01, ***P<0.001 *vs.* AS of the same sex. $^{+++}$P<0.001 male *vs.* female. ANOVA followed by Bonferroni's post-hoc test (n=3).


a
Male
% GH
% PRL
% Cells
50
40
30
20
10
0
***
*
AS
AS/dw
dw/dw


b
Female
% Cells
60
50
40
30
20
10
0
+++
+++
+++
**
***
AS
AS/dw
dw/dw

### 3.2.3 Experiment 3: FACS Analysis of Anterior Pituitary Cells from AS and *dw/dw* Rats

10 week old rats (AS and *dw/dw*; males and females; n=4) were stunned and decapitated. The anterior pituitaries were dispersed separately in a volume of 1.5ml and then separated into 3 aliquots of 500µl. The aliquots were fixed and stained for either GH, PRL or both antigens simultaneously and analysed by FACS. Controls, in which the primary antibody was replaced with normal serum (NMS or NRS), were included in each analysis. The following parameters were collected from 10,000 cells per sample;

1. forward scatter is a measure of cell size. It is determined by the amount of time it takes for the cell, moving at a constant rate, to pass through a laser beam.
2. side scatter measures the complexity or granularity of the cell. The more secretory granules etc., the more the laser beam is deflected by the cell.
3. fluorescence. Data can be collected in different channels to allow for more than one wavelength of fluorescence. The experiments in this thesis use single or double fluorescence in the form of fluorescein isothionate (FITC), phycoerythrin (PE).

Figure 3.6 shows an example of the performance of FACS analysis using a single fluorescent marker (in this case for GH). Panel (a) shows a typical scatter diagram for total anterior pituitary cells, with forward scatter displayed on the x-axis and side scatter on the y-axis. The black area represents cell debris, and was excluded from the data analysed (gated out). Panel (c) shows the use of NMS to illustrate and set the background level of non-specific fluorescence. Panel (d) shows a typical fluorescence plot of single GH FACS with a quadrant placed to separate the background fluorescence from signal. The numbers on the fluorescence plots (c and d) illustrate the percentage of cells (excluding debris) that have a positive signal for PE fluorescence (plotted on the Y-axis), in this case, 35% of the cells stain for GH compared to a background of 0.1%. These GH positive cells are shown in green in panel (d), and the scatter of this population alone is shown in panel (b). The few cells in the upper right quadrant of the fluorescence plots are from auto-fluorescence of cells and not

specific staining. Auto-fluorescent cells typically form a diagonal line, as fluorescence is detected in both channels.

Figure 3.7 shows a comparison with a similar FACS analysis for GH cells in *dw/dw* rats. Although a *dw/dw* pituitary contains less cells than an AS pituitary, all FACS data is collected from 10,000 cells per sample. The bottom panels (c and d) compare the fluorescence plots of cells from adult male AS and *dw/dw* rats. The reduction in the proportion of cells staining for GH in the *dw/dw* is readily visible (35% *vs.* 7.3%). The top panels (a and b) show the scatter characteristics of the GH positive cells. It can be seen that the GH cells in the *dw/dw* had lower side scatter (Y-axis), representing a less granular population of cells (also shown as a histogram in Figure 3.10). This supports the hypothesis that the somatotrophs in *dw/dw* rats synthesize and store less GH.

Figure 3.8 shows a similar single FACS analysis for PRL. Again, panel (a) shows a typical scatter diagram for anterior pituitary cells, with the cell debris indicated in black and gated out. Panel (c) shows the use of NRS to set the background level of fluorescence and panel (d) shows a typical fluorescence plot of single FACS for PRL (FITC fluorescence is plotted on the X-axis). The bottom right quadrant, indicated in green represents cells stained for PRL and the scatter of these cells alone is shown in panel (b). The PRL cells showed a lower forward and side scatter than the GH cells shown in shown in Figure 3.6, suggesting that these cells may be smaller and less granular (see also figure 3.10 for side scatter).

Figure 3.9 shows the comparison between FACS analysis for PRL in AS and *dw/dw* cells. The bottom panels (c and d) show examples of the fluorescence plots of cells from adult male AS and *dw/dw* rats stained for PRL. A higher proportion of cells stained for PRL in the *dw/dw* cells than the AS (41% *vs.* 20%). The top panels (a and b) illustrate the scatter characteristics of the PRL positive cells, however the differences are easier to see in figure 3.10. The main increase in PRL cells in the *dw/dw*, appears to be in the low side scatter area of the histogram, although an increase is seen throughout the histogram.

Figure 3.11 shows an example of cells stained simultaneously for GH and PRL. Again, panel (a) shows a typical scatter diagram for anterior pituitary cells, with the cell debris gated out. Panel (c) shows the simultaneous use of NMS and NRS to set the background level of fluorescence. The double staining method differs slightly, as a three layer system is used to stain GH (illustrated in Fig 2.1). This results in the GH stained and the background fluorescence having a higher signal in the PE channel than was seen in the two layer system. Panel (d) shows a typical double fluorescence plot, with the top right quadrant, indicated in green, representing mammosomatotrophs. As in previous examples, panel (b) shows the scatter characteristics of the double positive cells. The mammosomatotrophs seemed to show a wide distribution of scatter, perhaps with a bias towards low forward scatter, indicating that many of the cells may be fairly small.

Figure 3.12 shows the comparison between double FACS analysis of AS and *dw/dw* cells. The bottom panels (c and d) show examples of the fluorescence plots of cells from adult male AS and *dw/dw* rats. The top panels (a and b) show the scatter characteristics of the mammosomatotrophs. The "lobster" shaped plots, typical of double stained pituitary cells, were caused by the heterogeneity in scatter of the cell population. It can be readily seen that *dw/dw* cells had more PRL staining cells (lower right quadrant of panel d), whereas AS cells had more GH staining (upper left quadrant of panel c). In most cases, however, double FACS was only used for the quantification of double positive cells as the shape of the fluorescence plot made placing the quadrant marker difficult. For this reason, when quantifying cell types, the dispersed cells from each pituitary were divided into three tubes for single (total GH staining and total PRL staining) and double (mammosomatotrophs) FACS analysis. The proportion of cells staining for GH only or PRL only were than calculated from all of these results.

A series of FACS analyses were then performed on individually dispersed pituitaries from groups of AS and *dw/dw* male and female rats (n=4), and the results are shown in figure 3.13. Differences in GH or PRL only and double stained cells are indicated within the relevant bars, differences in total GH or

total PRL staining cells are indicated above the bars. Due to the large number of parameters, full details of the results and levels of significance are also presented in table 3.1a.

The proportion of GH staining cells (both GH only and total GH) was, as expected, deficient in the *dw/dw* compared to the AS in both male and female rats. In contrast, the proportion of PRL staining cells (both PRL only and total PRL) was higher in the *dw/dw* compared to the AS in both male and female rats. Interestingly, no differences in the proportion of mammosomatotrophs existed between the AS and *dw/dw* rats in either males or females. This means that the proportion of GH cells that also contained PRL in the *dw/dw* was higher than the AS. A sex difference in total PRL staining proportions existed in AS rats, the females having a higher proportion than the males. The proportion of cells staining for PRL only, however was not significantly different between AS males and females. There was no sex difference in lactotrophs in the *dw/dw*, or somatotrophs in either strain. In both AS and *dw/dw* rats, the males had a significantly higher proportion of mammosomatotrophs than the females.

Although this data shows the percentage of PRL cells to be increased, *dw/dw* pituitaries are smaller than those from AS rats. When dispersing pituitaries for culture (see chapter 5), AS pituitaries consistently yielded 4 million cells whereas *dw/dw* pituitaries yielded 2.5 million. Using these factors the total of each cell type per pituitary may be estimated. This data is also presented table 3.1b.

After correcting for pituitary size, the deficiency in GH cells, of course, still exists. However, the increase in the proportion of lactotrophs in the *dw/dw* was balanced by the reduced pituitary size in both male and female animals. In addition, the estimated total mammosomatotroph number was about 25% lower in male *dw/dw* pituitaries than male AS pituitaries. There was, however, no significant difference in mammosomatotrophs in female animals. Therefore, although the *dw/dw* exhibits a profound deficiency in classical somatotrophs, the mammosomatotroph population is not severely reduced.

Figure 3.15 compares the percentages of GH and PRL staining cells obtained by immunocytochemistry and FACS. The two methods use the same primary antibodies, so have the same antigen specificity. Apart from the GH measurement in AS females which was slightly lower by FACS than immunocytochemistry, there were no significant differences between the results obtained by the two methods, thus for analysis of cell percentages, FACS analysis seems to be an appropriate method.

**Figure 3.6: Single FACS analysis for GH.**

Dispersed anterior pituitary cells from an AS male rat were stained for GH and analysed by FACS. The primary antibody was replaced with NMS as a control.

a. Scatter characteristics of anterior pituitary cells.
b. Scatter characteristics of GH positive cells.
c. Fluorescence plot of NMS control.
d. Fluorescence plot of GH stained pituitary cells.

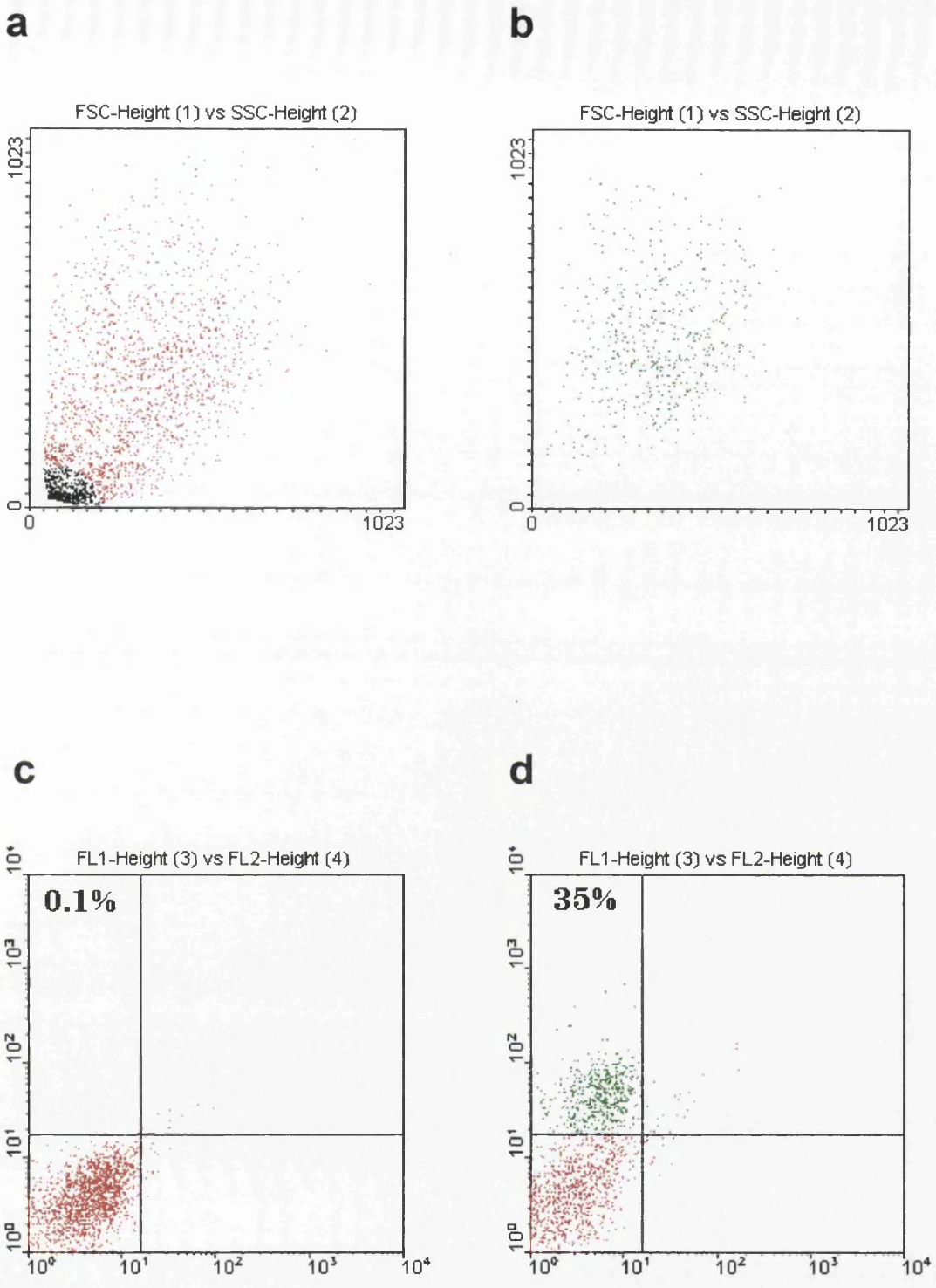

a
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
b
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
c
FL1-Height (3) vs FL2-Height (4)
0.1%
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
d
FL1-Height (3) vs FL2-Height (4)
35%
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$

**Figure 3.7: Comparison of single FACS analysis of AS and *dw/dw* pituitary cells for GH.**

Dispersed anterior pituitary cells from an AS and *dw/dw* male rats were stained for GH and analysed by FACS.

a. Scatter characteristics of GH positive cells in AS pituitary cells.
b. Scatter characteristics of GH positive cells in *dw/dw* pituitary cells.
c. Fluorescence plot of GH stained AS pituitary cells.
d. Fluorescence plot of GH stained *dw/dw* pituitary cells.

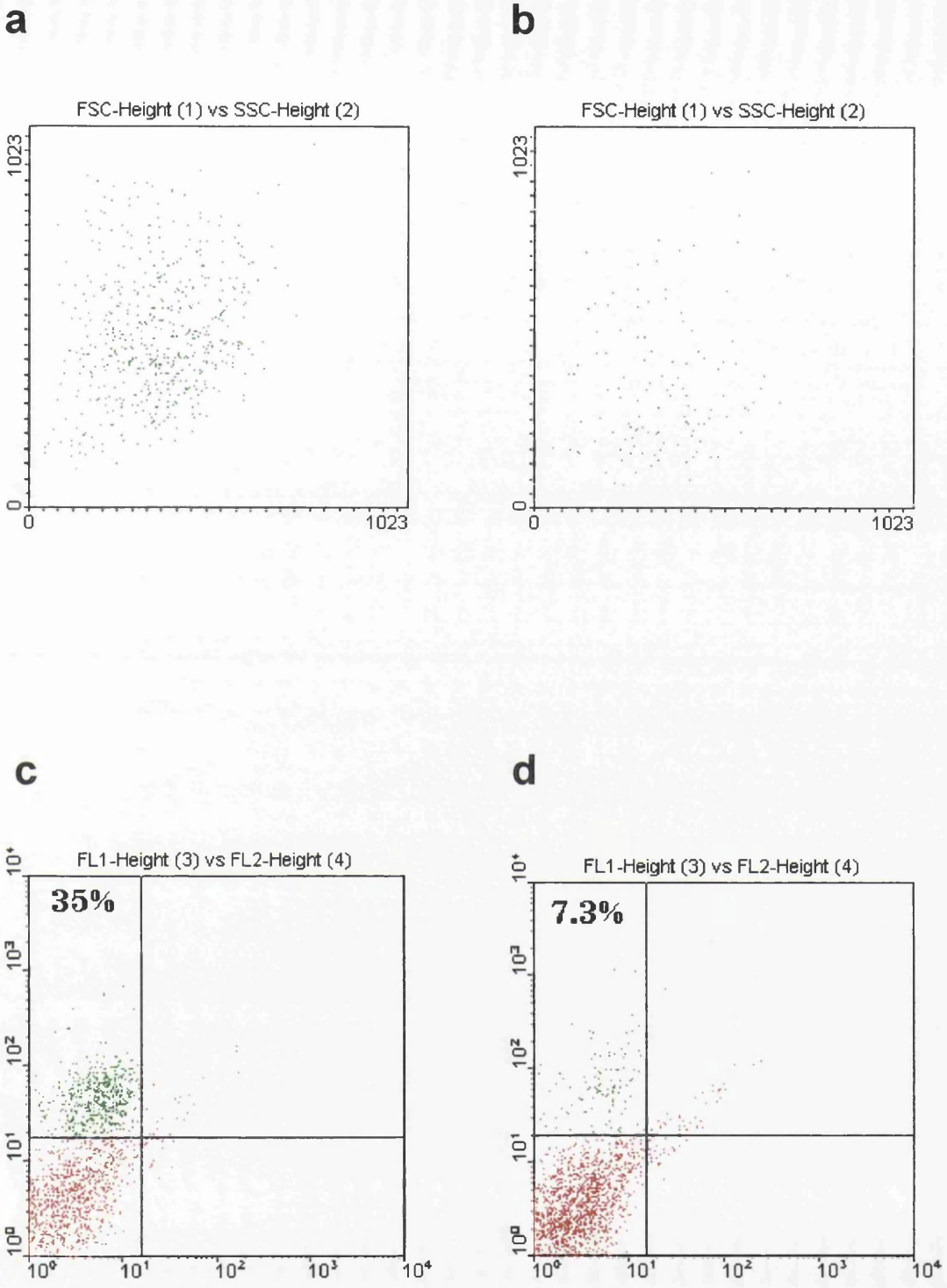

a
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
b
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
c
FL1-Height (3) vs FL2-Height (4)
35%
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
d
FL1-Height (3) vs FL2-Height (4)
7.3%
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$

**Figure 3.8: Single FACS analysis for PRL.**

Dispersed anterior pituitary cells from an AS male rat were stained for PRL and analysed by FACS. The primary antibody was replaced with NRS as a control.

a. Scatter characteristics of anterior pituitary cells.
b. Scatter characteristics of PRL positive cells.
c. Fluorescence plot of NRS control.
d. Fluorescence plot of PRL stained pituitary cells.

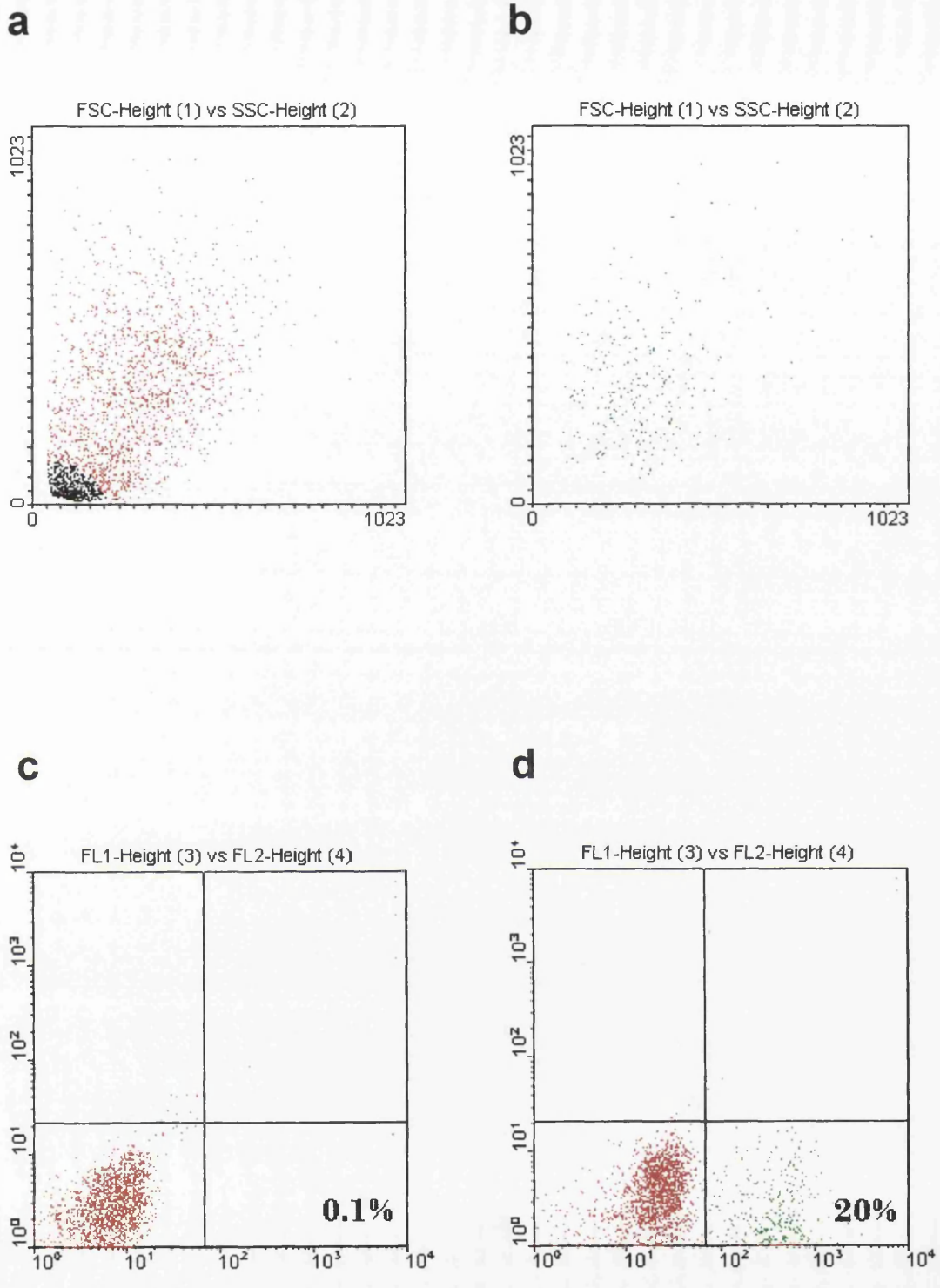

a
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
b
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
c
FL1-Height (3) vs FL2-Height (4)
0.1%
d
FL1-Height (3) vs FL2-Height (4)
20%

**Figure 3.9: Comparison of single FACS analysis of AS and *dw/dw* pituitary cells for PRL.**

Dispersed anterior pituitary cells from an AS and *dw/dw* male rats were stained for PRL and analysed by FACS.

a. Scatter characteristics of PRL positive cells in AS pituitary cells.
b. Scatter characteristics of PRL positive cells in *dw/dw* pituitary cells.
c. Fluorescence plot of PRL stained AS pituitary cells.
d. Fluorescence plot of PRL stained *dw/dw* pituitary cells.

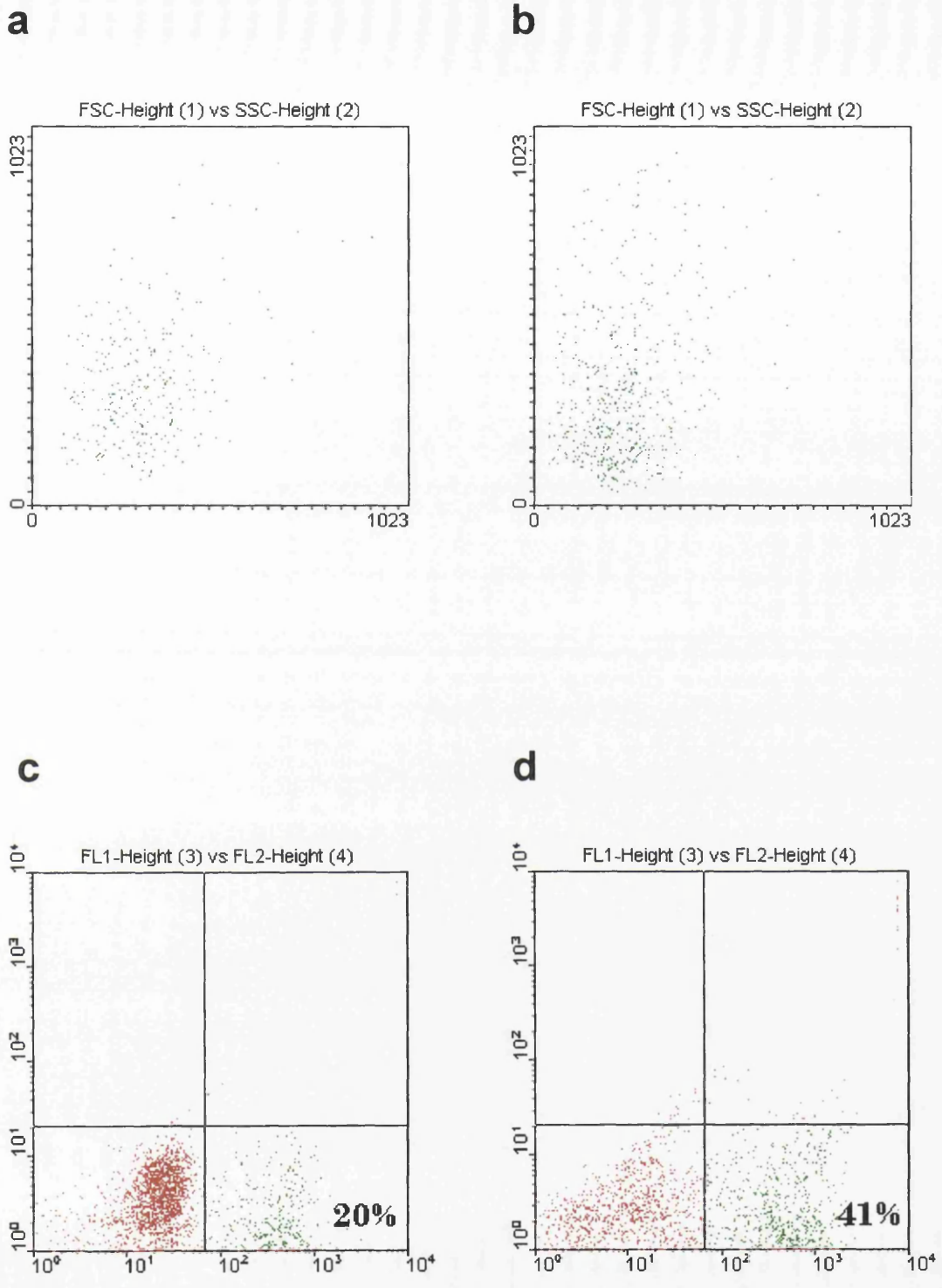

a
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
b
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
c
FL1-Height (3) vs FL2-Height (4)
20%
d
FL1-Height (3) vs FL2-Height (4)
41%

**Figure 3.10: Side scatter of AS and *dw/dw* GH and PRL staining cells.**

The GH positive and PRL positive cells from AS and *dw/dw* pituitaries (highlighted in green in figures 3.7 and 3.9) re-expressed as histograms.

a. GH positive cells in AS pituitary cells.
b. PRL positive cells in AS pituitary cells.
c. GH positive cells in *dw/dw* pituitary cells.
d. PRL positive cells in *dw/dw* pituitary cells.

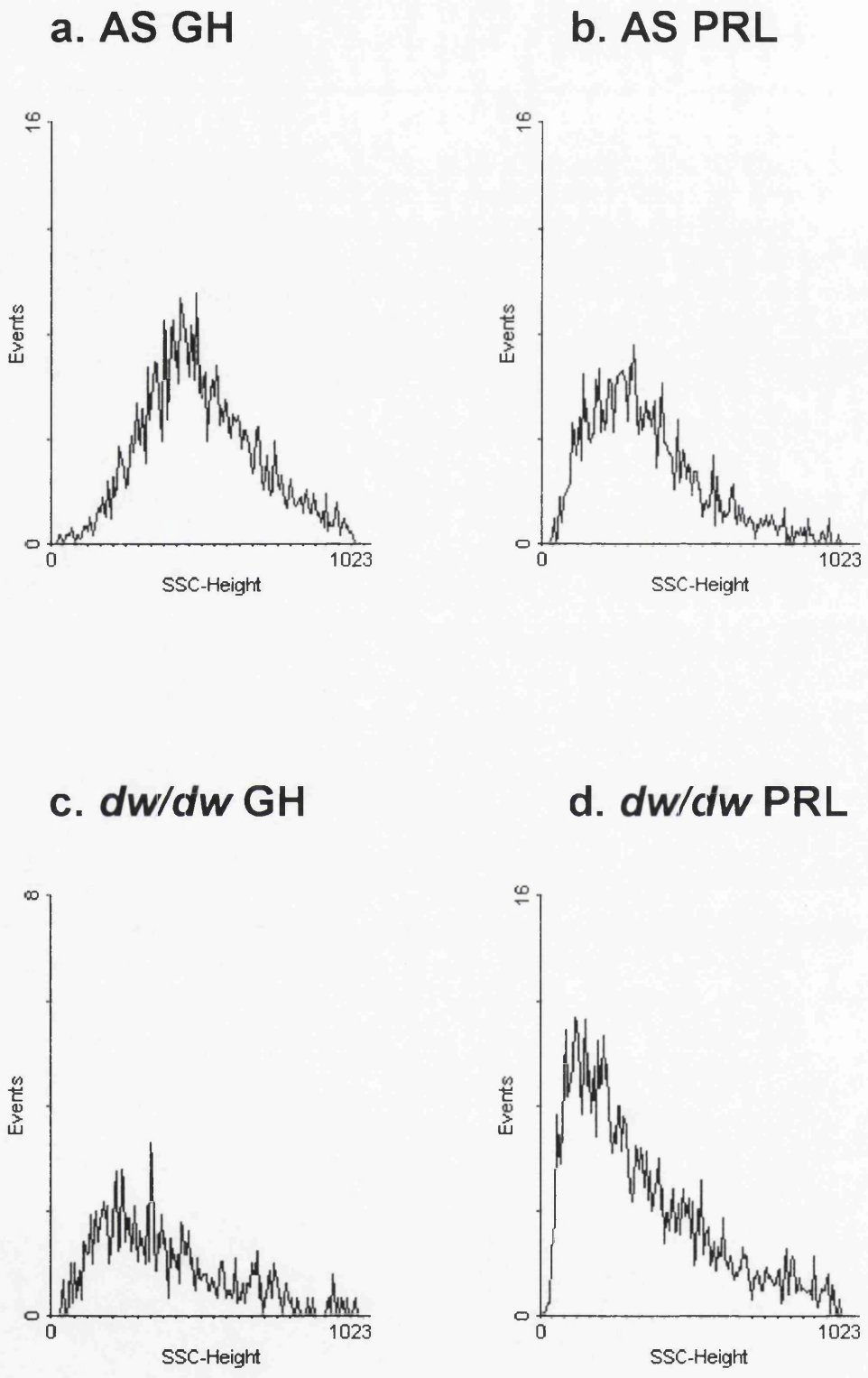

a. AS GH
b. AS PRL
c. dw/dw GH
d. dw/dw PRL
Events
SSC-Height
0
1023
16
8

**Figure 3.11: Double FACS analysis for GH and PRL.**

Dispersed anterior pituitary cells from an AS male rat were stained for GH and PRL simultaneously and analysed by FACS. The primary antibodies was replaced with NMS and NRS as a control.

a. Scatter characteristics of anterior pituitary cells.
b. Scatter characteristics of double positive cells.
c. Fluorescence plot of NMS and NRS control.
d. Fluorescence plot of double stained pituitary cells.

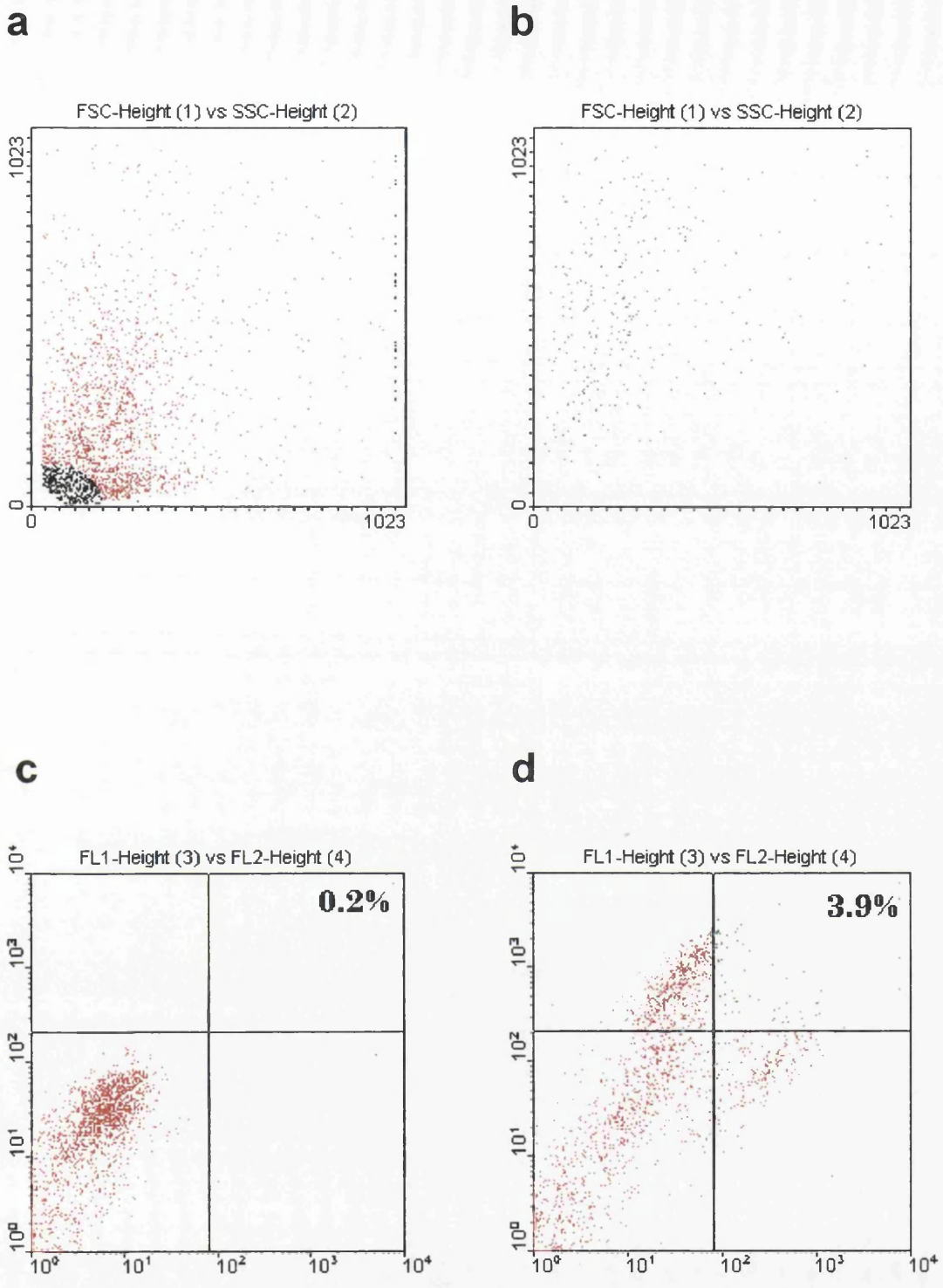

a
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
b
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
c
FL1-Height (3) vs FL2-Height (4)
0.2%
d
FL1-Height (3) vs FL2-Height (4)
3.9%

**Figure 3.12: Comparison of double FACS analysis of AS and *dw/dw* pituitary cells for GH and PRL.**

Dispersed anterior pituitary cells from an AS and *dw/dw* male rats were stained for PRL and analysed by FACS.

a. Scatter characteristics of double positive cells in AS pituitary cells.
b. Scatter characteristics of double positive cells in *dw/dw* pituitary cells.
c. Fluorescence plot of double stained AS pituitary cells.
d. Fluorescence plot of double stained *dw/dw* pituitary cells.

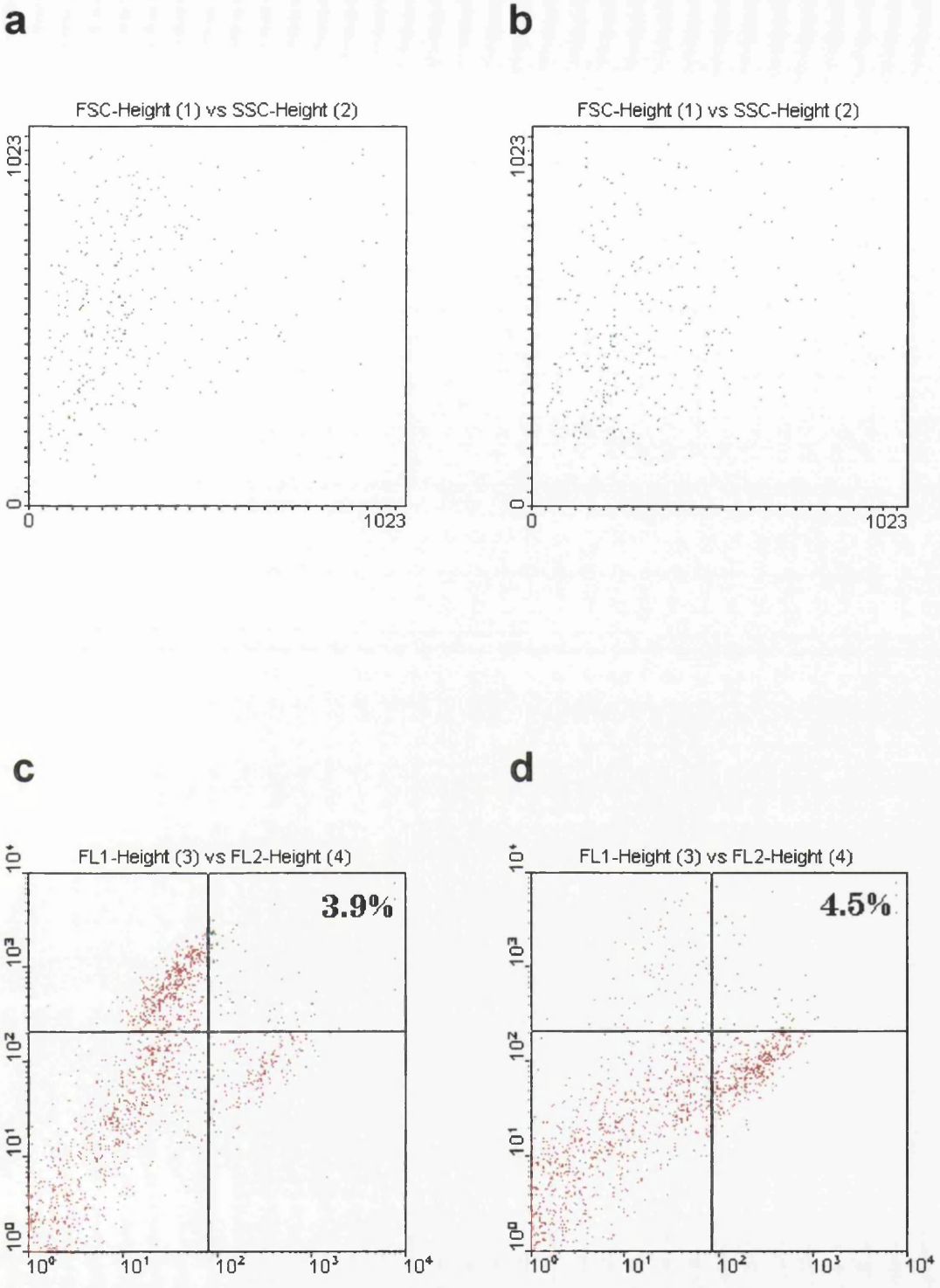

a
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
b
FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023
c
FL1-Height (3) vs FL2-Height (4)
3.9%
d
FL1-Height (3) vs FL2-Height (4)
4.5%

**Table 3.1a: Percentages of cell types in AS and *dw/dw* rats.**

The results are calculated from FACS analysis for single GH-, single PRL- and simultaneous GH and PRL- staining cells for each pituitary. This data is also shown in figure 3.13.

ANOVA followed by Bonferroni's post-hoc test (n=4).

**Table 3.1b: Total numbers of cell types in AS and *dw/dw* rats.**

The total cell numbers per pituitary (in millions) were estimated by assuming the total cell numbers to be 4 million cells per AS pituitary and 2.5 million cells per *dw/dw* pituitary. This data is also shown in figure 3.14.

ANOVA followed by Bonferroni's post-hoc test (n=4).

| | GH Total | PRL Total | Double | GH only | PRL only |
|---|---|---|---|---|---|
| AS Male | 31.73% ± 2.47 | 19.99% ± 1.27 | 6.14% ± 0.22 | 25.88% ± 2.48 | 13.94% ± 1.52 |
| AS Female | 28.65% ± 2.77 | 32.22% ± 1.60 | 2.70% ± 0.25 | 24.99% ± 3.41 | 29.11% ± 2.03 |
| *dw/dw* Male | 11.84% ± 1.08 | 38.56% ± 2.28 | 7.45% ± 0.46 | 4.4% ± 1.36 | 31.10% ± 2.59 |
| *dw/dw* Female | 5.67% ± 0.43 | 45.60% ± 2.34 | 3.73% ± 0.37 | 1.93% ± 0.48 | 41.87% ± 2.20 |
| AS Male *vs.* Female | N/S | P< 0.01 | P< 0.001 | N/S | P< 0.01 |
| *dw/dw* Male *vs.* Female | N/S | N/S | P< 0.001 | N/S | N/S |
| Male AS *vs.* *dw/dw* | P< 0.001 | P< 0.001 | N/S | P< 0.001 | P< 0.001 |
| Female AS *vs. dw/dw* | P< 0.001 | P< 0.01 | N/S | P< 0.001 | P< 0.05 |

| | GH Total ($\times 10^6$) | PRL Total ($\times 10^6$) | Double ($\times 10^6$) | GH only ($\times 10^6$) | PRL only ($\times 10^6$) |
|---|---|---|---|---|---|
| AS Male | 1.27 ± 0.1 | 0.80 ± 0.05 | 0.25 ± 0.01 | 1.02 ± 0.1 | 0.56 ± 0.06 |
| AS Female | 1.15 ± 0.11 | 1.29 ± 0.06 | 0.11 ± 0.01 | 1.00 ± 0.14 | 1.16 ± 0.08 |
| *dw/dw* Male | 0.30 ± 0.03 | 0.96 ± 0.06 | 0.19 ± 0.01 | 0.11 ± 0.03 | 0.78 ± 0.06 |
| *dw/dw* Female | 0.14 ± 0.01 | 1.14 ± 0.06 | 0.09 ± 0.01 | 0.05 ± 0.01 | 1.05 ± 0.06 |
| AS Male *vs.* Female | N/S | P< 0.001 | P< 0.001 | N/S | P< 0.001 |
| *dw/dw* Male *vs.* Female | N/S | N/S | P< 0.001 | N/S | N/S |
| Male AS *vs.* *dw/dw* | P< 0.001 | N/S | P< 0.05 | P< 0.001 | N/S |
| Female AS *vs. dw/dw* | P< 0.001 | N/S | N/S | P< 0.001 | N/S |

**Figure 3.13: Percentages of GH-, PRL-, and Double- staining cells in AS and *dw/dw* rat pituitaries.**

Anterior pituitary cells were stained for GH, PRL or both antigens simultaneously and analysed by FACS. The stack graphs show the proportion of GH (a) and PRL (b) in the cell population. The white areas represent the percentage of cells staining only for GH or PRL and the blue areas represent the mammosomatotroph population. Thus the total height of the bar represents the total GH or PRL staining cells. This data is also shown in table 3.1a.

Differences in GH or PRL only and double stained cells are indicated within the relevant bars, differences in total GH or total PRL staining cells are indicated above the bars. **P<0.01, ***P<0.001 *vs.* AS of the same sex. $^{+}$P<0.05, $^{++}$P<0.01 male *vs.* female. ANOVA followed by Bonferroni's post-test (n=4).

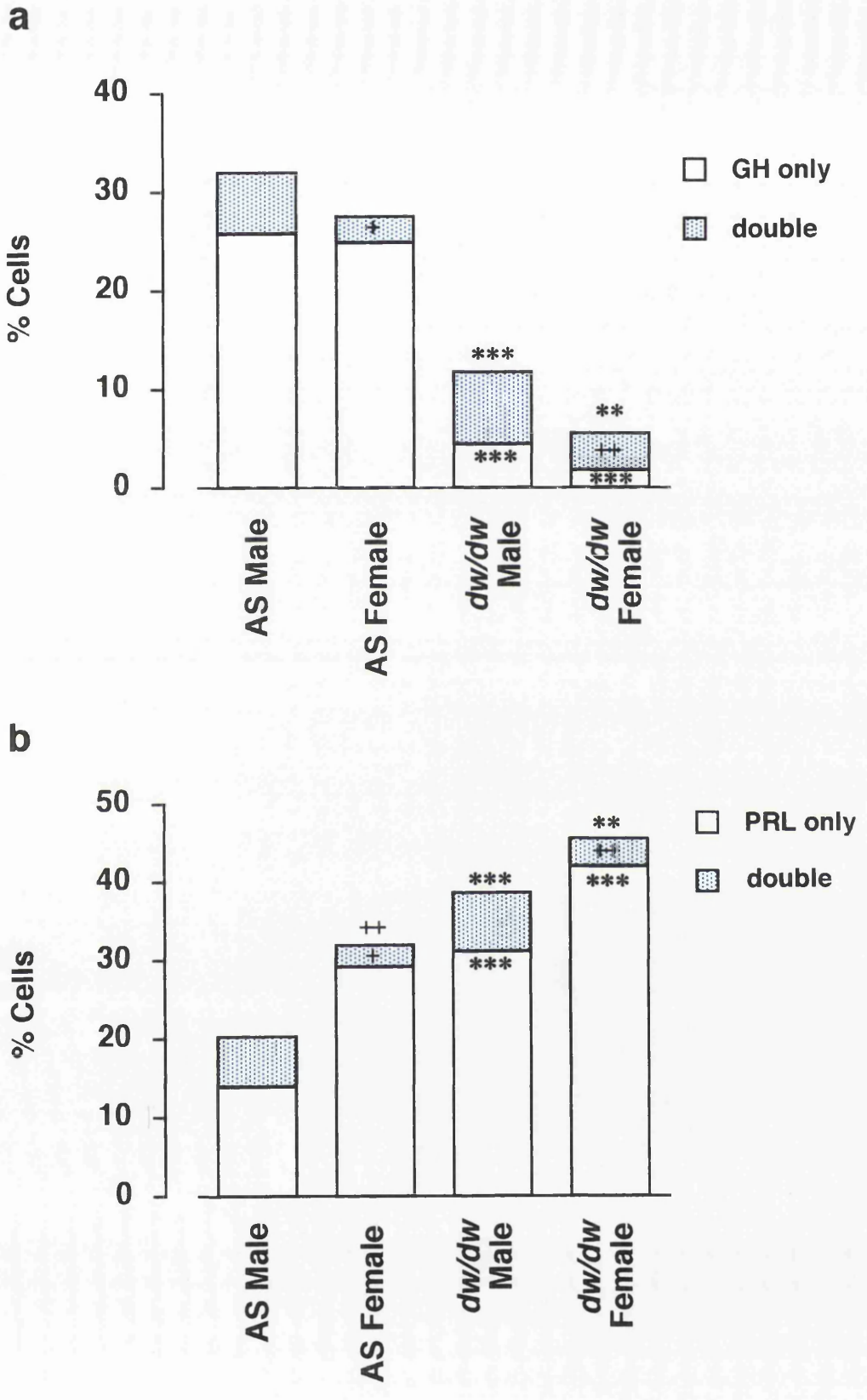

a
40
30
20
10
0
% Cells
GH only
double
***
**
++
+
***
***
AS Male
AS Female
dw/dw Male
dw/dw Female
b
50
40
30
20
10
0
% Cells
PRL only
double
**
++
++
+
***
***
***
AS Male
AS Female
dw/dw Male
dw/dw Female

**Figure 3.14: Estimated numbers of GH-, PRL-, and double-staining cells in AS and *dw/dw* rat pituitaries.**

The data from figure 3.13 was re-expressed as total cells per pituitary. Differences in GH or PRL only and double stained cells are indicated within the relevant bars, differences in total GH or total PRL staining cells are indicated above the bars. This data is also shown in table 3.1b.

*P<0.05, ***P<0.001 *vs.* AS of the same sex. $^{+++}$P<0.001 male *vs.* female. ANOVA followed by Bonferroni's post-test (n=4).

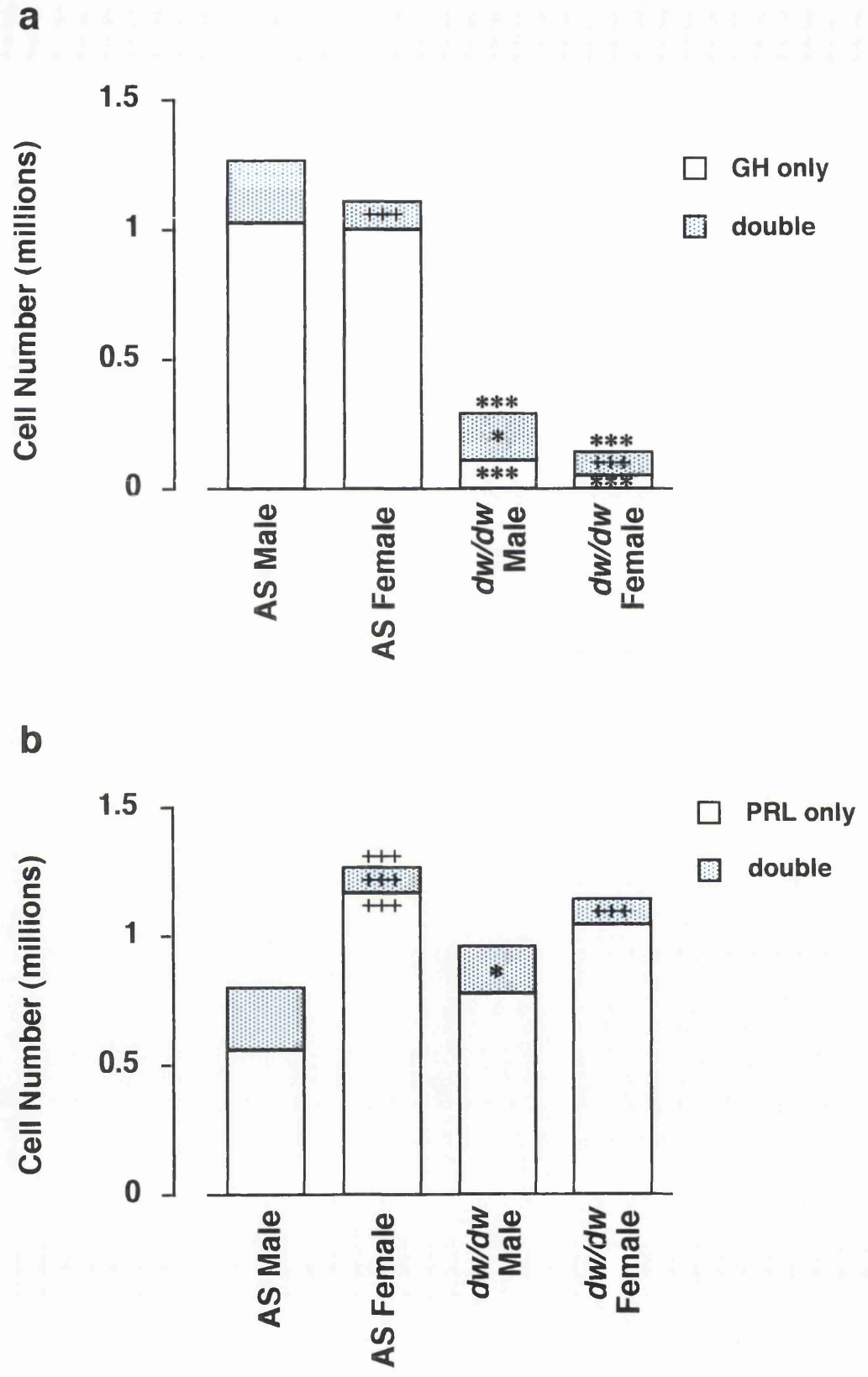
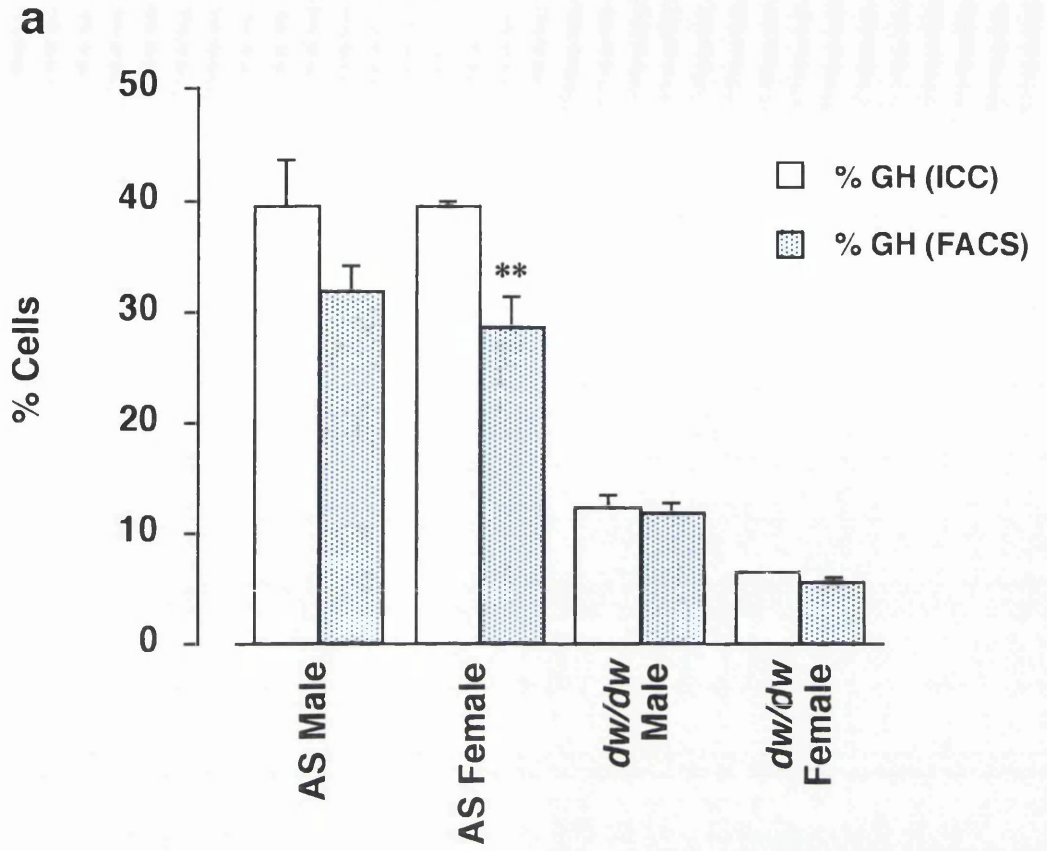
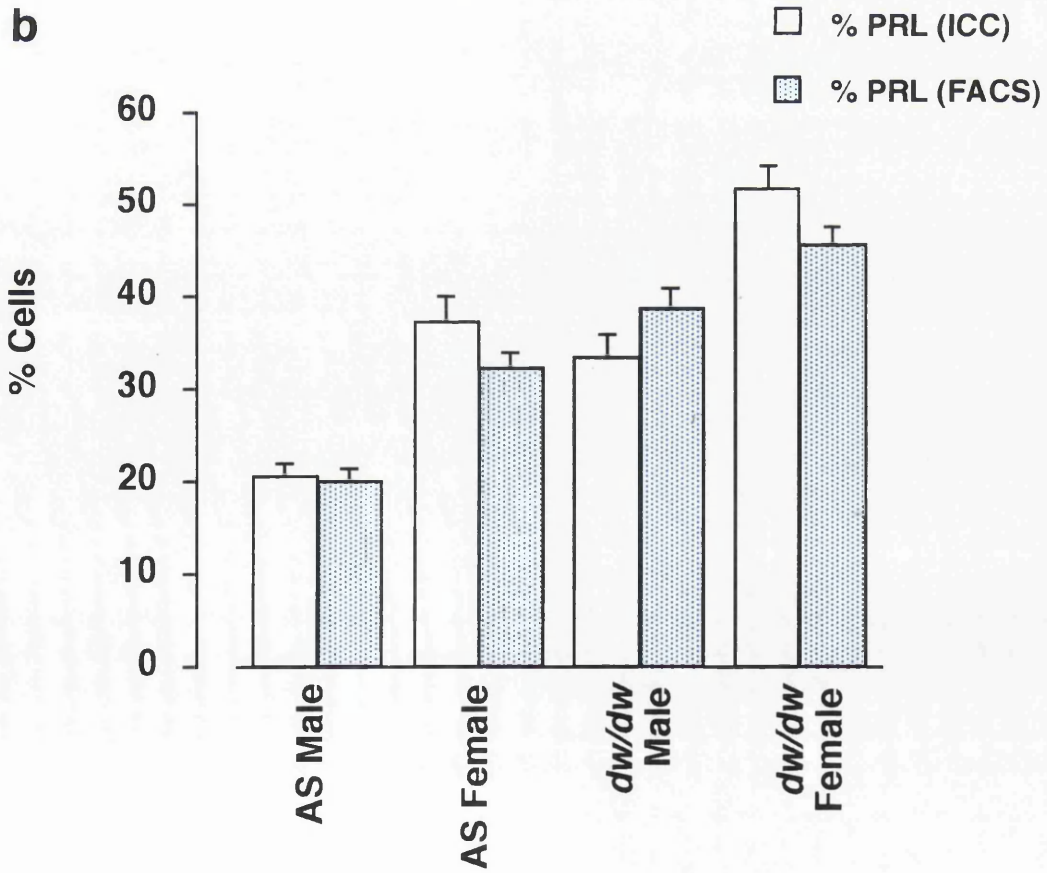

a
Cell Number (millions)
1.5
1
0.5
0
GH only
double
AS Male
AS Female
dw/dw Male
dw/dw Female
b
Cell Number (millions)
1.5
1
0.5
0
PRL only
double
AS Male
AS Female
dw/dw Male
dw/dw Female

**Figure 3.15: Comparison of results obtained by FACS and immunocytochemistry.**

The percentage of total GH staining cells (a) and total PRL staining cells (b) shown in figures 3.5 (immunocytochemistry) and 3.13 (FACS) are re-plotted as a comparison.

**P<0.01, *vs.* AS of the same sex. ANOVA followed by Bonferroni's post-test (Immunocytochemistry: n=3; FACS: n=4).


a
50
40
30
20
10
0
% Cells
% GH (ICC)
% GH (FACS)
**
AS Male
AS Female
dw/dw Male
dw/dw Female


b
% PRL (ICC)
% PRL (FACS)
60
50
40
30
20
10
0
% Cells
AS Male
AS Female
dw/dw Male
dw/dw Female

## 3.3 DISCUSSION

One of the aims of the work in this chapter was to investigate whether the heterozygote has a slight intermediate phenotype due to gene dosage. The body weight of the *dw/dw* rats was about 40% (male) and 30% (female) less than AS controls. The heterozygotes, instead of having an intermediate phenotype, were slightly but significantly heavier than AS controls. Although the *dw* mutation originated in a colony of Lewis rats, it was subsequently bred onto an AS background. Despite the mutant and the controls being of the same strain, the *dw/dw* line has subsequently been inbred from a population of homozygotes, much more than the AS colony, which could lead to other genetic differences between the two lines over time. Out-breeding of the AS and *dw/dw* could therefore lead to a degree of hybrid vigour, which could explain the increased body weight in the $F_1$ rats. This theory is supported by the fact that heterozygotes appeared more active than either AS or *dw/dw* rats.

Previous studies have shown a slight intermediate with respect to Pit-1 and GH mRNA (Houston *et al*, 1991). However, the data in this chapter has shown that there was no intermediate phenotype with reference to GH or PRL content or cell number. Thus any deficiency in GH mRNA does not appear to extend to the protein. In addition, a reduction in Pit-1 mRNA does not appear to result in a reduction in somatotroph number. From these data, the observed differences between AS and AS/*dw* rats at a biochemical level (Houston *et al*, 1991) does not result in differences in hormone output.

In the early reports concerning the *dw/dw*, the control animals were heterozygote litter-mates (Charlton *et al*, 1988; Bartlett *et al*, 1990) as *dw/dw* animals were initially bred from heterozygote parents. There are both advantages and disadvantages in using heterozygote controls. When *dw/dw* rats are bred from *dw/dw* parents, the birth weight will be affected by maternal size. In addition, *dw/dw* rats treated with GH during pregnancy produced pups which were heavier at birth and had a subsequently increased growth rate and Wistar rats treated with GH antiserum during pregnancy showed reduced fetal and placental size as well as reduced lactation (Spencer *et al*, 1994) indicating a

role for maternal GH or IGF-1. The breeding of *dw/dw* and heterozygote controls from the same crosses guarantees that these factors are the same between groups. However, if there is a gene dosage related phenotype in the heterozygotes, then using AS rats would be a better control and this has been done in subsequent studies in this laboratory.

The most significant finding in this chapter, is the fact that the *dw/dw* rats contained significantly more PRL than the AS, in both male and females. This is in discordance with the initial characterization of this model (Charlton *et al*, 1988) who found no significant difference in the PRL levels between *dw/dw* and heterozygous litter-mate controls. In this, and a later publication (Bartlett *et al*, 1990), the increased concentration of PRL was found to be merely compensating for the reduced pituitary size. Because of my PRL RIA data, and the SDS-PAGE studies described in chapter 6, this laboratory reinvestigated this problem. In studies carried out for other purposes, in parallel to my own, we have confirmed my results in other groups of animals. For example Phelps *et al* (1997) also report elevated PRL contents in *dw/dw* pituitaries, compared to AS; AS: 2.66 ± 0.2μg/pituitary *vs. dw/dw*: 5.32 ± 0.5 (Phelps *et al*, 1997): AS: 4.09 ± 0.75 *vs. dw/dw*: 8.16 ± 1.45 (present study). However, despite higher pituitary stores of PRL, the plasma concentration of PRL is not significantly higher in the *dw/dw* than the AS (D.F. Carmignac and G.B. Thomas, unpublished data), so it is unclear what physiological consequence this higher pituitary PRL has on the animals.

The SDR rat and the little mouse mutations would both be expected to cause isolated GH deficiencies, however both also show mild deficiencies in PRL. Due to the common lineage of GH and PRL cells, it is not surprising that an anomalous GH axis also leads to alterations in the PRL axis. In addition, GH deficiency influences the feedback mechanisms acting at the hypothalamus, for example, the level of GRF mRNA is increased in the *dw/dw* rat, (Bennett *et al*, 1997), the SDR rat (Kamegai *et al*, 1997) and the Little mouse (Frohman *et al*, 1989). There is also interplay between the GH and PRL axis at the hypothalamic level, for instance some GRF neurones also express TH, the rate limiting enzyme in DA synthesis (Meister *et al*, 1986). However, despite the

reduced GH in all three models, the *dw/dw* rat differs from the SDR rat and the Little mouse in that the PRL axis appears to be up-regulated rather than down-regulated. Thus it is likely that the PRL phenotype in the *dw/dw* is not merely a consequence of the GH deficiency. This contradicts the hypothesis that the somatotroph specific GRF signalling defect is the cause of the phenotype (Downs and Frohman, 1991), in favour of a defect affecting both somatotrophs and lactotrophs. Since the GH axis already shows a reduction before birth (Carmignac *et al*, 1993b; Zeitler *et al*, 1994), the phenotype is likely to be due to alteration of a developmental switch controlling the balance of the closely related GH and PRL axes. Such a defect may be explained by a mutation affecting the mammosomatotroph precursor cell, which is the progenitor to both somatotrophs and lactotrophs.

The data has shown, by both immunocytochemistry and FACS, that lactotrophs form a higher percentage of the cells in male and female *dw/dw* pituitaries compared to AS controls. This is in agreement with Kineman *et al* (1989) who found an increase in the percentage of lactotrophs in the *dw/dw* by RHPA. However, due to the smaller pituitary size of the *dw/dw*, a higher percentage of lactotrophs may not reflect an actual increase in the total lactotroph number in the pituitary. Attempts were made to correct for this in this chapter, although in retrospect I should have performed cell counts on the dispersed pituitaries or DNA measurements to determine the total cell number of each pituitary. When dispersing cells for primary culture (chapter 5), AS male pituitaries consistently yielded 4 million cells and *dw/dw* male pituitaries yielded 2.5 million cells per pituitary. These values are in agreement with Downs and Frohman (1991): AS: 4.37 million ± 0.23 *vs.*, *dw/dw*: 2.71 ± 0.12. Both sets of cell numbers show the *dw/dw* pituitary to have about 60% of the cell number of the AS. This does not, however, allow for sex differences in the total cell numbers, and thus must be treated as an estimate. Birge *et al* (1967) reported that the pituitary weight of adult female Sprague-Dawley rats was greater than males, suggesting there may be a difference in total cells number.

The total GH and PRL cell numbers per pituitary, estimated from the FACS data, revealed that there was no significant differences between lactotroph

number from AS and *dw/dw* rats. Thus the increased percentage of lactotrophs was merely compensating for the reduced pituitary size. The increase in pituitary PRL content without an increase in total lactotroph numbers implies that each cell contains more PRL. This was reflected in the more intense immunocytochemical staining of lactotrophs using fast red. However, the scatter characteristics of PRL positive cells did not show higher side scatter in the *dw/dw* as would be expected. In contrast, the main increase in cells appeared to be low side scatter cells, suggesting a less granular population. It is possible that the structure of the *dw/dw* and AS lactotrophs differ. A cell containing more hormone may show a lower side scatter if the extra stored hormone stretches the boundaries of the granules or the cell.

Previous reports have shown sex differences in GH and PRL cell number (Leong *et al*, 1985) and pituitary content (Birge *et al*, 1967) such that GH cell number and content was significantly higher in the male, and PRL cell number and content was significantly higher in the female. The sex differences in GH and PRL content in this chapter are in agreement with Birge *et al*, (1967), although the differences were not always significant. For instance only the male AS/*dw* had significantly more GH per pituitary than the female, whereas the AS and *dw/dw*, but not the AS/*dw* males had significantly less PRL per pituitary than the females. The percentage of lactotrophs measured by immunocytochemistry were significantly higher in AS, AS/*dw* and *dw/dw* females than males, but there were no significant sex differences in somatotroph number. When measured by FACS analysis, the percentage of lactotrophs was also significantly higher in AS females than males, although in this case there was no significant sex difference in the *dw/dw* pituitaries. Again, no significant sex difference was seen in somatotroph number. Although the sex differences seen in GH and PRL content and lactotroph number were not always significant, differences were seen in AS, AS/*dw* and *dw/dw* at different times, thus there is no evidence that any genotype is more prone to show a sex difference. In addition, where the differences were not significant (including the somatotroph number, which was not significant either by immunocytochemistry or FACS) the trend was in the same direction as previous reports (Birge *et al*, 1967; Leong *et al*, 1985) and thus may have been significant if more animals had been analysed.

Mammosomatotrophs have previously been detected in pituitary cells by sequential RHPA (Frawley *et al*, 1985; Leong *et al*, 1985) and immunogold staining (Nikitovitch-Winer *et al*, 1989). In addition, the mammosomatotroph population has been calculated from the difference between staining by mixed GH and PRL primary antibodies and the sum of single staining in both immunocytochemistry (Frawley *et al*, 1985) and FACS (Shinkai *et al*, 1995). The detection of mammosomatotrophs by these methods was reviewed in chapter 1. In this study, FACS analysis was chosen due to the availability of the technology and convenience of the method.

There were no significant differences in proportions of mammosomatotroph between AS and *dw/dw*, although this translated to an estimated 25% reduction in the total mammosomatotroph number in the male *dw/dw* compared to AS while there were still no significant differences between AS and *dw/dw* females. Thus this subpopulation of GH producing cells is less reduced than classical somatotrophs (staining for GH only), which were an estimated 9 fold and 20 fold deficient in male and female *dw/dw* pituitaries compared to AS. One possible explanation is the developmental blockage of somatotroph commitment from mammosomatotroph precursors. As mentioned earlier, a developmental defect would explain the phenotype more fully as both the early onset of GH deficiency and the accompanying PRL phenotype could be caused by such a defect.

The severely reduced somatotroph number without much, or any, reduction in mammosomatotrophs resulted in more than 50% of the GH containing cells in male and female *dw/dw* rats also containing PRL. Mammosomatotrophs can possess receptors typical of both somatotrophs and lactotrophs, and signal the release of both GH and PRL. Examples of this have been reported in acromegalic patients, where GRF caused release of PRL (Losa *et al*, 1985) and TRH released GH (Irie and Tsushima, 1972). Carmignac *et al* (1998) report that female or $E_2$ treated male *dw/dw*, but not AS, rats released PRL in response to GHRP-6. As GHS receptors are only found on cells expressing GH (Smith *et al*, 1997), this release is likely to be from mammosomatotrophs. The total number of mammosomatotrophs in the female *dw/dw* is not significantly different to the AS, however they respond differently to GHRP-6. This suggests that the

mammosomatotrophs in *dw/dw* rats are different to those in the AS. In addition, induction of PRL release in response to GHRP-6 by $E_2$ in *dw/dw* and not AS rats suggests different responses to sex steroids. The effects of $E_2$ on the proportion of cell types was compared in AS and *dw/dw* rats and the results are presented in chapter 4.

The detection of mammosomatotrophs by immunogold (Nikitovitch-Winer *et al*, 1989) involved the localization of GH and PRL by different sizes of colloidal gold to allow double positive cells to be seen by electron-microscopy. The mammosomatotrophs they described were smaller than either somatotrophs or lactotrophs. These cells contained granules of varying sizes and the appearance of the nuclei indicated that the cells were functionally active. Interestingly, GH and PRL were found to be co-localized in the same secretory granules. Nikitovitch-Winer *et al* (1989) hypothesised that these cells represented a precursor cell. Many of the double staining cells had low forward scatter, suggesting they may be small in size. However, larger cells were also present in the mammosomatotroph population. There is evidence that dual expressing cells also represent an intermediate cell type involved in transdifferentiation as well as a precursor cell. These intermediate cells are less likely to be small, and may represent the larger cells shown in the scatter plots in this chapter. Another report from the same group as the previous paper (Papka *et al*, 1986), in which double staining is not restricted to the smaller cells.

GH positive cells from AS rats had a higher side scatter than PRL positive cells. This correlates well with the reported densities of these cell types (Scheikl *et al*, 1985); somatotrophs are denser than lactotrophs suggesting they have more stored hormone and thus will be more granular. In this chapter I have shown that an AS male pituitary contains almost two orders of magnitude more GH than PRL, but this is stored in a similar number of cells, correlating well with the observed differences in side scatter. In addition, the distribution of side scatter of *dw/dw* somatotrophs is less than AS somatotrophs. Downs and Frohman (1991) calculated that a *dw/dw* somatotroph has 40% of the GH store of an AS somatotroph, again correlating well with the observed scatter characteristics.

Due to somatotrophs and lactotroph heterogeneity, separation of pituitary cells by density often revealed a bimodal distribution (Snyder *et al*, 1997; Lindstrom and Savandhal, 1996; Hu and Lawson 1994). There was no obvious bimodal distribution of somatotrophs or lactotrophs in their scatter characteristics, however it may be that the number of cells counted were not high enough to reveal subpopulations.

In this chapter, cell types have been quantified using both immunocytochemistry and FACS analysis. The two methods yielded similar results for both GH and PRL cells in AS and *dw/dw* rats. The two methods use the same primary antibody, so will have the same antigenic specificity but there are technical differences between the methods which need to be considered when choosing which is more appropriate for a particular study.

The main difference between the methods is that immunocytochemistry stains the cells in tissue sections, whereas FACS stains cells in suspension. If there is some heterogeneity in the distribution of a cell type through the pituitary, this will be lost with FACS analysis, in favour of a more accurate mean for the entire pituitary. For instance, lactotrophs exist in the pituitary in a defined pattern (Papka *et al*, 1986; Boockfor and Frawley, 1987). Outer zone and inner zone populations exist, separated by an area devoid of PRL staining cells. In addition the outer zone of the pituitary contained more mammosomatotrophs in lactating rats (Boockfor and Frawley, 1987). This spatial distribution can be investigated with immunocytochemistry but is obviously lost in FACS analysis. However, if an accurate percentage is required, the heterogeneity may be a disadvantage if immunocytochemistry is used, as which areas were counted would influence the numbers obtained.

The tissue preparation differed between the two methods. Pituitaries for immunocytochemistry were fixed immediately after removal from the animal, whereas they underwent enzymatic dispersion for FACS. Cells were counted making the assumption that the dispersion method is not selectively damaging to particular cell types. Alternatively, cells may have released their hormone during dispersal and then not stain leading to distorted cell counts.

Certain proteins may lose antigenicity after fixation. In both methods, the fixative is the same, but the whole pituitaries were fixed for longer than individual cells so that the fixative could penetrate to the centre of the tissue. If the fixative destroys the antigen, then the shorter fixation time would be more appropriate. There is, however, no evidence for GH or PRL being affected by paraformaldehyde fixation, thus either method would be applicable.

When collecting the data, 10,000 cells were counted per sample by FACS, however only 1000-2000 cells were counted in immunocytochemistry. In addition the FACS data was collected by computer and more parameters were stored. Collecting data from immunocytochemistry was more labour intensive and also relied on deciding subjectively if a cell is positive or negative. However, the cells are easily identified by eye, whereas the FACS machine will record from all particles within the gates defined at the beginning of the run, including blood cells and debris. Deciding where to place the gates can introduce a subjective element into FACS data collection. Furthermore, placing the quadrant to define the cut off fluorescence is also subjective. FACS is more commonly performed by immunologists, on much more uniform cell populations. These cells yield fluorescence plots where the positive and negative cells fall into more discrete populations. Pituitary cells are a highly heterogeneous population and can be more difficult to analyse if the populations appear to overlap. In addition, it was often impossible to place the quadrant on the "lobster" shaped plot acquired from double staining so that one analysis could quantify GH only-, PRL only- and double- staining cells. For this reason, the double staining was used only to determine the mammosomatotroph population, in combination with separate single GH and single PRL staining of other aliquots of the same cells. The background fluorescence when using double staining was higher due to the difference in the methods used. Single GH staining involved a two layer method, using a PE-conjugated second antibody. When using double staining, the GH was stained using a three layer system where a biotinylated second antibody is subsequently labelled with PE-SA. By using this modified method, placing the quadrant was made slightly easier.

To summarize, the data in this chapter has shown:

1. The adult *dw/dw* rat has a higher pituitary PRL content than the AS, although the lactotroph number appears to be unaltered.
2. The mammosomatotroph number is similar, or only slightly reduced, in the *dw/dw* compared to AS rats.
3. The AS/*dw* heterozygote does not show an intermediate phenotype for GH or PRL cell number or hormone content.
4. FACS analysis is a viable and valuable method in the determination of cell numbers in the pituitary.

Somatotrophs and lactotrophs are both derived from mammosomatotroph precursors. The block of somatotroph commitment, coupled with the increase in PRL content in the face of severe GH deficiency, suggests a defect in the differentiation mechanisms of this cell lineage.

# EFFECTS OF AGE, SEX AND HORMONAL STATUS ON THE CELL TYPES IN AS, TGR AND *DW/DW* RATS

## 4.1 INTRODUCTION

In the previous chapter, the distribution of somatotrophs and lactotrophs in adult AS and *dw/dw* rats was investigated. I showed that in addition to a deficiency in the GH axis, the *dw/dw* possesses an increase in PRL content suggesting that the phenotype is not restricted to the somatotrophs. As somatotrophs and lactotrophs have a common lineage (Hoeffler *et al*, 1985), the work presented in this chapter investigated the possibility that a developmental defect exists in the *dw/dw* rat. The proportion of cells staining for GH, PRL and both hormones was quantified at various ages using FACS analysis. The TGR rat is a second rat model which exhibits somatotroph hypoplasia (Flavell *et al*, 1996), and as such, may act as a useful comparison to the *dw/dw*. While the *dw/dw* fails to proliferate it's somatotrophs in response to GRF, the TGR has no inherent pituitary defect. Instead, the somatotrophs are not stimulated to proliferate due to the down-regulation of GRF by the hGH transgene. In this developmental study, the pituitary cell types present in male and female AS, TGR and *dw/dw* rats were investigated.

The balance between somatotrophs and lactotrophs in the adult is not static. Differences in the proportions of the cell types have been shown between virgin, pregnant and lactating rats (Porter *et al*, 1990) and steroids have been shown to influence the interconversion of the cell types (Kineman *et al*, 1992). The second experiment in this chapter was designed to compare the influence of $E_2$ *in vivo* on AS and *dw/dw* male rats, using the methods I have developed.

## 4.2 EXPERIMENTAL RESULTS

### 4.2.1 Experiment 1: Developmental Study of Pituitary Cell Types in AS, TGR and *dw/dw* rats

In this experiment AS, TGR and *dw/dw* rats were analysed at 9, 16, 23, 45, 70 and 145 days of age. The animals were weighed on the day of sacrifice and were killed by schedule 1 methods as appropriate for their weight; 9, 16 and 23 day

old rats were killed by cervical dislocation. 45, 70 and most of the 145 day old rats were stunned and decapitated. The AS and TGR male rats at 145 days were killed by $CO_2$ asphyxiation.

In the older rats, the anterior pituitary was dissected out and placed in warm HBSS for transport from the animal facility to the lab. In the case of 9, 16 and 23 day rats, the whole pituitary was taken. The pituitaries were dispersed individually for FACS analysis (sections 2.2.1 and 2.4.2). 45-145 day pituitaries were dispersed in 1.5ml and then the cell suspension was divided between three tubes (for single GH, single PRL and double FACS analysis). 9-23 day pituitaries were dispersed in 0.5ml and processed for double FACS analysis only.

The data presented are from 3-8 animals per group, with the exception of the 16 day animals. Due to technical problems with the genotyping of the AS/TGR animals, only two TGR males and a single AS female were identified. The DNA extracts did not yield any specific bands after PCR, thus it is possible that the DNA had degraded. Therefore, the data from this age group is presented without statistical analysis. Each parameter at each age group was tested by ANOVA followed by Bonferroni's post-hoc test for selected pairs.

The growth curves of the rats (Figure 4.1) show the extent of the growth retardation of the two models compared to the AS. There was no significant difference in body weight until 23 (*dw/dw* female) or 45 (TGR male and female, *dw/dw* male) days. Figure 4.2 shows that there was no significant sex difference in body weight of any of the models until 70 days. A slight (non-significant) sex difference in body weight is seen in the *dw/dw* at 45 days, compared to the AS and TGR, where the growth curves of each sex were still superimposed.

When cells are analysed by FACS, in addition to the fluorescence, the scatter characteristics are also stored. Forward scatter (x-axis of scatter plots) is a measure of cell size, while side scatter (y-axis) relates to the complexity or granularity of the cell. Figure 4.3 shows the scatter profiles of AS male pituitary cells over a range of ages. The cells had low side scatter at 9 days, relating to the low granularity, and this increased during development. The less heterogeneous cells from younger animals resulted in more distinct populations on the

fluorescence plots. Thus, all the staining populations could be determined from double stained cells.

The total GH staining cells (ie. including somatotrophs and mammosomatotrophs) for each group are shown in figure 4.4. As expected, the percentage of GH cells in the *dw/dw* was significantly lower in both males and females at all ages studied. There was also a reduction in the number of GH cells in the male TGR, significant at 45 and 145 days only. No significant differences were seen between female AS and TGR rats at any age. The AS females at 45 and 145 days also had significantly fewer GH positive cells than the males. The adult *dw/dw* females appeared to have fewer GH cells than the males (although this was not significant at any age). The difference increased with age, reaching more than a 50% reduction at 145 days. While the percentages of GH staining cells remained fairly stable throughout development, the total cell number would have increased dramatically over the age range. Unfortunately, the total cell numbers were not recorded in this study.

There was, surprisingly, a significantly higher percentage of total PRL staining cells in the *dw/dw* males and female at all ages tested (Figure 4.5). This increase was most striking at 9 days, where the percentage was 4 fold higher in the *dw/dw* than the AS. This was reduced to a 3 fold increase at 23 days and was less than double at 145 days. Significant sex differences were only seen in AS and TGR rats at 70 days. No significant differences between AS and TGR rats (male or female) were detected at any age.

The double positive (mammosomatotroph) cells are shown in figure 4.6. *Dw/dw* males and females had a significantly higher percentage than the AS at 9 days and lower than the AS at 145 days At other ages, AS and *dw/dw* rats had similar percentages of mammosomatotrophs. At 70 days, AS and *dw/dw* females had significantly lower percentage of double staining cells than their respective males.

By re-plotting the data with each graph comparing the ages of each group, any trend with increasing age can be seen. The percentage of double staining cells (Figure 4.7) tended to increase with age. The trend was slightly less apparent in

*dw/dw* females. It should be noted that this is still expressed as the percentage of total cells. The pituitaries increase in size with age, thus the effect trend would actually be more dramatic if shown as total mammosomatotroph numbers.

These data are expressed as stack graphs in figures 4.8 and 4.9, allowing GH and PRL only, double and total GH and PRL populations to be compared directly. Figure 4.8 shows that from 23 days onwards, the GH and PRL cells make up a similar proportion of the pituitary, the excess in lactotrophs compensating for the proportional lack of somatotrophs.

At 9 days, the increase in lactotrophs (as was shown in figure 4.5) is even more striking when separated into PRL only- and double-staining cells. As was shown in figure 4.6, the proportion of double staining cells in the *dw/dw* was about four times as high as in the AS, however the percentage of PRL only staining cells was a massive 10 fold greater in the *dw/dw* than the AS.

Each graph in figure 4.9 is a visual representation of the changes in proportion of GH and PRL cells occurring over the development of each of the rat models. The progressive increase in total PRL staining cells (top and middle bars) and PRL-only staining cells (top bars only) in each model can be clearly seen. Similarly, the increase in mammosomatotrophs (middle bars) with age is also visible. This is coupled with a slight, gradual decrease in total GH cells (middle and bottom bars) in the *dw/dw* (more so in the female than the male), such that the proportion of GH cells also containing PRL rises from 20% at 9 days to 35% in the male and 75% in the female at 145 days. The total GH staining population in the AS and TGR rats remained at a steady proportion throughout development.

Figure 4.10 shows examples of fluorescence plots of double stained male AS and *dw/dw* pituitary cells at 9, 23 and 70 days. As with figure 4.9, this is a visual representation of the development of the cell proportions in these models. By panning down the page, it is possible to observe the cell populations developing. The upper left quadrant (GH-only cells) clearly shows the deficiency in the *dw/dw* compared to the AS at all ages. Similarly, the lower right quadrant (PRL-only cells) shows the higher proportion of lactotrophs in the *dw/dw* compared with the AS at all ages, in particular, the striking lack of PRL staining

cells in the 9 day AS. Notice that the fluorescence populations fall more easily into the quadrants in the 9 and 23 day animals, rather than the "lobster" shaped plots more typical to pituitary cells from older rats. Thus, all the parameters could be determined from this analysis without the need for single GH and single PRL staining.

FACS analysis, thus convincingly shows the differences that develop with time in these models.

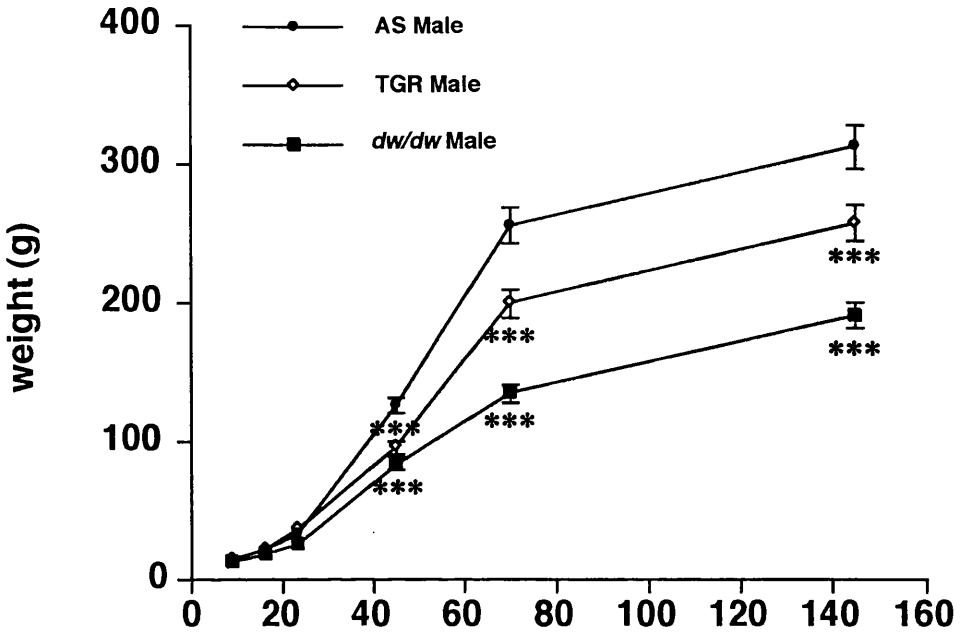
**Figure 4.1: Growth curves of AS, TGR and *dw/dw* rats.**

a. Male AS, TGR and *dw/dw* rats.

b. Female AS, TGR and *dw/dw* rats.

*P<0.05, ***P<0.001 *vs.* AS of the same sex. ANOVA followed by Bonferroni's post-hoc test (n=3-8).

a


AS Male
TGR Male
dw/dw Male
weight (g)
400
300
200
100
0
0
20
40
60
80
100
120
140
160
***
***
***
***
***
***

b

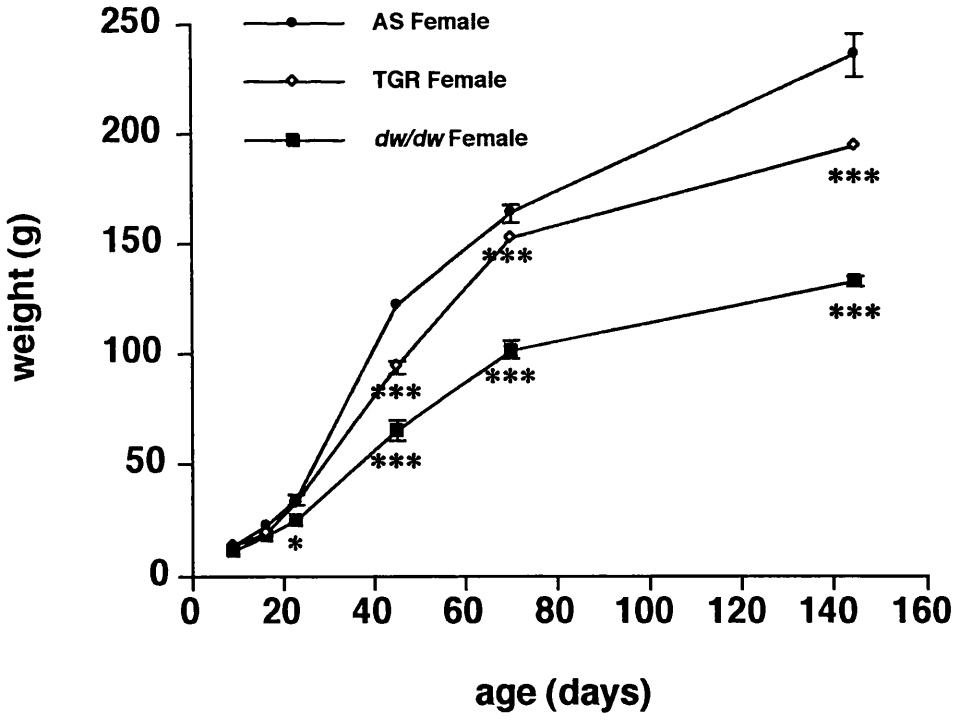

AS Female
TGR Female
dw/dw Female
weight (g)
250
200
150
100
50
0
0
20
40
60
80
100
120
140
160
age (days)
*
***
***
***
***
***

**Figure 4.2: Growth curves of AS, TGR and *dw/dw* rats.**

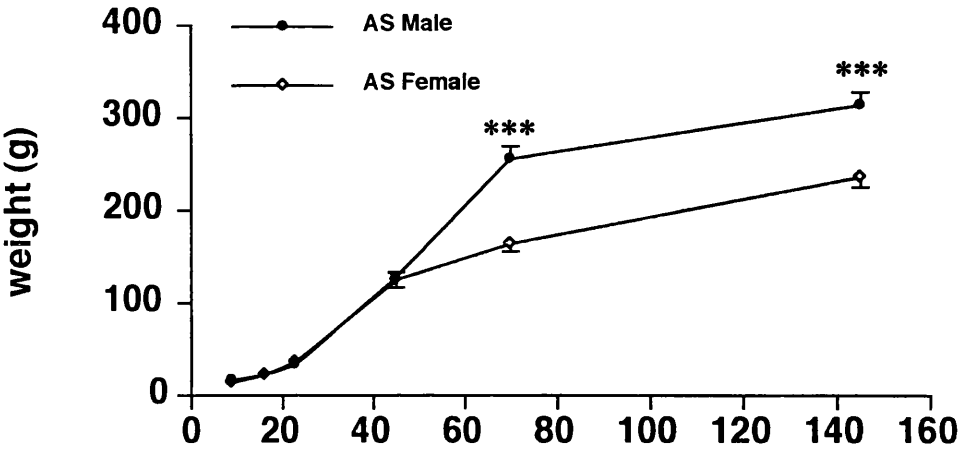
a. AS male *vs.* female,

b. TGR male *vs.* female,

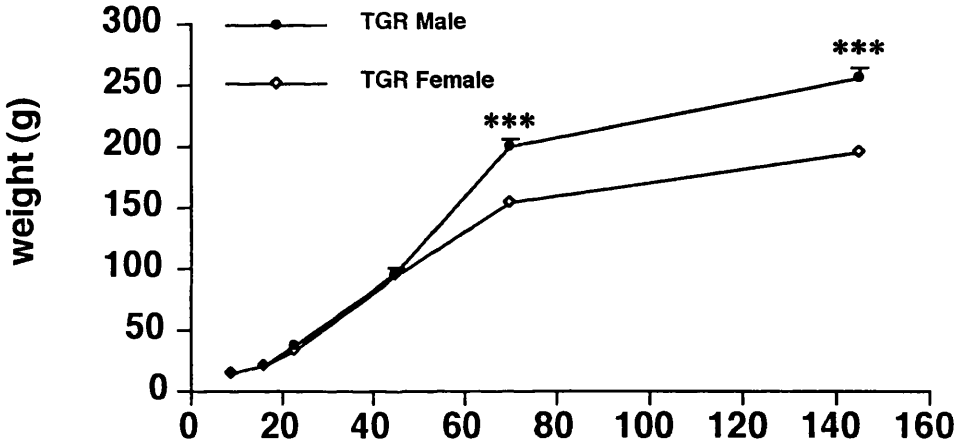
c. *dw/dw* male *vs.* female.

***P<0.001 Male *vs.* female. ANOVA followed by Bonferroni's post-hoc test (n=3-8).

a


AS Male
AS Female
***
***
weight (g)
400
300
200
100
0
0
20
40
60
80
100
120
140
160

b


TGR Male
TGR Female
***
***
weight (g)
300
250
200
150
100
50
0
0
20
40
60
80
100
120
140
160

c

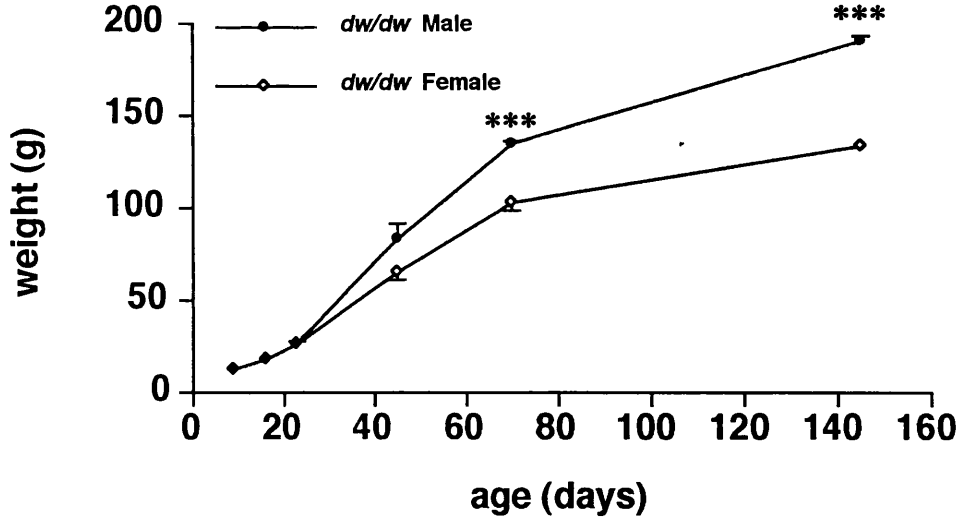
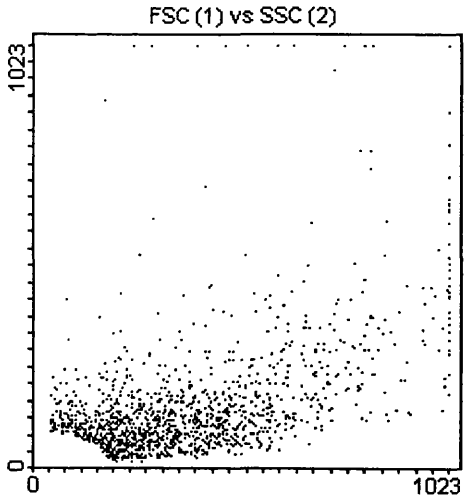

dw/dw Male
dw/dw Female
***
***
weight (g)
200
150
100
50
0
0
20
40
60
80
100
120
140
160
age (days)

**Figure 4.3: Scatter characteristics of pituitary cells from AS male rats of various ages.**

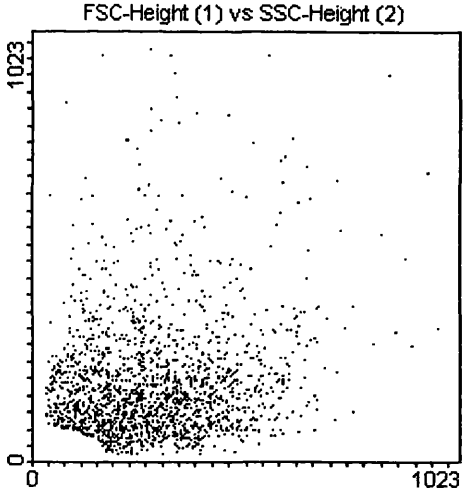
Dispersed pituitary cells from AS male rats were analysed by FACS, Forward scatter (cell size) is shown on the x-axis, while side scatter (granularity) is shown on the y-axis.

a. 9 days,
b. 23 days,
c. 45 days.

## a. 9 Days




## b. 23 Days




## c. 45 Days

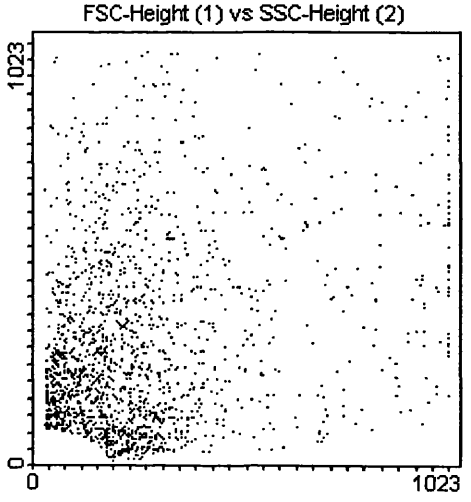


**Figure 4.5: Total PRL staining cells during development of AS, TGR and *dw/dw* rats.**

Dispersed anterior pituitary cells were stained for PRL and analysed by FACS. The graphs compare the PRL staining populations between AS, TGR and *dw/dw* male and female rats at various ages.

***P<0.001 *vs.* AS of the same sex. ++ P<0.01 male *vs.* female. ANOVA followed by Bonferroni's post-hoc test (n=3-8).

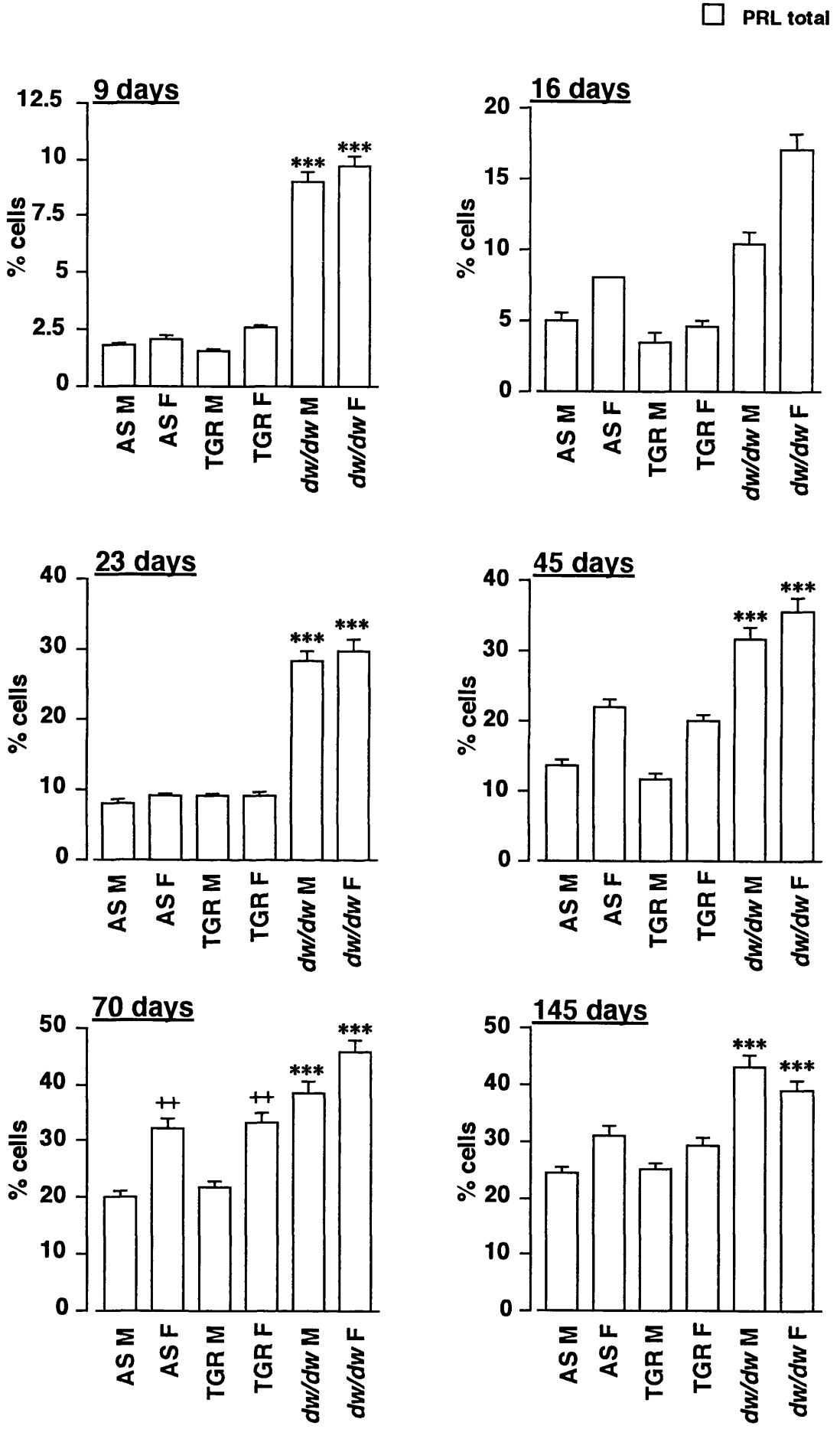

PRL total
9 days
16 days
23 days
45 days
70 days
145 days
% cells
AS M
AS F
TGR M
TGR F
dw/dw M
dw/dw F
***
++

**Figure 4.6: Double staining cells during development of AS, TGR and *dw/dw* rats.**

Dispersed anterior pituitary cells were stained for GH and PRL and analysed by FACS. The graphs compare the double staining populations between AS, TGR and *dw/dw* male and female rats at various ages.

*P<0.05, **P<0.01, ***P<0.001 *vs.* AS of the same sex. $^{+}$ P<0.05, $^{++}$ P<0.01 male *vs.* female. ANOVA followed by Bonferroni's post-hoc test (n=3-8).

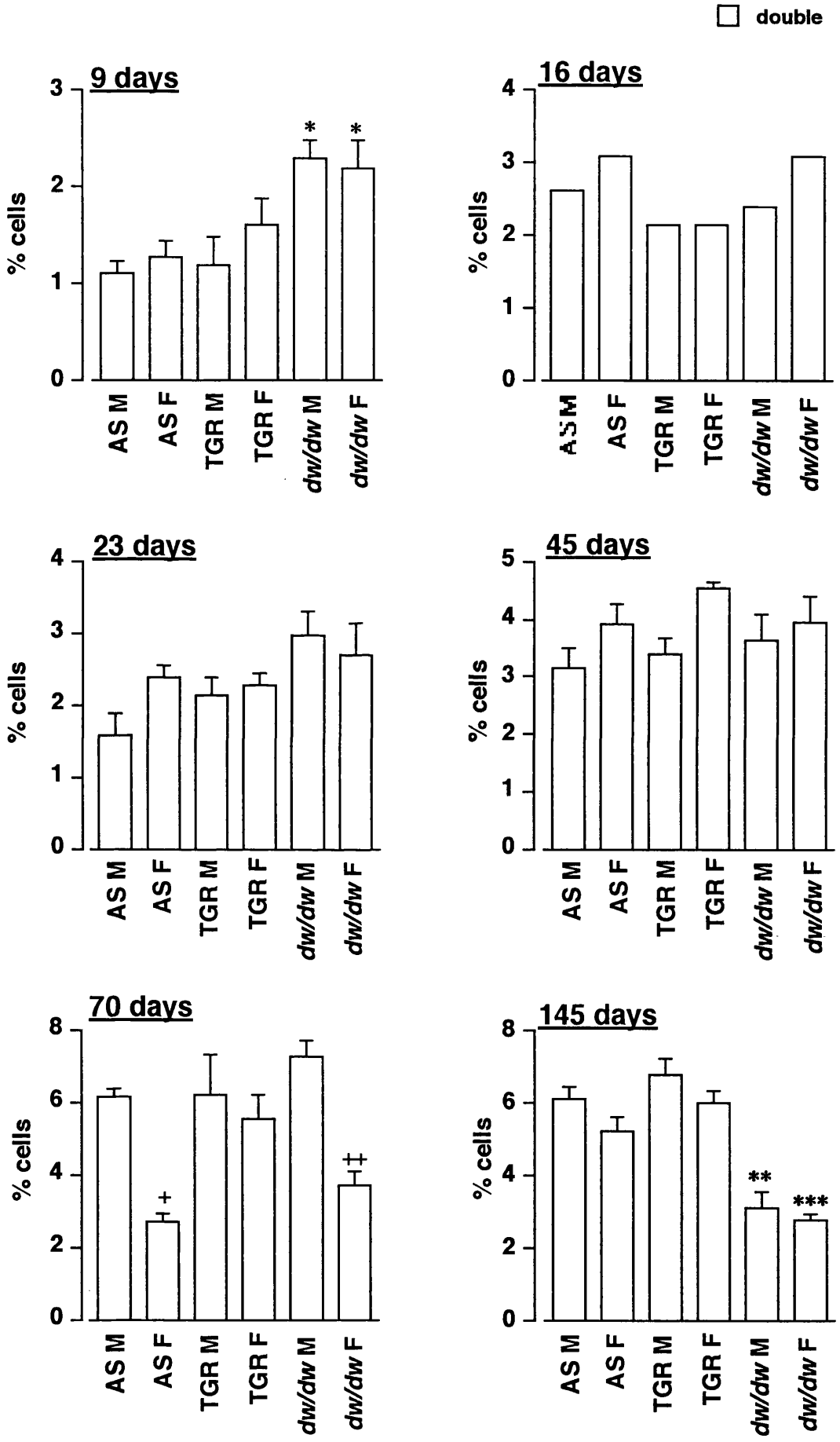

double
9 days
16 days
23 days
45 days
70 days
145 days
% cells
AS M
AS F
TGR M
TGR F
dw/dw M
dw/dw F
*
+
‡
**
***

**Figure 4.7: Double staining cells during development of AS, TGR and *dw/dw* rats.**

Dispersed anterior pituitary cells were stained for GH and PRL and analysed by FACS. The graphs compare the double staining populations during development of each group.

ANOVA followed by post-test for significant trend; all cases except the *dw/dw* females: $P < 0.0001$, *dw/dw* female: $P = 0.056$.

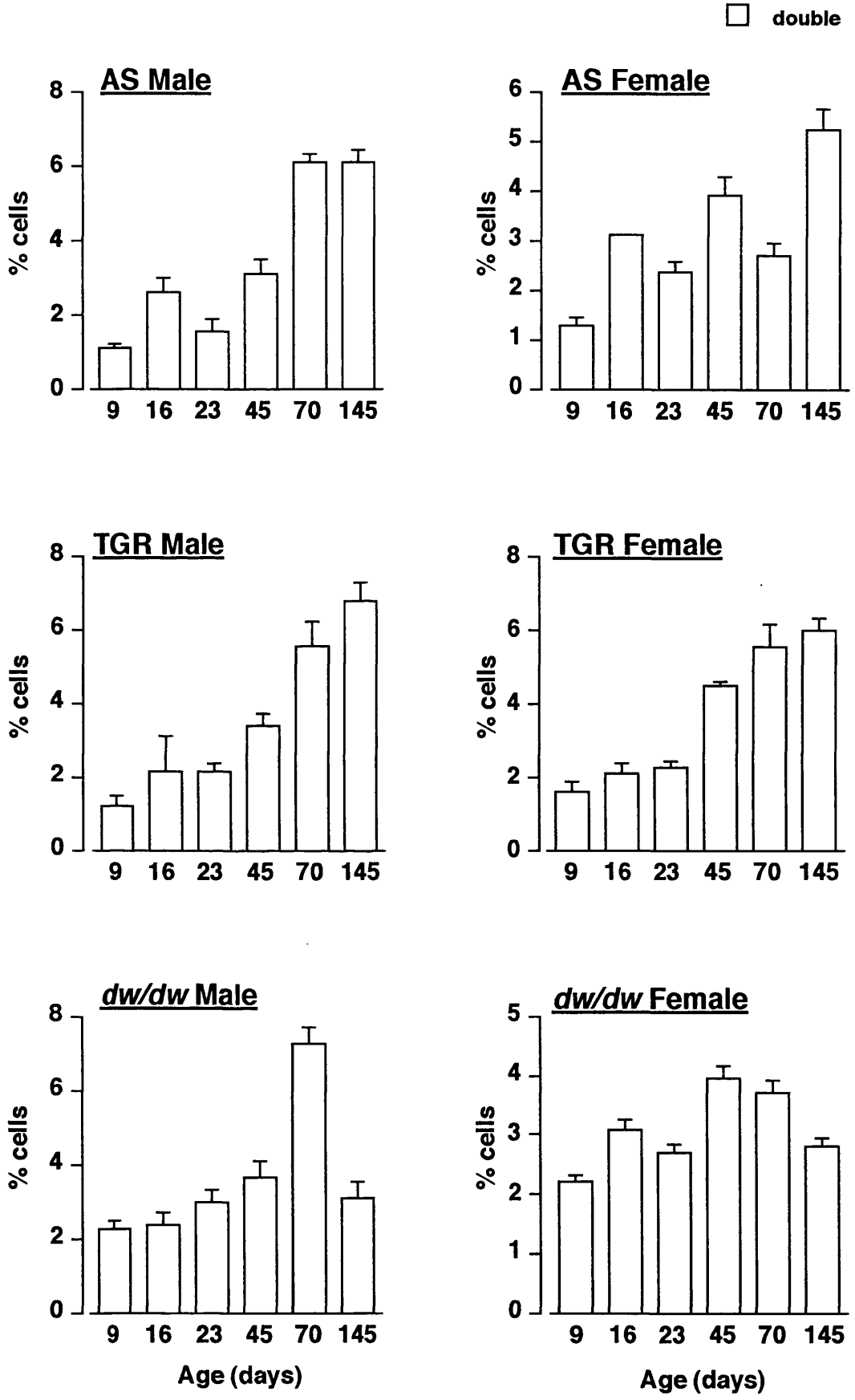

double
AS Male
AS Female
TGR Male
TGR Female
dw/dw Male
dw/dw Female
% cells
Age (days)
9 16 23 45 70 145

**Figure 4.8: GH-, PRL- and double staining cells during development of AS, TGR and *dw/dw* rats.**

The data from figures 4.4, 4.5 and 4.6 are combined as stack graphs, comparing the relative proportions of cell types in AS, TGR and *dw/dw* male and female rats throughout development.

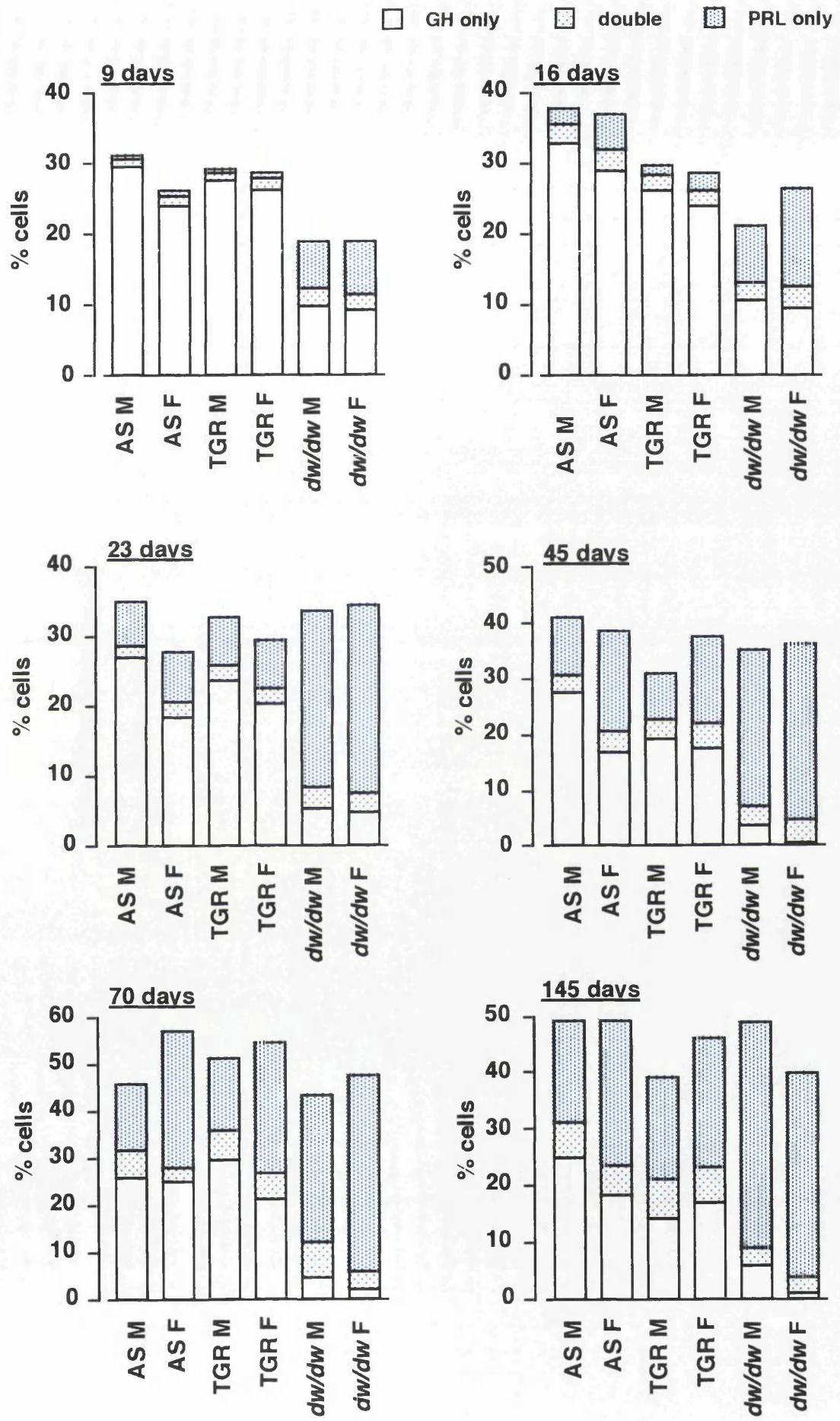

GH only
double
PRL only
9 days
16 days
23 days
45 days
70 days
145 days
% cells
AS M
AS F
TGR M
TGR F
dw/dw M
dw/dw F

**Figure 4.9: GH-, PRL- and double staining cells during development of AS, TGR and *dw/dw* rats.**

The data from figures 4.4, 4.5 and 4.6 are combined as stack graphs, comparing the relative proportions of cell types throughout development in each group.

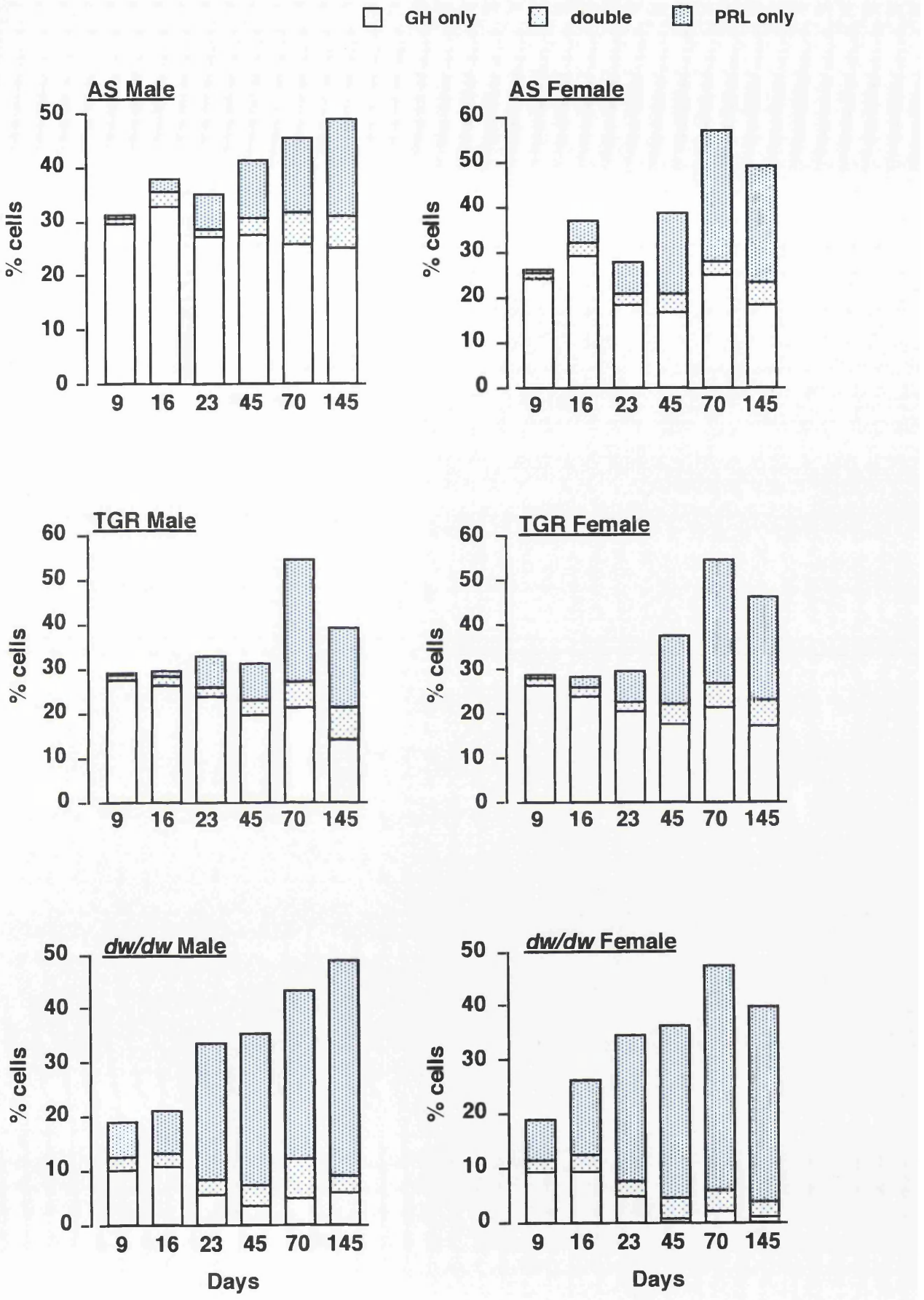

GH only
double
PRL only
AS Male
AS Female
TGR Male
TGR Female
dw/dw Male
dw/dw Female
% cells
Days
9
16
23
45
70
145

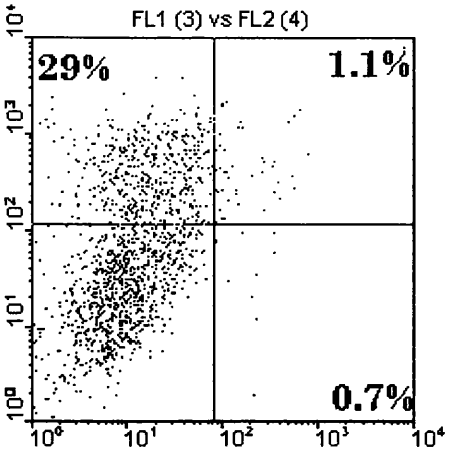
**Figure 4.10: Examples of fluorescence plots of pituitary cells from AS and *dw/dw* males at various ages.**

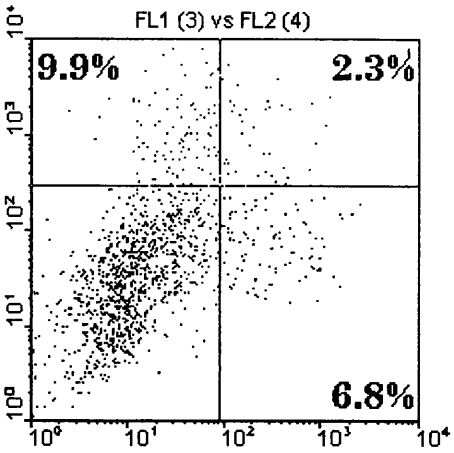
Dispersed pituitary cells were stained for GH and PRL and analysed by FACS. PE fluorescence (GH) is shown on the x-axis, while FITC fluorescence (PRL) is shown on the y-axis.

## AS

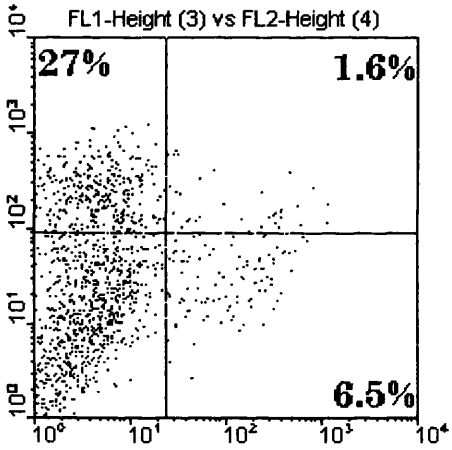
## *dw/dw*

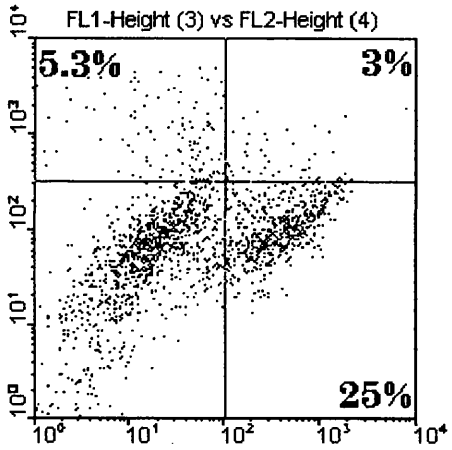
### 9 days



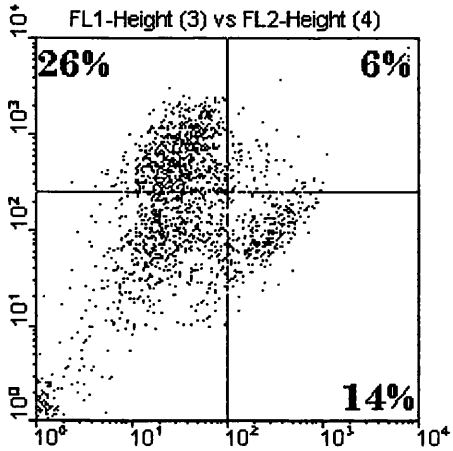



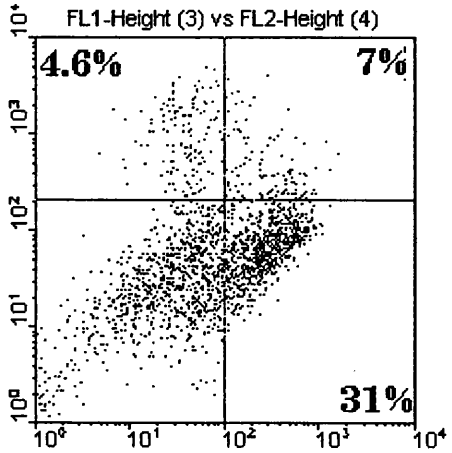

### 23 days




### 70 days



### 4.2.2 Experiment 2: Effects of Oestrodiol Treatment on AS and *dw/dw* Male Rats

Male AS and *dw/dw* rats at 7-8 weeks of age were treated with $E_2$ (25mg/day) for 10 days by placing a slow release pellet (Innovative Research of America, Ohio) subcutaneously, under halothane anaesthesia. The surgery was kindly performed by Danielle Carmignac. Control male and female animals were completely untreated as it has been previously shown that sham operated animals show no alteration in pituitary physiology (Carmignac *et al*, 1993a, 1998). Male rats were weighed at the beginning and end of the 10 days.

After the 10 days, the animals were stunned and decapitated, and the anterior pituitaries processed for FACS analysis. As with the previous experiment, the pituitaries were dispersed in 1.5 ml and then aliquoted into three tubes for single GH, single PRL and double FACS analysis.

Figure 4.11a shows the body weights of the AS and *dw/dw* male rats before and after the 10 day $E_2$ treatment. After the 10 days, both AS and *dw/dw* $E_2$ treated males were significantly lighter than controls. The $E_2$ treated AS and *dw/dw* rats significantly lost weight over the 10 days compared to the controls which had significantly gained weight. The change in body weight of each group is shown in figure 4.11b.

Figure 4.12 shows the total GH- and total PRL- staining cells. As in the previous experiment, the *dw/dw* males and females showed reduced somatotroph and increased lactotroph percentages. The females of each model had a significantly lower percentage of somatotrophs than the males, but no significant sex difference was seen in the lactotroph populations. $E_2$ treatment had no significant effects on the total GH- or total PRL-staining cells in either AS or *dw/dw* male rats.

Figure 4.13a shows that there were no significant differences in double staining cells between sexes, strains or with $E_2$ treatment. Figure 4.13b shows the same data as a stack graph. The percentage of cells staining for GH-only in the *dw/dw* male, although small, was 3 fold that of the female ($4.07 \pm 0.49$ *vs.* $1.37 \pm 0.23$; $P<0.001$). After $E_2$ treatment, the *dw/dw* male had significantly fewer GH-

only staining cells than the control males (1.42 ± 0.31: $P<0.001$), at a similar level as the female. The percentage of GH only-cells in the female AS was also significantly less than the male ($P<0.001$), however $E_2$ treatment had no effect the GH-only staining cell population in this model. Thus $E_2$ appeared to alter the proportions of the cell types in the *dw/dw* male, but not the AS.

Figure 4.14 shows scatter plots, gated on the PRL positive cells only, of AS and *dw/dw* control and $E_2$ treated male rats. Each dot on the scatter plot represents one PRL positive cell. The number of points on the *dw/dw* plots appear higher, reflecting the increased proportion of lactotrophs in this model. In both the AS and *dw/dw*, $E_2$ treatment results in an increased proportion of the lactotrophs falling into the high forward and side scatter area of the plot (towards the upper right), indicating an increase in size and granularity of these cells.

**Figure 4.11: Body weights of AS and *dw/dw* males before and after $E_2$ treatment.**

Male AS and *dw/dw* rats were $E_2$ treated (25μg/day) for 10 days. Controls were completely untreated. Treated and control rats were weighed at the beginning and end of the 10 days. Panel (a) shows the body weights at each time point, while panel (b) shows the change in weight for each group.

*P<0.05, **P<0.01 day 0 *vs.* day 10. Paired students t-test. $^{+}$P<0.05 $E_2$ treated *vs.* control. ANOVA followed by Bonferroni's post-hoc test (n=4).

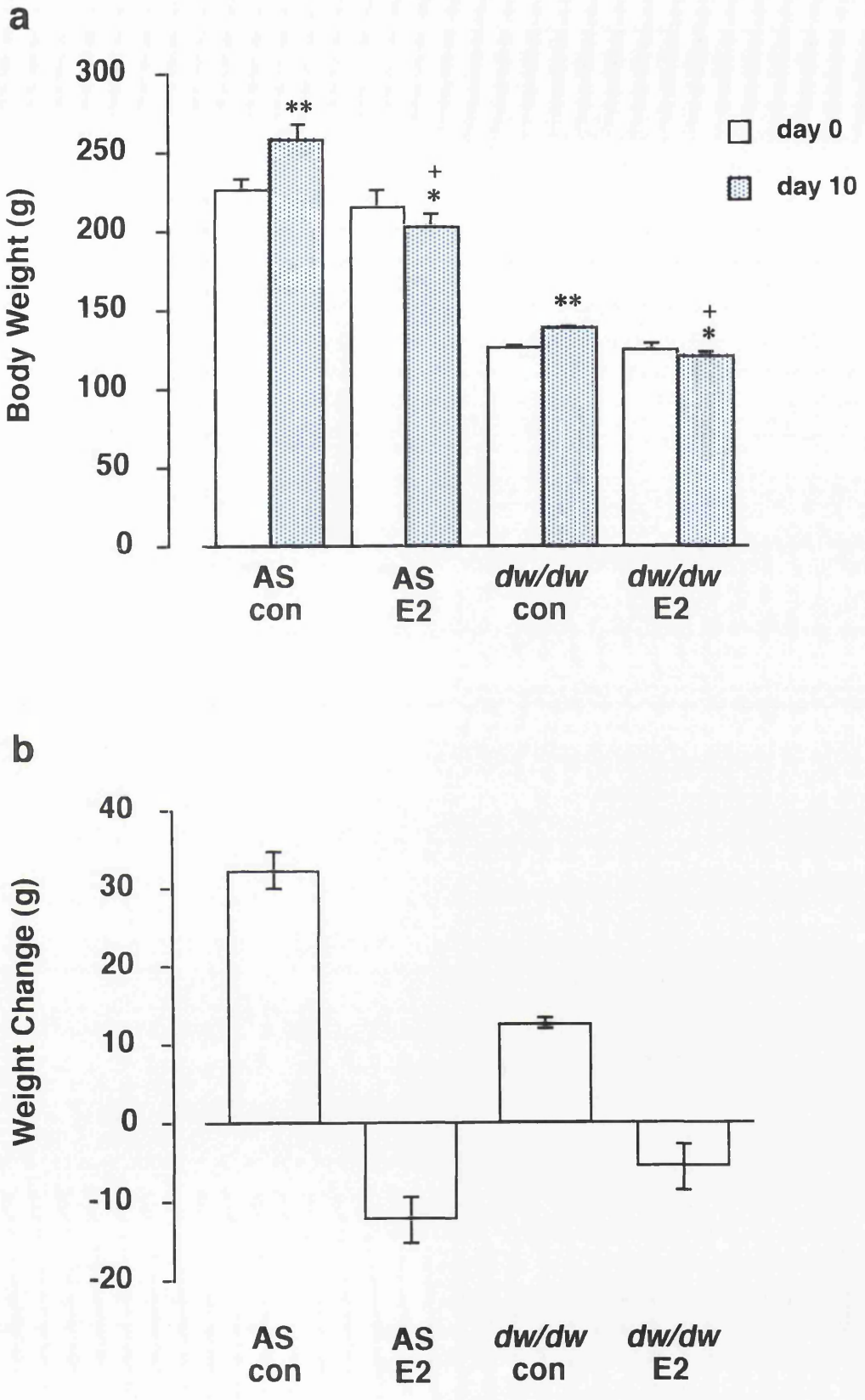
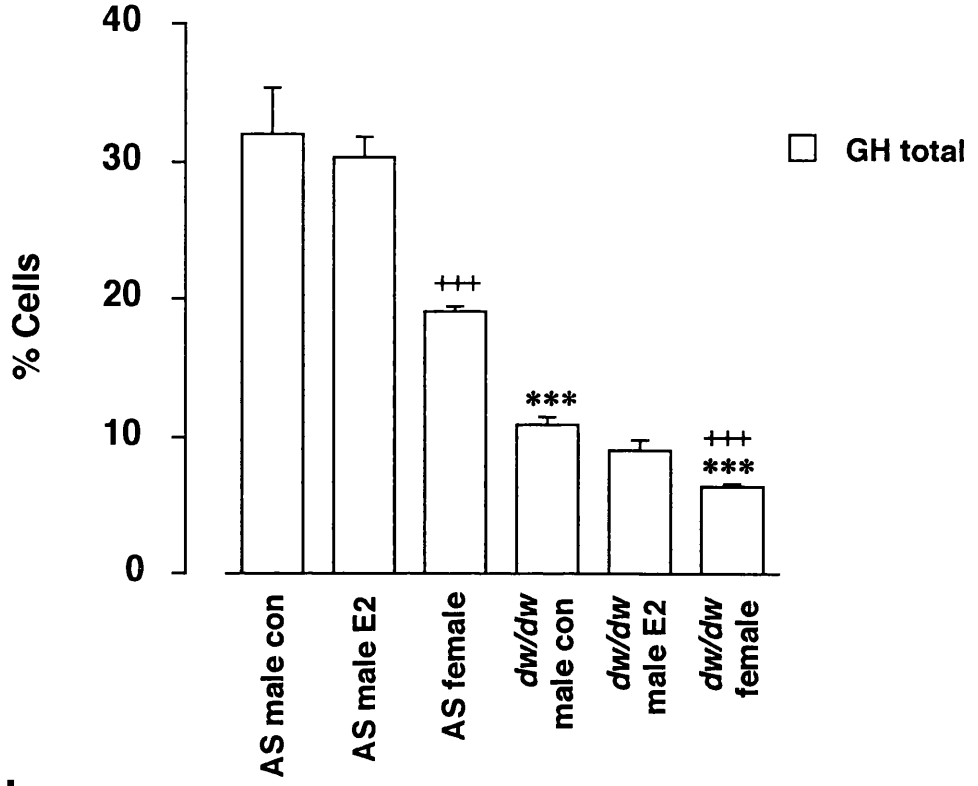

a
Body Weight (g)
300
250
200
150
100
50
0
day 0
day 10
**
+
*
**
+
*
AS con
AS E2
dw/dw con
dw/dw E2
b
Weight Change (g)
40
30
20
10
0
-10
-20
AS con
AS E2
dw/dw con
dw/dw E2

**Figure 4.12: Effect of $E_2$ treatment on total GH- and PRL- staining cells in AS and *dw/dw* male rats.**

Dispersed anterior pituitary cells from $E_2$ treated male AS and *dw/dw* rats, and male and female controls were stained separately for GH and PRL and analysed by FACS. Panel (a) shows the total GH staining cells, while panel (b) shows the total PRL staining cells.

***P<0.001 *dw/dw vs.* AS of the same sex. $^{+++}$P<0.001 male *vs.* female. ANOVA followed by Bonferroni's post-hoc test (n=4).

a


40
30
20
10
0
% Cells
GH total
+++
***
+++
***
AS male con
AS male E2
AS female
dw/dw male con
dw/dw male E2
dw/dw female

b

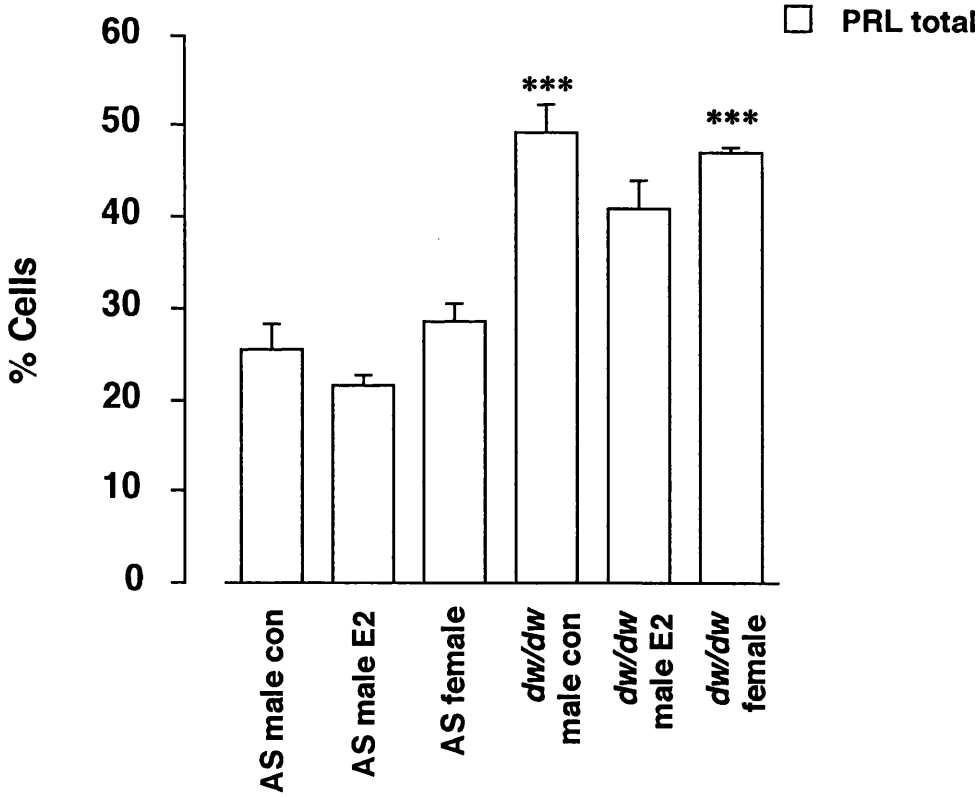

60
50
40
30
20
10
0
% Cells
PRL total
***
***
AS male con
AS male E2
AS female
dw/dw male con
dw/dw male E2
dw/dw female

**Figure 4.13a: Effect of $E_2$ treatment on total double staining cells in AS and *dw/dw* male rats.**

Dispersed anterior pituitary cells from $E_2$ treated male AS and *dw/dw* rats, and male and female controls were stained for GH and PRL and analysed by FACS.

ANOVA followed by Bonferroni's post-hoc test (n=4).

**Figure 4.13b: Effect of $E_2$ treatment on GH-, PRL- and double staining cells in AS and *dw/dw* male rats.**

The data from figure 4.12 and figure 4.13a are re-plotted as a stack graph, showing the GH-, PRL- and double staining populations simultaneously.

***P<0.001 *vs.* male control of the same strain. ANOVA followed by Bonferroni's post-hoc test (n=4).

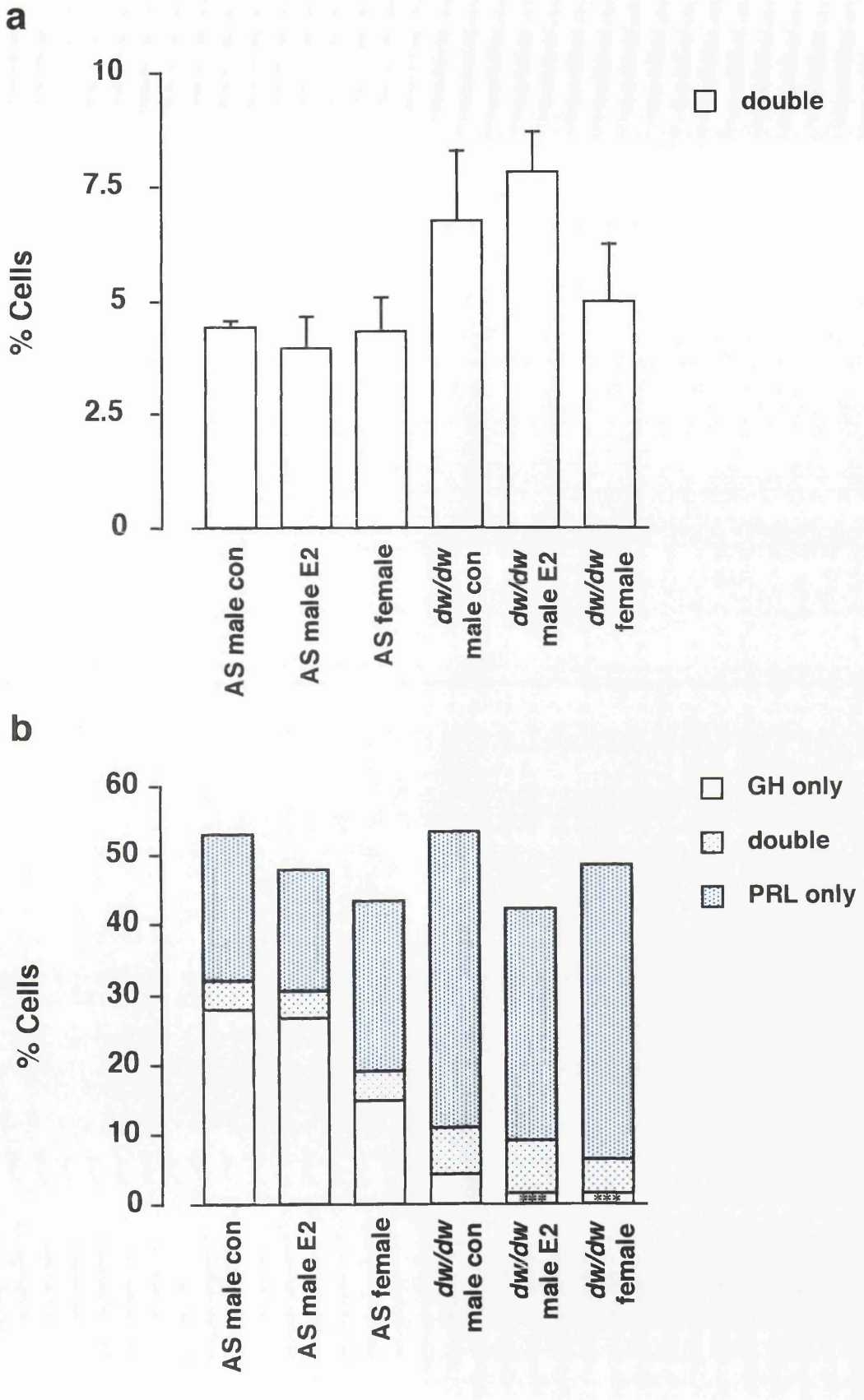
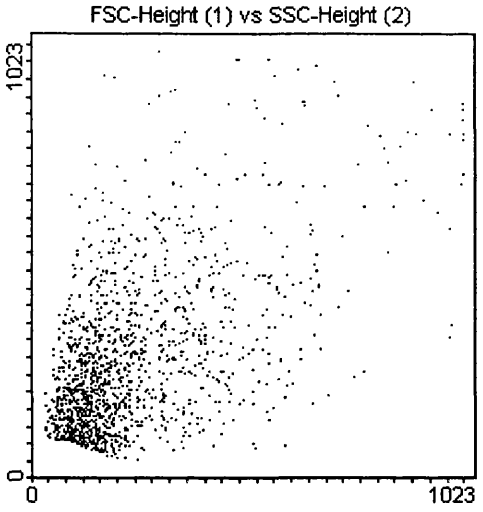

a
10
7.5
5
2.5
0
% Cells
double
AS male con
AS male E2
AS female
dw/dw male con
dw/dw male E2
dw/dw female
b
60
50
40
30
20
10
0
% Cells
GH only
double
PRL only
AS male con
AS male E2
AS female
dw/dw male con
dw/dw male E2
dw/dw female

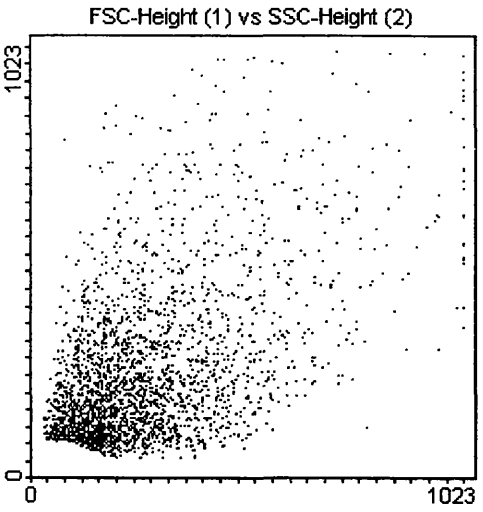
**Figure 4.14: Scatter characteristics of gated PRL staining cells from control and $E_2$ treated male AS and *dw/dw* rats.**

Dispersed anterior pituitary cells from control and $E_2$ treated male rats were stained for PRL and analysed by FACS. The PRL staining cells were electronically gated, and their scatter characteristics plotted (x-axis: forward scatter; y-axis: side scatter).

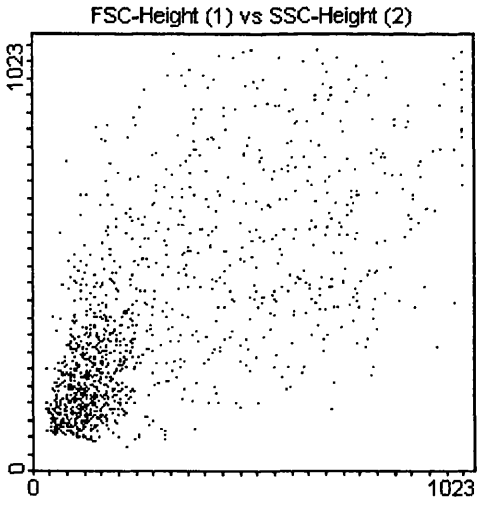
**AS Control**



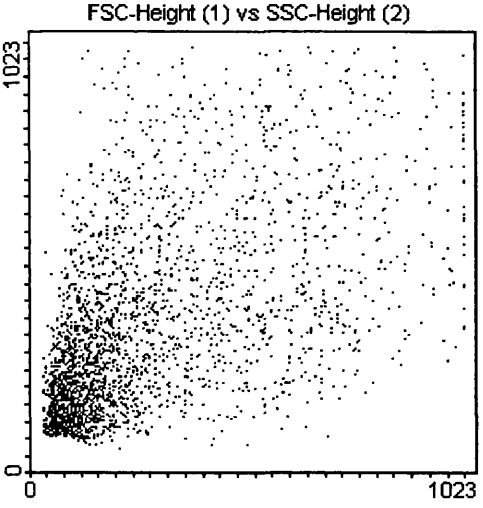

***dw/dw* Control**




**AS $E_2$**




***dw/dw* $E_2$**



## 4.3 DISCUSSION

The most important finding of the first experiment in this chapter is the greatly increased proportion of PRL cells in the *dw/dw* pups. In contrast to the adult, where the increase in percentage is probably compensatory for the reduced pituitary size, the increase in the younger animals is likely to represent an actual increase in lactotroph number. At 10 days, the total cell number in the *dw/dw* pituitary is about 60% of control animals, similar to the adult proportions (Zeitler *et al*, 1994). Assuming this proportion is maintained throughout development, at 9 days when the percentage of lactotrophs is 4 fold higher in the *dw/dw*, the total lactotroph number will be 2.4 fold increased. At 23 days, the 3 fold increase in percentage would translate to a 1.8 fold increase in absolute numbers. By 45 days, the total lactotroph number would be 1.5 fold higher in the *dw/dw* male, but the female would have equal numbers in AS and *dw/dw*. Thus during development, the increase in lactotroph numbers in the *dw/dw* changes from a real increase to a higher percentage due to the reduced pituitary size. It is interesting that the total percentage of GH and PRL staining cells is similar between AS and *dw/dw* pituitaries after 23 days. It would appear that the increased percentage of PRL staining cells is compensating for the GH deficiency, although only partially as the total cell number is reduced in the *dw/dw*.

Hoeffler *et al* (1985) reported that the explosive increase in PRL secretors in rats occurs between 3 and 4 days post-partum thus it would be interesting to compare AS and *dw/dw* rats at this time. The rapid increase in PRL secreting cells is thought to be stimulated by a milk-borne peptide which mimics basic FGF (Porter and Frawley, 1991; Porter *et al*, 1993, 1994). The increased PRL positive population in *dw/dw* rats could be explained by an increased sensitivity to this factor, although this is unlikely, as it appears to act by removing a translational block in cells already expressing PRL mRNA (Frawley and Miller, 1989). The cells destined to produce PRL already contain PRL mRNA at birth (Frawley and Miller, 1989), thus the defect in the *dw/dw* leading to the increased proportion is likely to come into effect before this time. In addition, the somatotroph phenotype in the *dw/dw* rat is also established before birth, as the GH content in the *dw/dw* has been shown to be already reduced by e18 (Zeitler *et al*, 1994).

In addition, the proportion of cells staining for GH remains fairly stable in AS and TGR pituitaries, while only declining slightly with age in the *dw/dw*. As both the AS and *dw/dw* pituitaries expand during development, it therefore follows that the rate of expansion of AS and *dw/dw* somatotrophs is similar, and that the reduction in somatotrophs at each age is mainly due to the initial deficiency present by birth (Carmignac *et al*, 1993b; Zeitler *et al*, 1994).

Previous theories for the eitiology of the *dw/dw* phenotype suggest a defect in the GRF signalling pathway of the somatotroph (Brain *et al*, 1991; Zeitler *et al*, 1994), or more specifically an inability of $G_s\alpha$ to stimulate adenylate cyclase (Downs and Frohman, 1991). The data in this chapter questions this hypothesis as lactotroph differentiation is independent of GRF signalling (Lin *et al*, 1993), thus the phenotype can not be fully explained by such a defect. Furthermore, the phenotype of the *dw/dw* rat is established before birth (Carmingnac *et al*, 1993; Zeitler *et al*, 1994) when GRF signalling is not required for somatotroph ontogeny (Lin *et al*, 1993). I propose that the defective signalling pathway is a consequence of a developmental defect occurring earlier in pituitary development which affects both somatotroph and lactotroph phenotype.

It has been previously shown that the percentage of somatotrophs detectable by immunocytochemistry in the TGR is reduced compared to AS rats (Flavell *et al*, 1996; Thomas *et al*, 1996). The inclusion of the TGR rat in this study was primarily as a control for secondary somatotroph hypoplasia, however the proportion of GH staining cells were found to be significantly reduced only in 45 and 145 day old male rats. The differences in the results from the two methods could be due to differences in the sensitivity of the methods. For instance, there may be a population of somatotrophs in the TGR that express GH$\alpha_t\alpha$ very low level detectable by FACS but not by immunocytochemistry.

Another possible explanation for the discrepancy in the GH data from immunocytochemistry and FACS analysis, is the immunocytochemistry quantifies cells from a single plane at a time. The size of the cell may distort the proportions of each cell type, as larger cells would be visible in more consecutive sections, while a smaller cell would be visible in fewer sections. Thus, a population of large cells would result in more of them being visible on an

individual section. In the case of TGR somatotrophs, the reduced GRF stimulation could conceivably result in smaller cells, as they would contain less GH. This is supported by the fact that the GH content of the pituitary is more severely deficient than the reported somatotroph number (Flavell *et al*, 1996). Thus the somatotroph deficiency may appear to be more deficient by immunocytochemistry, if the somatotrophs were smaller. FACS, however, is not subject to these cell size artefacts.

It should be noted that some of the groups consisted of only 3 or 4 rats. As mentioned earlier, this was partly due to technical difficulties encountered during genotyping TGR hemizygotes. In addition, it was difficult to obtain larger groups of animals of similar ages in colonies of rats whose breeding rate was limited by space in the animal house. It is possible that differences between groups (for instance GH staining cells in AS and TGR rats) were overlooked due to the low number of animals per group and had larger groups been used, the differences may have reached significance.

The ability of FACS analysis to detect mammosomatotrophs is one reason why the method was chosen. The higher proportion of mammosomatotrophs at 9 days is in keeping with the data of Hoeffler *et al* (1985), as early lactotrophs are derived from dual secreting cells. In older animals, the mammosomatotroph population in AS and *dw/dw* rats was not significantly different. Thus, although the total GH positive cells were severely deficient in the *dw/dw*, the mammosomatotroph population was unaffected and the deficiency lies only in the classical somatotrophs (GH-only staining).

I found that the proportion of mammosomatotrophs increased with age in AS, TGR and *dw/dw* rats. A similar study using female Wistar rats of 3-27 months also found an increase in mammosomatotrophs with age by FACS analysis (Shinkai *et al*, 1995). Their data show mammosomatotroph numbers reaching 20% of the pituitary at 27 months, accompanied by a decrease in GH only staining cells. Although the age range is different, the data in this chapter show a similar pattern, with a gradual decrease in GH only staining cells with age in AS, TGR and *dw/dw* rats. The only exception is the *dw/dw* female, which showed no significant trend in mammosomatotroph number. This appears to be

related to the fact that most of the GH positive cells also contain PRL, suggesting that the mammosomatotrophs are derived from somatotrophs. In the older female *dw/dw* rats, there are no more GH-only staining cells to be recruited.

The stage of estrous cycle has been shown to alter the proportion of somatotrophs, lactotrophs and mammosomatotrophs in the bovine pituitary gland (Kineman *et al*, 1991). If this is also true for the rat, this may have an effect on the results of this study as the stage of the cycle for each group of female rats was not determined in my study. Puberty in the rat is at around 40 days, thus only the results from 45 days and over will be affected. The growth curves of the male and female *dw/dw* rats started to diverge at 45 days, whereas the AS and TGR curves were still superimposed at this time, suggesting that *dw/dw* rats enter puberty at a younger age. However, data from our lab has shown that puberty (determined by vaginal opening) is actually delayed in the *dw/dw*, due to the GH deficiency (E.F. Gevers, Personal Communication).

The second experiment in this chapter addressed the effects of $E_2$ treatment *in vivo* for 10 days in AS and *dw/dw* rats. $E_2$ treatment increases the PRL synthesis in existing lactotrophs and also recruits PRL production in somatotrophs (Goth *et al*, 1996; Leiberman *et al*, 1982; Boockfor *et al*, 1986). The increased production of PRL can be seen in AS and *dw/dw* rats by the increase in granularity shown in the scatter plots, in agreement with Hatfield and Hymer, (1985a). The recruitment of somatotrophs to produce PRL was readily evident in the *dw/dw* male, where 2/3 of the GH only positive cells became mammosomatotrophs after $E_2$ treatment, resembling the female controls. This could also be viewed as GH cells making a lot of PRL. Therefore, my work suggest that $E_2$ treatment in male *dw/dw* rats "pushes" them to a PRL predominant phenotype. Considering the hypothesis of Frawley and Boockfor (1991), that all acidophils have the ability to interconvert between somatotroph and lactotroph phenotypes in a dynamic equilibrium, the *dw/dw* rat rests closer to the lactotroph side than the AS rat, and can be pushed even closer by $E_2$ treatment.

In this laboratory, Carmignac *et al* (1998) have recently compared the responses of AS and *dw/dw* rats to GHRP-6. An increase in PRL release was seen in

response to GHRP-6 in *dw/dw* females and $E_2$ treated AS males, but not control males or ovarectomized females. This $E_2$ dependant PRL release was not detected in AS females or $E_2$ treated males, supporting the increased $E_2$ sensitivity of the *dw/dw*. As GHRP-6 stimulated PRL release was coupled with TRH stimulated GH release, it was suggested that mammosomatotrophs were the target cells in the *dw/dw*. This increased $E_2$ dependant plasticity of the cell types in the *dw/dw* compared to the AS provides functional evidence, supporting the cell number data of this chapter that, in addition to somatotrophs, *dw/dw* PRL cells differ from those in the AS.

The body weights of *dw/dw* rats are about 40% less than the AS rats, so 25μg/day of $E_2$ equates to a higher dose per kg body weight in the *dw/dw* than the AS. It is unlikely that this increase in dose would explain the increased response in the *dw/dw* than the AS, as the dose would be maximal in both models (maintaining 160pg/ml plasma in AS rats (Carmignac *et al*, 1993a)). As in previous studies (Goth *et al*, 1996), $E_2$ treatment of AS and *dw/dw* males caused them to lose weight. This weight loss can be attributed, at least in part, to reduced food intake as control rats with their food intake restricted to match the intake of $E_2$ treated males also lose weight (Birge *et al*, 1967).

To summarize, the work in this chapter compared the populations of somatotrophs, lactotrophs, and their precursor and intermediate mammosomatotrophs during development and in response to $E_2$ treatment. The *dw/dw* rats showed a marked increase in their lactotroph population early in development, in contrast to the well documented deficiency in somatotrophs. Although the lactotroph number was probably only increased in rats 23 days or less, the data in chapter 3 showed that the PRL content of the pituitary remains increased into adulthood.

These results question the current theory as to the defect in this model, and taken together with other data, leads me to propose that the observed signalling deficiency in *dw/dw* somatotrophs is a consequence of an earlier developmental defect, not the cause of this model. The equilibrium between the GH / PRL cell populations rests closer to the PRL phenotype. In addition, although both AS and *dw/dw* rats showed increased granularity only *dw/dw* rats show conversion

of somatotrophs to mammosomatotrophs. Thus, not only is the balance between GH and PRL cells during development altered, but the sensitivity of the system to $E_2$ is also greater in the *dw/dw* pituitary.

# ANALYSIS OF CELL TYPES *IN VITRO*

## 5.1 INTRODUCTION

In previous chapters, I have described the phenotypes of the rat models. To investigate differences in the behaviour of the cell types, they were removed from the *in vivo* environment and placed into tissue culture. Tissue culture allows the cells to be isolated from the influences of the hypothalamus and easily manipulated or challenged with various factors to investigate their responses under controlled conditions. *In vivo* studies are complicated by the interactions of various systems in the animal model whereas *in vitro* studies can investigate whether a factor has direct effects on the system, and thus the results can be easier to interpret. Of course, the disadvantage is that the properties of the pituitary cells may change with time in culture, and this must be borne in mind.

This chapter describes results from experiments in which anterior pituitary cultures were made from AS, *dw/dw*, AS/*dw* heterozygote and TGR rats. The *dw/dw* somatotroph has been shown to be defective in its cAMP response to GRF (Downs and Frohman, 1991) and this was further investigated in this chapter. In chapter 3 I confirmed the AS/*dw* heterozygote to have a normal distribution of GH and PRL cell types and pituitary contents, however this model had not yet been studied thoroughly in tissue culture. In this chapter pituitaries from AS/*dw* heterozygotes were also studied in culture and their GRF responses investigated to see if they were normal or hyporesponsive *in vitro*.

The *dw/dw* somatotroph number is thought to be reduced due to the inability to proliferate in response to GRF. In contrast, the TGR rat possesses an unexpanded somatotroph population without any underlying somatotroph defect. Instead, the ultrashort loop feedback of the hGH transgene on the GRF neurones results in a reduced stimulatory influence on the pituitary somatotrophs. Thus, the TGR may be a useful comparison to the *dw/dw*. In addition, because the TGR phenotype is of hypothalamic origin, primary culture of pituitary cells would show the effects of removing those influences.

## 5.2 EXPERIMENTAL RESULTS

### 5.2.1 Treatments

5.2.1.1 Growth Hormone Releasing Factor

0.15mM hGRF $N^{leu}27(1\text{-}29)NH_2$ in 0.1M acetic acid was stored in 20µl aliquots at -20°C. 1µM stock was made by diluting an aliquot in 3ml D-MEM + 1% BSA. This working stock was filter sterilized and stored at -20°C in 200µl aliquots for up to 3 months.

5.2.1.2 Somatostatin

Somatostatin was dissolved in $dH_2O$ prior to use, filter sterilized and diluted to 50nM in D-MEM + 0.1% BSA.

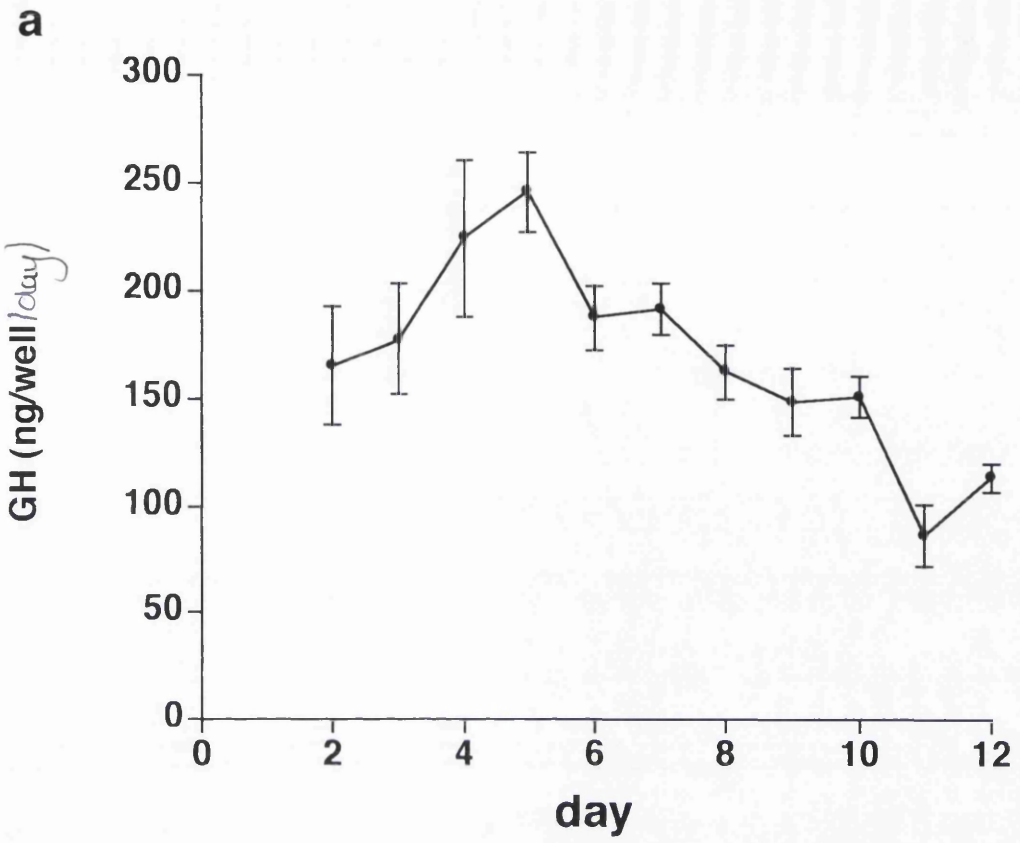
### 5.2.2 Experiment 1: Effects of Duration of Culture on Basal GH and PRL Release

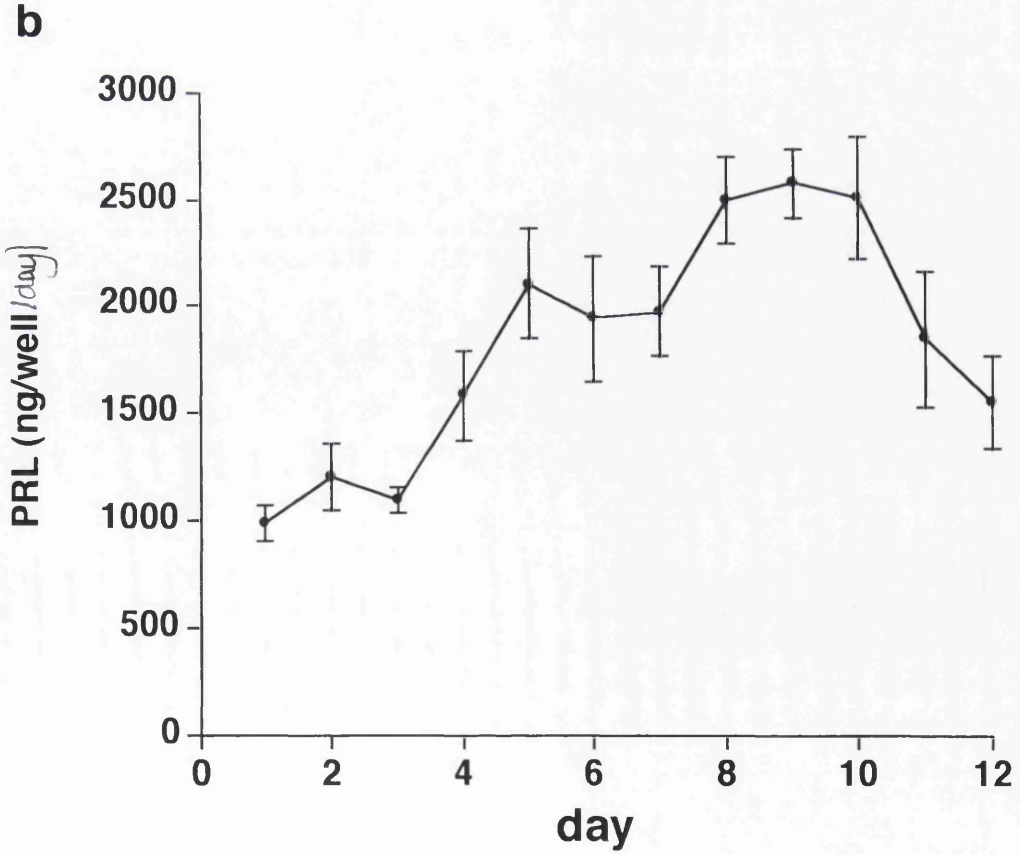
The aim of this experiment was to determine the effects of duration of culture on GH and PRL release. Anterior pituitary cells from male AS rats were cultured in 24 well plates, at the equivalent of ¼ pituitary per well. The medium was changed daily and stored at -20°C for GH and PRL RIA.

Figure 5.1 illustrates the release of GH and PRL from cultured AS anterior pituitary cells. As is well known, the lactotrophs removed from their normal environment of inhibitory dopamine show an increase in PRL release with time, continuing for 8-10 days of culture in this case. After this time, the PRL release started to decline The release of GH also increased and declined, however the peak in GH release occurred at day 5 of culture, several days earlier than the peak in PRL release, implying that somatotrophs may not survive so well in culture as lactotrophs. Even when GH release was maximal, it should be noted that PRL release was an order of magnitude higher than this. This is in contrast to the pituitary contents, where the PRL stores were about 2 orders of magnitude lower than GH (see chapter 3). Due to the peak in GH release being on day 5, this was chosen as a standard duration of culture for treatment and analysis.

**Figure 5.1: Effects of duration of culture on GH and PRL release in AS anterior pituitary primary culture.**

AS anterior pituitary cells were cultured at ¼ pituitary per well in 24 well plates. The medium was replaced at 12pm (±10 minutes) each day, and stored at -20°C until analysis. The graphs show GH (a) and PRL (b) measured by RIA and expressed as hormone released per day in ng/well (n=4).


a
GH (ng/well/day)
300
250
200
150
100
50
0
0
2
4
6
8
10
12
day


b
PRL (ng/well/day)
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
day

### 5.2.3 Experiment 2: Immunocytochemistry of Primary Cultures

The aim of this experiment was to quantify the proportion of GH and PRL staining cells after 5 days of culture. Dispersed anterior pituitary cells were plated at ¼ pituitary per well in 24 well plates on poly-L-lysine coated coverslips. After 5 days of culture, the cells were washed twice in PBS and fixed for 30mins in 4% paraformaldehyde in PBS for GH and PRL immunocytochemistry. Results from two separate experiments are illustrated;

1. AS, AS/*dw* and *dw/dw* males.
2. AS, TGR and *dw/dw* males.

Figure 5.2 shows the cell counts for GH and PRL in the cultures. In panel (a), the *dw/dw* culture had markedly fewer GH staining cells than the AS control and that the GH cell number in the heterozygote culture was not significantly different from the AS, again suggesting that there is not an intermediate phenotype with respect to GH and PRL cell number. There were no significant differences between the PRL cell populations, expressed as a percentage of total cells.

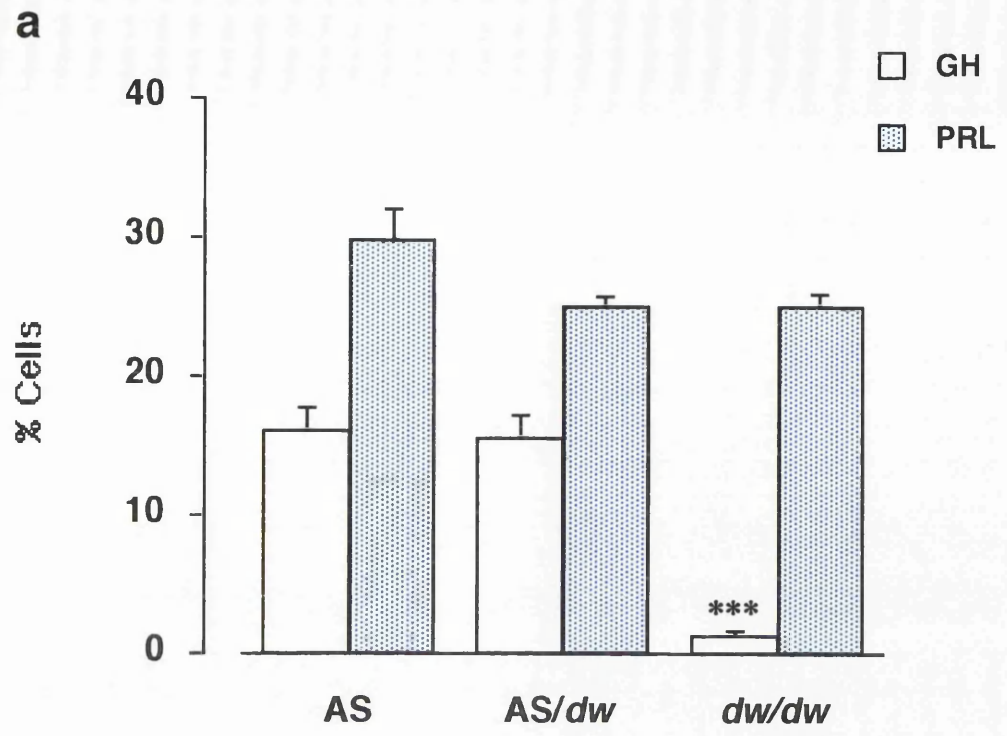
Panel (b) shows the results of the second experiment. Both the TGR and *dw/dw* cultures had significantly fewer GH cells than the AS culture, the deficiency in the *dw/dw* being more severe. Again, there were no significant differences between the PRL cell populations.

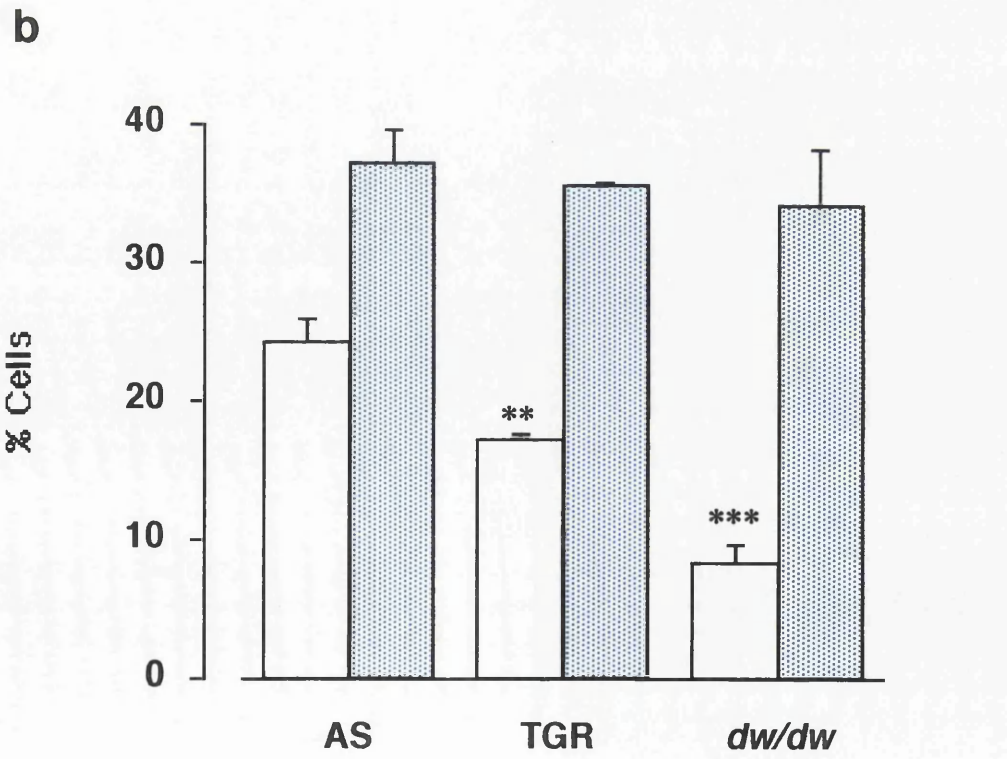
**Figure 5.2: GH and PRL cell numbers in cultured pituitary cells.**

Anterior pituitary cells were cultured for 5 days at ¼ pituitary per well on poly-L-lysine coated coverslips. The cells were washed in PBS, fixed and stained for GH or PRL by immunocytochemistry. The number of cells stained for GH or PRL as a percentage of total in the culture was estimated by counting $2mm^2$ on each of 4 coverslips. The results from 2 experiments are shown.

a. AS, AS/ *dw* and *dw/dw* males.
b. AS, TGR and *dw/dw* males.

**P<0.01, ***P<0.001 *vs.* AS. ANOVA followed by Bonferroni's post-test (n=4).


a
GH
PRL
40
30
20
10
0
% Cells
***
AS
AS/dw
dw/dw


b
40
30
20
10
0
% Cells
**
***
AS
TGR
dw/dw

### 5.2.4 Experiment 3: Time Course of GRF Responses in Primary Culture

The aim of this experiment was to determine the time course of the response to GRF. Anterior pituitary cells from male AS rats were cultured in 24 well plates, maintained for 5 days and then treated with 10nM GRF for varying times.

For GH release studies, 10nM GRF treated and control wells were incubated for 20, 60 and 180 minutes. At the end of the treatment, the medium was removed and assayed for GH. For cAMP accumulation studies, the cells were treated with 10nM GRF for 5, 15, 30, 60 and 180 minutes. Separate control wells for each time were not needed as a zero time point served as a control. At the end of the treatment the cells were extracted in acid alcohol and assayed for cAMP.

Figure 5.3a shows the basal and GRF stimulated release of GH at different time points. Although a significant difference can be seen at 60 minutes, 180 minutes showed a greater fold increase over basal release. This was subsequently chosen as a standard treatment for release studies.

Figure 5.3b shows that the time course of cAMP accumulation was different to that of GH release. The level of cAMP was significantly higher than the control after just 5 minutes, reached a maximum level by 15 minutes and remained elevated for the duration of the 3 hour experiment. All subsequent cAMP investigations were incubated for 30 minutes.

**Figure 5.3: Time course for GRF stimulated GH release and cAMP accumulation.**

AS anterior pituitary cells were cultured at ¼ pituitary per well for 5 days.

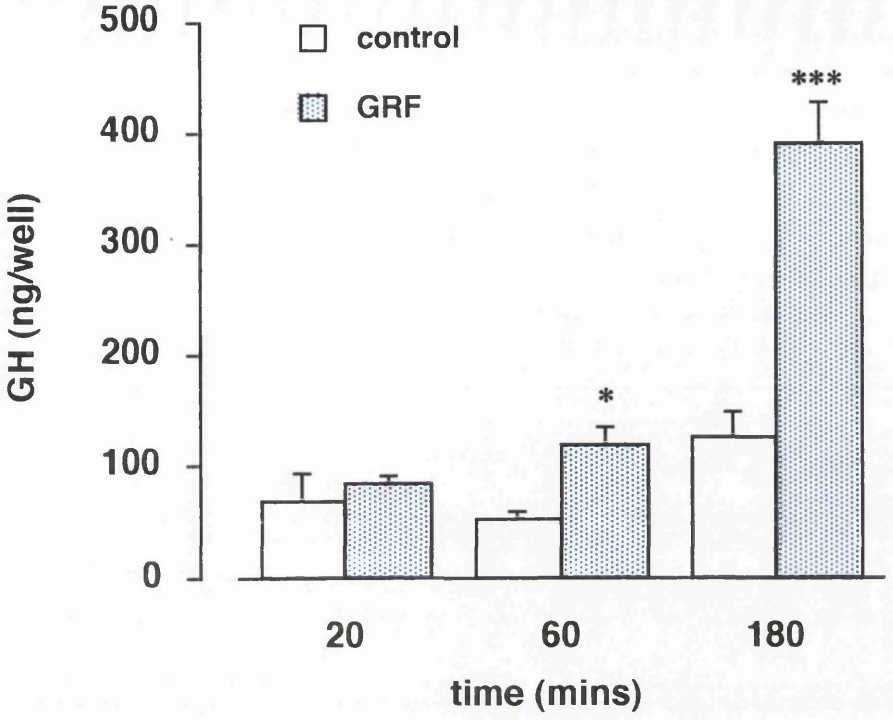
For the GH release time course (a), cells were incubated with or without 10nM GRF for 20, 60 or 180 minutes. The medium was then removed and assayed for GH. Although a significant release was apparent at 60 minutes, a larger fold release over basal occurred at 180 minutes.

*P<0.05, ***P<0.001 Control *vs.* GRF at each time point. ANOVA followed by Bonferroni's post-test (n=4).

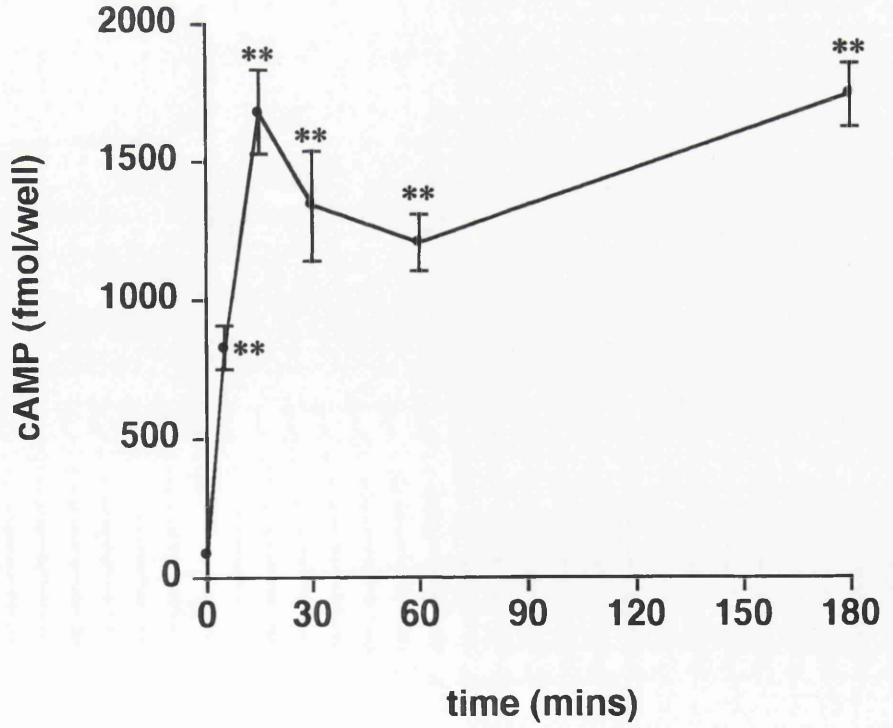
For the cAMP time course (b), cells were incubated with GRF for 0,5,15,30, 60 and 180 minutes. The medium was removed and the cells extracted in acid alcohol and assayed for cAMP. The level of cAMP was significantly higher than basal at 5 minutes, reached a maximum at 15 minutes and remained elevated for the duration of the 180 minute experiment.

**P<0.01 *vs.* control. ANOVA followed by Dunnet's multiple comparison (n=3).

a


500
400
300
200
100
0
GH (ng/well)
control
GRF
*
***
20
60
180
time (mins)

b


2000
1500
1000
500
0
cAMP (fmol/well)
**
**
**
**
**
0
30
60
90
120
150
180
time (mins)

### 5.2.5 Experiment 4: GRF and SS Treatment of Cultures: Secretory Response

The aim of this experiment was to investigate the secretory responses of cultures derived from AS, TGR and *dw/dw* rats to 3 hour treatments with GRF or SS.

Cultures were prepared at ¼ pituitary per well in 24 well plates. After 5 days, the cells were treated for 3 hours with GRF or SS. The medium was then removed and assayed for GH and PRL. In some experiments, the cells were washed twice and extracted in PBS + CPI for GH- and PRL- RIA.

The dose response curves for GRF shown in figure 5.4a show that male AS, TGR and *dw/dw* cultures all released GH in a dose dependant manner, although the amplitude and sensitivity differed between the models. The *dw/dw* cells, although less responsive and sensitive, still responded to GRF indicating that the defect in this model does not completely abolish the secretory response to GRF. The $ED_{50}$ were calculated as 0.01-0.03nM for both AS and TGR male cultures, and 0.3-1nM for the *dw/dw*. 10nM GRF was sufficient to evoke a near maximal GH release and was therefore subsequently used as a standard treatment. The data is also shown in Figure 5.4b as fold release over basal. The relative release from the *dw/dw* culture at maximum stimulation is not as severely deficient when expressed this way. At 100nM GRF (maximal GH release in both AS and *dw/dw* cultures in this experiment), the AS showed a 4.9 fold increase in GH release over basal compared to a 3.5 fold increase in the *dw/dw*. The TGR responded to 100nM GRF with a 4.4 fold increase over basal, similar to the AS.

PRL was also assayed in the same medium and the results plotted in figure 5.5a. The basal PRL release of the TGR culture was greater than the AS or *dw/dw*. This was surprising considering that TGR rats have a reduced PRL pituitary content *in vivo*. It appears that the TGR cells in culture underwent a rebound response, such that the PRL deficiency became an excess. In this experiment, all three cultures showed no PRL response to GRF. At the 1000nM GRF, both the AS and the TGR showed slightly reduced GH and PRL release compared to lower doses, suggesting that at this dose it may have toxic or non-specific effects.
In contrast, the PRL release in a similar experiment performed at a different time is shown in figure 5.5b. All three cultures showed variation between doses

(AS, *dw/dw*: P<0.01, TGR: P<0.05). Both AS (P<0.001) and *dw/dw* (P<0.0001) showed a significant linear trend, indicating a dose dependant release of PRL. The magnitude of the release was different between the AS and *dw/dw*, the latter reaching 9 fold over basal and the AS only a doubling of basal. The reason for the PRL release in response to GRF in this experiment is not clear. The GH release in this second experiment, although more variable, showed similar results to those shown in figure 5.4.

The GRF dose response experiment was also carried out on cultures derived from female rats and the results presented in figure 5.6. The three female cultures also released GH in a dose dependant manner (tested for linear trend: all P<0.0001). Like the male cultures, the AS and TGR showed similar responses to GRF, while the *dw/dw* culture exhibited lower basal and stimulated GH release.

The basal PRL release from AS, TGR and *dw/dw* female cultures was 8 (AS), 3.5 (TGR) and 17 (*dw/dw*) fold higher than from the male cultures prepared in parallel (figure 5.5b). While the AS and *dw/dw* PRL release were similar in the male cultures and the TGR about 3 fold higher, the female cultures showed a different pattern. The female *dw/dw* culture released about twice as much PRL as the AS culture, while the TGR released an intermediate amount. None of the female cultures showed significant variation or linear trend in their PRL release, with increasing GRF.

In some experiments, the cells were extracted for GH- and PRL- RIA. Four wells of cells (either control or 10nM GRF treated) were pooled in one extract (4 wells was equivalent to one pituitary as the cultures were seeded at ¼ pituitary per well). The GH and PRL were measured by RIA and expressed as percentage soluble protein. Because the wells were pooled, each experiment yielded only one control and one GRF treated cell extract. Figure 5.7 shows the result from several experiments combined (AS: n=10, TGR: n=2, *dw/dw*: n=5). The AS cultures showed a significant reduction in GH content after GRF treatment (Paired student's t-test). The *dw/dw* cultures contained significantly less GH than the AS culture but no significant reduction in GH was seen after GRF treatment. The GH content of the TGR culture was not significantly different to AS and the reduction after GRF treatment was not significantly different,

although this may have been because the TGR data only represented duplicate experiments.

The PRL contents showed no difference between control and GRF treated extracts. The mean PRL content in the *dw/dw* culture extracts appeared several fold higher than in the AS, although due to the larger variance in the *dw/dw* data this failed to reach significance. The PRL content of the TGR, although double than the AS, also was not significantly different.

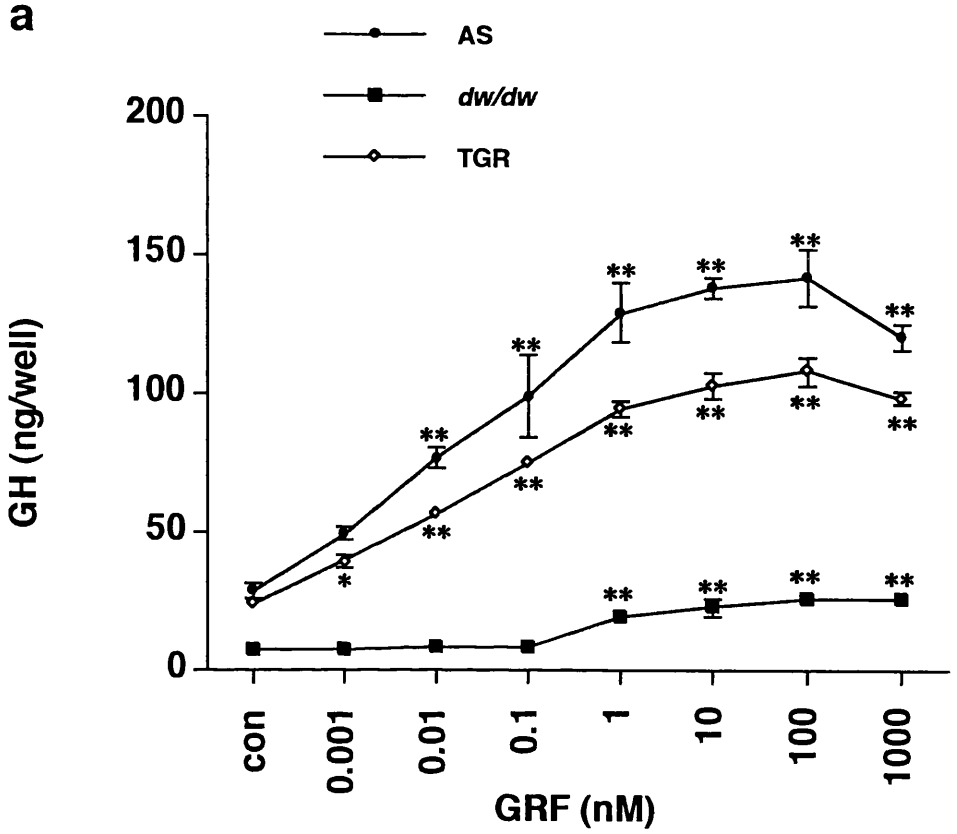
The responses to SS were also compared between AS and *dw/dw* male cultures. Figure 5.8 shows the GH and PRL release from control and 50nM SS treated cultures over 3 hours. GH release (note the different scales for AS and *dw/dw* GH release) is inhibited in both AS and *dw/dw* cultures. Both AS and *dw/dw* cultures also showed significant inhibition of PRL release in response to SS. However, the pattern of inhibition differed between AS and *dw/dw* cultures. AS cultures showed 70% inhibition of GH and 25% inhibition of PRL, whereas *dw/dw* cultures showed about 50% inhibition of both GH and PRL in response to SS.

**Figure 5.4: GRF dose response in primary cultures of male pituitary cells: GH release.**

Anterior pituitary cells from male AS, *dw/dw* and TGR rats were cultured at ¼ pituitary per well in 24 well plates for 5 days. The cells were incubated for 3 hours with 0.001 - 1000nM GRF and the medium was then assayed for GH. The results are displayed as ng/well (a) and fold over basal secretion (b).

*P<0.05, **P<0.01 *vs.* control. ANOVA followed by Dunnet's multiple comparison (n=3).

a


AS
dw/dw
TGR
GH (ng/well)
200
150
100
50
0
con
0.001
0.01
0.1
1
10
100
1000
GRF (nM)

b

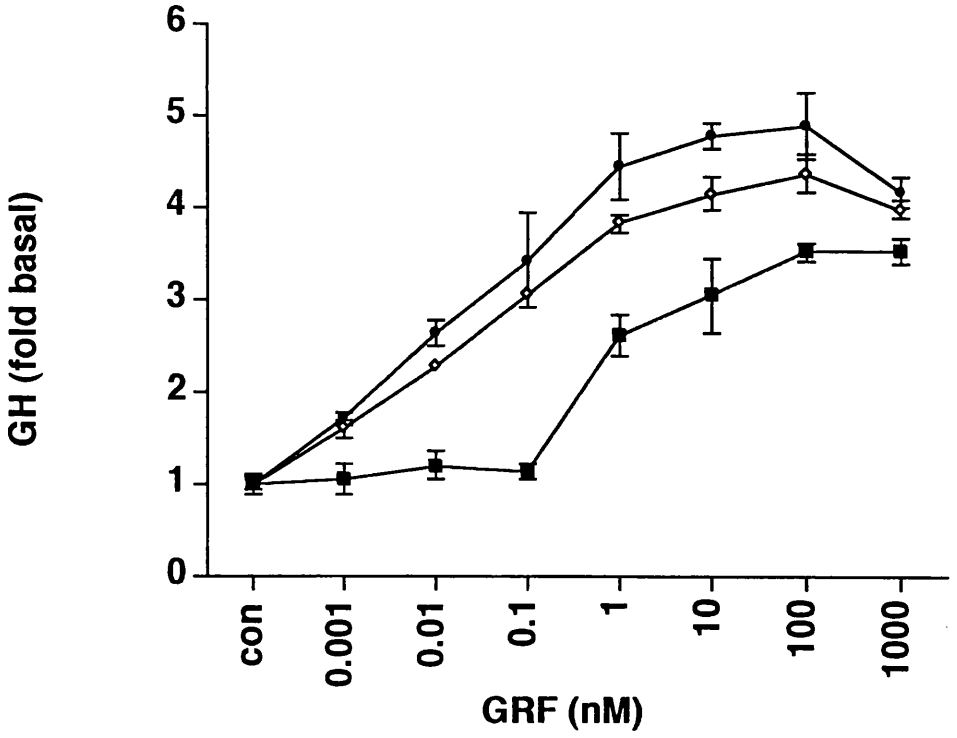
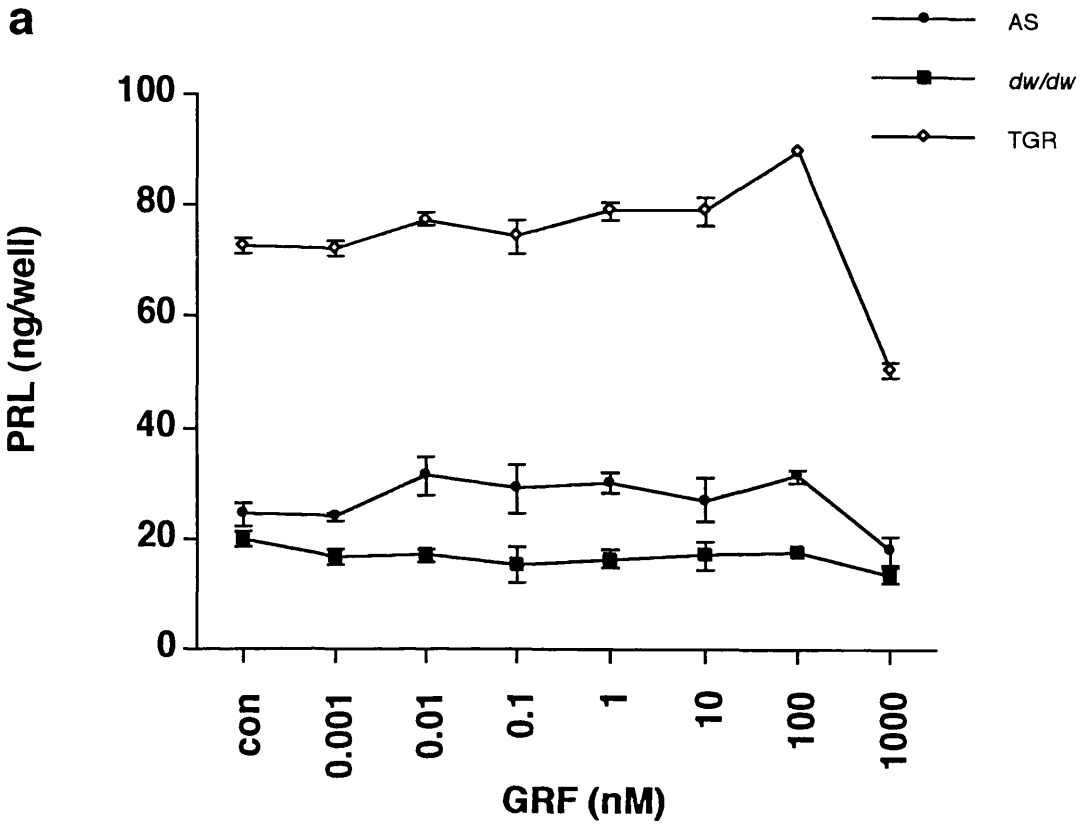

GH (fold basal)
6
5
4
3
2
1
0
con
0.001
0.01
0.1
1
10
100
1000
GRF (nM)

**Figure 5.5: GRF dose response in primary cultures of male pituitary cells: PRL release.**

Anterior pituitary cells from male AS, *dw/dw* and TGR rats were cultured at ¼ pituitary per well in 24 well plates for 5 days. The cells were incubated for 3 hours with 0.001 - 1000nM GRF and the medium was then assayed for PRL. The facing graphs show the PRL release from the same experiment illustrated in figure 5.4 (a) and a similar experiment performed at a different time (b).

*P<0.05, **P<0.01 *vs.* control. ANOVA followed by Dunnet's multiple comparison (n=3).


a
AS
dw/dw
TGR
PRL (ng/well)
100
80
60
40
20
0
con
0.001
0.01
0.1
1
10
100
1000
GRF (nM)

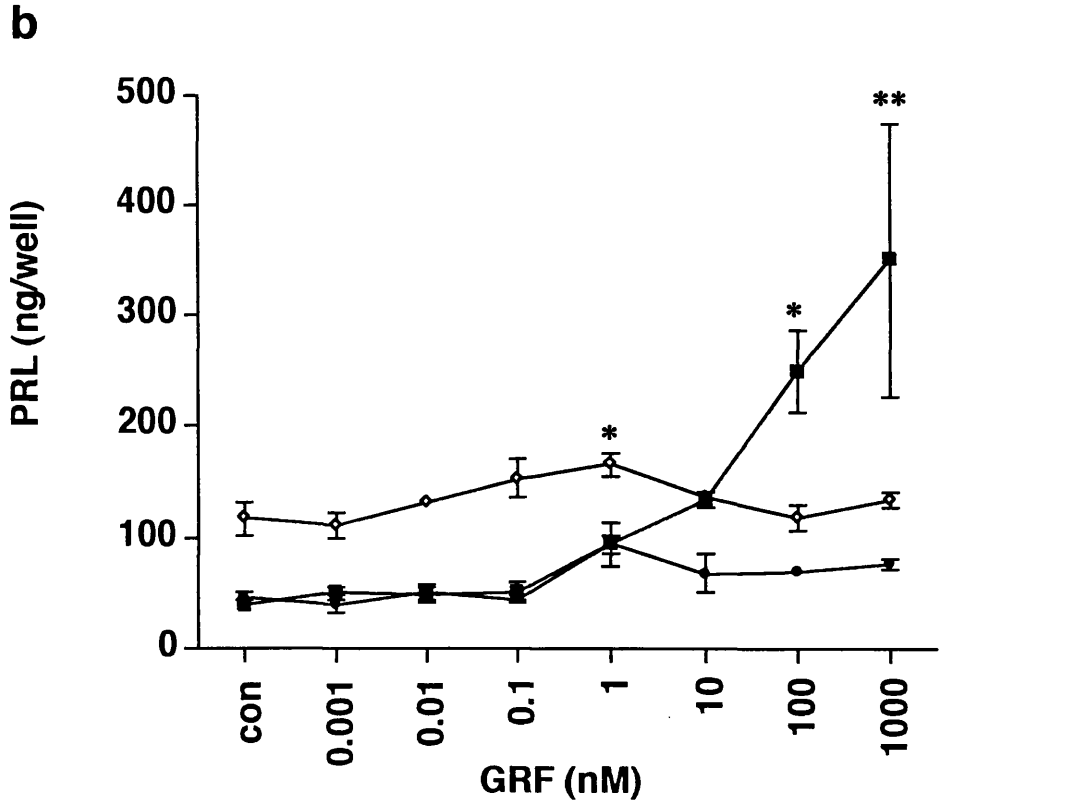
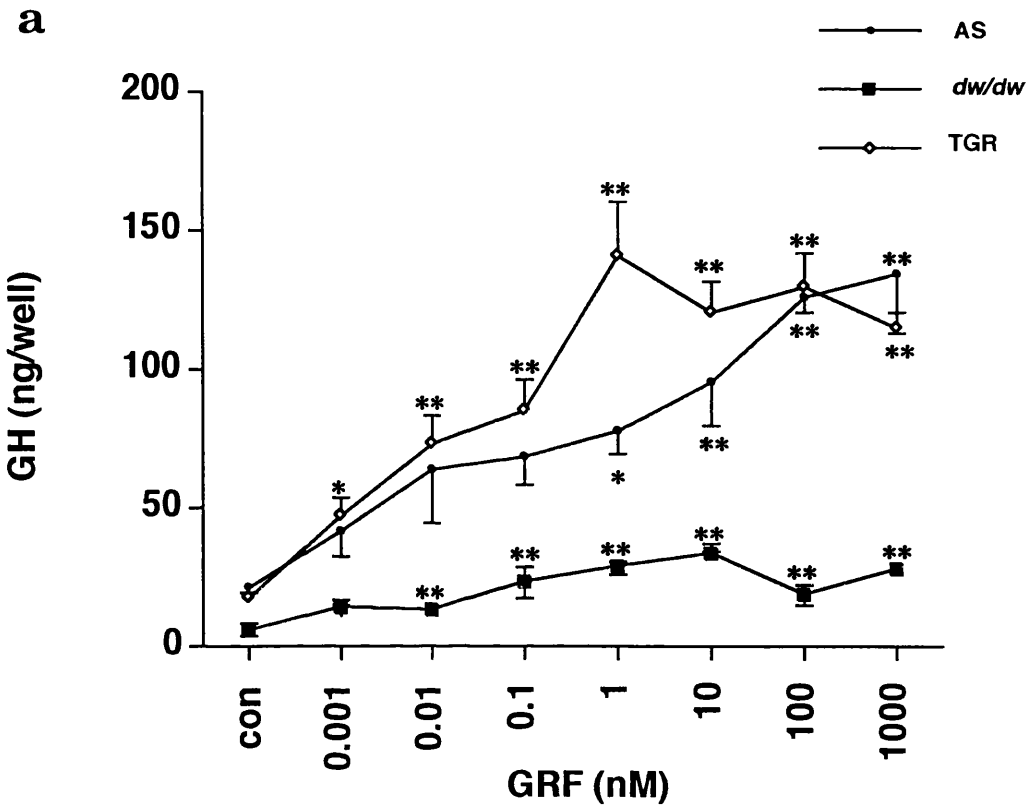

b
PRL (ng/well)
500
400
300
200
100
0
*
*
**
con
0.001
0.01
0.1
1
10
100
1000
GRF (nM)

**Figure 5.6: GRF dose response in primary cultures of female pituitary cells: GH and PRL release.**

Anterior pituitary cells from female AS, *dw/dw* and TGR rats were cultured at ¼ pituitary per well in 24 well plates for 5 days. The cells were incubated for 3 hours with 0.001 - 1000nM GRF and the medium was then assayed for GH (a) and PRL (b).

*P<0.05, **P<0.01 *vs.* control. ANOVA followed by Dunnet's multiple comparison (n=3).


a
AS
dw/dw
TGR
GH (ng/well)
200
150
100
50
0
con
0.001
0.01
0.1
1
10
100
1000
GRF (nM)

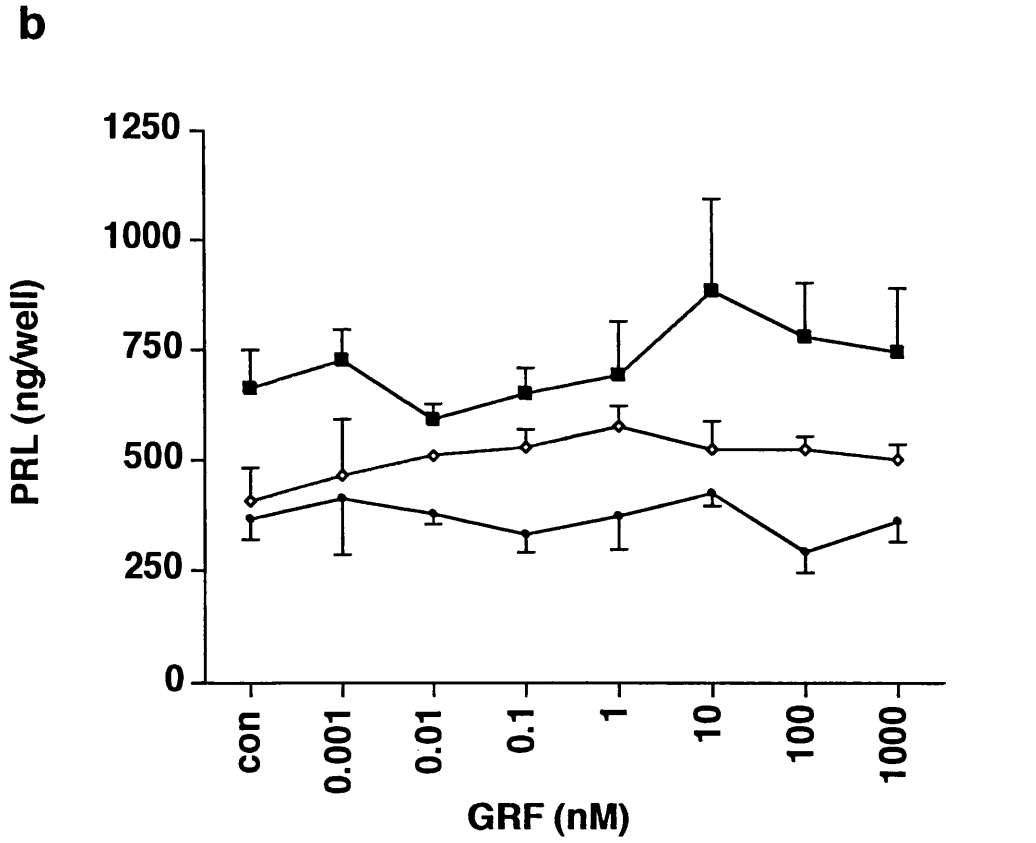
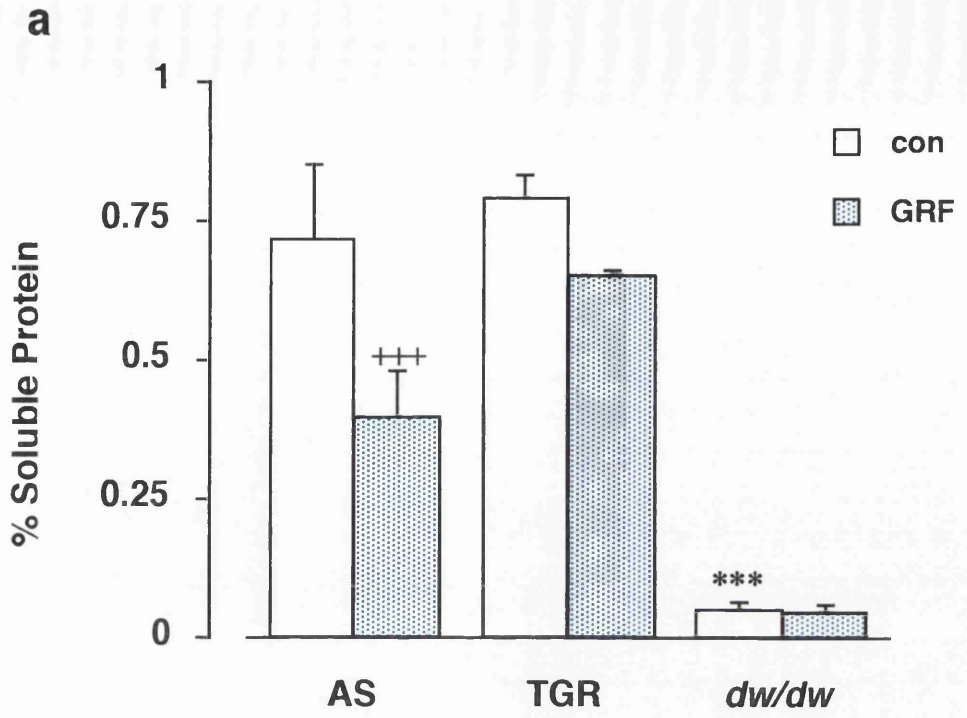

b
PRL (ng/well)
1250
1000
750
500
250
0
con
0.001
0.01
0.1
1
10
100
1000
GRF (nM)

**Figure 5.7: GH and PRL content of pituitary cultures.**

Four wells of cells (either control or 10nM GRF treated) were pooled in one extract (4 wells was equivalent to one pituitary seeded at ¼ pituitary per well). The GH and PRL were measured by RIA and expressed as percentage soluble protein. Because the wells were pooled, each experiment yielded only one control and one GRF treated cell extract. The graphs show the GH (a) and PRL (b) contents from several experiments combined.

***P<0.001 *vs.* AS. Welch Alternate t-test. $^{+++}$P<0.001 control *vs.* GRF. Student's t-test. (AS: n=10, TGR: n=2, *dw/dw*: n=5).


a
1
0.75
0.5
0.25
0
% Soluble Protein
con
GRF
+++
***
AS
TGR
dw/dw

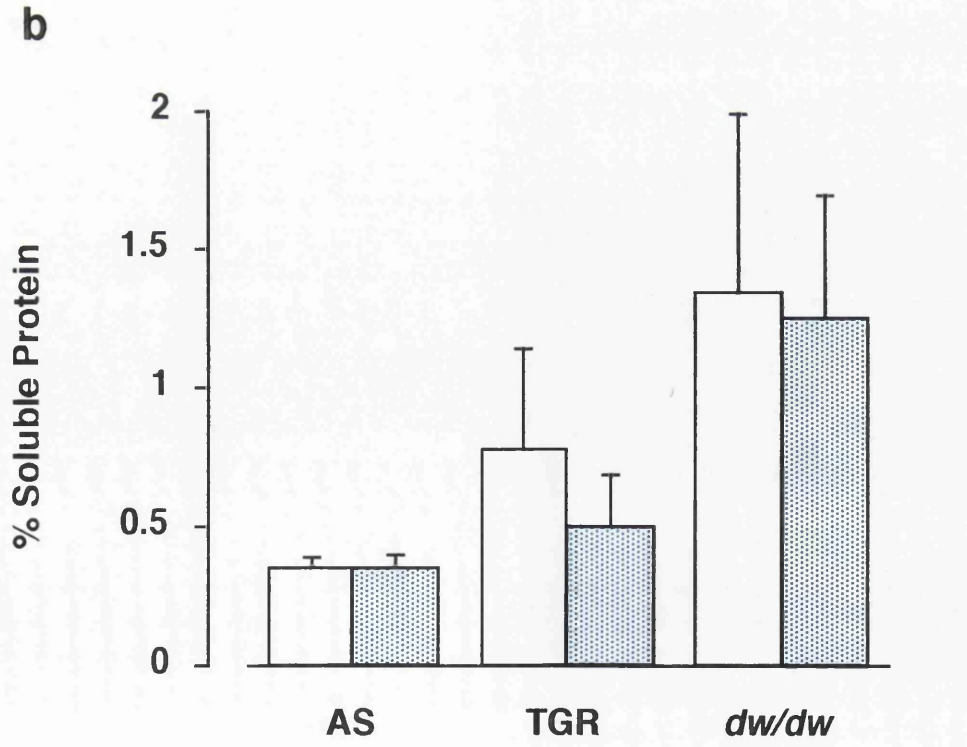
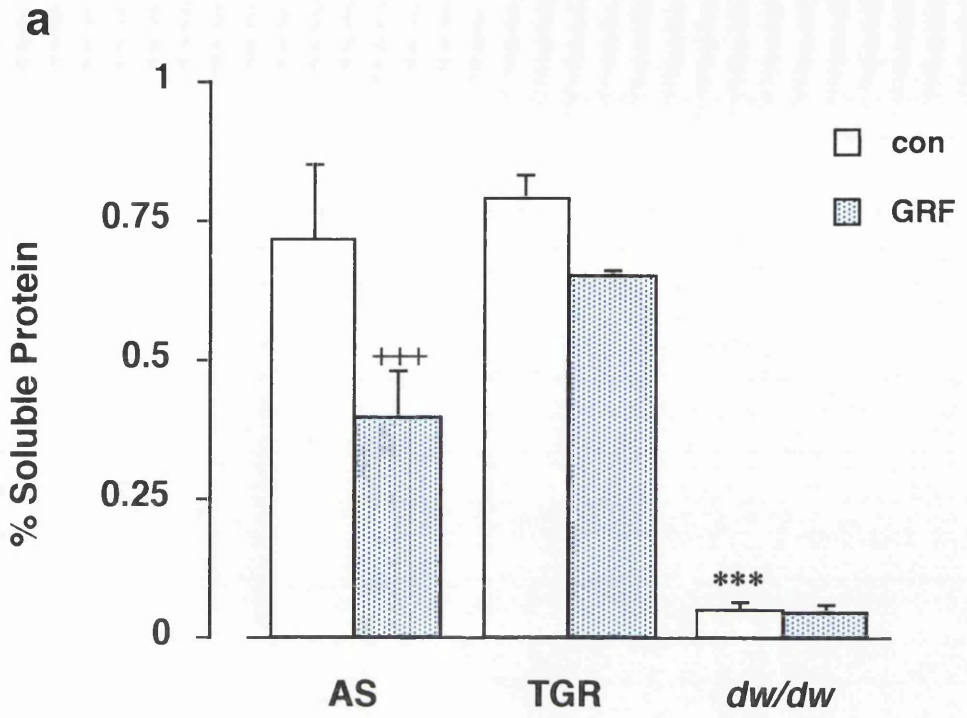

b
2
1.5
1
0.5
0
% Soluble Protein
AS
TGR
dw/dw

**Figure 5.7: GH and PRL content of pituitary cultures.**

Four wells of cells (either control or 10nM GRF treated) were pooled in one extract (4 wells was equivalent to one pituitary seeded at ¼ pituitary per well). The GH and PRL were measured by RIA and expressed as percentage soluble protein. Because the wells were pooled, each experiment yielded only one control and one GRF treated cell extract. The graphs show the GH (a) and PRL (b) contents from several experiments combined.

***P<0.001 *vs.* AS. Welch Alternate t-test. $^{+++}$P<0.001 control *vs.* GRF. Student's t-test. (AS: n=10, TGR: n=2, *dw/dw*: n=5).


a
% Soluble Protein
1
0.75
0.5
0.25
0
con
GRF
+++
***
AS
TGR
dw/dw

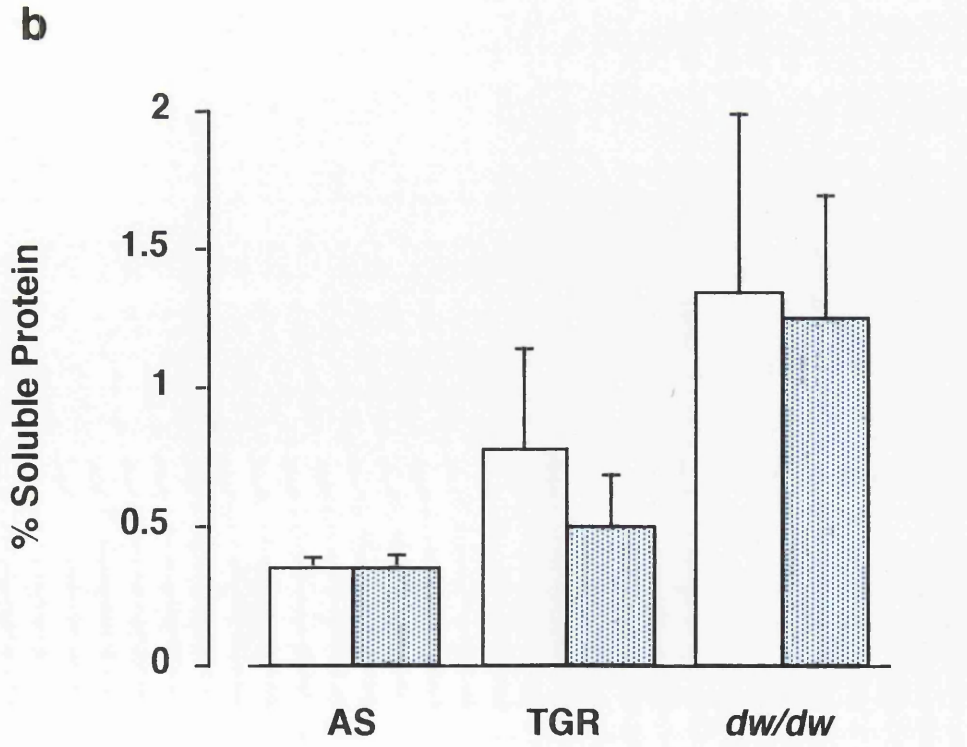
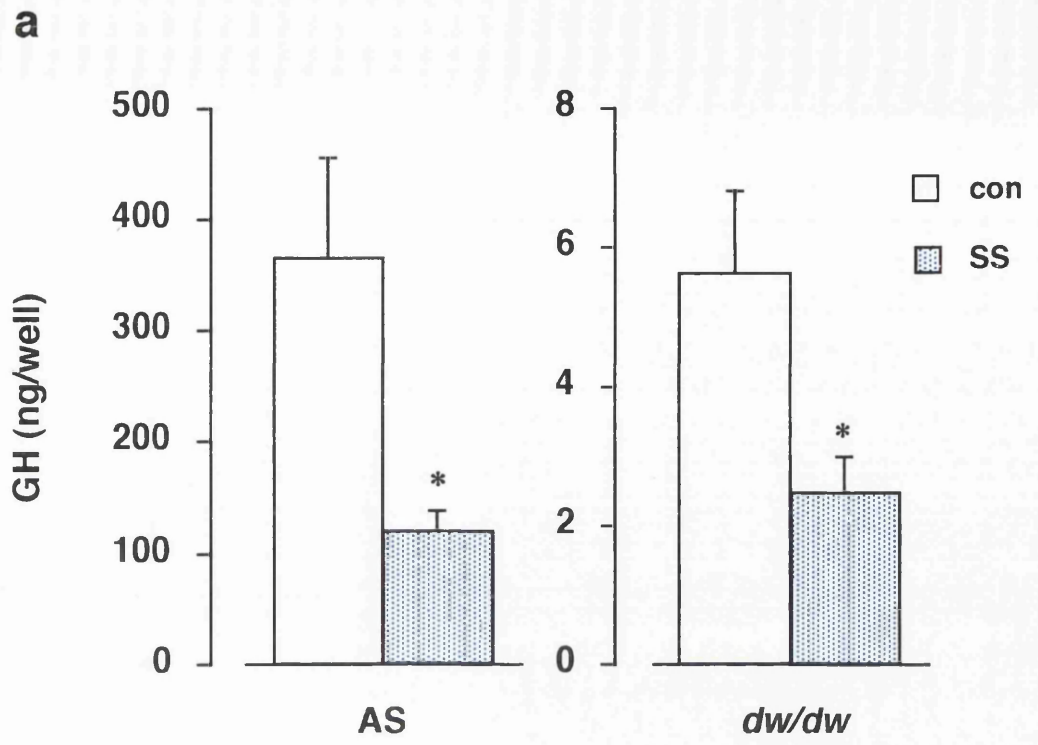
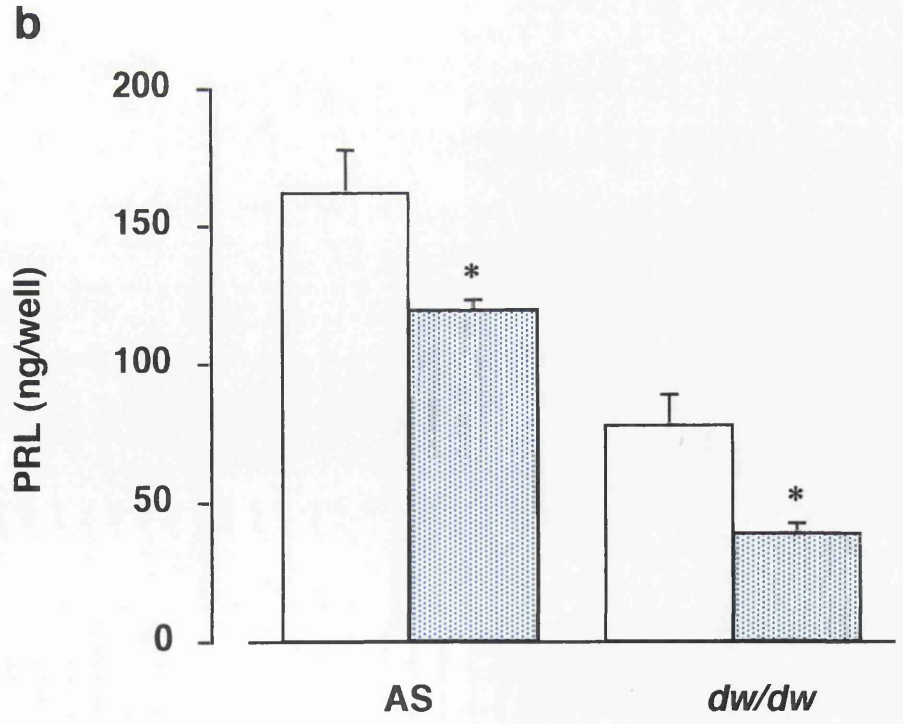

b
% Soluble Protein
2
1.5
1
0.5
0
AS
TGR
dw/dw

**Figure 5.8: Effect of somatostatin on GH and PRL release in AS and *dw/dw* cultures.**

♂ Anterior pituitary cultures were treated for 3 hours with or without 50nM SS and the medium assayed for GH (a) and PRL (b).

*P<0.05 Control *vs.* SS. Welch Alternate t-test (n=5-6).


a
GH (ng/well)
500
400
300
200
100
0
8
6
4
2
0
con
SS
*
*
AS
dw/dw


b
PRL (ng/well)
200
150
100
50
0
*
*
AS
dw/dw

### 5.2.6 Experiment 5: GRF Treatment of Cultures: cAMP Accumulation

This experiment was also a dose response determination, this time for cAMP accumulation. The cells were plated at ¼ pituitary per well, maintained for 5 days and treated with varying concentrations of GRF for 30minutes. The cells were extracted and assayed for cAMP. Three separate experiments are illustrated;

1. AS *vs.* TGR males
2. AS *vs. dw/dw* males
3. AS *vs.* AS/*dw* males

Figures 5.9 and 5.10 show the cAMP accumulation dose response to GRF. The first point to note is the different sensitivity of response compared to the secretory response. The AS culture was already starting to respond at 0.001nM GRF with a release of GH (see figure 5.4), however at least 100 fold more than this was needed to give a detectable rise of cAMP. In most cases the curves did not reach a plateau with the range of doses used, so an $ED_{50}$ could not be determined.

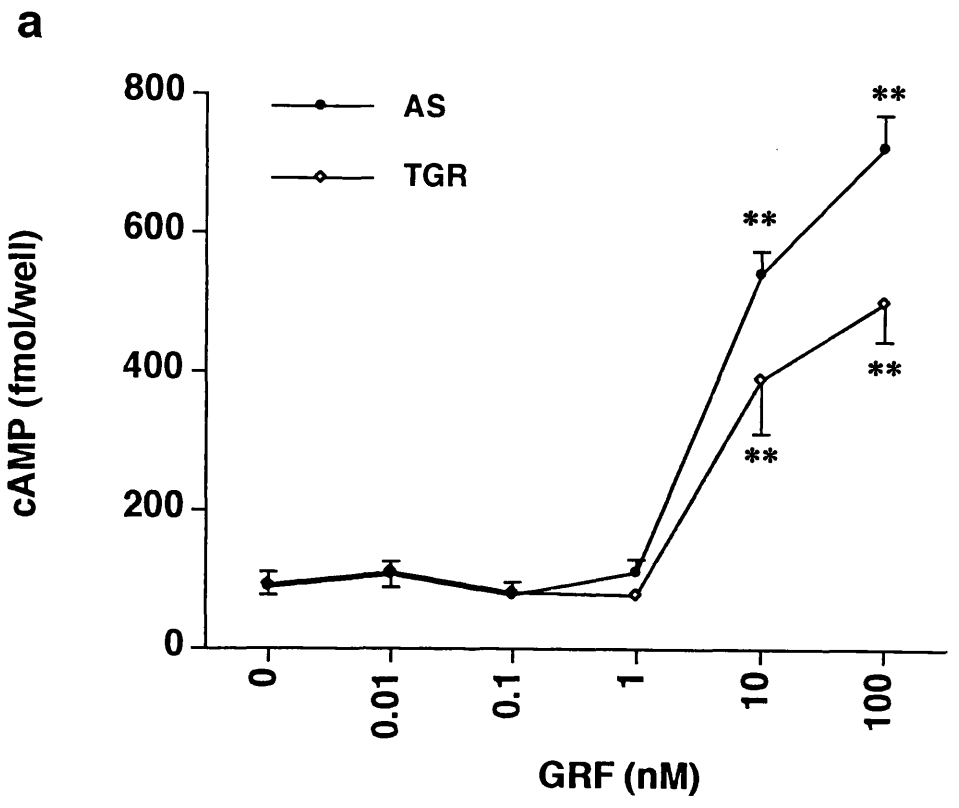
The TGR (figure 5.9a) showed a similar sensitivity as the AS, but the amplitude of response was less probably reflecting the reduced somatotroph population. Figure 5.9b shows the comparison of AS and *dw/dw* cultures in a separate experiment. The *dw/dw* culture did not show any detectable response to GRF at the range of doses used. Figure 5.10 shows two replicate experiments comparing the cAMP accumulation of AS and heterozygote male cultures. In both cases, the sensitivity and amplitude of response were similar in AS and AS/*dw* cultures, thus there is no evidence for an intermediate phenotype in the cAMP response to GRF in heterozygotes.

**Figure 5.9: GRF dose response in primary cultures of AS, TGR and *dw/dw* male pituitary cells: cAMP accumulation.**

Primary cultures were treated for 30 minute with varying doses of GRF. The cells were extracted in acid alcohol and assayed for cAMP. The results from two experiments are shown.

a. AS *vs.* TGR
b. AS *vs. dw/dw*

**P<0.01 *vs.* control. ANOVA followed by Dunnet's multiple comparison. The data was also post-tested for linear trend to show dose dependant release (n=3).


a
800
600
400
200
0
cAMP (fmol/well)
AS
TGR
**
**
**
**
0
0.01
0.1
1
10
100
GRF (nM)

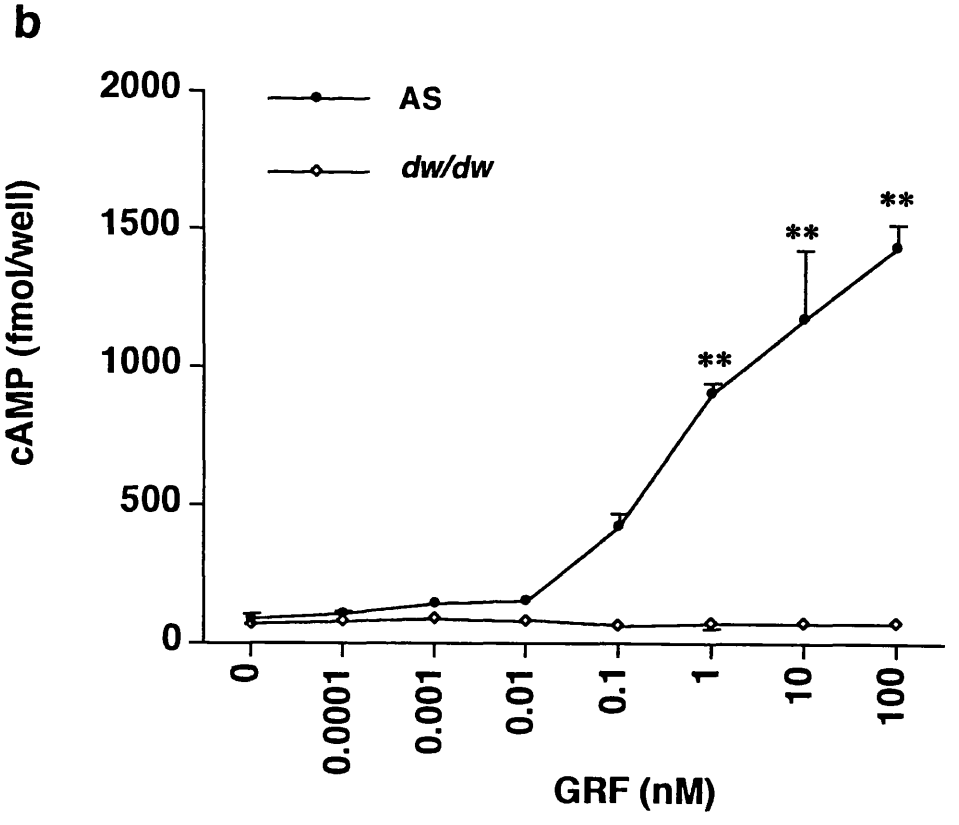
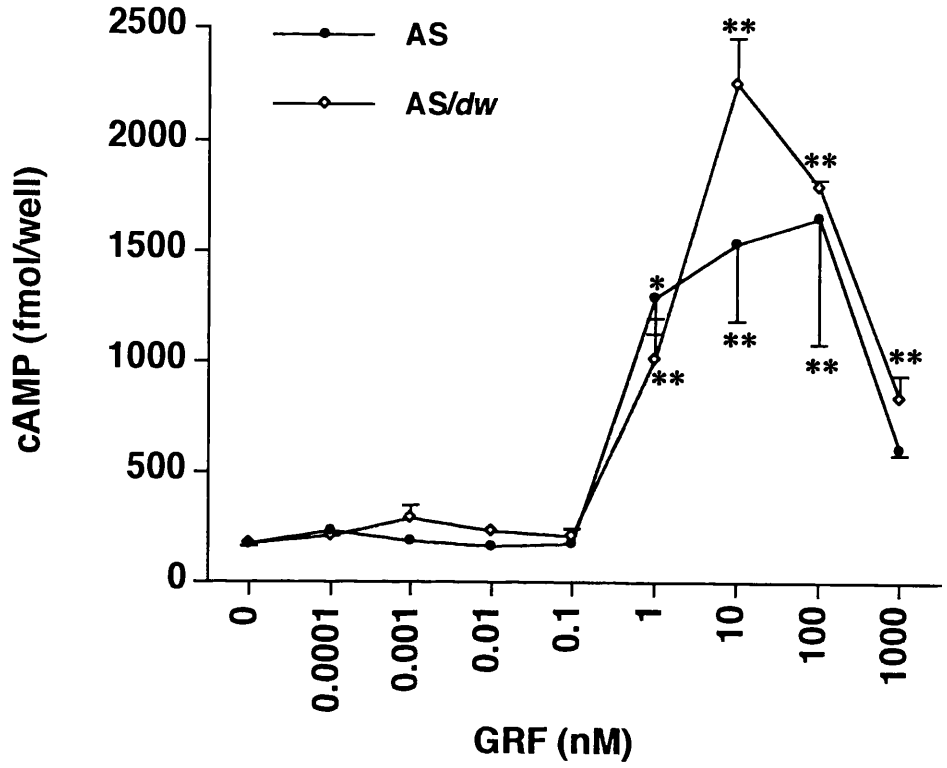

b
2000
1500
1000
500
0
cAMP (fmol/well)
AS
dw/dw
**
**
**
0
0.0001
0.001
0.01
0.1
1
10
100
GRF (nM)

**Figure 5.10: GRF dose response in primary cultures of AS and AS/*dw* heterozygote male pituitary cells: cAMP accumulation.**

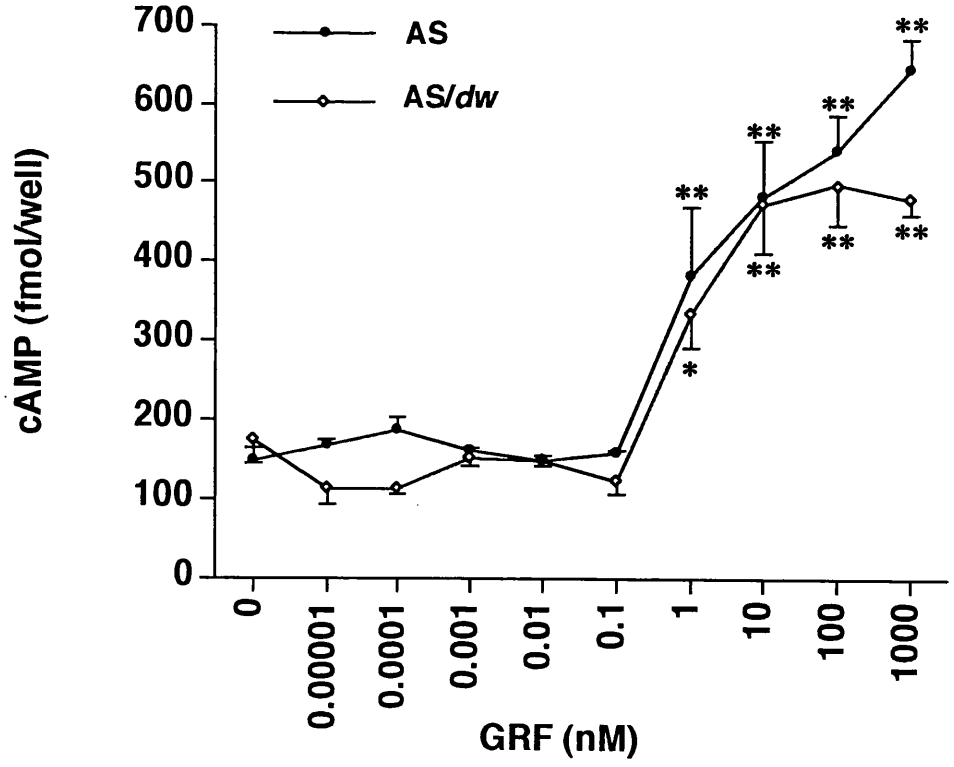
Primary cultures were treated for 30 minute with varying doses of GRF. The cells were extracted in acid alcohol and assayed for cAMP. The results from two replicate experiments comparing AS and AS/*dw* cultures are shown.

*P<0.05, **P<0.01 *vs.* control. ANOVA followed by Dunnet's multiple comparison. The data was also post-tested for linear trend to show dose dependant release (n=3).

a


AS
AS/dw
cAMP (fmol/well)
2500
2000
1500
1000
500
0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
GRF (nM)

b


AS
AS/dw
cAMP (fmol/well)
700
600
500
400
300
200
100
0
0
0.00001
0.0001
0.001
0.01
0.1
1
10
100
1000
GRF (nM)

### 5.2.7 Experiment 6: GRF Treatment of Mixed AS and *dw/dw* Cells: cAMP Accumulation

It was possible that the differences observed were simply because that the low somatotroph number in the *dw/dw* cultures made the detection of cAMP accumulation difficult. Thus, the aim of this experiment was to study cultures adjusted to contain reduced numbers of normal somatotrophs. This culture could be compared to *dw/dw* cultures to control for effects secondary to low somatotroph numbers.

AS and *dw/dw* anterior pituitaries were dispersed and combined in varying proportions and plated at 500,000 cells per well in 24 well plates. Thus, as well as cultures containing just AS and just *dw/dw* pituitary cells, wells were also plated with, for instance, 50% AS cells and 50% *dw/dw* cells. The culture was maintained for 5 days and incubated for 30 minutes with or without 10nM GRF. The medium was removed and the cells extracted in acid alcohol for cAMP RIA. A preliminary experiment was performed including a broad range of percentages to characterize the behaviour of cells of mixed origin in culture. A second experiment was then performed including only low percentages of AS cells as well as AS and *dw/dw* controls.

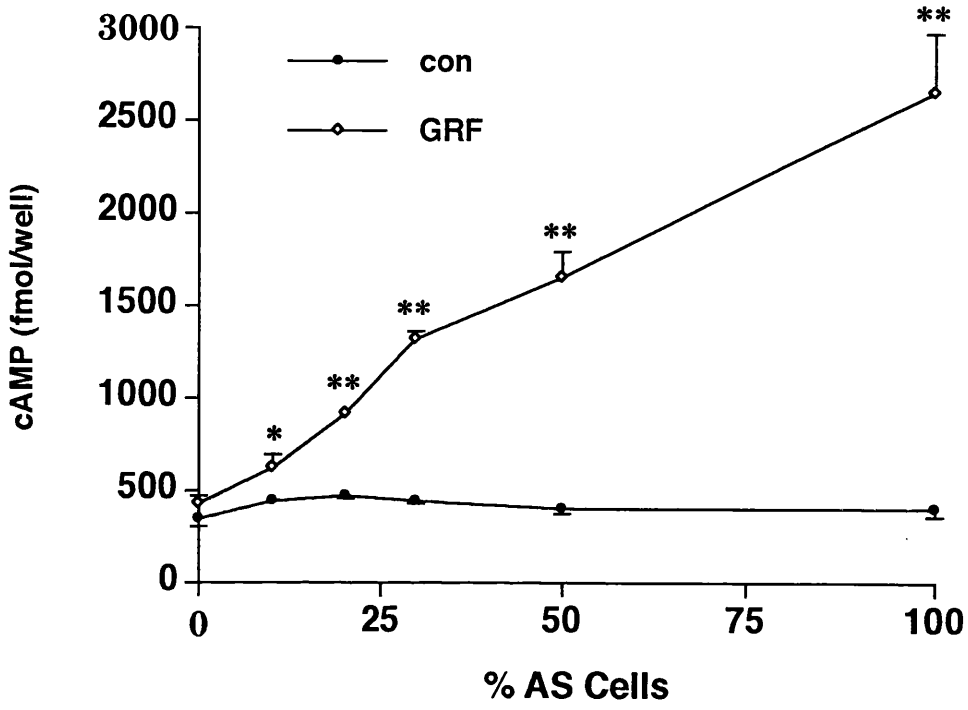
In the preliminary experiment (figure 5.11a), the accumulation of cAMP in response to GRF was linear with respect to increasing percentage of AS cells. This can also be seen in figure 5.11b, although in this instance, the experiment was designed to focus on lower percentages of AS cells. Even at 5% AS cells (equivalent to 2% somatotrophs), the rise in cAMP was significant. This means a GRF stimulated cAMP response should be apparent in *dw/dw* rats if their somatotrophs were simply reduced in number. Since they are not, this implies an intrinsic deficit in this aspect of GRF signalling in *dw/dw* somatotrophs.

**Figure 5.11: Effect of 10nM GRF on cAMP accumulation in cultures of mixed cells from AS and *dw/dw* pituitaries.**

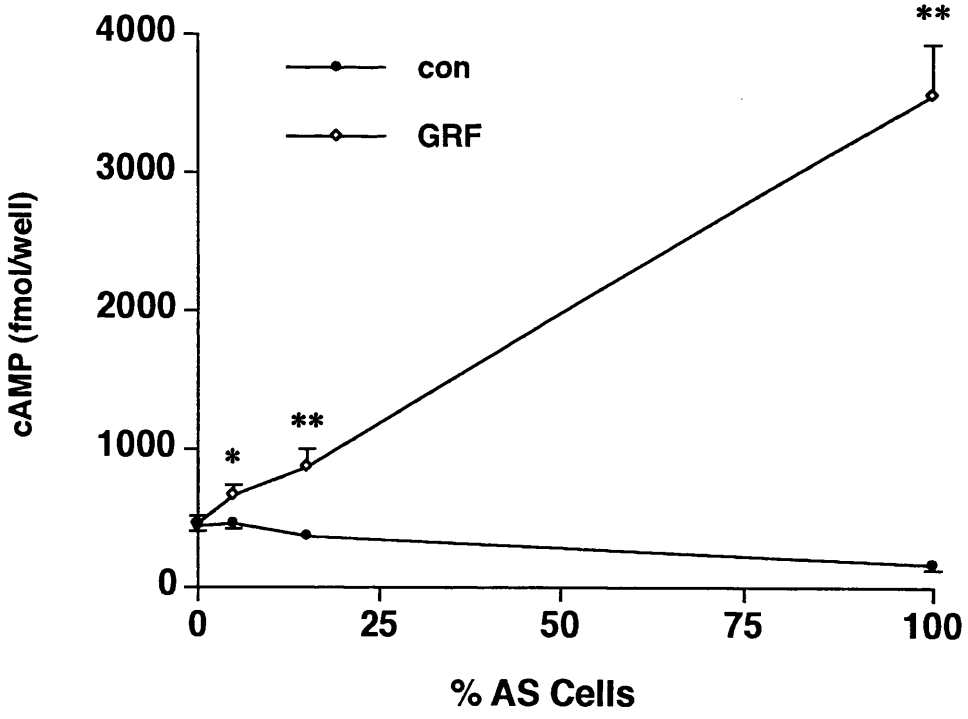
Anterior pituitary cells from AS and *dw/dw* rats were plated in various proportions at 500,000 cells per well and cultured for 5 days. The cells were treated with or without 10nM GRF for 30 minutes and the cells were then extracted in acid alcohol for cAMP RIA. The facing graphs show the cAMP accumulation plotted against the percentage of AS cells in the culture.

*P<0.05,**P<0.01 control *vs.* GRF for each cell mixture. Alternate Welch t-test (n=3).

a


3000
2500
2000
1500
1000
500
0
cAMP (fmol/well)
con
GRF
*
**
**
**
**
0
25
50
75
100
% AS Cells

b


4000
3000
2000
1000
0
cAMP (fmol/well)
con
GRF
*
**
**
0
25
50
75
100
% AS Cells

## 5.3 DISCUSSION

Endocrine cells in monolayer culture do not proliferate rapidly, less than 0.5% labelling when treated with tritiated thymidine (Leiberman *et al*, 1982; Billestrup *et al*, 1986). However, cytosine arabinoside treatment of cultures reduced the number of lactotrophs in culture, indicating that mitosis is involved in expansion of the lactotroph population in culture (Frawley and Hoeffler, 1988). Lactotroph differentiation is reported to be post-mitotic (Frawley and Miller III, 1989; Borrelli *et al*, 1989), thus this proliferation may represent expansion of the mammosomatotroph precursor with subsequent cell commitment. Fibroblast-like cells are the main proliferating cells during culture (Leiberman *et al*, 1982), reaching confluence after about 3 days in my cultures. The variation in secretion of GH and PRL over the culture period, therefore was mainly due to the increase in secretion and/or gene expression by individual cells. Day 5 was chosen as a standard culture period as the GH secretion reaches a peak at this time under the culture conditions used. After this time, the decline in GH could be due to either a reduction in secretion per cell, cell death or a combination of the two.

At the end of the five day culture period, the cell numbers were quantified by immunocytochemistry. The results for AS, *dw/dw* and heterozygote cultures were consistent with the adult pituitary section immunocytochemistry and FACS analysis data detailed in chapter 3. The GH cell number was deficient in the *dw/dw* but not in the heterozygote and the PRL cell numbers showed no significant differences. The data in chapter 3 showed that *dw/dw* pituitaries contained 2-3 fold more PRL than AS pituitaries in both male and female rats. The PRL contents of the cultures maintained this pattern, with *dw/dw* cultures containing about 3 fold more PRL than AS cultures, although this failed to reach significance due to the large variance in the *dw/dw* data. Although the GH and PRL contents of the culture extracts are expressed as percentage of soluble protein, the levels of protein were not significantly different between AS and *dw/dw* culture extracts (AS: 209μg ± 12.6 *vs. dw/dw*: 245 ± 22.8), thus this 3 fold difference is also applicable to PRL content per pituitary equivalent (4 wells). It is interesting that the difference in PRL content between AS and *dw/dw* pituitaries is maintained in culture. This implies that the PRL

phenotype in the *dw/dw* is inherent to the pituitary rather than in response to feedback on the hypothalamus due to GH deficiency.

The immunocytochemistry was performed in two separate experiments, both of which included AS and *dw/dw* cultures. Although the relative proportions were similar (ie. reduced somatotroph number in the *dw/dw* without a reduction in lactotrophs), the actual percentages differed between the experiments. The somatotroph and lactotroph proportions were all higher in the experiment shown in figure 5.2b than 5.2a, in particular the GH staining cells in the *dw/dw* were about 6 fold higher. Although the coverslips that the cells were grown on were poly-L-lysine coated, I encountered problems with cells floating off during the staining procedure. This should not have influenced the percentages of cell types, as the cells tended to come off in a layer and not individually, although the possibility that different cell types had better adhesive properties than others cannot be excluded. There was variability between experiments relating to whether or not the cells tended to float off, suggesting that the coating was more effective in some experiments than others. This may have affected the way the cells behaved in culture either by altering the proportion of endocrine cells that adhered to the coverslip, or by altering the amount of proliferation of fibroblast-like cells, which would reduce the percentages of endocrine cells.

The proportion of GH staining cells in the TGR culture was 30% less than that in the AS, similar to the results obtained from immunocytochemistry of pituitary sections (Flavell *et al*, 1996: TGR: 31% ± 1 *vs.* litter-mate controls: 45 ± 1). Although the somatotroph number was still deficient, the GH content of the culture extracts was not significantly different to the AS culture. This implies that each TGR somatotroph would contain more GH than an AS somatotroph in culture. Similarly, the percentage of lactotrophs was not significantly different between AS and TGR cultures, again reflecting the situation *in vivo* (Flavell *et al*, 1996). The PRL content, although 60% of control *in vivo* (Flavell *et al*, 1996), was double that of controls in culture extracts (although this did not reach significance), again implying that the PRL content per lactotroph would be higher than in AS cells. This suggests that hormone synthesis has increased in both somatotrophs and lactotrophs in culture.

An ultra-short feedback loop exists in the TGR, resulting in somatotroph hypoplasia and GH deficiency due to reduced stimulation of the pituitary by GRF. In addition, the PRL deficiency is also thought to have hypothalamic origins, secondary to hGH expression. DA immunofluorescence has recently been found to be increased in the TIDA DA neurones in the TGR (Phelps *et al*, 1997). Similarly, DA was found to increased hGH, but not bGH transgenic mice (Phelps and Bartke, 1997). As hGH, but not bGH, is lactogenic in mice and rats, it is likely that this is causing an increase in DA by feeding back on PRL receptors. This increased hypothalamic DA would then lead to a reduced PRL content of the pituitary, seen in the TGR. While TGR pituitary cells are normal, and may be expected to behave as AS cells in culture, it is possible that the different hypothalamic origins may have lead to differences in sensitivity to various factors.

The GH release dose response to GRF for the male TGR showed a similar sensitivity and fold increase, although the amount of GH released was lower at basal and all doses of GRF. This suggests that although the GH content of the culture was no longer deficient compared to the AS culture, the basal and stimulated GH release appeared to still be reduced, reflecting the deficiency in GH cell number. However, in addition to the recovery in PRL content, the PRL release from the male TGR culture was higher than the AS culture. Thus removing the TGR pituitary cells from the higher DA inhibitory environment appeared to have caused a rebound response which is greater than in AS cultures.

The increased prolactin release from female derived cultures for all three strains of rat is in agreement with Hoefer *et al* (1984), who concluded that the PRL population in culture is strongly dependant on the pituitary donor. The PRL release showed no response to GRF in the female cultures or in the first set of male cultures. However, a significant variation was seen in all three of the second set of male cultures and a significant dose dependant trend was seen in the AS and *dw/dw* cultures. The *dw/dw* reached a striking 9-fold stimulation of PRL release, compared to AS and TGR cultures where the maximum increase was only 2 fold. GRF stimulation of PRL could be explained by interconversion of GH and PRL expressing cells. The GH cells may be making some PRL, or

alternatively PRL cells may be expressing GRF receptors. The reason for the difference between the first and second set of male cultures may have been the fetal calf serum in the culture medium. Initially, an unidentified batch of serum, present in the lab prior to the beginning of this work, was used. As it was unlabelled, it was not possible to replace it with the same batch. Experiments carried out with the second batch of fetal calf serum showed a greater tendency for PRL release in response to GRF. It has been shown that $E_2$ treatment recruits cells expressing GH only to become mammosomatotrophs (Lieberman *et al*, 1982; Boockfor *et al*, 1996) thus a possible explanation is that the second batch of fetal calf serum contained an oestrogenic compound. If this was the case, the effect may be eliminated by the use of charcoal stripped sera to reduce the steroid content of the medium. The medium also contained phenol red, which can also be oestrogenic (Hubert *et al*, 1986).

It is interesting that the *dw/dw* culture responded more than the AS, mirroring the increased sensitivity of the *dw/dw* males to $E_2$ shown in chapter 4. It is also curious that the female cultures, carried out in parallel with the second batch of male cultures show no GRF stimulated PRL release. Perhaps the equilibrium in these cultures is further towards the classical lactotroph phenotype, thus not responsive to GRF. However, these results emphasise that it may be an oversimplification to assume that the only cells in culture that respond to GRF are somatotrophs.

An alternative explanation would be that GRF was acting non-specifically on other receptors. PACAP, which has been shown to release GH, PRL, LH and ACTH in superfused rat pituitary cells, shares some homology to GRF (Miyata *et al*, 1989) thus it is possible that non-specific effects may be mediated *via* the PACAP receptor. PACAP receptors are pharmacologically classified as type I or type II (Journot *et al*, 1995). Type II PACAP receptors bind vasoactive intestinal peptide (VIP) with similar affinity to PACAP, and are therefore also called VIP receptors. While PRL release in *dw/dw* cultures was occasionally encountered, cAMP accumulation was never detected. However, PACAP and VIP both signal *via* cAMP accumulation. It is not known if *dw/dw* pituitary cells are able to respond to PACAP with increased cAMP. However, corticotrophs in the *dw/dw* pituitary can accumulate cAMP in response to CRF, showing that adenylate

cyclase can be stimulated in other pituitary cell types. Stimulation of *dw/dw* pituitary cells with PACAP would therefore be expected to raise cAMP levels in other cell types than somatotrophs. Whether it also stimulates the *dw/dw* somatotroph would depend on where the GRF signalling pathway is uncoupled. It is thought that Gsα is unable to stimulate adenylate cyclase (Downs and Frohman, 1991), in which case the *dw/dw* somatotrophs may also be unable to respond to PACAP. It would therefore be interesting to challenge *dw/dw* cultures with PACAP to investigate cAMP accumulation and hormone release. This experiment may be useful for the further characterization of the defect in this model.

Whatever the response of the somatotrophs, it is likely that the other cell types would respond to PACAP with an increase in cAMP. In addition, VIP has been shown to induce cAMP accumulation (Gourdji *et al*, 1979). As the *dw/dw* culture never responded to GRF with an increase in cAMP, it is unlikely that non-specific effects were mediated *via* these receptors. TRH however, signals *via* PI turnover (Sutton *et al*, 1982) and is therefore a more likely mediator of any non-specific GRF effects.

The possible feminization of the cultures may also explain the unexpected inhibition of PRL release by SS (Kimura *et al*, 1986; Goth *et al*, 1996). $E_2$ treatment of ovarectomized rats caused the up-regulation of the estrogen dependant somatostatin receptor subtype SSTR2, and down-regulation of SSTR5, allowing PRL release to be inhibited by SS by reducing cAMP (Kimura *et al*, 1986, 1989, 1998). The more pronounced effect in the *dw/dw* (50% inhibition of PRL *vs.* 30% in the AS) may again reflect the increased response of *dw/dw* pituitary cells to $E_2$.

The GRF dose response obtained for GH release in AS male cultures was similar to Fukata *et al* (1985), who also obtained an $ED_{50}$ of 0.01-0.03nM GRF and a five fold increase in GH release at maximal stimulation. The sensitivity of the *dw/dw* culture was reduced with an $ED_{50}$ of 0.3-1nM GRF, more than 10 fold higher than the AS culture. This is slightly different to the data of Downs and Frohman (1991), who reported that the difference in sensitivity was only 2.5 fold. It should be noted, however, that their culture medium (α-Modified Eagles

medium + 10% horse serum) differed to mine and that the cells were maintained for only 3 days before GRF challenge.

Although the basal and GH release is markedly deficient in the *dw/dw* cultures compared to AS, when expressed as percentage of content, there were no significant differences (Downs and Frohman, 1991), suggesting that the deficiency in basal GH release is mainly due to the reduced GH stores. To compare the relative responses to GRF, the data were plotted as fold stimulation over basal. The difference in the fold increase at maximal stimulation gave similar results to Downs and Frohman (1991), who reported a 25% decrease in the response of the *dw/dw* culture. The 3.5 fold increase in the *dw/dw* GH release was 30% lower than the 4.9 fold increase in the AS. Kineman *et al* (1989) failed to show a difference in response between AS and *dw/dw* by RHPA, however plaque size is only a semi-quantitative measure of hormone release. Nevertheless, the magnitude of the responses (4 fold increase in plaque size) were similar to those shown in this chapter.

The GH secretory response and the cAMP accumulation represent distinct pathways (Barinaga *et al*, 1985). This is reflected by the data in this chapter in several ways. The time course of the GH release and cAMP accumulation were shown to differ, the cAMP accumulation reaching maximal within 15 minutes but a much longer time was necessary for large differences between basal and GRF stimulated GH release. Similar time course experiments were performed by Downs and Frohman (1991), who showed maximal cAMP accumulation at 15-30 mins and optimal duration for GH release at 4 hours. In addition, the threshold sensitivity is higher for cAMP accumulation than GH release as 0.001nM GRF elicited a GH release response but at least 0.1nM GRF was necessary for any detectable increase in cAMP in AS cultures.

The GH gene expression and somatotroph proliferation pathway, dependant on cAMP accumulation, is more severely deficient in the *dw/dw* than the GH secretion, which is still mostly intact. Secretion involves other signalling mechanisms as well as cAMP accumulation and is strongly inhibited by SS. PI turnover is also thought to be involved in GRF stimulated GH release in ovine (Wu *et al*, 1997; Sartin *et al*, 1996) and rat (Cuttler *et al*, 1995; Canonico *et al*,

1983) pituitary cells, however preliminary attempts to measure PI turnover in AS and *dw/dw* cultures were unsuccessful (data not shown). Although there are reports of SS reducing cAMP (Michel *et al*, 1983; Tentler *et al*, 1997), Kimura *et al* (1989) could not detect any reduction in cAMP in anterior pituitary membrane preparations from ovarectomized rats, suggesting that SSTR2 receptors on the somatotroph do not signal through cAMP and is therefore more inhibitory to GH secretion than GH synthesis or somatotroph proliferation. This agrees with Barinaga *et al* (1985), who measured GH transcription directly and found it not to be inhibited by SS.

In contrast to the relatively mild deficiency in GH release, the *dw/dw* culture failed to show any detectable cAMP accumulation in response to GRF. Downs and Frohman (1991) showed a severe deficiency in cAMP stimulation, although this was not complete as the *dw/dw* cultures did show a small cAMP accumulation in response to GRF in their hands. Addition of dibutyryl cAMP, however, caused a comparative release of GH from both AS and *dw/dw* cultures (Downs and Frohman, 1991). This suggests that not only can cAMP cause a release of GH despite the distinct pathways, but also that the pathway beyond cAMP is intact in the *dw/dw*. The reduced sensitivity of the *dw/dw* to release GH in response to GRF may also suggest that the cAMP dependant pathway plays some role in GH secretion. This may be indirect, as GRF-R expression is up-regulated by the cAMP dependant pathway (Lin *et al*, 1992; Horikawa *et al*, 1996). The GRF-R mRNA has been shown to be reduced in the *dw/dw* pituitary (Carmignac *et al*, 1996), although this does not necessarily reflect a reduction in receptor protein per somatotroph.

In order to investigate the contribution of reduced somatotroph number to the cAMP deficiency, I produced cultures containing AS and *dw/dw* cells mixed in different proportions. Initially, a broad range of proportions was tested to characterize the behaviour of cultures of mixed origins. The cAMP accumulation plotted against percentage of AS cells in the culture was linear. This suggests that there were no changes in response due to somatotroph density, implying that no major paracrine effects were occurring. The second experiment showed that a culture seeded at just 5% AS cells showed a significant cAMP response to GRF (this is equivalent to about 2% normal somatotrophs, as AS pituitaries

contain about 40% somatotrophs). In chapter 3, the somatotroph population of male *dw/dw* rats was shown to be about 12%. Therefore, if the somatotrophs had been normal a significant cAMP accumulation would have been seen, supporting the hypothesis that the response of each individual somatotroph is impaired. The linear response of the accumulation with somatotroph number confirms that the calculation performed by Downs and Frohman (1991) is valid. From the relative responses in AS and *dw/dw* cultures and the relative somatotroph numbers in the two models, they calculated that each somatotroph had a 6-10 fold reduced response. It was not possible to perform the same calculation on my data, as I found no detectable rise in cAMP in *dw/dw* cultures.

Although the GH content of the pituitary was not significantly different to controls and there was no reduction in body weight, the AS/*dw* may have reduced GH mRNA (Houston *et al*, 1991; Houston, 1992). As GH gene transcription is cAMP dependant I wanted to investigate whether this model showed an intermediate phenotype for cAMP accumulation. Due to the striking difference between AS and *dw/dw* responses, this parameter may be considered the most likely to show an intermediate phenotype. However, both the sensitivity and the maximum response were similar between AS and AS/*dw* cultures, showing that the defect is fully recessive with regard to this parameter also.

This chapter has outlined some of the differences between AS and *dw/dw* pituitaries at the cellular level. In particular the absence of cAMP accumulation in response to GRF, without the same impact on the GH secretion, highlighting the different signalling mechanisms involved in these systems. The signalling defect, however, may be a symptom of the phenotype and not the cause as the *dw/dw* shows others differences to the AS apart from the lack of cAMP accumulation. For instance, the elevated PRL content of culture extracts and increased tendency for PRL release in response to GRF.

A mammosomatotroph cell line exists ($P_0$) that releases both GH and PRL in response to GRF (Kashio *et al*, 1990). In addition, this cell line shows reduced cAMP accumulation compared to primary cultures (Kashio *et al*, 1990), resembling the phenotype of the *dw/dw* pituitary cells. GRF stimulation of $P_0$ cells and a similar cell line derived from *dw/dw* pituitary cultures (DP cells)

increased cAMP by only 2-4 fold in both cases (Brain *et al*, 1991). It is interesting that the responses of $P_0$ and DP cells do not differ greatly despite their origins being *dw/dw* and control cultures. It is possible that both these cell lines represent immortalized mammosomatotroph precursor cells and similar cells may be present in the mature *dw/dw* due to a developmental block preventing somatotroph commitment.

# SOMATOTROPH SPECIFIC PROTEIN MARKERS

## 6.1 INTRODUCTION

This chapter describes some attempts made to identify protein markers, other than GH, for somatotroph function. GH content of somatotrophs fluctuates according to it's relative synthesis and release, thus it could be considered an unreliable marker.

Such a search has been done before. Yokoya and Friesen (1986) identified 17 secreted proteins of 13-22kDa from monolayer pituitary cultures, by 2D gel-electrophoresis, whose secretion was influenced by GRF and SS. 14 of these showed rGH-LI and probably represented GH variants and fragments. In addition, three 16kDa non GH-LI proteins were identified, with isoelectric points (pI) of 7.6, 7.0 and 6.7. Six of the GH-LI proteins and one of the non GH-LI (pI 7.6) were synthesized rapidly *de novo*, shown by pulse chase analysis, and three of the GH-LI and two of the non immunoreactive proteins were found in cell extracts.

An antibody which was raised to one of the three 16kDa proteins (designated peptides 23, 24 and 25), recognized all three, suggesting that homology exists between these proteins (Yamamoto *et al*, 1992) which show no homology with GH (Tachibana *et al*, 1988). Sequencing of peptide 23 revealed complete sequence homology with pancreatitis-associated protein (PAP), a member of the C-type lectin superfamily (Katsumata *et al*, 1995). Immunoreactivity was localized to a sub-population of endocrine cells in the rat pyloric gland and pancreatic islets in addition to the anterior pituitary. Double immunofluorescence revealed peptide 23 to be located only in GH containing cells, however only about 15% of somatotrophs showed peptide 23 like immunoreactivity. To act as a marker, a protein would have to be expressed in all somatotrophs, thus this is not a good candidate.

In another study, Ivarie *et al* (1980) detected a 16kDa protein in $GH_3$ cells which was increased in response to $T_3$ or dexamethasone. This non GH-LI protein was later found to be a carboxy-terminal cleavage product of GH (Davis *et al*, 1987).

Another reported candidate protein is Fetal Antigen 1 (FA1), a 32-38kDa glycoprotein in the EGF superfamily (Larsen *et al*, 1996). This secreted protein was co-localized with GH in 5 separate human pituitary samples. The mouse FA1 was purified from amniotic fluid, and found to be a 42-50kDa glycoprotein with 74% homology to human FA1, and 100% homology to translated cDNA for preadipocyte factor 1 (Pref-1: Krogh *et al*, 1997). Immunocytochemistry localized Pref-1 to the endocrine structures of the mouse pancreas, adrenal and pituitary gland (Bachmann *et al*, 1996).

An obvious candidate for a somatotroph specific protein marker is the GRF receptor. GRF-R mRNA has been shown to be reduced in the *dw/dw* pituitary (Carmignac *et al*, 1996), however detection of the protein would be impractical due to vanishingly low levels.

French *et al* (1996) recently reported that, within the anterior pituitary of the mouse, the interleukin-1 (IL-1) receptor was restricted to the somatotrophs. The IL-1 receptor exists as two subtypes, IL-1RI (80kDa) and IL-1RII (68kDa), the latter of which has a shorter carboxy terminus, and is thought to be non-signalling. Both subtypes were shown to be co-localized with GH in the mouse. If this is also the case in the rat, IL-1R could be used as a marker protein for somatotrophs. IL-1 binding sites have been shown to be present in the anterior pituitary of the rat by binding studies (Marquette *et al*, 1995). Binding sites were also detected in the posterior pituitary in the rat, which were not seen in the mouse by *in situ* hybridization (Cunningham *et al*, 1992).

Reports on the effects of IL-1 on hormone secretion are contradictory. The major effect *in vivo* is on the hypothalamo-pituitary-adrenal (HPA) axis. It is thought that IL-1 effects on the HPA are mainly hypothalamic (Tsagarakis, *et al*, 1989; Barbanel *et al*, 1990; Suda *et al*, 1990; Navarra *et al*, 1991) and IL-1 binding sites have been localized on the walls of the third ventricle (Marquette *et al*, 1995). However, CRF treatment has been shown to up-regulate the IL-1R levels in the mouse pituitary (Takao *et al*, 1995) and also on the corticotroph cell line AtT-20 (Takao *et al*, 1994). Direct ACTH release is also reported from pituitary cells in culture (Bernton *et al*, 1987; Kehrer *et al*, 1988) and from perfused pituitary cells (Beach *et al*, 1988). Uehara *et al* (1987) did not find release of any pituitary

hormones in culture, apart from a small synergism of IL-1 with CRF on ACTH release.

The effect on GH secretion is also under dispute. In contrast to Uehara *et al* (1987), there are several reports of direct GH release from pituitary cells in culture (Bernton *et al*, 1987; Kehrer *et al*, 1988), from perfused pituitary cells (Beach *et al*, 1988) and by RHPA (Niimi *et al*, 1994). The *in vivo* effects on the growth axis are even more contradictory. Both stimulation (Rettori *et al*, 1987) and inhibition (Wada *et al*, 1995) of GH release are reported. Payne *et al* (1992) found that small doses of IL-1β (2.5ng) caused a stimulation and large doses (25ng) administered icv caused an inhibition of GH release. This can be explained by the fact that IL-1β causes a stimulation of both GRF and SS (Honegger *et al*, 1991; Scarborough *et al*, 1989).

This chapter covers some work aiming to screen for new somatotroph specific proteins. Due to the deficiency in somatotrophs in the *dw/dw*, proteins were screened for that were more abundant in AS than *dw/dw* pituitary and pituitary culture extracts. In addition, proteins up-regulated by GRF, which were likely to be involved in somatotroph function, were screened for using radioactive amino acid incorporation studies. This chapter also reports some preliminary work done to investigate the possibility of IL-1R expression within the rat anterior pituitary being restricted to the somatotroph, as it is in the mouse.

## 6.2 EXPERIMENTAL RESULTS

### 6.2.1 Experiment 1: SDS-PAGE of AS and *dw/dw* Pituitary Extracts

Anterior pituitaries from male AS and *dw/dw* rats were extracted in 1ml D-MEM + CPI. The soluble protein content of the extracts was measured by Lowry protein assay. The equivalent 10μg of protein per extract was separated on a miniature 12% acrylamide gels and visualized by Coomassie blue or silver stain plus.

Figure 6.1 shows AS and *dw/dw* extracts separated by SDS-PAGE and visualized by Coomassie blue. The GH band was fainter in the *dw/dw* than AS lane, however the PRL band was considerably stronger in the *dw/dw* than AS

lane. It was this consistent finding that prompted us to re-investigate the PRL axis in the *dw/dw* as described in chapters 3 and 4.

Figure 6.2 shows a larger SDS-PAGE gel, this time silver stained. Again lanes 1 and 2 are AS and *dw/dw* anterior pituitary extracts. Between the 66.2kDa and 45kD markers, some differences in the proteins can be seen. These will be referred to hereafter as ~50kDa bands. Band 1 was darker in the AS than in the *dw/dw*. Band 2, just below band 1, was darker in the *dw/dw* than the AS. There were however, no obvious bands missing in the *dw/dw* extract.

**Figure 6.1: SDS-PAGE of AS and *dw/dw* pituitary extracts: Coomassie blue.**

10μg of protein were separated on a miniature 12% acrylamide gel by SDS-PAGE and stained by coomassie blue.

M: Marker.

1. AS extract.
2. *dw/dw* extract.

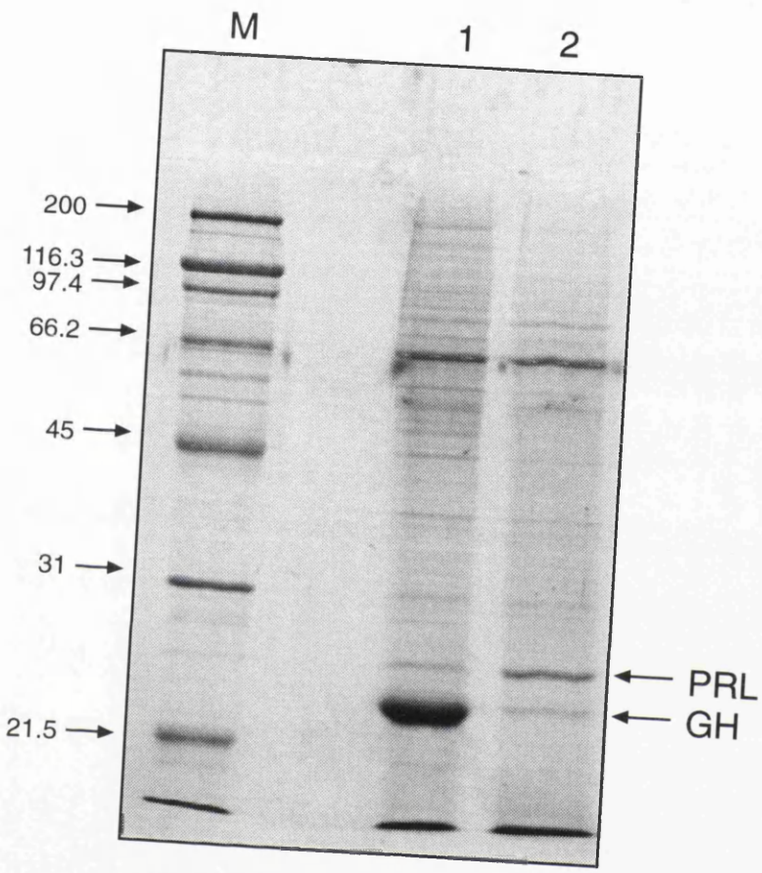
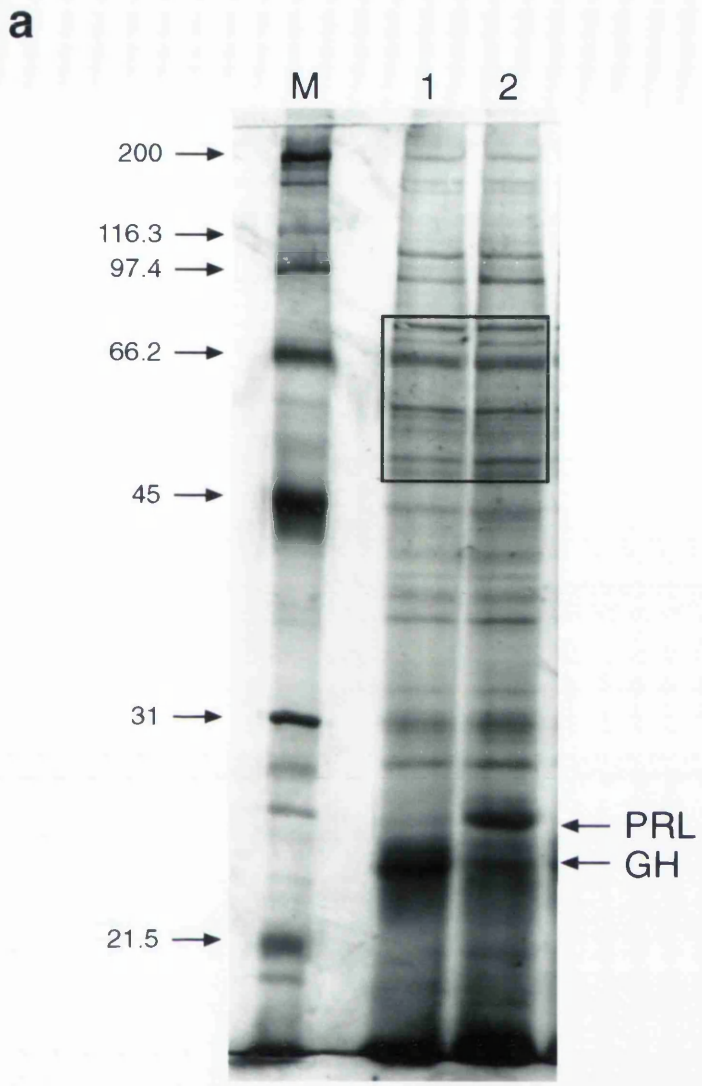

M
1
2
200
116.3
97.4
66.2
45
31
21.5
PRL
GH

**Figure 6.2: SDS-PAGE of AS and *dw/dw* pituitary extracts: Silver stained.**

10μg of protein were separated on a large 12% acrylamide gel by SDS-PAGE and silver stained. Panel (b) shows the area between the 66.2 and 45kDa markers enlarged.

M: Marker.
Lane 1: AS extract.
Lane 2: *dw/dw* extract.


a
M
1
2
200
116.3
97.4
66.2
45
31
21.5
PRL
GH
b
1
2
66.2
1
2

### 6.2.2 Experiment 2: Double Radiolabel of Primary Cultures

Differential protein synthesis in the presence or absence of GRF was investigated by double radio-labelled amino acid incorporation. The strategy involved the use of different isotopes ([$^{35}$S]methionine or [$^{3}$H]methionine) included in different treatments (methionine free D-MEM with or without 10nM GRF) and is summarized in figure 6.3a.

Anterior pituitaries were cultured for 5 days in 6-well plates at one pituitary per well. The cells were washed twice with methionine free D-MEM and then incubated in 1ml methionine free D-MEM with or without 10nM GRF and with 0.74MBq [$^{3}$H]methionine or [$^{35}$S]methionine per well for three hours. Where the specific activity of the labelled methionine differed, cold methionine was added with the higher specific activity methionine to balance the treatments. The experiment was performed in both possible permutations, ie. $^{3}$H.control *vs.* $^{35}$S.GRF and $^{35}$S.control *vs.* $^{3}$H.GRF in order to control for differential amino acid incorporation. Proteins unaffected by GRF would be labelled to the same extent by each isotope, however proteins whose synthesis is stimulated by GRF would label more strongly with the isotope included in the GRF treatment. At the end of the treatment, the medium was collected separately (and pooled later), but the cells from control and GRF treated wells were pooled during extraction in D-MEM + CPI.

The extracts were separated by reverse phase HPLC and 1 minute fractions collected. 200µl of the fractions were transferred to scintillation vial inserts and 5ml Ready Safe scintillation cocktail added (Beckman, Fullerton, CA). The vials were capped, inverted to mix and the $^{35}$S and $^{3}$H counted for up to 10 mins per vial (Beckman LS 5000 CE). Due to an overlap in the energies of $^{35}$S and $^{3}$H, some of each isotope would be detected in the counting window of the other. By counting a standard curve containing just $^{35}$S and just $^{3}$H, a correction factor was determined to eliminate this overlap, which was more significant for $^{35}$S counts detected in the $^{3}$H channel than the converse;

$$^{35}\text{S counts in the } ^{3}\text{H channel} = 0.612 \times {}^{35}\text{S cpm}^{0.953}$$

$$^{3}\text{H counts in the } ^{35}\text{S channel} = {}^{3}\text{Hcpm} \times 0.01$$

Once the corrected counts had been calculated, the ratio of counts per minute (cpm) of the isotope with GRF: cpm of the control isotope were plotted against fraction number. GRF stimulated proteins would be visible as peaks superimposed on a baseline ratio.

Figure 6.4 shows an example the results obtained from the $^{3}H$ and $^{35}S$ counts of the HPLC fractions. The corrected counts per fraction in the $^{3}H$ and $^{35}S$ channels are shown by the bars and the ratio of GRF cpm: control cpm shown by the line. A peak in this ratio would indicate a protein incorporation specifically stimulated by GRF, however, there were no peaks in the ratio that were consistent in all experiments. In all cases the $^{3}H$.control *vs.* $^{35}S$.GRF showed a peak at fraction 19-20, whereas a trough was seen in the $^{35}S$.control *vs.* $^{3}H$.GRF experiments. This suggests that this was an isotope related artefact as the $^{35}S$ counts were always higher in this fraction. This artefact was even more apparent in the conditioned medium plots (Figure 6.5). 250µl each of medium from control and GRF wells (either $^{3}H$.control + $^{35}S$.GRF or *vice versa*) were mixed and the proteins separated by HPLC. The ratio of the isotopes showed a very erratic baseline, and most of the peaks on one plot ware mirrored by troughs on the other. The only peak that appeared to be more consistent is at fraction 47, and represents GH itself. Even this peak, which should act as a positive control, was not present in all experiments.

250µl each of $^{3}H$ and $^{35}S$ treatment media (without incubating on cells) were also separated by HPLC. Figure 6.6 shows the cpm per fraction of $^{35}S$ and $^{3}H$ in the fractions. Fractions 11-27 showed more $^{35}S$ than $^{3}H$, indicating that the artefact was present in the treatment medium and was not related to the cells.

Due to the difficulties encountered with this method, and the variability of the results, this strategy was abandoned in favour of a similar method (outlined in figure 6.3b) where the protein extracts were separated by SDS-PAGE, described in experiment 3.

**Figure 6.3: Flow diagram of double and single radiolabelled amino acid incorporation experiments.**

Primary cultures were plated at 1 pituitary per well in 6 well plates and maintained for 5 days. The cells were washed twice with methionine free D-MEM and then incubated for 3 hours with the treatments shown on the figure. The medium was then removed and the cells washed in ice cold PBS and then extracted as shown for subsequent protein analysis by HPLC or SDS-PAGE. Each group consisted of 3 wells, thus in the case of the double label experiment, each extract contained protein from 6 wells of cells. The medium from the double label experiment was also combined and analysed by HPLC.

a. Experiment 2: double label.
b. Experiment 3: single label.

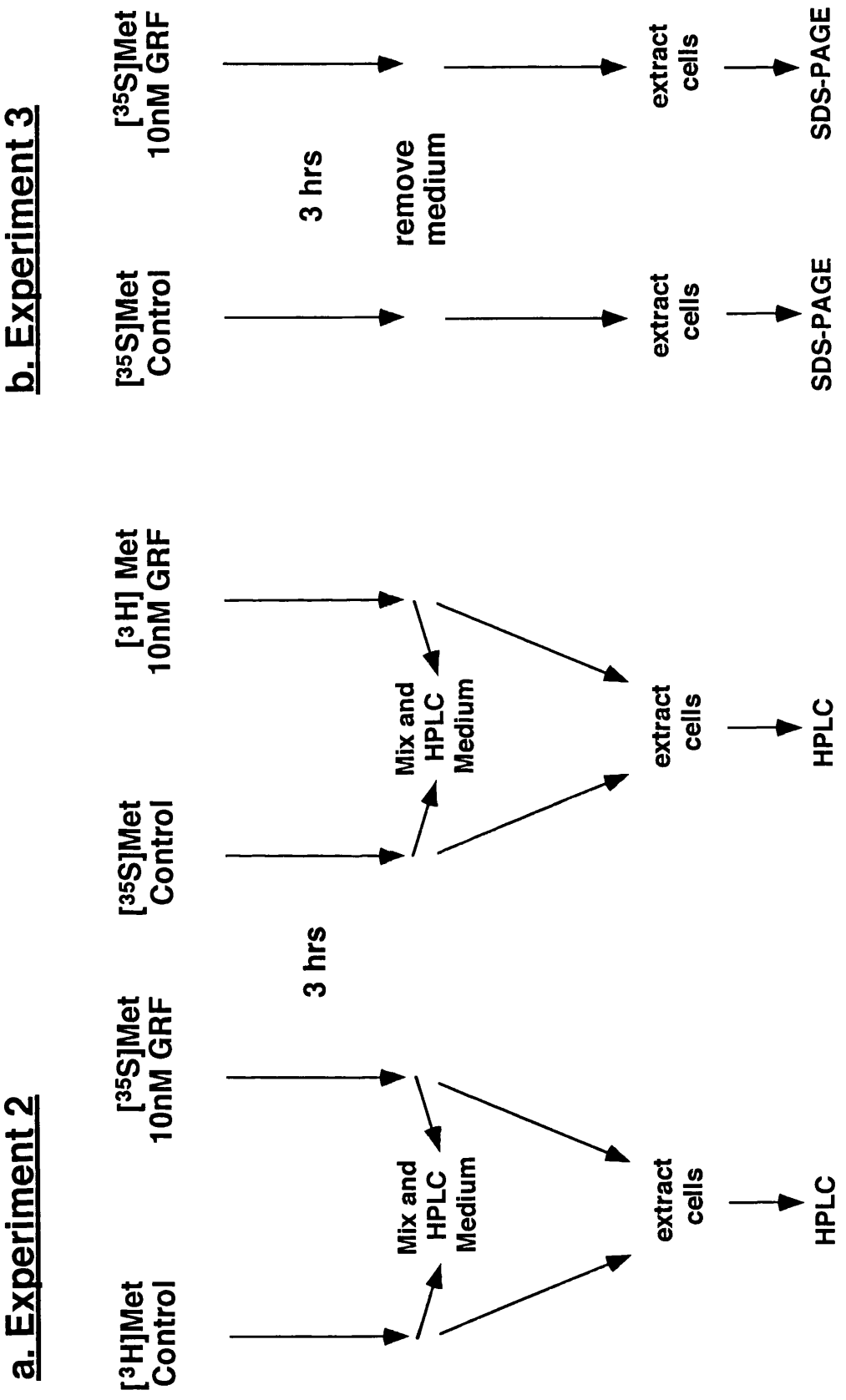
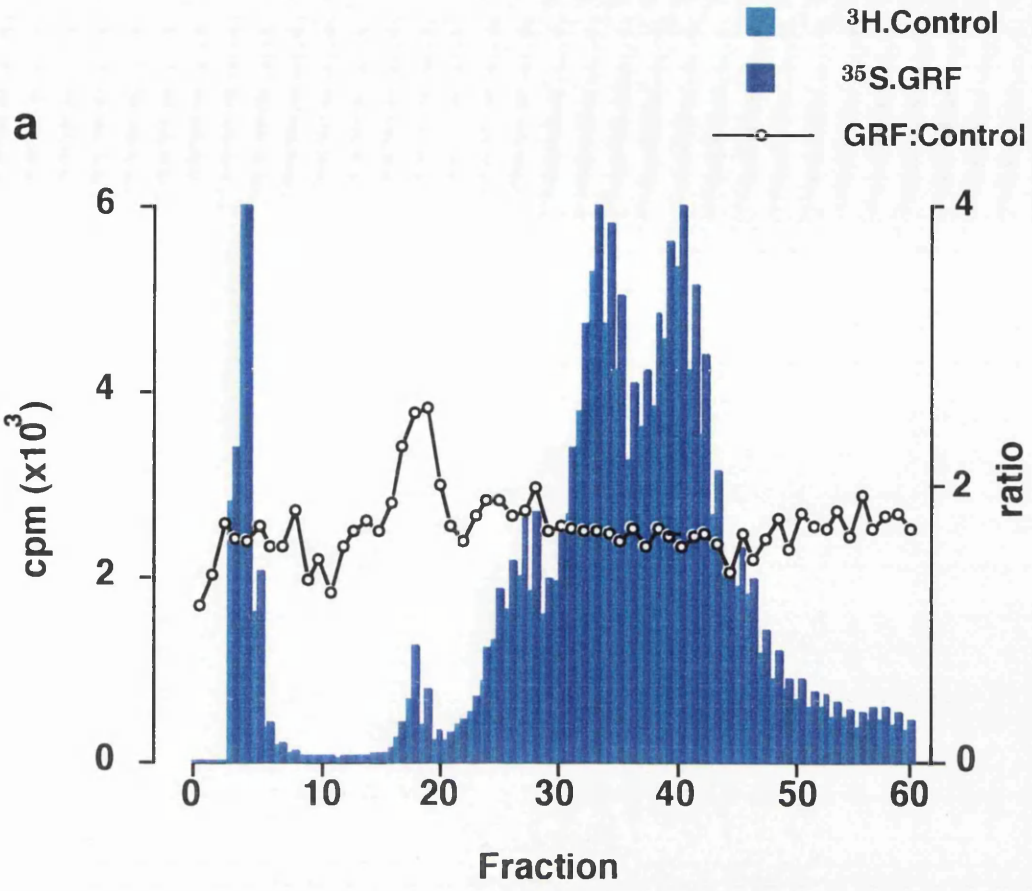

a. Experiment 2
[3H]Met Control
[35S]Met 10nM GRF
3 hrs
Mix and HPLC Medium
extract cells
HPLC
[35S]Met Control
[3 H] Met 10nM GRF
Mix and HPLC Medium
extract cells
HPLC
b. Experiment 3
[35S]Met Control
[35S]Met 10nM GRF
3 hrs
remove medium
extract cells
SDS-PAGE
extract cells
SDS-PAGE

**Figure 6.4: HPLC of double radiolabelled AS primary culture extracts.**

100μg of protein were separated by HPLC. 1 minute fractions were collected and 200μl of each were counted for $^{3}H$ and $^{35}S$. The total counts per fraction for $^{3}H$ and $^{35}S$ are shown by bars and the ratio of GRF cpm: Control cpm is shown by the line. Therefore peaks in this ratio represent proteins more heavily labelled in the GRF group.

a. $^{3}H$.Con *vs.* $^{35}S$.GRF

b. $^{35}S$.Con *vs.* $^{3}H$.GRF


a
$^{3}$H.Control
$^{35}$S.GRF
GRF:Control
cpm (x10$^{3}$)
ratio
Fraction
0
2
4
6
0
10
20
30
40
50
60

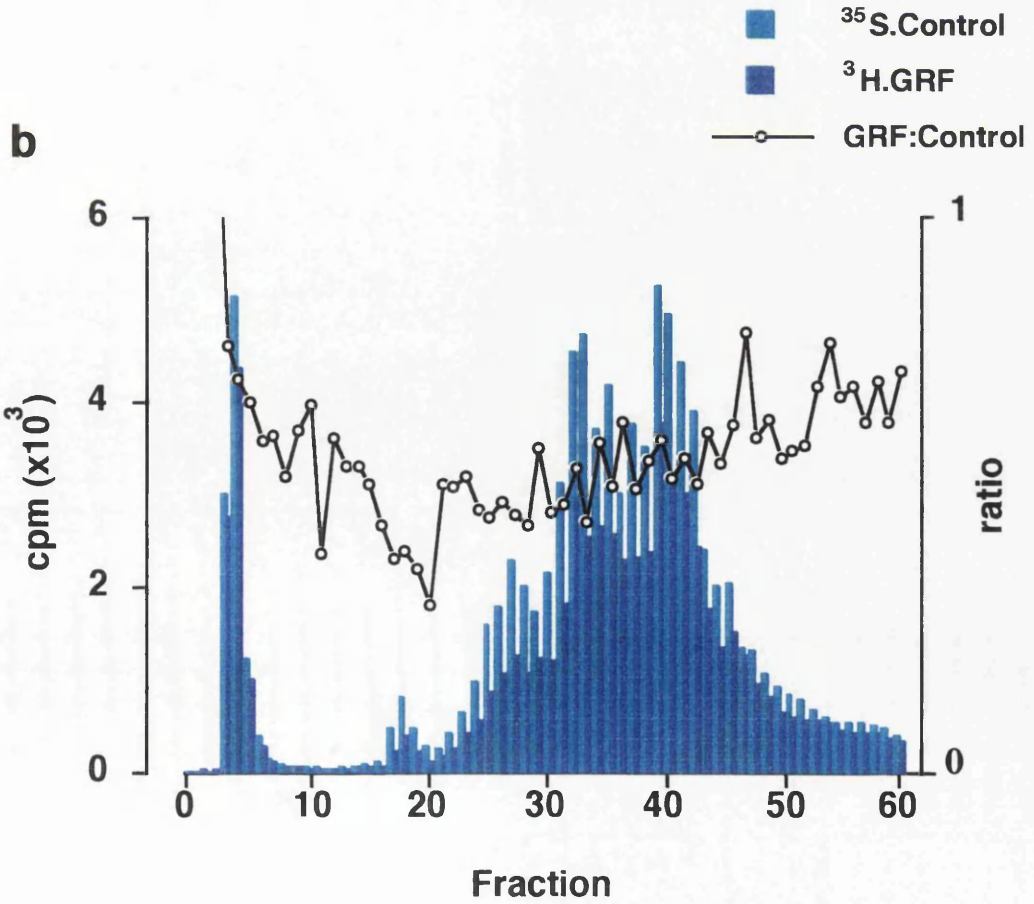
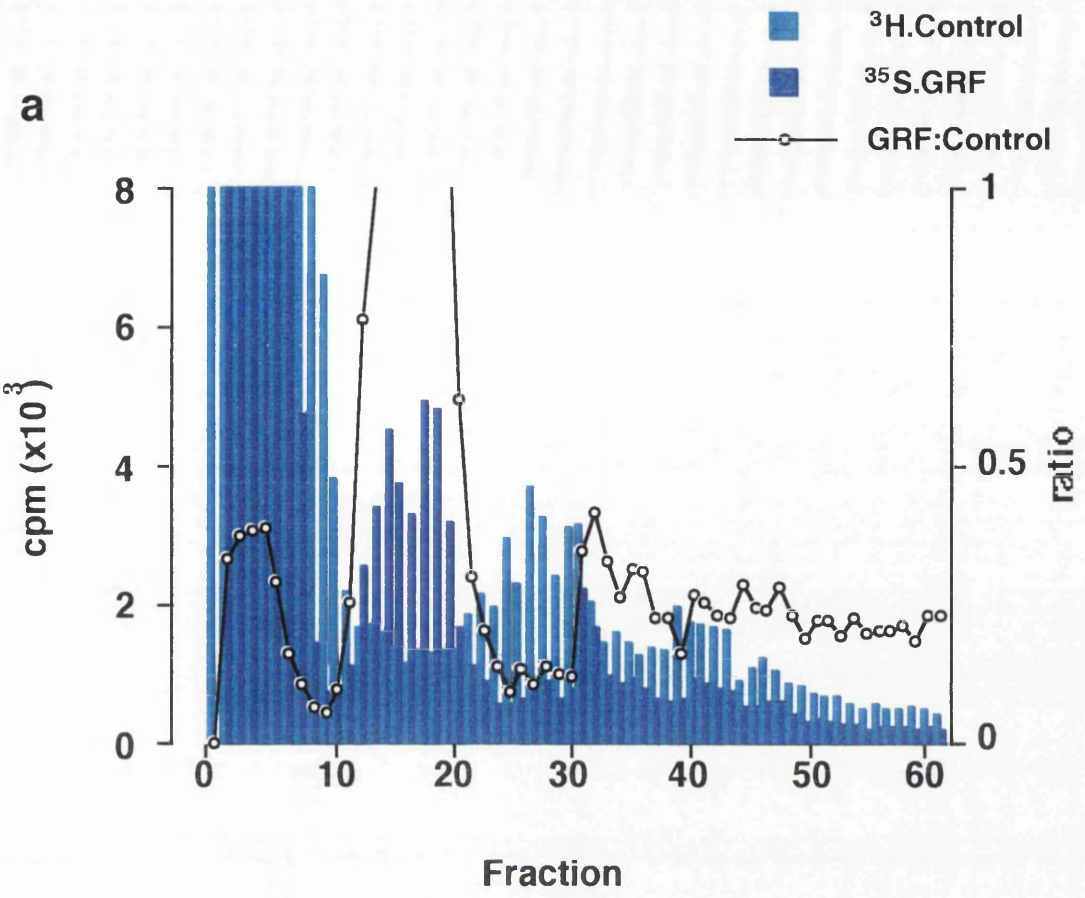

b
$^{35}$S.Control
$^{3}$H.GRF
GRF:Control
cpm (x10$^{3}$)
ratio
Fraction
0
1
2
4
6
0
10
20
30
40
50
60

**Figure 6.5: HPLC of double radiolabelled AS primary culture conditioned medium.**

250µl of control and GRF medium (with different isotopes) were mixed and separated by HPLC. 1 minute fractions were collected and 200µl of each were counted for $^{3}H$ and $^{35}S$. The total counts per fraction for $^{3}H$ and $^{35}S$ are shown by bars and the ratio of GRF cpm: Control cpm is shown by the line. Therefore peaks in this ratio represent released proteins more heavily labelled in the GRF group.

a. $^{3}H$.Con *vs.* $^{35}S$.GRF

b. $^{35}S$.Con *vs.* $^{3}H$.GRF


a
$^{3}$H.Control
$^{35}$S.GRF
GRF:Control
cpm (x10$^{3}$)
ratio
Fraction
0
2
4
6
8
0
0.5
1
0
10
20
30
40
50
60

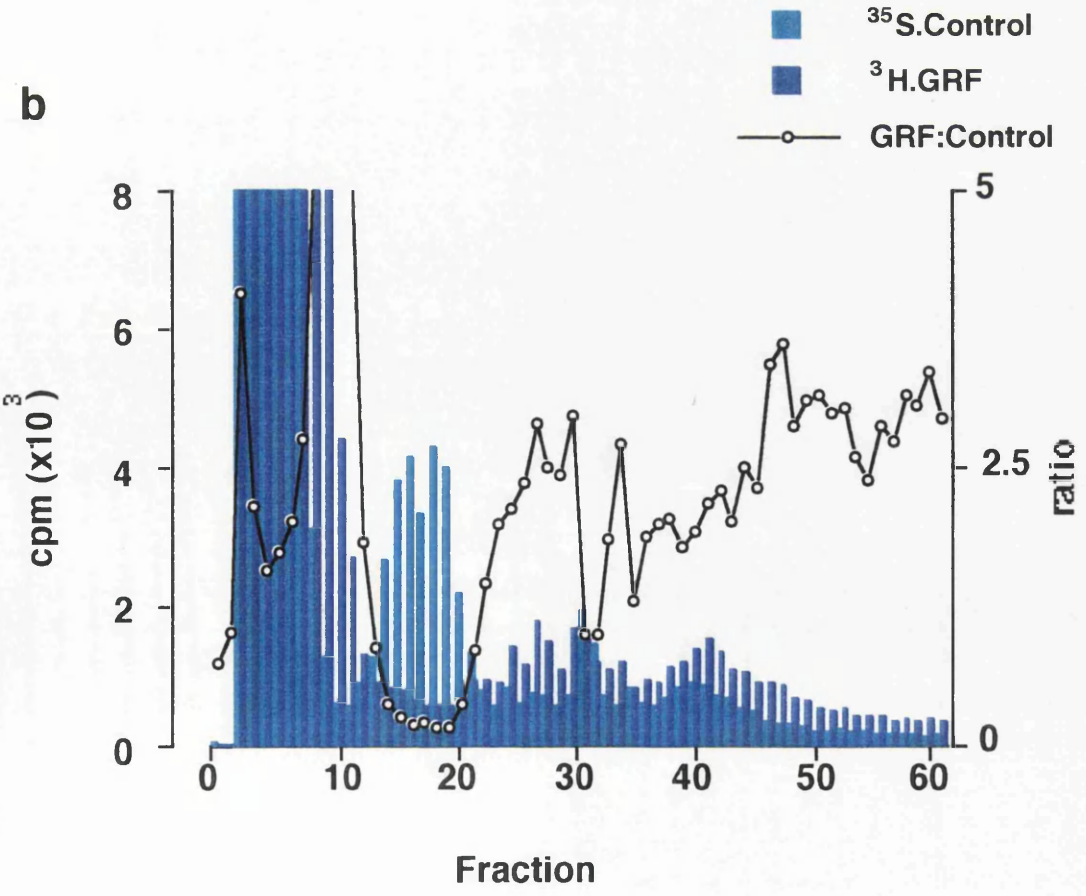
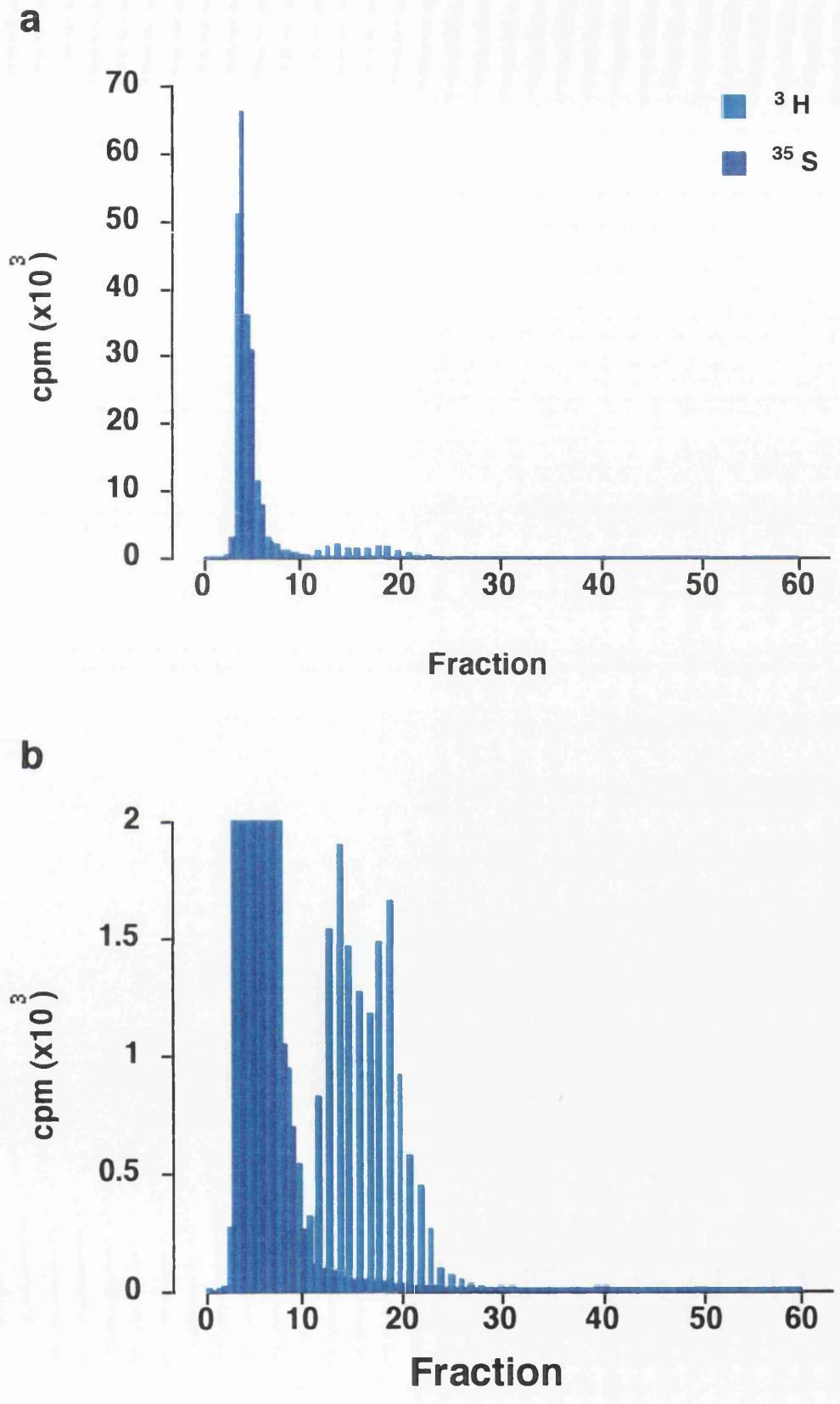

b
$^{35}$S.Control
$^{3}$H.GRF
GRF:Control
cpm (x10$^{3}$)
ratio
Fraction
0
2
4
6
8
0
2.5
5
0
10
20
30
40
50
60

**Figure 6.6: HPLC of [$^{3}$H] and [$^{35}$S] methionine treatment medium.**

250µl of each [$^{35}$S] and [$^{3}$H] methionine treatment medium (without incubating on cells) were mixed and separated by HPLC. 1 minute fractions were collected and 200µl of each were counted for $^{3}$H and $^{35}$S. The total counts per fraction for $^{3}$H and $^{35}$S are shown by bars. The scales of the Y-axis differ between panels (a) and (b), which show the same data.


a
70
60
50
40
30
20
10
0
cpm (x10 3)
0
10
20
30
40
50
60
Fraction
3 H
35 S
b
2
1.5
1
0.5
0
cpm (x10 3)
0
10
20
30
40
50
60
Fraction

### 6.2.3 Experiment 3: Single Radiolabel of Primary Cultures

Anterior pituitaries were cultured for 5 days in 6-well plates at one pituitary per well. The cells were washed twice with methionine free D-MEM and then incubated in methionine free D-MEM with or without 10nM GRF and with 0.74MBq [$^{35}$S]methionine per well for three hours. The medium was collected and the cells extracted in D-MEM + CPI. 10μg protein was separated by SDS-PAGE, and the gel soaked in 1M sodium salycilate (BDH) for 15 mins, dried and laid down on Biomax MR single emulsion film (Kodak, Rochester, NY) for 1-7 days as necessary.

Figure 6.7a shows an autoradiograph of SDS-PAGE separated $^{35}$S labelled extracts from AS cultures. The most noticeable difference between the lanes is the lighter GH band in the GRF lane, suggesting that the labelled GH was released from the cells. In addition, a band running just ahead of the 97.4kDa marker (hereafter referred to as ~95kDa) was more intensely labelled in the GRF extracts than the control extracts. When this experiment was repeated using *dw/dw* cultures, (figure 6.7b), the ~95kDa protein also appeared to be up-regulated by GRF, although the band intensity appeared to be lower than in GRF stimulated AS extracts. In addition, notice the reduced intensity of the GH bands in the *dw/dw* extracts, illustrating the reduced rate of GH synthesis is the *dw/dw* culture.

Figure 6.8 shows a silver stained acrylamide gel from a similar experiment. In this case, the ~95kDa band was up-regulated by GRF only in the AS culture. The *dw/dw* culture showed a fainter band in the control extract than the AS, and no increase in intensity after GRF treatment. In addition, there was a band running just behind the 31kDa marker which was darker in the AS lanes than the *dw/dw* lanes. This band appeared to represent a protein of about 35kDa. It is difficult to say if the intensity differs between the control and GRF treated extract, as there was a pressure mark from handling the gel at that point on the control lane. The band running with the 66.2kDa marker, which was darker in the AS GRF and the *dw/dw* control lanes was BSA, probably due to insufficient washing of the culture after the removal of the treatment medium which contained 0.1% BSA.

In order to identify the candidate proteins, attempts were made to extract the ~95KDa band and the GH band from the acrylamide gel as described in section 2.6.7. The GH band was used as a positive control for the method as it is a smaller, more abundant protein and should be more easily isolated. The concentrated proteins obtained were run on SDS-PAGE and silver stained. Unfortunately, no staining of the protein was seen, indicating that the extraction procedure was not successful.

**Figure 6.7: Autoradiograph of SDS-PAGE separated $^{35}S$ labelled extracts: AS.**

20μg of protein were separated on a large 12% acrylamide gel. The gel was soaked in 1M sodium salicylate for 15 minutes, dried and laid down on film for 3 days.

a. AS
Lane 1: AS Control
Lane 2: AS GRF

b. *dw/dw*
Lane 3: *dw/dw* Control
Lane 4: *dw/dw* GRF

The GH band is lighter in the GRF treated than control AS lane, indicating that the newly labelled GH was released. The *dw/dw* lanes have much fainter GH bands, illustrating the reduced GH synthesis in *dw/dw* cultures. The bottom panels show areas of the gels enlarged to more easily show the up-regulation of a protein running just ahead of the 97.4kDa marker.

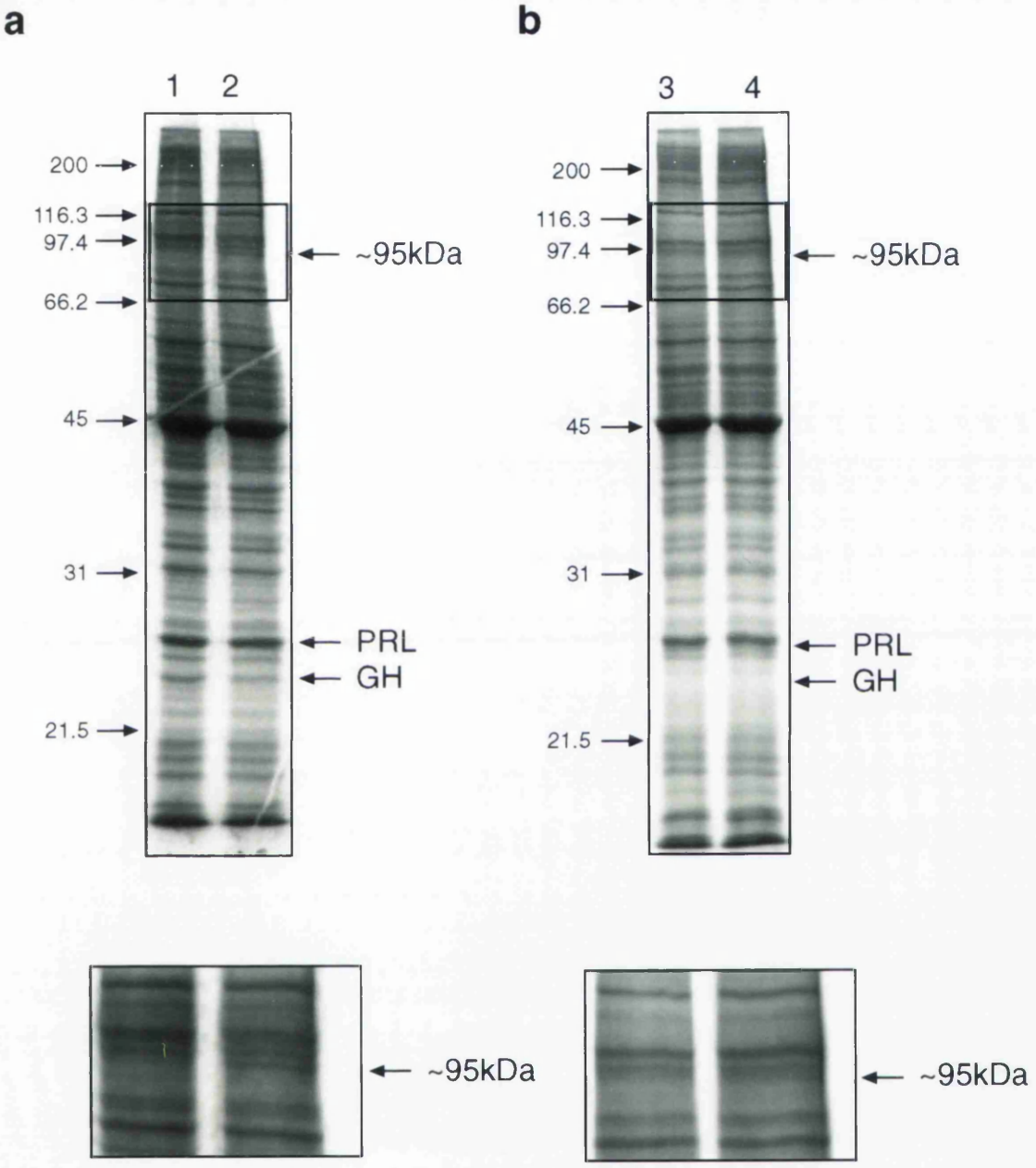
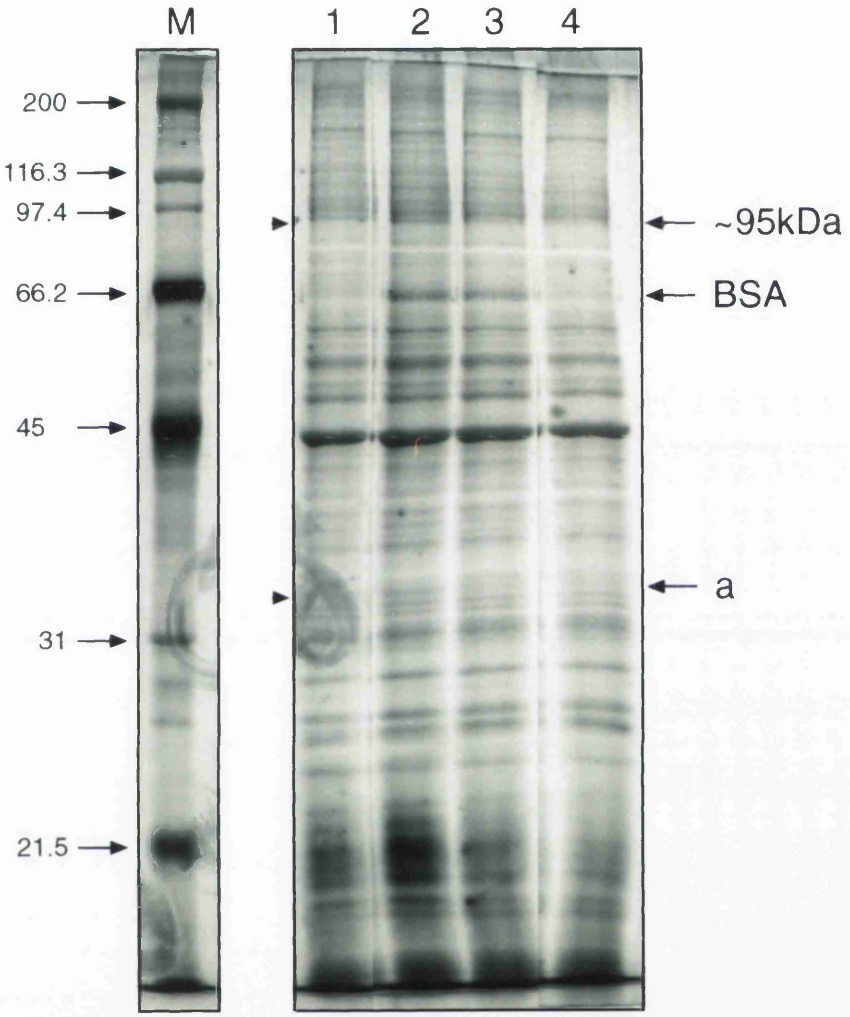

a
b
1
2
3
4
200
116.3
97.4
66.2
45
31
21.5
~95kDa
PRL
GH

**Figure 6.8: SDS-PAGE of AS and *dw/dw* primary culture extracts: Silver stained.**

AS and *dw/dw* pituitary cells were cultured for 5 days and then incubated for 3 hours with or without 10nM GRF. The cells were washed and extracted in D-MEM + CPI. 10μg of protein was separated by SDS-PAGE on a large 12% acrylamide gel and subsequently silver stained.

M: Marker
Lane 1: AS Control
Lane 2: AS GRF
Lane 3. *dw/dw* Control
Lane 4. *dw/dw* GRF


M
1
2
3
4
200
116.3
97.4
66.2
45
31
21.5
~95kDa
BSA
a

### 6.2.4 Experiment 4: Investigation of the Interleukin-1 Receptor as a Candidate Somatotroph Marker

Attempts were made to investigate the presence of IL-1R by immunocytochemistry, FACS analysis and Western blotting using an IL-1RI (M20) rabbit polyclonal antibody. The antibody was raised against the carboxy terminus of the precursor of mouse IL-1R type I and is therefore not cross-reactive with the non-signalling type II receptor which has a shorter carboxy terminus.

Several attempts were made at immunocytochemistry for IL-1R. The starting method was as described for GH and PRL in section 2.4.3. The secondary antibody was the same as for PRL immunocytochemistry: biotinylated swine-anti-rabbit used at 1:200. The primary antibody is immuno-reactive with both rat and mouse protein, so mouse pituitaries were also used in an attempt to repeat the observations of French *et al* (1996). Rat spleen was also used as a control tissue, as it is known that it possesses IL-1R.

Some progress was made in optimising the conditions of immunocytochemistry for IL-1R by trying different permutations of the method. It was found that TBS used for GH and PRL immunocytochemistry did not allow staining of IL-1R. Instead, PBS was used (9.1mM $Na_2HPO_4$, 1.7mM $NaH_2PO_4.2H_2O$, 150mM NaCl, pH to 7.4 with NaOH). It was also necessary to omit the triton wash, which is used in the GH and PRL immunocytochemistry to permeablize the membranes and allow the antibodies access to the cytosol. This may be because IL-1R is a membrane receptor protein and needs the membrane to be intact for staining to occur. The method worked best with the trypsin wash included, a step that breaks cross-linking of the antigen caused by the fixation process.

The preparation of the tissue was also investigated. As well as paraffin embedded tissue, fresh frozen tissue sections were also used. These were post-fixed with either 4% paraformaldehyde or acetone. In addition, different methods were tried for visualization of the antigen, (DAB and fast red). The method of fixation or the method of visualization did not affect the results.

The primary antibody was titrated between 0.1-2μg/ml as suggested on it's accompanying data sheet. It was found that 0.5μg/ml was the lowest effective concentration for immunocytochemistry. Even after optimization of the method, staining was restricted to very few cells in rat spleen, rat pituitary and mouse pituitary. The results were also inconsistent and not reproducible. Examples of the staining obtained in spleen and rat pituitary are shown in figure 6.9.

FACS analysis was carried out under various conditions. Rat anterior pituitary cells and rat spleen cells were prepared by dispersion either with or without trypsin, as described in section 2.2.1. The cells were stained for IL-1R using either 1μg/ml or 5μg/ml primary antibody. The secondary antibody was again the same as for PRL staining: FITC conjugated swine-anti-rabbit used at 1:20. The background staining was determined using NRS instead of the primary antibody.

Figure 6.10 shows FACS plots of anterior pituitary cells for IL-1R under different conditions. The dispersion was carried with or without trypsin followed by analysis in FACS buffer + azide or saponin. The cells stained in FACS buffer + azide after dispersion without trypsin (panel a) showed a slight increase in fluorescence above background with both 1 and 5μg/ml primary antibody. In all other combinations, increased fluorescence was only seen with 5μg/ml antibody. All further analyses were therefore carried out without trypsin or saponin.

Sodium azide may affect the binding of some antibodies, thus the use of FACS buffer with or without azide was compared. Figure 6.11 shows the comparison between FACS buffer alone or with azide on the staining of rat pituitary and spleen cells. The pituitary cells showed no difference between the 1 and 5μg/ml antibody either with or without azide. Although the fluorescence did not appear to be higher than the background (shown by M1 on the histograms), the profiles did show a shoulder on the histogram suggesting a higher fluorescence on a sub-population of 24-30% of pituitary cells (shown by M2 on the histograms). Furthermore, the background staining in this particular experiment was higher than normal suggesting that non-specific binding was occurring, possibly due to an old batch of NRS being used. It is therefore possible that there was fluorescence due to IL-1R staining, although the experiment would need to be

repeated to confirm this. The spleen cells did not show a shoulder in the fluorescence profiles, however the histograms were slightly shifted to the right of the background, the 5μg/ml antibody slightly more so than the 1μg/ml. It is not possible to say if the background staining of the spleen cells was higher than normal, as this was the only experiment including these cells. There were no differences between FACS buffer with or without azide on staining of either cell type, thus the poor staining could not be attributed to azide inhibiting antibody binding.

Double FACS analysis for GH and IL-1R was attempted by staining for intracellular GH in the presence of saponin followed by staining for IL-1R in FACS buffer + azide. The background staining with NRS and NMS replacing the primary antibodies is shown in figure 6.12a. This allowed the quadrant to be placed to set the background fluorescence. Panel (b) shows positive intracellular GH (y-axis) staining followed by background NRS staining (x-axis). The upper left quadrant contains the GH stained population consisting of 24% of the cells. The upper right quadrant contained 3% of the cells, representing the GH positive population that showed a FITC signal above the set background with NRS. Panel c shows positive intracellular GH staining followed by cell surface IL-1R staining. There is a slight increase in the percentage of cells in the upper right quadrant (double positives) compared to panel (b), although much less than expected if all the GH cells were to stain for IL-1R.

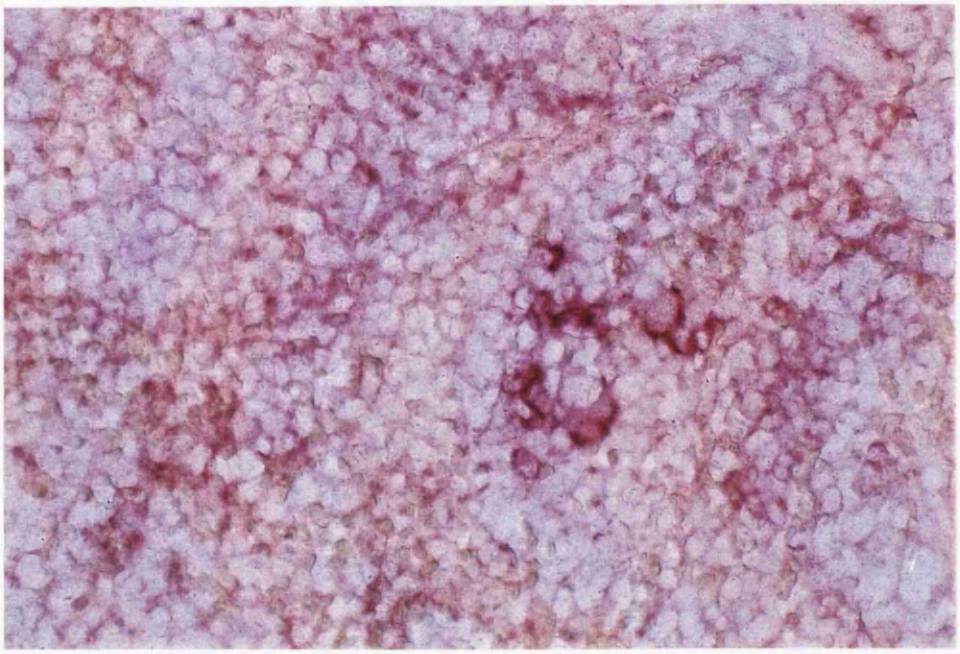
Attempts at Western analysis for IL-1R were also carried out. Pituitary extracts in D-MEM + CPI and culture extracts in RIPA + CPI along with the control peptide (that the antibody was raised against) were separated by SDS-PAGE and transferred to a PVDF membrane. The antigen was stained and visualized with DAB. Western analysis revealed a strong band in the lane with the control peptide running just ahead of the 66.2kDa BSA marker. Titration of the antibody showed that 0.5μg/ml primary antibody was sufficient for maximal staining. No specific (68 or 80kDa) bands were seen in the pituitary or culture extracts (data not shown).

**Figure 6.9: Immunocytochemistry for IL-1R in pituitary and spleen sections.**

a. IL-1R visualized by fast red in spleen sections.

b. IL-1R visualized by DAB in anterior pituitary and counterstained by haematoxylin.

a



b

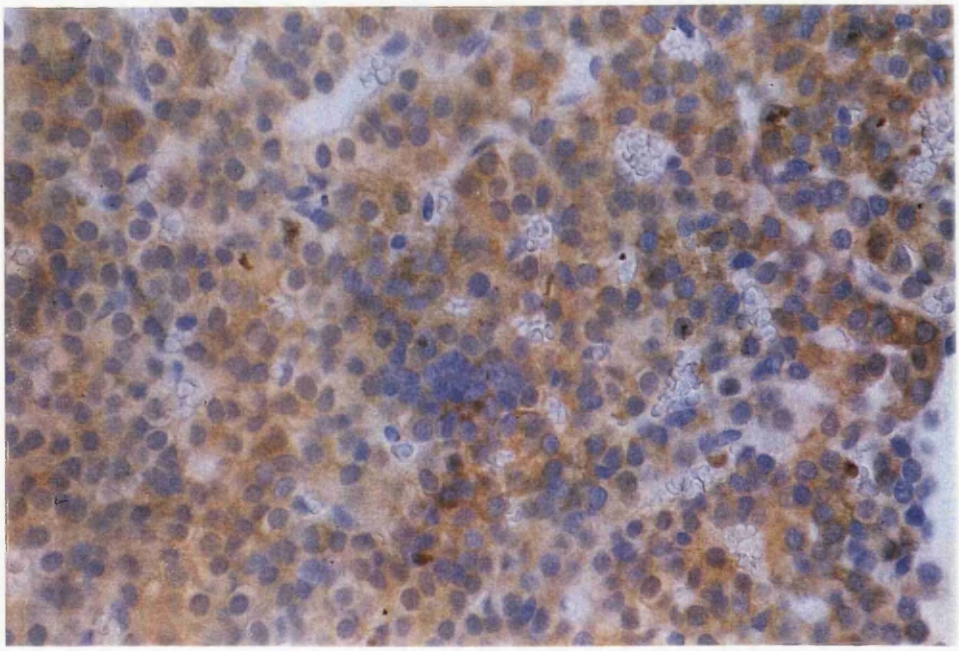


**Figure 6.10: FACS analysis of pituitary cells for IL-1R: Effect of dispersion method and buffer.**

Pituitary cells from AS rats were dispersed by collagenase and DNase with or without trypsin. The cells were fixed and then stained for IL-1R in FACS buffer + azide or FACS buffer + saponin. Both 5μg/ml (blue lines) and 1μg/ml (red lines) were used and the background FITC fluorescence (X-axis) was set using NRS instead of primary antibody (black line). M1 indicates the fluorescence above background.

a. Without trypsin, FACS buffer + azide.
b. With trypsin, FACS buffer + azide.
c. Without trypsin, FACS buffer + saponin.
d. With trypsin, FACS buffer + saponin.

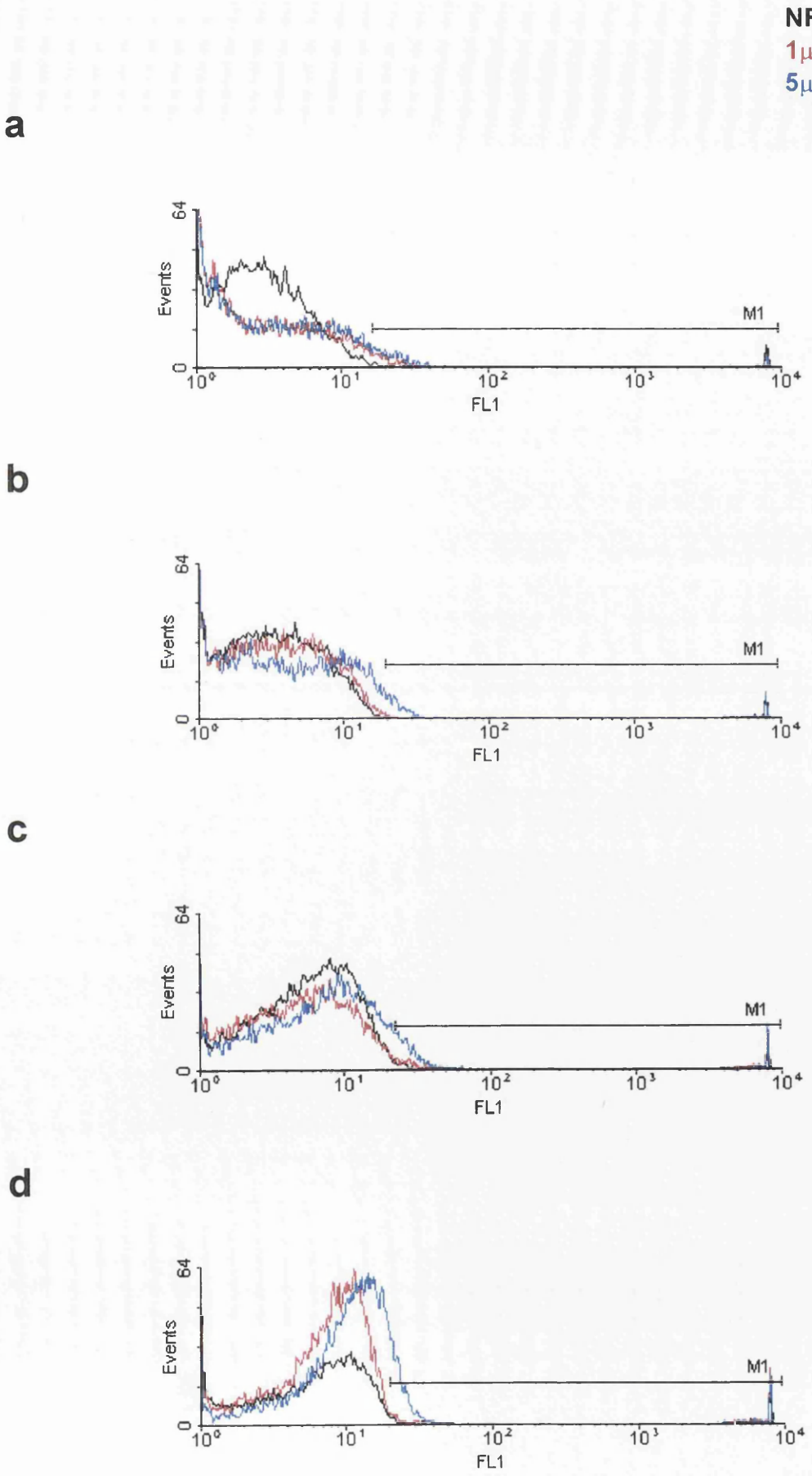

NRS
1μg/ml
5μg/ml
a
b
c
d
Events
64
0
M1
FL1
10⁰
10¹
10²
10³
10⁴

**Figure 6.11: FACS analysis of pituitary and spleen cells for IL-1R.**

Pituitary and spleen cells were dispersed without trypsin then fixed and stained for IL-1R in FACS buffer with or without sodium azide. Both 5μg/ml (blue lines) and 1μg/ml (red lines) were used and the background fluorescence was set using NRS instead of primary antibody (black line). M1 indicates the fluorescence above background. M2 represents the shoulder on the fluorescence histogram of pituitary cells, including 24-30% of the cells.

a. Pituitary cells, FACS buffer + azide.
b. Pituitary cells, FACS buffer.
c. Spleen cells, FACS buffer + azide.
d. Spleen cells, FACS buffer.

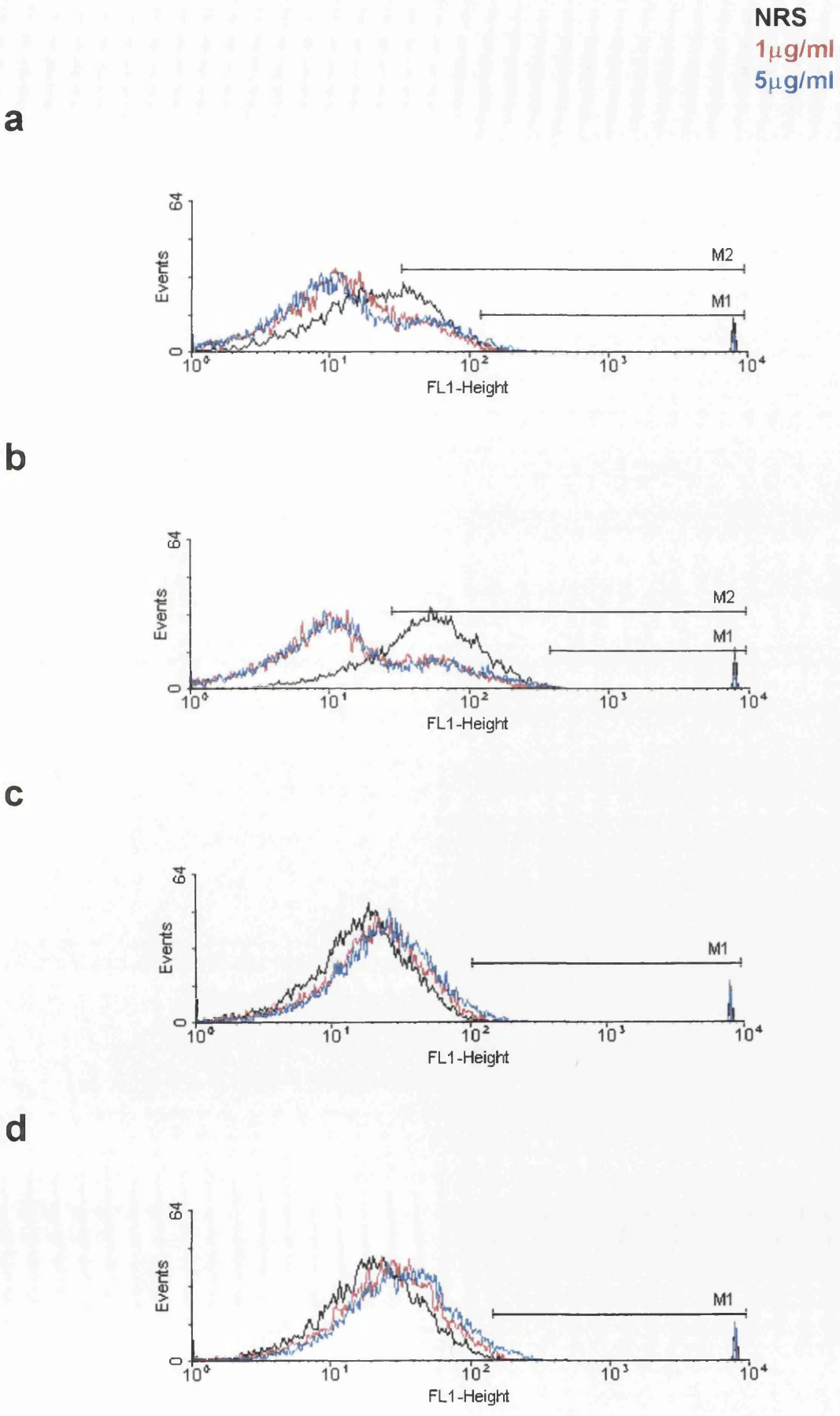
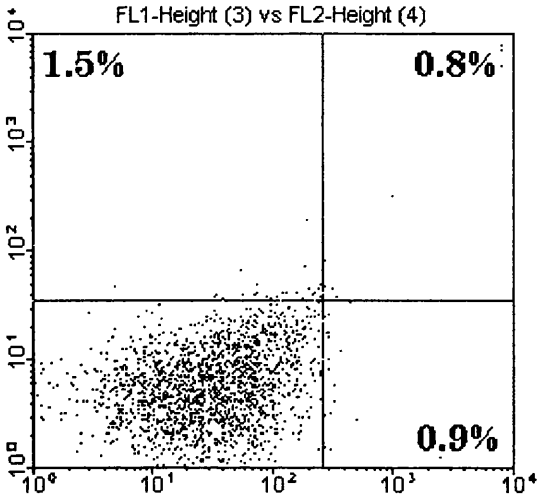

NRS
1μg/ml
5μg/ml
a
b
c
d
Events
64
0
M1
M2
FL1-Height

**Figure 6.12: FACS analysis of pituitary cells sequentially stained for GH and IL-1R.**

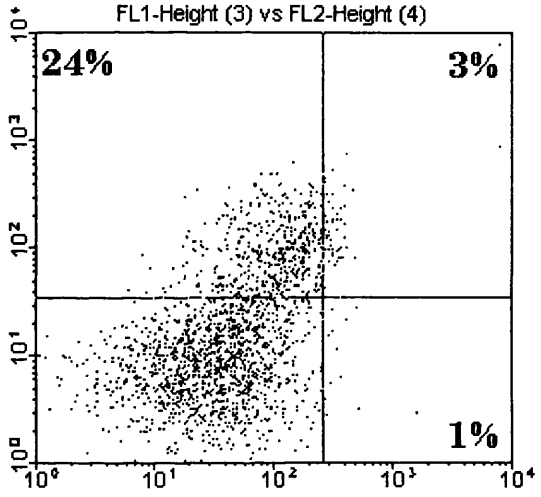
Anterior pituitary cells were fixed and then stained for GH in FACS buffer + saponin, followed by IL-1R in FACS buffer + azide. Primary antibodies were replaced with NMS and NRS respectively to set the background levels of PE (GH: y-axis) and FITC (IL-1R: x-axis) fluorescence.

a. NMS followed by NRS.
b. GH staining followed by NRS.
c. GH staining followed by IL-1R staining.

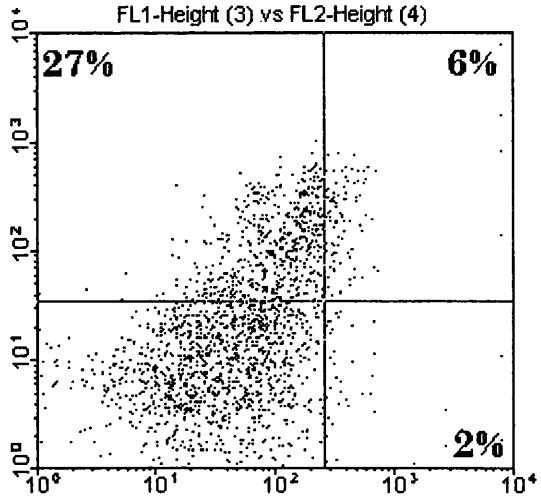
**a**


FL1-Height (3) vs FL2-Height (4)
1.5%
0.8%
0.9%

**b**


FL1-Height (3) vs FL2-Height (4)
24%
3%
1%

**c**


FL1-Height (3) vs FL2-Height (4)
27%
6%
2%

## 6.3 DISCUSSION

The first observation in this chapter was the visualization by SDS-PAGE of the GH and PRL levels in pituitary extracts. This semi-quantitative method was used as early as 1965, where Jones *et al* compared pituitary extracts from male and female rats. They showed a sex difference in the GH and PRL band intensities by disc electrophoresis and also manipulated these band intensities by gonadectomy and steroid replacement. The identities of the bands were confirmed by bioassay (tibial assay and pigeon crop sac assay). In this study, I have compared the relative band intensities of GH and PRL in AS and *dw/dw* male rats. The band intensity for GH in the *dw/dw* was much lower than the AS, while the PRL band was darker in the *dw/dw*. It is important to note that equal amounts of protein (by Lowry assay) were loaded, equivalent to a higher proportion of the *dw/dw* pituitary. In addition, the band intensities are only a semi-quantitative measure of the level of protein. However, allowing for these factors, the relative band intensities still suggested that the amount of PRL in the *dw/dw* extract was higher than the AS extract. It was following this result that the PRL levels were re-evaluated by RIA. These results, presented in chapter 3, confirmed the PRL content to be to be increased in the *dw/dw* even after correction for pituitary size.

Silver staining of acrylamide gels is much more sensitive than coomassie blue, allowing detection of proteins at ng quantities. When AS and *dw/dw* pituitary extracts were compared, slight differences were seen at ~50kDa. There was a reciprocal difference in the intensity of two bands very close together ie. band 1 was more abundant in the AS and band 2 was more abundant in the *dw/dw*. One possible explanation for this is that band 1 is a somatotroph protein and band 2 is a lactotroph protein. These may be totally unrelated proteins that just happen to run at a similar speed by SDS-PAGE. Alternatively, they may be splice variants of a protein expressed differently in the two related cell types. Another explanation is that they are the same protein, but the *dw/dw* form is mutated in some way.

The double label strategy, described in experiment 3, is theoretically a very elegant method which would reveal GRF stimulated proteins. However, the

results obtained were inconsistent and the isotope related artefacts made the data difficult to interpret. The most obvious protein that would be stimulated by GRF is GH itself. Stachura *et al* (1989) showed that newly synthesized GH is released in preference to older stores, thus there should have been a peak in the ratio of counts around fraction 47 in the double label medium plots. Even this internal control was not always present, suggesting that the method lacks sensitivity. It is not so surprising that the peak at fraction 47 is lacking from the double labelled extract plots, as any extra GH labelled may have been released. The release of newly formed GH is supported by the fact that the GH band in the $^{35}S$ labelled extracts separated by SDS-PAGE was more heavily labelled in the control lane than the GRF lane.

GH elutes during the latter part of the acetonitrile gradient, representing one of the larger proteins that could be separated by HPLC. The 17 GRF and SS regulated proteins described by Yoyoka and Friesen (1986) were all under 22kDa, and theoretically should have been in the extracts or conditioned medium samples. However, the relative abundance of these proteins differed, the lowest representing 0.05% of the signal of GH in radioactive amino acid incorporation experiments. The double label method produced results with too much noise and not enough signal to consistently produce a peak representing GH itself, so it is not surprising that it was unable to detect other candidate protein markers which may be at low abundance.

The single radio-label incorporation followed by SDS-PAGE separation was more successful, however. A candidate GRF stimulated protein was found at ~95kDa, which was also GRF stimulated in the *dw/dw* cultures. The increase in band intensity was less in the *dw/dw*, consistent with the reduced somatotroph number. If this protein is up-regulated in *dw/dw* somatotrophs, it is unlikely to be down stream of cAMP.

However, it is possible that a more severe deficiency would be expected considering the severity of the somatotroph reduction. This raises the possibility that the up-regulation in the *dw/dw* is not restricted to the somatotrophs. It was shown in chapter 5 that under certain circumstances, GRF released PRL as well as GH, indicating that non-specific effects can occur in culture. In the silver

stained extracts, the ~95kDa was fainter in the *dw/dw* control than the AS control. In addition, it was only up-regulated in the AS culture. This may support the non-specific actions in the *dw/dw* $^{35}S$ labelling experiments showing that the effect was not consistent. However, the silver stain and the autoradiograph are detecting different parameters. The silver stained gel shows the total content of the particular protein, whereas the autoradiograph shows only the protein made during the incubation period. If the amount of protein made during the incubation period is the same, but the total content indicated by silver staining is less, it is conceivable that the protein may be less stable in the *dw/dw*.

In addition, a protein running just behind the 31kDa marker (~35kDa protein) was shown to be more intensely silver stained in AS culture extracts than *dw/dw*. This protein appears to be a similar size to Pit-1. While there are conflicting reports stating that Pit-1 mRNA is (Houston *et al*, 1991) or is not (Zeitler *et al*, 1993) reduced in the *dw/dw* pituitary, there is no literature concerning the levels of Pit-1 protein. Pit-1 protein is present in lactotrophs and thyrotrophs, in addition to somatotrophs, which would contribute to the total Pit-1. However, different splice variants have been shown to differ in their promoter specificity (Vila *et al*, 1993; Theill *et al*, 1992; Voss *et al*, 1993; Haugen *et al*, 1994). It may be that the distribution of splice variants differs between AS and *dw/dw* pituitaries. The involvement of Pit-1 splice variants in the *dw/dw* phenotype would be consistent with a somatotroph deficiency without a reduction in other cell types. It may also explain the observed increase in the PRL axis.

The ~95kDa and ~35kDa proteins, the and the ~50kDa doublet are larger than any protein identified by Yokoya and Friesen (1986), who found no differences in proteins above 22kDa in their study. Therefore, while my studies have revealed new candidate marker proteins, I have not succeeded in repeating the observations of previous studies. However, smaller proteins would run near the bottom of the gels, thus a higher acrylamide content may be more successful in identifying these previously reported proteins.

Although five of the GRF and SS regulated proteins previously identified (Yoyoka and Friesen, 1986) were also found in cell extracts, the main emphasis

was on secreted proteins. Although secreted proteins are convenient to measure by blood sampling, the content within the cells is more likely to be variable. The aim of my study was to identify marker proteins whose abundance in the somatotroph is stable, thus the emphasis on this work was cell extracts rather than conditioned medium samples.

Attempts to isolate proteins from acrylamide gel were unsuccessful. An alternative method would have been electro-elution, although this was not tried as the equipment was not available. The isolation of the protein from the gel slice would allow amino acid sequencing to be performed to identify the proteins. It is unlikely, however that a single protein would have been isolated from the gel slice, due to the number of proteins present in a total cell extract. The proximity of the bands to each other would make cutting the gel without including contaminant proteins virtually impossible. A second purification step would produce better separation of the proteins. 2D gel electrophoresis has been utilized to identify the GRF and SS regulated proteins (Yoyoka and Friesen, 1986) and also to identify differentially expressed proteins in pituitary and thyroid in the *rdw* hypothyroid mouse (Oh-Ishi *et al*, 1998), illustrating the power of the method in this kind of study. In addition to repeating the observations of Yoyoka and Friesen (1986) in GRF treated cultures, 2D gel electrophoresis may have also enabled more differentially expressed proteins to be identified between AS and *dw/dw* pituitary extracts.

In addition to screening for new somatotroph marker proteins, the latter part of this chapter covered some preliminary work to further characterize a previously identified candidate. The recent localization of IL-1R to the somatotrophs in the anterior pituitary of the mouse (French *et al*, 1996) raised the possibility that it may be cell specific in the rat also. However, even after optimising the method, immunocytochemistry only stained a few cells. This is in discordance with the high levels of binding sites for IL-1β in the rat found by Marquette *et al* (1995) suggesting that the protein is not being effectively stained by my method.

To prove that the IL-1R is somatotroph specific, double immunocytochemistry for IL-1R and GH is necessary. However, the triton wash which is necessary for intracellular staining of GH abolished the IL-1R staining. A similar problem was

encountered with FACS analysis. The intracellular staining for GH in the presence of saponin would probably prevent staining of IL-1R on the cell surface after the removal of the saponin. The triton in immunocytochemistry and the saponin in FACS permeablize the membrane. It is possible that the membrane did not reform and thus the IL-1R was not available for staining.

The single FACS analysis for IL-1R did not give high enough fluorescence for a separate peak, but only a shoulder on the fluorescence plot at best. The shoulder, however, did represent 24-30% of the cells, similar to the GH staining population shown in figure 6.13 and in previous chapters. The antibody used is not listed as recommended for FACS analysis, so maybe the lack of sufficient signal to produce a separate peak is to be expected.

The inability to detect IL-1R by western analysis suggests that it is not in the extract. This may be due to the extraction method not being optimized for this protein. The extraction methods used were non-specific and designed to obtain a broad range of proteins. However, RIPA buffer should extract membrane proteins due to it's detergent content. Another possibility is that the protein may be present on the cell surface at low levels, making it's detection in cell extracts difficult. The strong staining of the control peptide proves that the problem is not the detection system.

To summarize, it was not possible to prove the somatotroph specificity of IL-1R in the rat, although the data shown is in agreement with it's expression within the anterior pituitary. Furthermore, new candidate proteins were detected by SDS-PAGE.

1. A protein of about 95kDa was found to be up-regulated by GRF, but is probably not restricted to the somatotrophs.
2. Two proteins of about 50kDa were found to be differentially expressed in AS and *dw/dw* rat pituitaries. These may be totally unrelated or they could be alternate forms of the same protein.
3. A protein of about 35kDa was found to more abundant in AS than *dw/dw* culture extracts.

All the bands detected are larger than previously reported candidate proteins (Yokoya and Friesen, 1986; Ivarie *et al* 1989), which are mainly below 22kDa. Attempts to isolate the proteins for further characterization were unsuccessful for technical reasons.

The screening of differentially expressed proteins is made difficult as the pituitary is made up of a mixture of cell types. The next chapter describes some work investigating the use of FACS sorting to enrich somatotrophs and lactotrophs from AS and *dw/dw* pituitary cells. Comparisons could then be made between somatotrophs and lactotrophs to screen for somatotrophs specific proteins. In addition, comparing AS and *dw/dw* somatotrophs may shed more light on the eitiology of this dwarf model.

# PURIFICATION OF PITUITARY CELLS FROM AS AND *DW/DW* RATS

## 7.1 INTRODUCTION

When comparing the *in vitro* response of AS and *dw/dw* somatotrophs, it is often difficult to attribute deficiencies in the *dw/dw* to cell number or to a deficiency per somatotroph. The work this chapter investigates the feasibility of purifying somatotroph populations from *dw/dw* pituitary cell suspensions, in order to compare them with purified AS somatotrophs and lactotrophs.

In the previous chapter, I described some attempts to identify somatotroph specific protein markers. The purification of pituitary cells provides an additional strategy, as cell specific proteins should be enriched in a purified cell population. Thus, as well as investigating the responses of the cells in culture, extracts of purified somatotrophs and lactotrophs from AS and *dw/dw* pituitaries were compared by SDS-PAGE to screen for differentially expressed proteins.

Methods of purification which rely on size, density or granularity would not be appropriate, as *dw/dw* somatotrophs differ in these characteristics from AS somatotrophs. As the secretory pathway in the *dw/dw* somatotroph is relatively intact, the cells would have GH antigen available on the cell surface in the same way as AS somatotrophs, thus FACS sorting was chosen to purify the cell types.

Previous publications have confirmed that sorted cells can be cultured and are still responsive to hypothalamic factors (St. John *et al*, 1986; Wynick *et al*, 1990b). Although GH and PRL cells sorts have been reported (Hatfield and Hymer, 1995; St. John *et al*, 1986; Wynick *et al*, 1990b), my work provides the first description of FACS sorting of somatotrophs from *dw/dw* anterior pituitaries.

## 7.2 EXPERIMENTAL RESULTS

The results in this chapter are divided in to three main categories. Experiments 1 and 2 provide validation for the FACS sorting of pituitary cells, by investigating the efficiency of sorting. Experiments 3 and 4 extend the work described in chapter 5, investigating the behaviour of AS and *dw/dw* pituitary cells in culture. Finally, experiments 5 and 6 complement the work described in chapter 6, further investigating differentially expressed proteins in search of somatotroph markers.

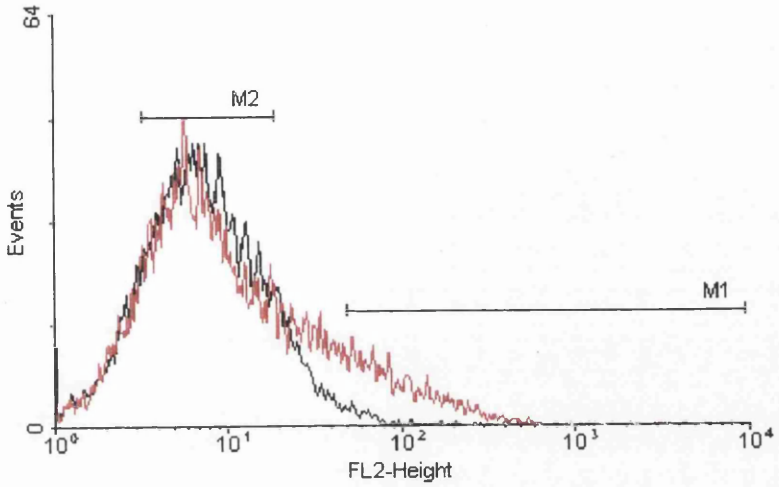
Figures 7.1 and 7.2 show the profiles of typical GH and PRL sorts of AS cells. The red line in panel (a) shows the fluorescence distribution of the stained cells before sorting compared to the normal serum controls in black. The region marked "M1" indicates the positive stained cells with respect to the normal serum control. "M2" indicates a population of cells not stained. M1 and M2 represent the GH or PRL enriched and depleted populations of cells that were collected. The rest of the cells, along with the small debris that was gated out, were sent to waste. The fluorescence histogram is not bimodal, therefore the positively stained populations form a shoulder. M1 is set as a compromise between yield and purity, as the more cells included in this region (by including cells with lower fluorescence), the higher the chance of also including unstained cells. In addition, it is possible for cells containing the target hormone to remain unstained if they were not secreting at the time, and thus be collected in the depleted population. Panel (b) of each figure shows the scatter profile of the cells before sorting. The distribution of positive stained cells (M1) are shown in red. Notice the lack of staining in the bottom left corner of the diagram which is predominantly cell debris.

**Figure 7.1: A typical GH sort.**

Panel (a) shows the fluorescence plot of AS cells stained for GH (red) compared to the NMS control (black). M1 and M2 represent GH enriched and depleted populations that are collected from the sort.

Panel (b) shows the scatter characteristics of AS pituitary cells, with the cells in the M1 region shown in red.

**a**


64
M2
Events
M1
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
FL2-Height

**b**

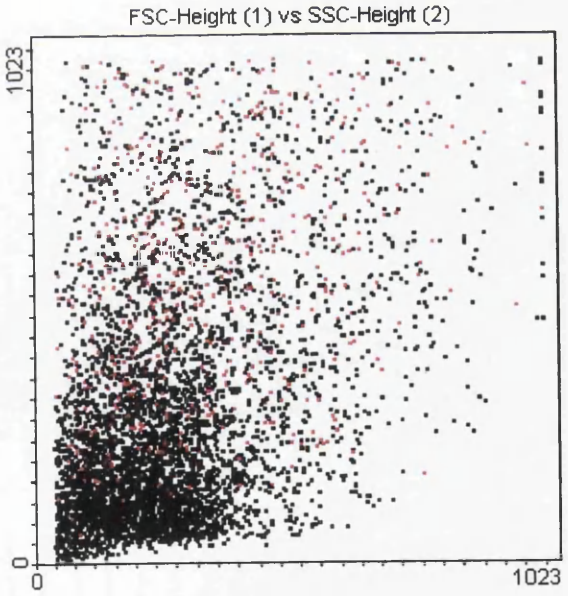
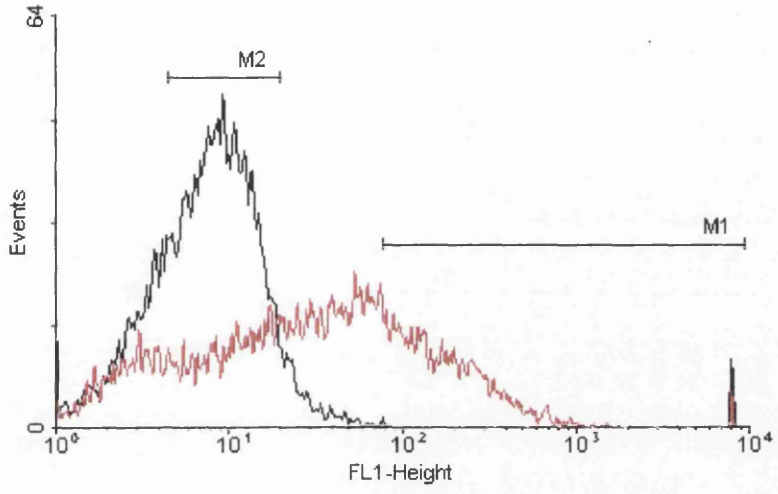

FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023

**Figure 7.2: A typical PRL sort.**

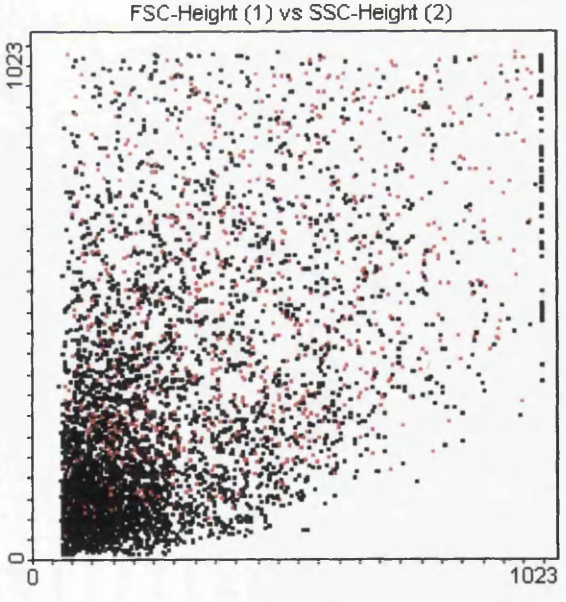
Panel (a) shows the fluorescence plot of AS cells stained for PRL (red) compared to the NRS control (black). M1 and M2 represent PRL enriched and depleted populations that are collected from the sort.

Panel (b) shows the scatter characteristics of AS pituitary cells, with the cells in the M1 region shown in red.

**a**


64
M2
M1
Events
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
FL1-Height

**b**


FSC-Height (1) vs SSC-Height (2)
1023
0
0
1023

### 7.2.1 Experiment 1: Evaluation of the Efficacy of FACS Sorting by Western Analysis

The aim of this experiment was to determine if FACS sorting caused an enrichment of cell types by comparing the hormone contents of the cell extracts. This experiment was carried out as two different non-sterile cells sorts;

1. AS male GH sort,
2. AS male PRL sort.

Anterior pituitaries from 18 rats were dispersed and stained for either GH or PRL. The cells were sorted by fluorescence with respect to the normal serum control. 1 million unsorted, GH or PRL enriched and GH or PRL depleted cells were centrifuged at 300g for 5 minutes and extracted in 200µl D-MEM + CPI for protein analysis. The extracts were concentrated to 10µl using a Microcon-3 (Amicon). The proteins were separated by SDS-PAGE and transferred to a PVDF membrane for Western analysis. The membrane was stained for GH and PRL simultaneously.

Figure 7.3 shows the Western blots showing the GH and PRL bands. Panel (a) shows the GH sort and the panel (b), the PRL sort. By comparing relative band intensities of the enriched and depleted cells, it can be seen that the GH enriched cell extract had a higher GH:PRL ratio than the GH depleted cell extract. Similarly, the band intensities indicate enrichment of PRL compared to GH in the PRL enriched cell extract, when compared to the PRL depleted cell extract. Although more quantitative comparisons of band intensity could have been made by densitometry, this experiment was intended to be only semi-quantitative, thus a visual comparison was sufficient.

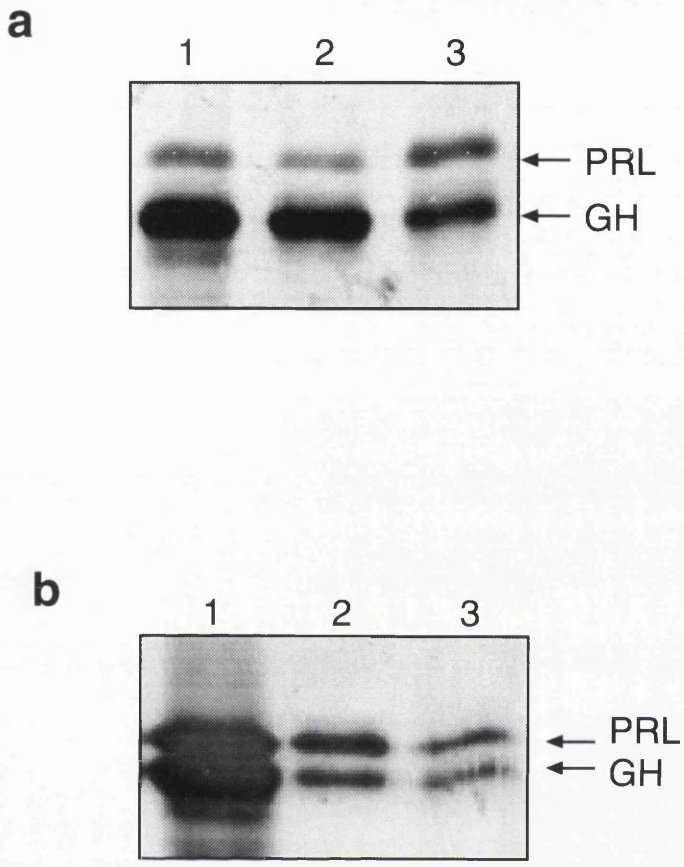
Although all the lanes represent the same cell number, the bands appear darker in the unsorted lanes, possibly due to the different methods of cell counting. The unsorted cells were haemocytometer counted, whereas the sorted cells were counted by the FACS machine during sorting.

**Figure 7.3: Western analysis of extracts of sorted cells.**

Pituitary cells were sorted for GH or PRL. 1 million unsorted, GH or PRL enriched and GH or PRL depleted cells were extracted in 200µl D-MEM + CPI, concentrated to 10µl and separated by SDS-PAGE. After transferring to PVDF membrane, GH and PRL were stained by Western analysis.

a. Lane 1: Unsorted
   Lane 2: GH enriched
   Lane 3: GH depleted.

b. Lane 1: Unsorted
   Lane 2: PRL enriched
   Lane 3: PRL depleted


a
1
2
3
PRL
GH
b
1
2
3
PRL
GH

### 7.2.2 Experiment 2: Culture of FACS Sorted Cells

The aim of this experiment was to quantify the degree of purification of the cells sorted, and to follow their progress when subsequently cultured. This experiment was performed as three different sterile cell sorts:

1. AS male PRL sort,
2. AS male GH sort,
3. *dw/dw* male GH sort.

Anterior pituitaries from 18 rats were dispersed and stained. The cells were sorted by fluorescence, under sterile conditions, with respect to the normal serum control. Eight wells each of unsorted, positive stained and negative stained cells were plated on poly-L-lysine coated coverslips in two 24 well plates. One of the plates was stained for GH and PRL (two wells each for each group) by immunocytochemistry the following day. The other plate was cultured for five days. The medium was changed daily and stored at -20°C until assayed for GH and PRL and the plate was processed for immunocytochemistry on day five.

Figures 7.4, 7.5 and 7.6 show the cell counts from the immunocytochemistry in experiment 1. GH and PRL stained cells and total cells from 4 areas of $1mm^2$ were counted on each of the two coverslips per group. The two coverslips represented a single population, thus an n of 8 was used to accurately estimate the distribution of cells in the population.

On day 1, the AS PRL sort (figure 7.4a) showed almost 75% PRL cells in the PRL enriched population compared to 42% in the unsorted population ($P<0.001$). The GH staining cells were reduced from 35% in the unsorted to 21% in the PRL enriched population ($P<0.05$). In contrast the PRL staining cells were reduced to 7% in the PRL depleted population ($P<0.001$).

After 5 days of culture (figure 7.4b), the AS PRL sort still showed lactotrophs enriched with respect to somatotrophs (3.5% *vs.* 2%) although this did not reach significance. The percentage of both GH and PRL staining cells was significantly lower in the PRL enriched than the unsorted population due the presence of more fibroblast-like cells. The PRL depleted cells still had fewer PRL staining

cells than the unsorted (6.8% *vs.* 12.7%), although this failed to reach significance due to the high variance of the data.

On day 1, the GH enriched cells from the AS GH sort (figure 7.5a) contained about twice as many GH staining cells as PRL staining (43% *vs.* 22%: $P<0.001$). This is in comparison to the unsorted, where GH and PRL staining cells were at the same abundance (35% *vs.* 32%), and the GH negative population which had significantly fewer GH staining cells than PRL staining cells (4.3% *vs.* 24%: $P<0.001$). Although the proportion of GH staining cells in the GH enriched population was not significantly greater than in the unsorted cells (43% *vs.* 35%), the percentage in the GH depleted population (4.3%) was significantly less than both the unsorted and GH enriched (both $P<0.001$).

After 5 days culture (figure 7.5b), the percentage of GH staining cells in the GH enriched population was higher than the unsorted (16.6% *vs.* 2.3%: $P<0.01$), but there were now more PRL staining cells than GH staining cells (23.3% *vs.* 16.6%: $P<0.01$). The GH depleted population still had significantly fewer GH staining cells than the GH enriched population (2.3% *vs.* 16.6%: $P<0.001$).

On day 1 the *dw/dw* GH sort (figure 7.6a) showed 45% GH staining cells in the GH enriched population compared to 10% in the unsorted cells ($P<0.001$). In addition, the PRL staining population was also depleted in the GH enriched population form 52% to 29% ($P<0.001$). The GH depleted population contained very few GH staining cells (1.3%), significantly fewer than both the unsorted and the GH enriched cells (both $P<0.001$).

On day 5 (figure 7.6b), the *dw/dw* GH enriched population still contained about twice as many somatotrophs than lactotrophs (6.2% *vs.* 2.8%: although this was not significantly different) compared to the unsorted cells which contained 5 fold more PRL staining than GH staining cells (14.5% *vs.* 2.9%: $P<0.001$). In addition, the somatotrophs in this population were still twice as abundant as in the unsorted population (6.2% *vs.* 2.9%), although this did not reach significance.

To summarize the immunocytochemistry data, all three sorts enriched the target cell in the GH or PRL enriched populations, while reducing the proportion in the

depleted populations. After 5 days of culture, the AS PRL enriched cells and the *dw/dw* GH enriched cells still showed enrichment with respect to the other cell type, although the GH enriched cells contained more PRL staining cells than GH staining at this time. The percentages of the cell populations were also more erratic at 5 days than 1 day. This was mainly due to differences in the proliferation of fibroblast-like cells.

Figures 7.7, 7.8, and 7.9 show the daily release of GH and PRL over the 5 day culture period in experiment 1. The release of GH and PRL (measured in ng per million cells per day) from unsorted, enriched and depleted populations is shown in the left hand panels (a, c and e) of each figure. The release of both hormones tended to be lower from the sorted cells than the unsorted (particularly the depleted population). In all three sorts, the release of both GH and PRL from sorted and unsorted cells declined during the 5 days, suggesting that the cells did not survive well in culture. It is possible that the folliculo-stellate (FS) cells, removed by the centrifugation through 4% BSA, are necessary for healthy cultures. To make the results easier to interpret, the data is also expressed in terms of relative release in right hand panels (b, d and f), where the release of the hormone not sorted for is normalized to 1.

The AS PRL enriched cells (figure 7.7) did not appear to show enhanced PRL release on day 1, although the relative amount increased throughout the culture period. The best enrichment of PRL release was on day 4, when the PRL release from the PRL enriched cells was 85% of the GH release compared to unsorted cells which released 4 times as much GH as PRL (representing more than a 3 fold enrichment in PRL:GH release). There was very little PRL released, relative to GH, from the PRL depleted cells at any time during the 5 days.

Over the first 4 days of culture, the AS GH enriched cells (figure 7.8) released 35-45 fold more GH than PRL, compared to the unsorted and GH depleted cells which released 4-8 fold more GH than PRL. Thus, the ratio of hormone release was increased in the GH enriched cells by about 5 fold on day 1 to 11 fold on day 4. On the 5$^{th}$ day, the ratio of GH:PRL release fell in both unsorted and GH enriched cells, although the GH release was still 5 fold higher than PRL in the GH enriched cells.

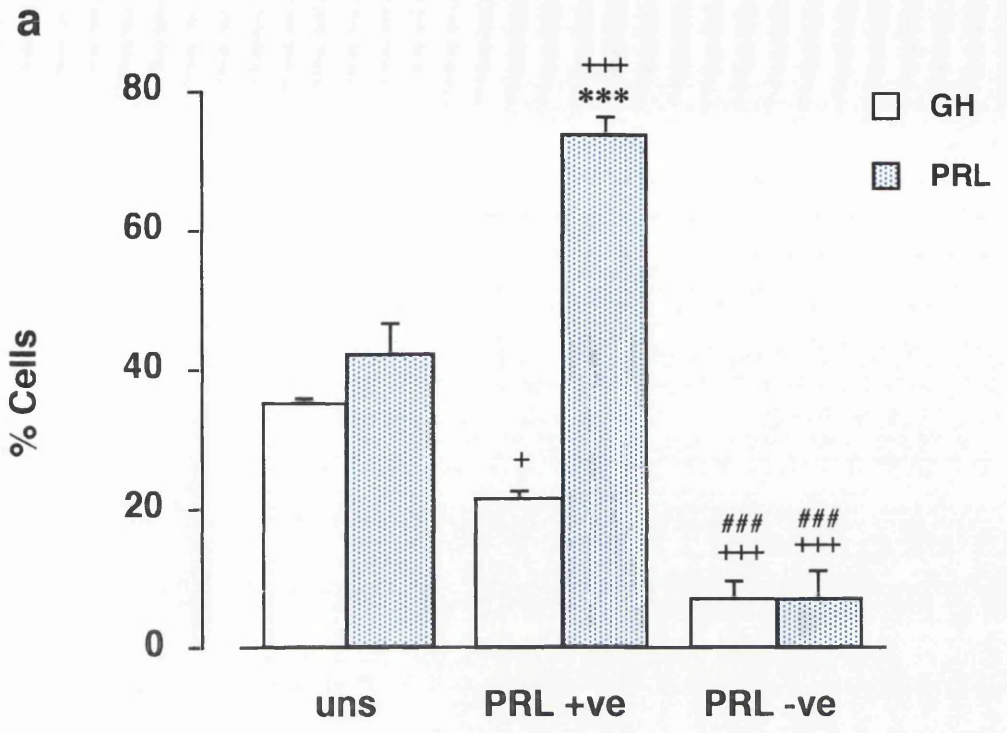
The *dw/dw* GH enriched cells (figure 7.9) also released relatively more GH than PRL than the unsorted cells for the entire culture time. The peak in enrichment of release was day 4, when the unsorted cells released half as much GH as PRL, whereas the GH enriched cells released 5 fold more GH than PRL, representing a 10 fold enrichment. Unexpectedly, the GH depleted cells appeared to increase in relative GH release throughout the 5 days. However, panel (e) shows that the amount of both is very small and as the PRL declined, the GH secretion remained relatively steady.

**Figure 7.4: Immunocytochemistry of cultured sorted cells: AS PRL sort.**

Unsorted, PRL enriched and PRL depleted cells were plated on poly-L-lysine coated coverslips and cultured for 1 and 5 days. At each time point, two coverslips of each cell population were stained for GH or PRL. GH and PRL stained cells and total cells from 4 areas of $1mm^2$ were counted on each of the two coverslips per group. The two coverslips represented a single population, thus an n of 8 was used to accurately estimate the distribution of cells in the population. The data are shown on the facing graphs.

a. Day 1
b. Day 5

***P<0.001 GH *vs.* PRL in the same population. $^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001 sorted *vs.* unsorted. $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 enriched *vs.* depleted. ANOVA, followed by Bonferroni's post-hoc test (n=8).


a
80
60
40
20
0
% Cells
GH
PRL
+++
***
+
###
+++
###
+++
uns
PRL +ve
PRL -ve

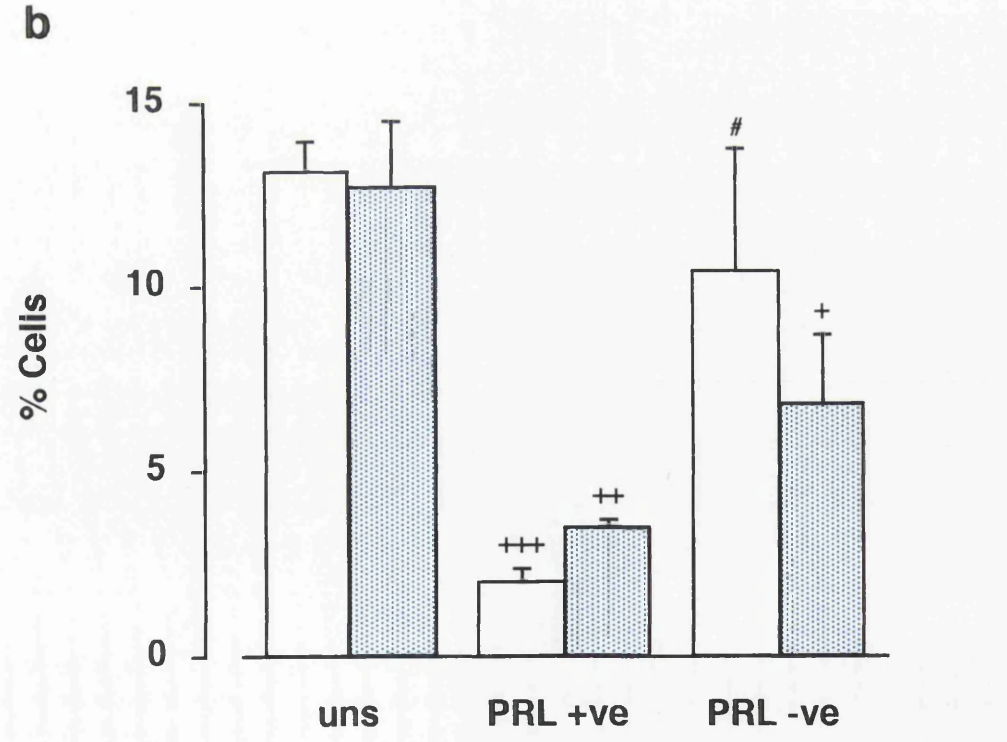
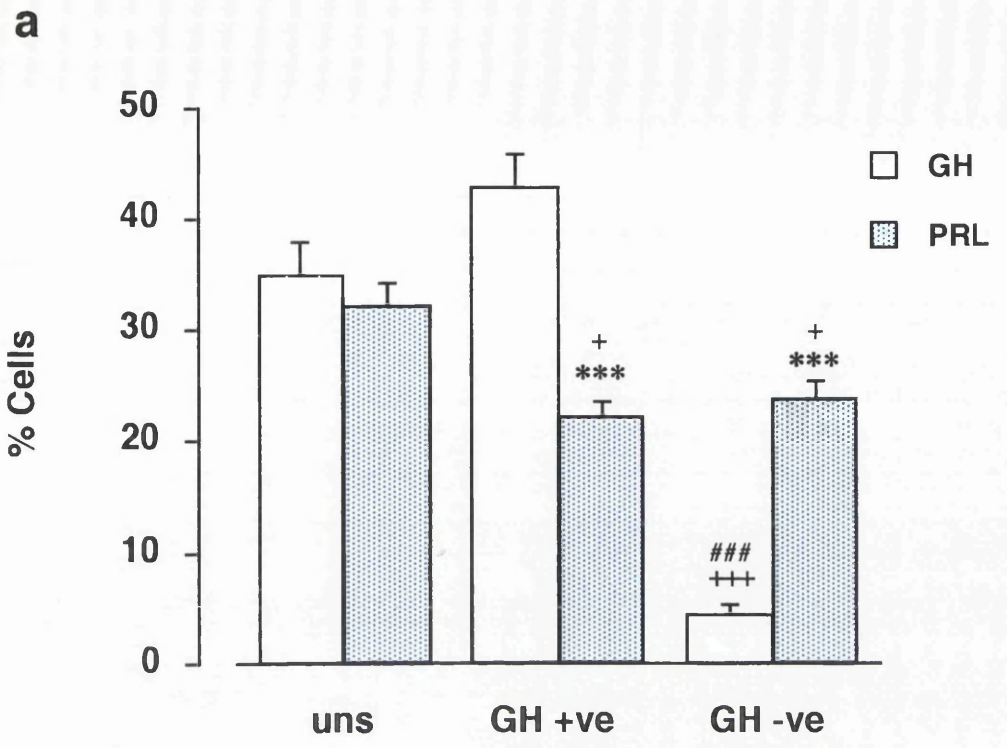

b
15
10
5
0
% Cells
#
+
+++
++
uns
PRL +ve
PRL -ve

**Figure 7.5: Immunocytochemistry of cultured sorted cells: AS GH sort.**

Unsorted, GH enriched and GH depleted cells were plated on poly-L-lysine coated coverslips and cultured for 1 and 5 days. At each time point, two coverslips of each cell population were stained for GH or PRL. GH and PRL stained cells and total cells from 4 areas of $1mm^2$ were counted on each of the two coverslips per group. The two coverslips represented a single population, thus an n of 8 was used to accurately estimate the distribution of cells in the population. The data are shown on the facing graphs.

a. Day 1
b. Day 5

**P<0.01, ***P<0.001 GH *vs.* PRL in the same population. $^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001 sorted *vs.* unsorted. ###P<0.001 enriched *vs.* depleted. ANOVA, followed by Bonferroni's post-hoc test (n=8).


a
50
40
30
20
10
0
% Cells
GH
PRL
+
***
+
***
###
+++
uns
GH +ve
GH -ve

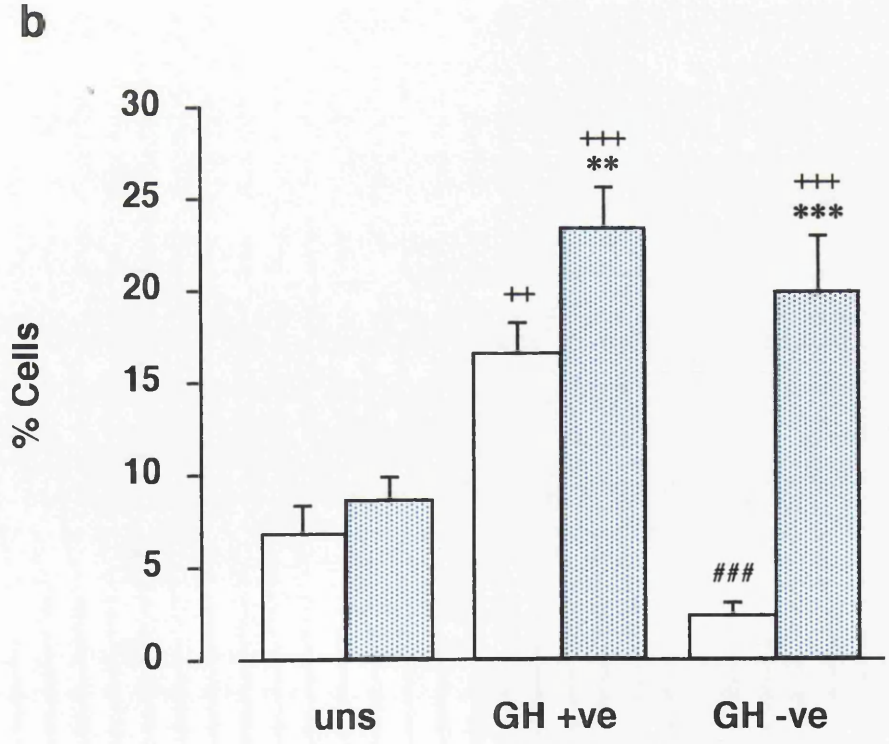
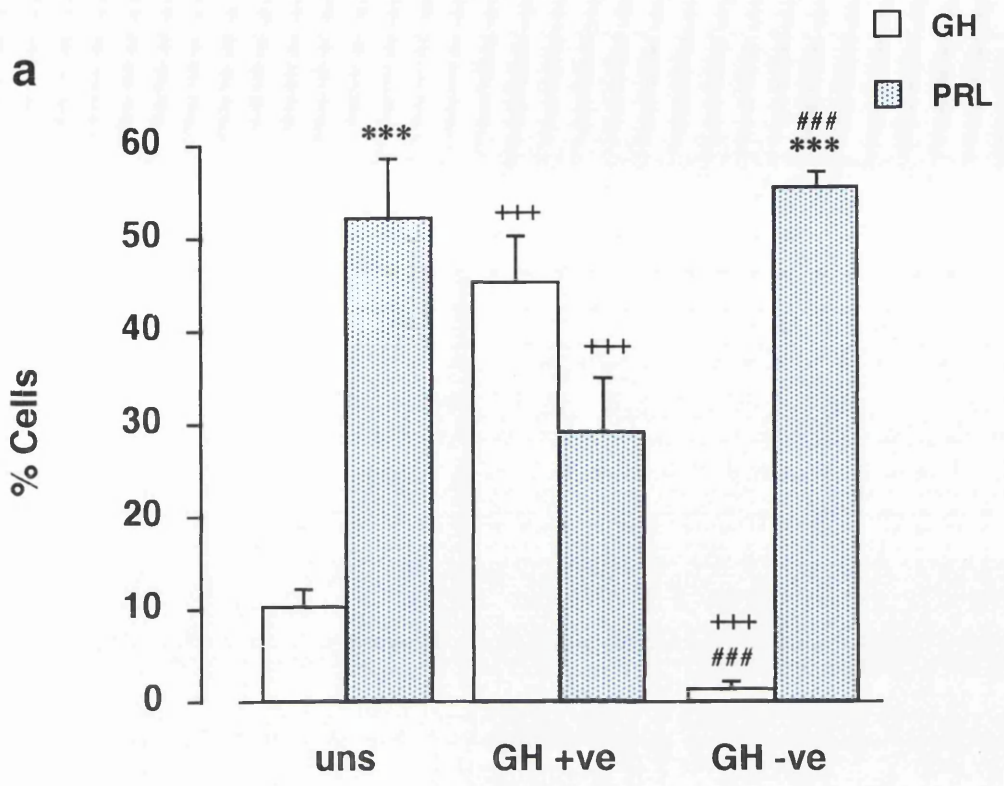

b
30
25
20
15
10
5
0
% Cells
+++
**
++
+++
***
###
uns
GH +ve
GH -ve

**Figure 7.6: Immunocytochemistry of cultured sorted cells: *dw/dw* GH sort.**

Unsorted, GH enriched and GH depleted cells were plated on poly-L-lysine coated coverslips and cultured for 1 and 5 days. At each time point, two coverslips of each cell population were stained for GH or PRL. GH and PRL stained cells and total cells from 4 areas of $1mm^2$ were counted on each of the two coverslips per group. The two coverslips represented a single population, thus an n of 8 was used to accurately estimate the distribution of cells in the population. The data are shown on the facing graphs.

a. Day 1
b. Day 5

***P<0.001 GH *vs.* PRL in the same population. $^{+++}$P<0.001 sorted *vs.* unsorted. ###P<0.001 enriched *vs.* depleted. ANOVA, followed by Bonferroni's post-hoc test (n=8).


a
GH
PRL
% Cells
60
50
40
30
20
10
0
***
+++
+++
+++
###
***
uns
GH +ve
GH -ve

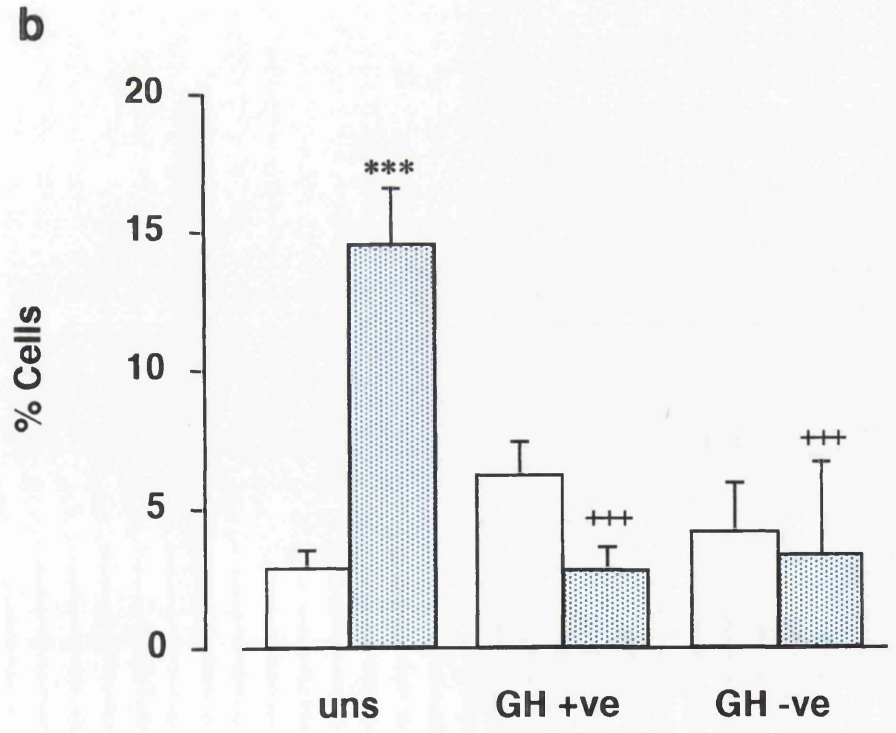

b
% Cells
20
15
10
5
0
***
+++
+++
uns
GH +ve
GH -ve

**Figure 7.7: Daily GH and PRL release from sorted cells: AS PRL sort.**

Unsorted, PRL enriched and PRL depleted cells were cultured in 24 well plates for 5 days (n=4). The medium was changed daily and assayed for GH and PRL. The data is shown as daily release in ng/well and with the GH release normalized to 1.

a. Unsorted: Daily release
b. Unsorted: Relative release
c. PRL enriched: Daily release
d. PRL enriched: Relative release
e. PRL depleted: Daily release
f. PRL depleted: Relative release

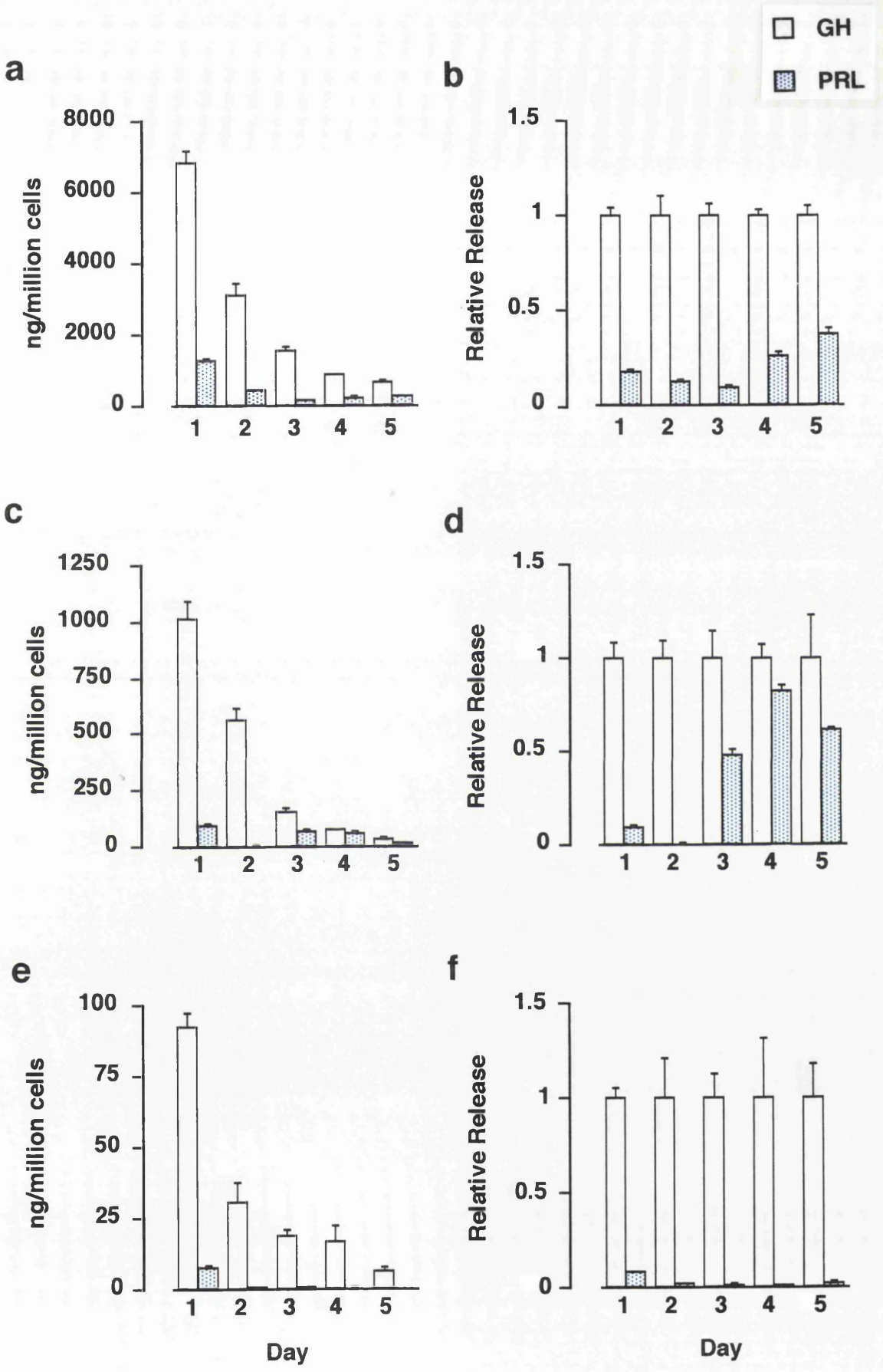

GH
PRL
a
b
c
d
e
f
ng/million cells
Relative Release
8000
6000
4000
2000
0
1250
1000
750
500
250
100
75
50
25
1.5
1
0.5
1 2 3 4 5
Day

**Figure 7.8: Daily GH and PRL release from sorted cells: AS GH sort.**

Unsorted, GH enriched and GH depleted cells were cultured in 24 well plates for 5 days (n=4). The medium was changed daily and assayed for GH and PRL. The data is shown as daily release in ng/well and with the PRL release normalized to 1.

a. Unsorted: Daily release
b. Unsorted: Relative release
c. GH enriched: Daily release
d. GH enriched: Relative release
e. GH depleted: Daily release
f. GH depleted: Relative release

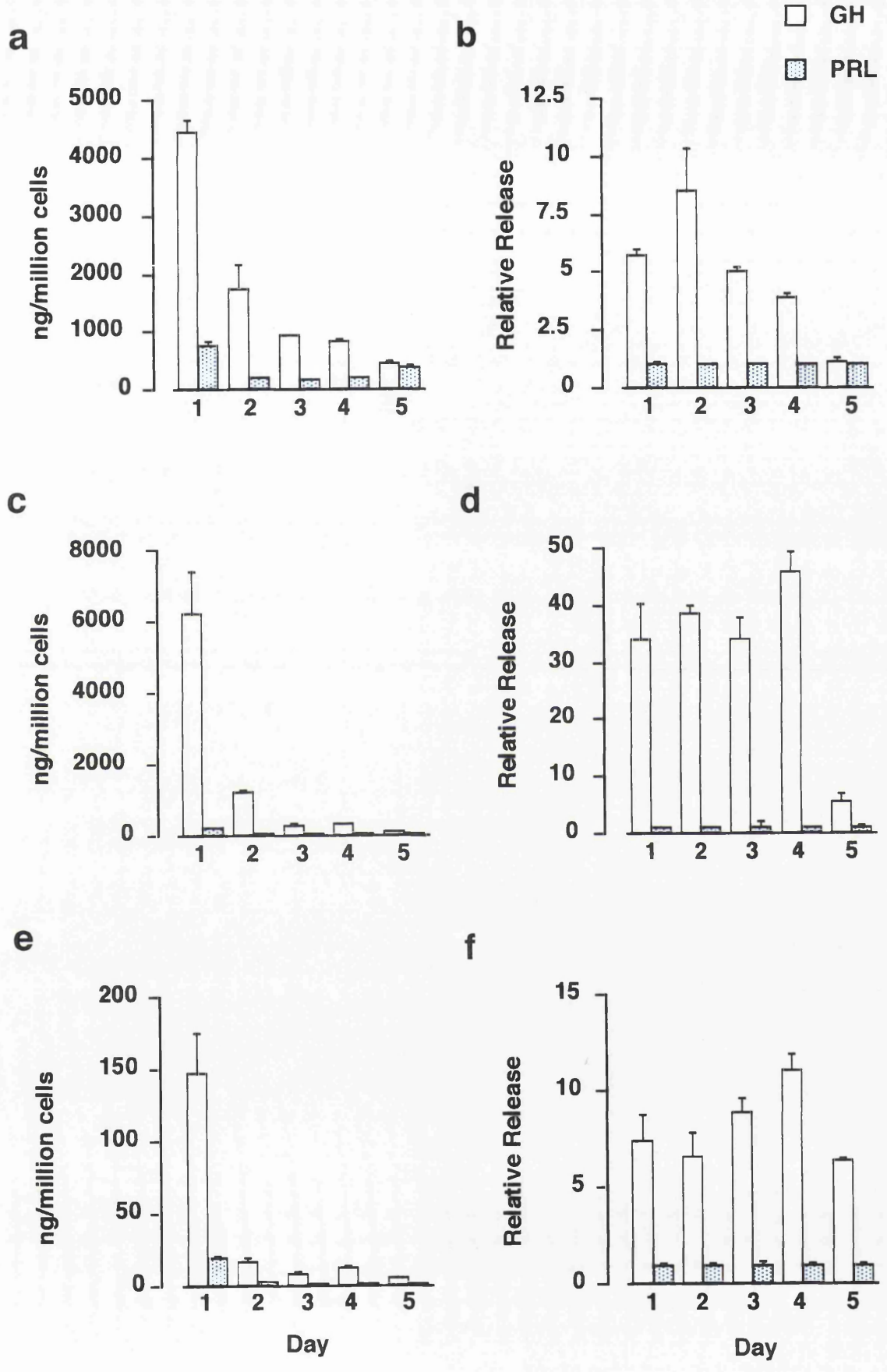
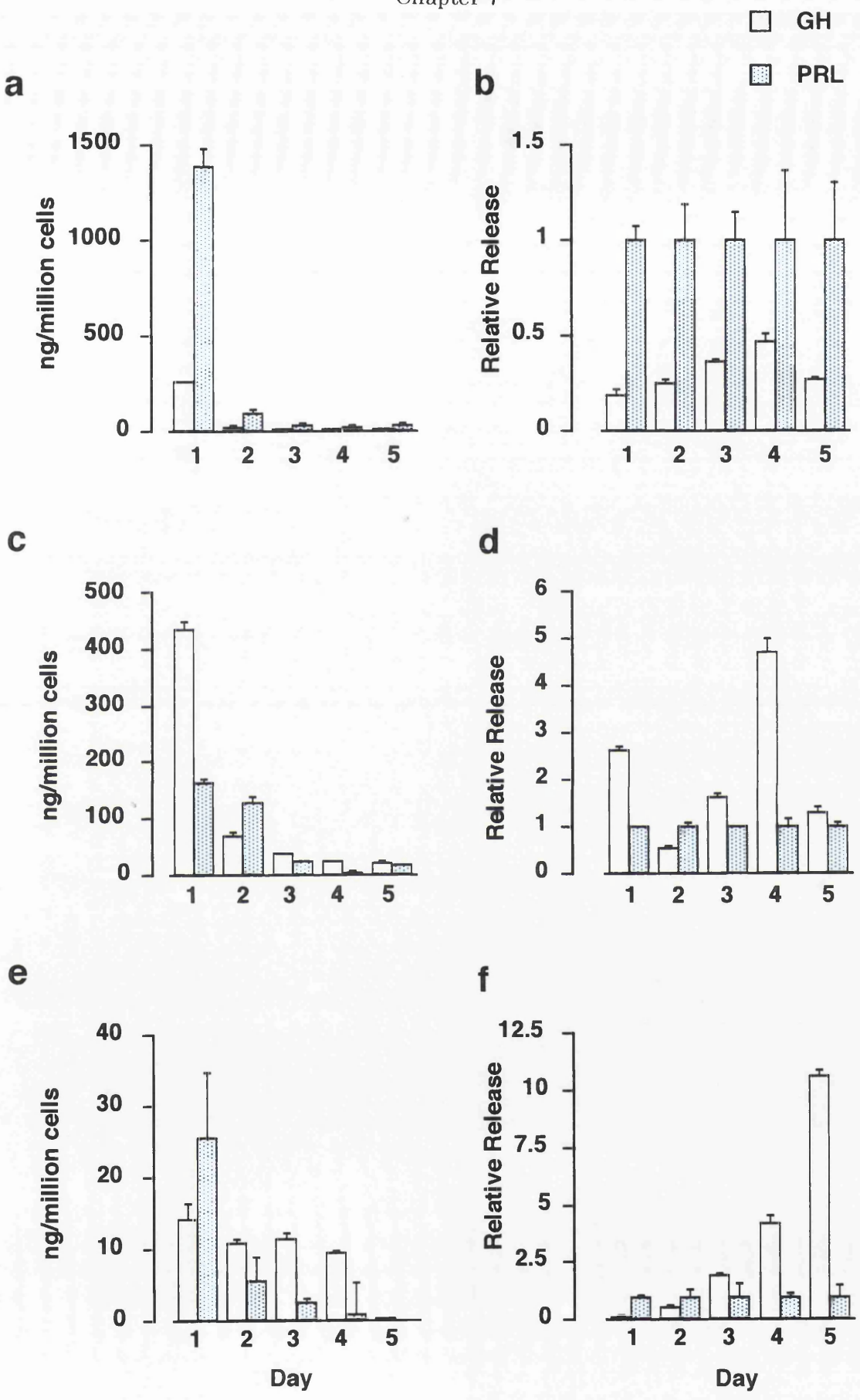

a
b
GH
PRL
ng/million cells
Relative Release
5000
4000
3000
2000
1000
0
12.5
10
7.5
5
2.5
0
1 2 3 4 5
c
d
8000
6000
4000
2000
0
50
40
30
20
10
0
e
f
200
150
100
50
0
15
10
5
0
Day

**Figure 7.9: Daily GH and PRL release from sorted cells: *dw/dw* GH sort.**

Unsorted, PRL enriched and PRL depleted cells were cultured in 24 well plates for 5 days (n=4). The medium was changed daily and assayed for GH and PRL. The data is shown as daily release in ng/well and with the GH release normalized to 1.

a. Unsorted: Daily release
b. Unsorted: Relative release
c. PRL enriched: Daily release
d. PRL enriched: Relative release
e. PRL depleted: Daily release
f. PRL depleted: Relative release


GH
PRL
a
ng/million cells
1500
1000
500
0
1 2 3 4 5
b
Relative Release
1.5
1
0.5
0
1 2 3 4 5
c
ng/million cells
500
400
300
200
100
0
1 2 3 4 5
d
Relative Release
6
5
4
3
2
1
0
1 2 3 4 5
e
ng/million cells
40
30
20
10
0
1 2 3 4 5
Day
f
Relative Release
12.5
10
7.5
5
2.5
0
1 2 3 4 5
Day

### 7.2.3 Experiment 3: GRF Treatment of FACS Sorted Cells: Secretory Response

The aim of this experiment was to investigate the secretory responses to GRF of sorted cells. This experiment was also performed as three individual sterile sorts;

1. AS female PRL sort,
2. AS male GH sort,
3. *dw/dw* male GH sort.

Four wells each of unsorted, GH or PRL enriched and depleted cells were plated in a 24 well plate and cultured for five days. At the end of this time, the culture was washed twice in treatment medium (D-MEM + 0.1% BSA). The wells were incubated in treatment medium with or without 10nM GRF for three hours in duplicate. The medium was removed and assayed for GH and PRL.

Figure 7.10a shows the release of GH and PRL with or without 10nM GRF over 3 hours of the AS PRL sort. The data is re-expressed relative to the GH control value in panel (b). The graphs do not have error bars as each point is a mean of only 2 wells, thus the significance of any responses cannot be statistically analysed. The first point to note is that GRF appeared to cause an increase in GH release, thus the cells were still viable. There also appeared to be a slight GRF stimulated PRL release in the PRL enriched cells which was not apparent in the unsorted or PRL depleted cells. In this experiment, the basal PRL release in the PRL enriched cells did not appear to be enriched with respect to GH release in comparison with the unsorted cells. The amount of GH and PRL released by the PRL depleted cells was much less than either the PRL enriched or unsorted cells, although when this was expressed as relative release, the PRL release appeared depleted relative to GH.

Figure 7.11 shows the data for the AS GH sort. The GH basal release appeared enriched 2 fold with respect to PRL when expressed as relative release. The GH depleted cells, as with the AS PRL sort, did not release much hormone. When this was expressed relative to the PRL control value the GH release appeared enriched, but again this was due to the very low PRL release from these cells. GRF stimulated GH release, but not PRL in all three populations. The relative

increase in GH release in response to GRF was similar in unsorted and GH enriched cells (2.9 and 2.5 fold), whereas it only resulted in a 1.5 fold increase in the GH depleted population.

The *dw/dw* GH sort (figure 7.12) resulted in the basal GH release being higher and the PRL release being lower in the GH enriched cells compared to the unsorted. These differences resulted in a four fold enrichment of GH release. The GH depleted cells appeared to release less GH than the unsorted, both relative to PRL and absolute GH per well. GRF resulted in 4.4, 5.7 and 3.8 fold increase in GH release in the unsorted, GH enriched and GH depleted populations respectively.

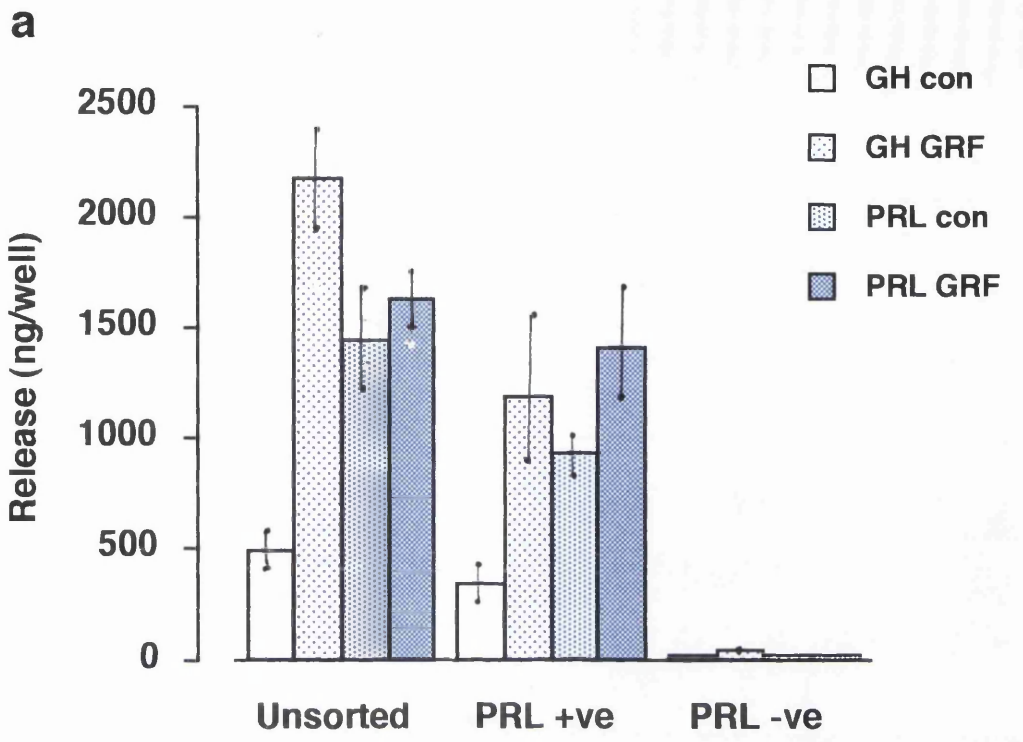
Thus to summarize, both the AS and *dw/dw* GH sorts resulted in enriched GH release on day 5, however the AS PRL sort did not show enrichment in PRL release. In addition, the data in this experiment has shown that sorted cells were still responsive to GRF in culture.

**Figure 7.10: GRF treatment of sorted cells: AS PRL sort. (♀).**

Unsorted, PRL enriched and PRL depleted cells were cultured for 5 days and then treated with or without 10nM GRF for 3 hours (n=2). The medium was assayed for GH and PRL.

a. GH and PRL release.
b. Relative GH and PRL release, GH control normalized to 1.

The bars indicate the raw duplicate data.


a
Release (ng/well)
2500
2000
1500
1000
500
0
GH con
GH GRF
PRL con
PRL GRF
Unsorted
PRL +ve
PRL -ve

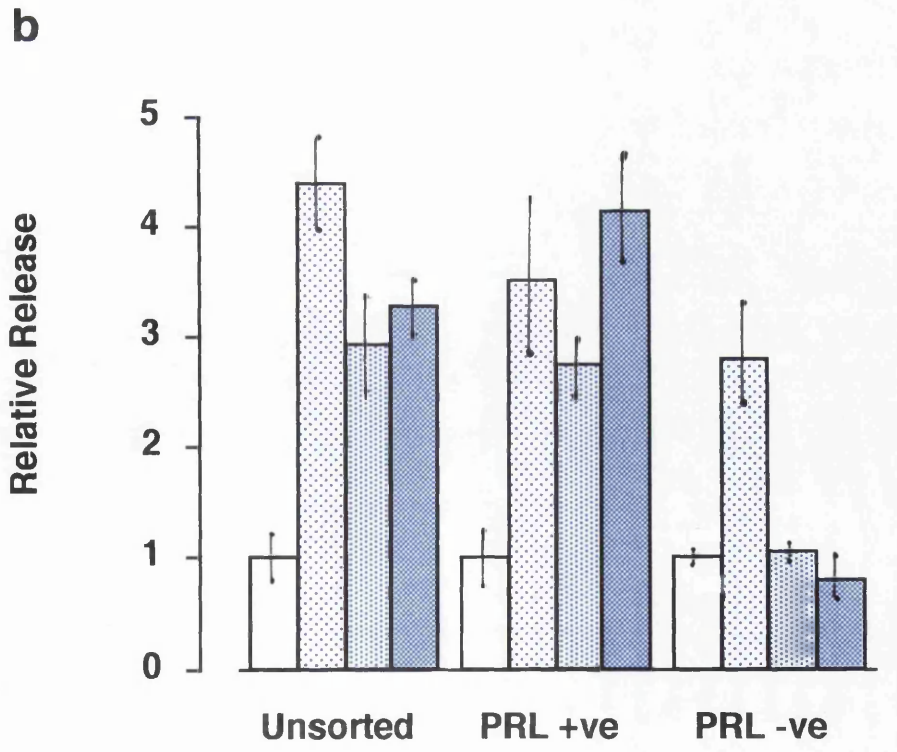
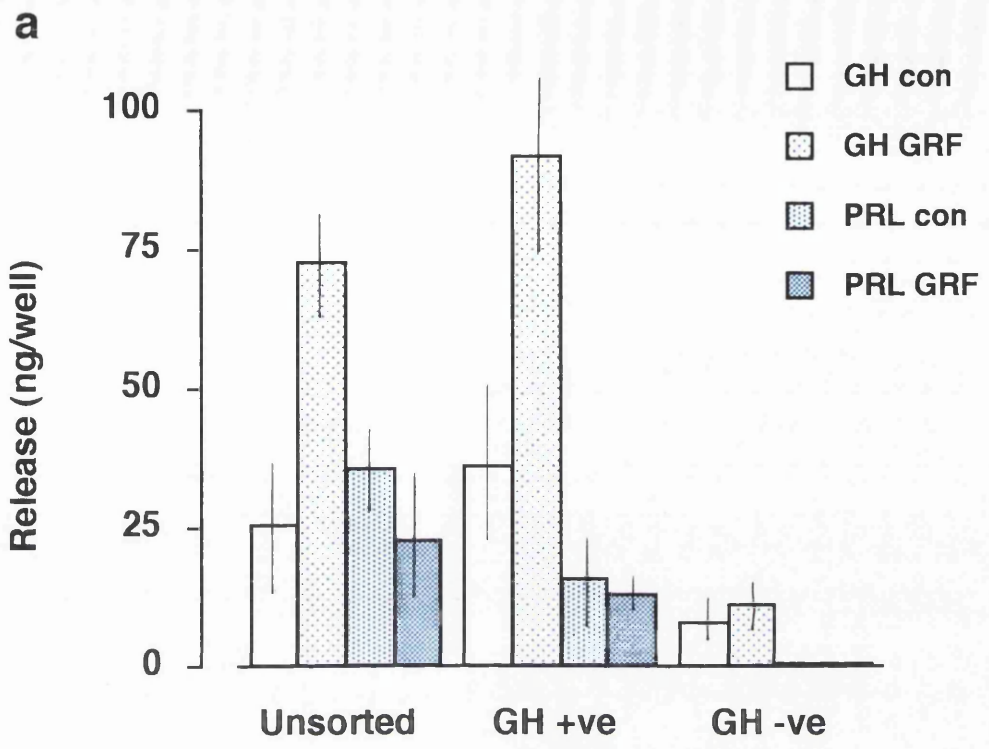

b
Relative Release
5
4
3
2
1
0
Unsorted
PRL +ve
PRL -ve

**Figure 7.11: GRF treatment of sorted cells: AS GH sort.** (♂)

Unsorted, GH enriched and GH depleted cells were cultured for 5 days and then treated with or without 10nM GRF for 3 hours (n=2). The medium was assayed for GH and PRL.

a. GH and PRL release.
b. Relative GH and PRL release, PRL control normalized to 1.

**The bars indicate the raw duplicate data.**


a
GH con
GH GRF
PRL con
PRL GRF
Release (ng/well)
100
75
50
25
0
Unsorted
GH +ve
GH -ve

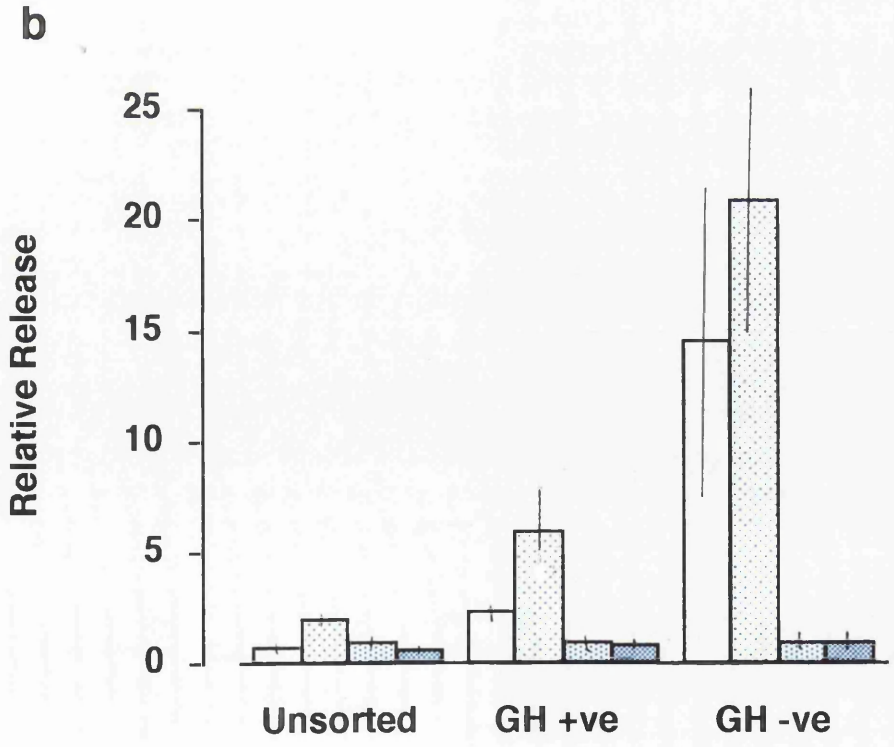
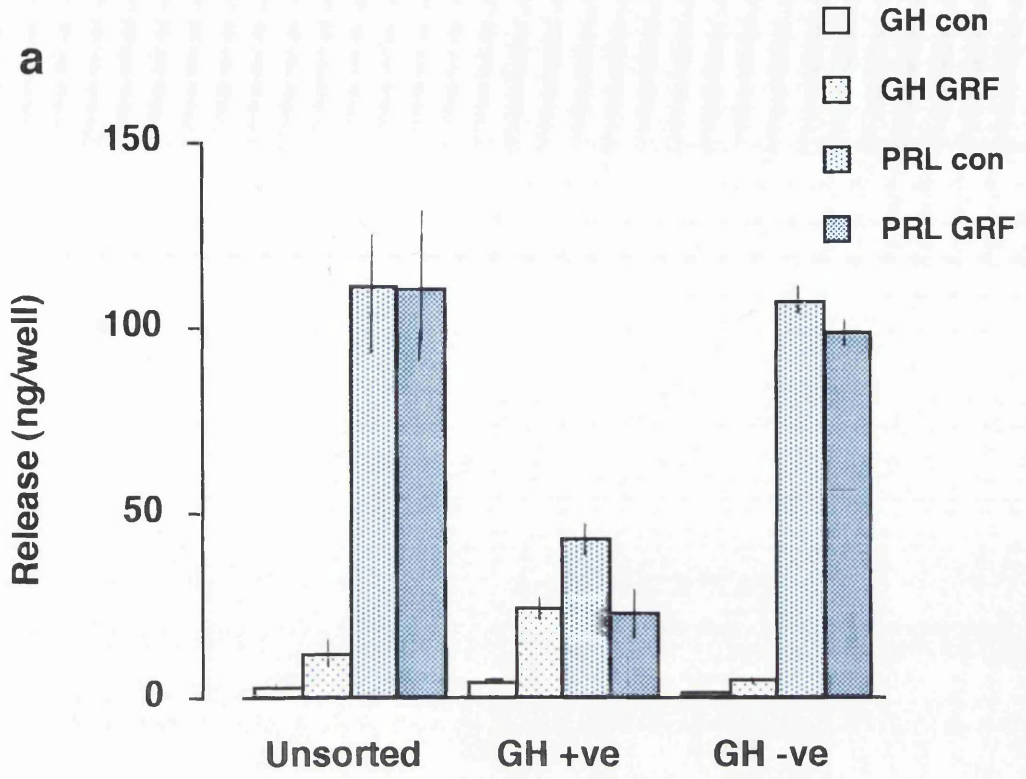

b
Relative Release
25
20
15
10
5
0
Unsorted
GH +ve
GH -ve

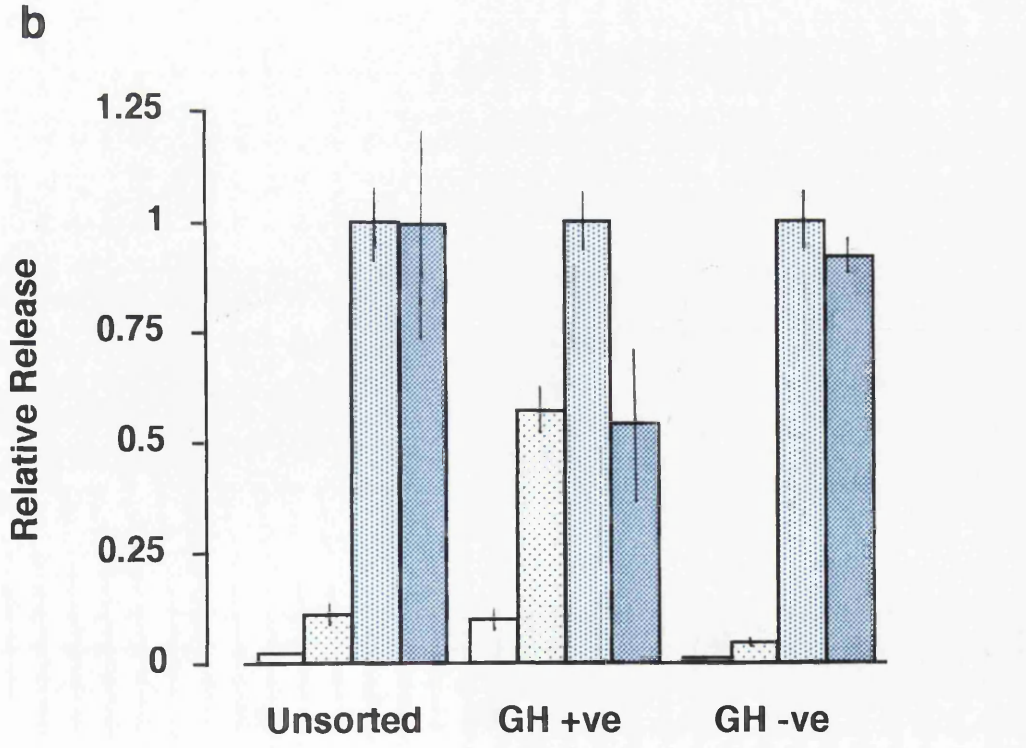
**Figure 7.12: GRF treatment of sorted cells: *dw/dw* GH sort. (♂)**

Unsorted, GH enriched and GH depleted cells were cultured for 5 days and then treated with or without 10nM GRF for 3 hours (n=2). The medium was assayed for GH and PRL.

a. GH and PRL release.
b. Relative GH and PRL release, PRL control normalized to 1.

The bars indicate the raw duplicate data.


a
GH con
GH GRF
PRL con
PRL GRF
Release (ng/well)
150
100
50
0
Unsorted
GH +ve
GH -ve


b
Relative Release
1.25
1
0.75
0.5
0.25
0
Unsorted
GH +ve
GH -ve

### 7.2.4 Experiment 4: GRF Treatment of FACS Sorted Cells: cAMP Accumulation

The aim of this experiment was to compare the cAMP accumulation in response to GRF in AS and *dw/dw* cells sorted for GH. Pituitary cells from 18 AS and 18 *dw/dw* male rats were sorted for GH. For each sort, four wells of unsorted, GH enriched and GH depleted cells were plated in a 24 well plate and cultured for five days. At the end of this time, the culture was washed twice in treatment medium (D-MEM + 0.1% BSA). The wells were then incubated in treatment medium with or without 10nM GRF for 30 minutes in duplicate. The cells were washed twice with ice cold PBS extracted in acid alcohol and assayed for cAMP.

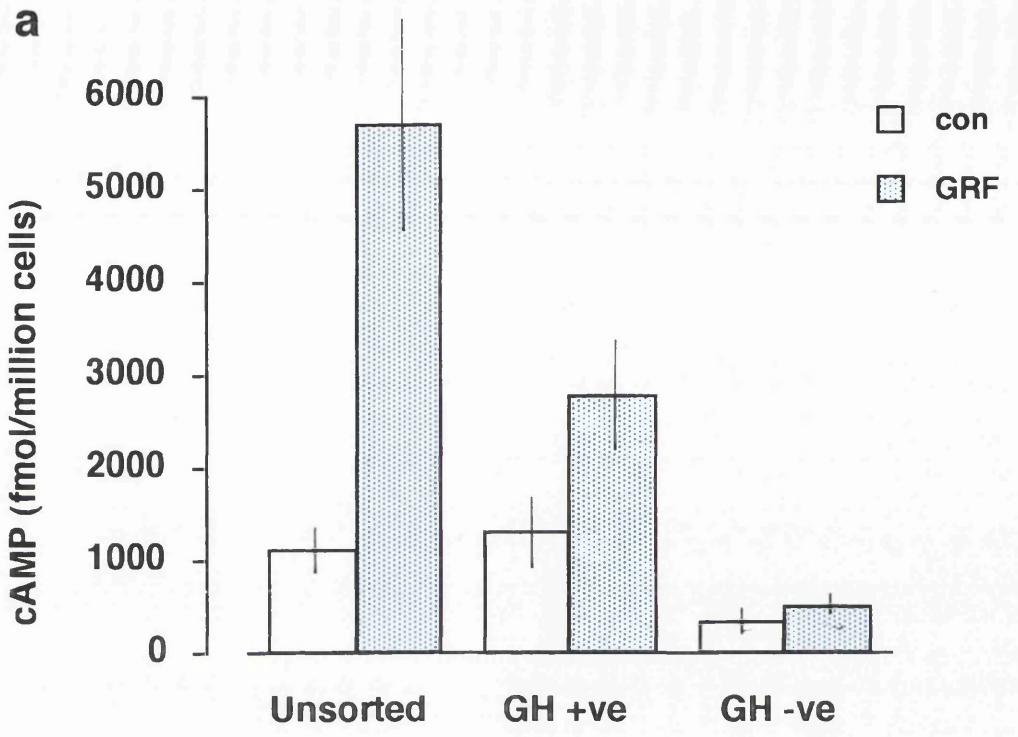
Figure 7.13 shows the cAMP accumulation in response to 10nM GRF for 30 mins in AS (panel a) and *dw/dw* (panel b) GH sorts. The basal cAMP accumulation in the GH enriched cells was similar to the unsorted for both AS and *dw/dw* cultures. The GH depleted cells had lower basal levels than the unsorted cells in both cases. The AS cells showed increased cAMP in response to GRF in both unsorted and GH enriched cells, however it was not possible to say if this was so for the GH depleted cells. There was a slight increase in cAMP in the unsorted *dw/dw* cells, although this is unlikely to be significant in the light of the extensive experiments on normal *dw/dw* primary cultures (described in chapter 5). Furthermore, the *dw/dw* GH enriched cells showed no increase in cAMP in response to GRF.

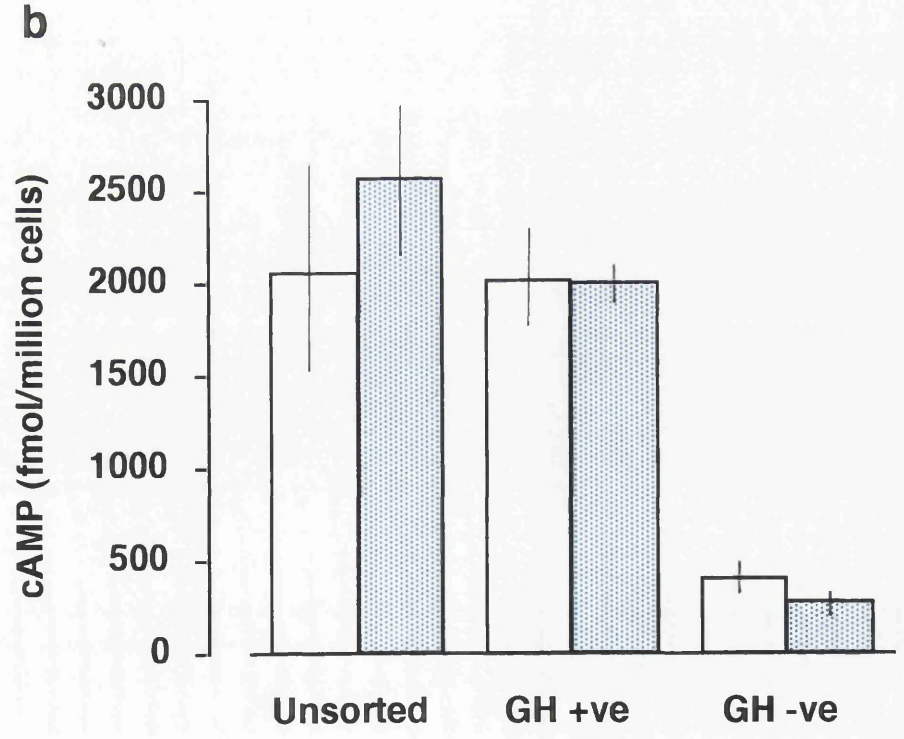
**Figure 7.13: Accumulation of cAMP in GH sorted AS and *dw/dw* cells.**

Unsorted, GH enriched and GH depleted cells from AS and dw/dw rats were cultured for 5 days and then treated with or without 10nM GRF for 30 minutes. The cells were extracted in acid alcohol and assayed for cAMP (n=2).

a. AS GH sort,
b. *dw/dw* GH sort.

The bars indicate the raw duplicate data.


a
6000
5000
4000
3000
2000
1000
0
cAMP (fmol/million cells)
con
GRF
Unsorted
GH +ve
GH -ve


b
3000
2500
2000
1500
1000
500
0
cAMP (fmol/million cells)
Unsorted
GH +ve
GH -ve

### 7.2.5 Experiment 5: SDS-PAGE of Extracts of FACS Sorted Cells to Screen for Differentially Expressed Proteins

The aim of this experiment was to screen for differentially expressed proteins in enriched somatotrophs and lactotrophs from AS and *dw/dw* pituitaries. For this experiment, no culture was necessary, so sterile conditions were not used. 1 million unsorted, GH or PRL enriched and depleted cells were centrifuged at 300g for 5 minutes and extracted in 200µl D-MEM + CPI for protein analysis. The proteins were concentrated, separated by SDS-PAGE and silver stained. The gel was dried and examined for differentially stained bands.

There are four separate gels illustrated, representing the four sorts (or pairs of sorts) carried out in this investigation;

1. AS GH sort,
2. AS and *dw/dw* GH sorts,
3. AS PRL sort,
4. AS and *dw/dw* PRL sorts.

The gel from the first AS GH sort is illustrated in figure 7.14a. The first point to note is that the GH depleted extract (lane 3) contained fewer bands than the unsorted or GH enriched. This was typical of GH or PRL depleted cell populations. This may be due to the low levels of protein in the extract, or the presence of debris. There were several bands showing different intensities between lanes, representing proteins that were enriched or depleted in the different cell populations. Between the 45 and 66.2kDa markers, one band (labelled a) was enriched in the GH depleted extract and possibly depleted in the GH enriched extract. Between the 45 and 31kDa markers (around 40kDa), there were three bands differing in intensity (labelled b, c and d), the largest being enriched in the GH enriched extract, and the smaller two being depleted. A band less than 21.5kDa (labelled e) was also depleted in the GH enriched extract.

The second gel, comparing GH sorts from AS and *dw/dw* pituitaries is shown in figure 7.14b. A band (labelled f) running just ahead of the 31kDa marker was depleted in the GH enriched extracts, and enriched in the GH depleted extracts from both AS and *dw/dw* sorts. In addition, comparison of the GH enriched lanes from AS and *dw/dw* cells could indicate proteins differing between AS and

*dw/dw* somatotrophs. However, the only visible difference between these lanes was the lower intensity of the GH band in the *dw/dw* lane.

Figure 7.15a shows the gel from a PRL sort of AS cells. The GH band was depleted in the PRL enriched extract, while there was no visible difference in the PRL band, thus PRL was enriched with respect to GH. There was also a band between the 45 and 66.2kDa markers which was enriched in the PRL enriched extract (labelled g). This may be the same band which was depleted in the first GH sort (band a in figure 7.14a).

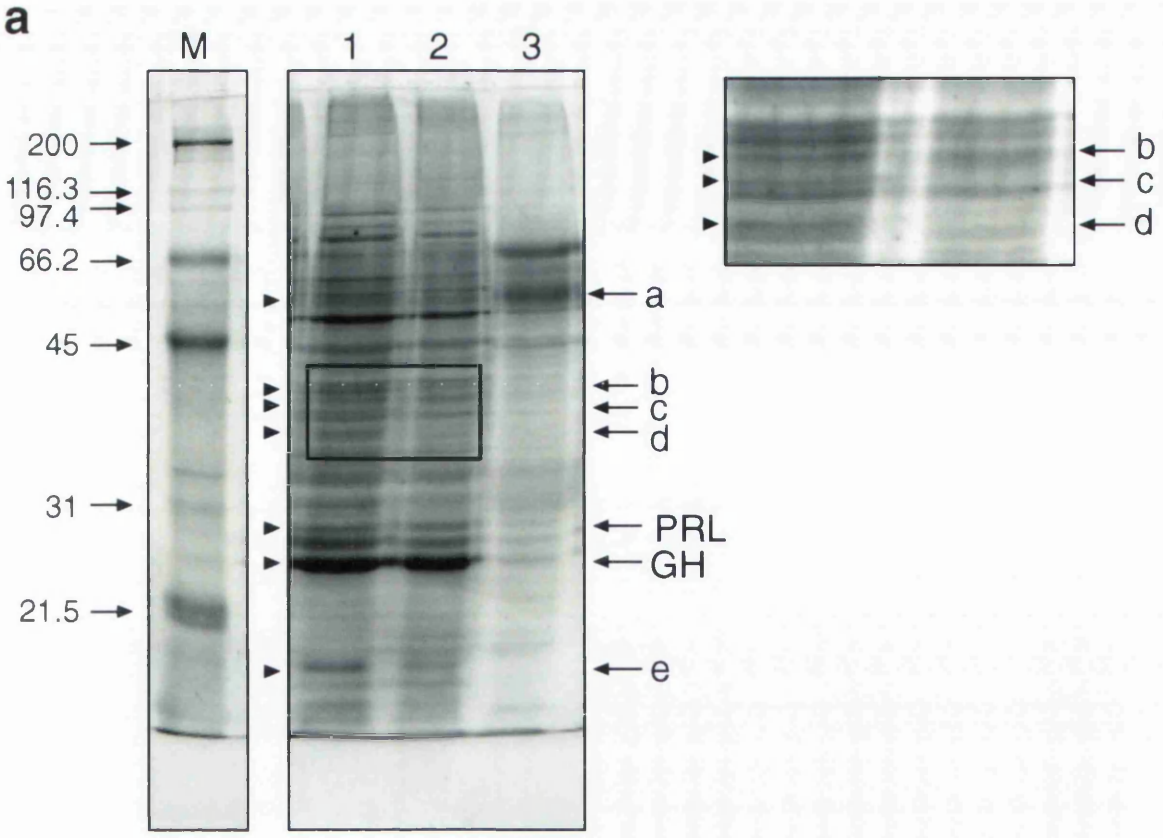
The AS and *dw/dw* PRL sorts are shown in figure 7.15b. It was slightly more difficult to identify differentially expressed proteins in this experiment as the PRL enriched extract had less protein than the unsorted in the AS sort, but more protein than the unsorted in the *dw/dw* sort. However, the band running just ahead of the 31kDa marker (f) was depleted in both AS and *dw/dw* PRL enriched extracts and enriched in the PRL depleted extracts.

**Figure 7.14: SDS-PAGE of sorted cells: GH sorts.**

Unsorted, GH enriched and GH depleted cells were extracted in 200µl D-MEM + CPI and concentrated to 10µl. The extracts were separated by SDS-PAGE and silver stained to screen for enriched and depleted proteins.

a. AS GH sort.
Lane 1: Unsorted
Lane 2: PRL enriched
Lane 3: PRL depleted.

b. AS and *dw/dw* GH sorts.
Lane 1: AS unsorted
Lane 2: AS GH enriched
Lane 3 AS GH depleted.
Lane 4: *dw/dw* unsorted
Lane 5: *dw/dw* GH enriched
Lane 6: *dw/dw* GH depleted.


a
M
1
2
3
200
116.3
97.4
66.2
45
31
21.5
a
b
c
d
PRL
GH
e
b
c
d

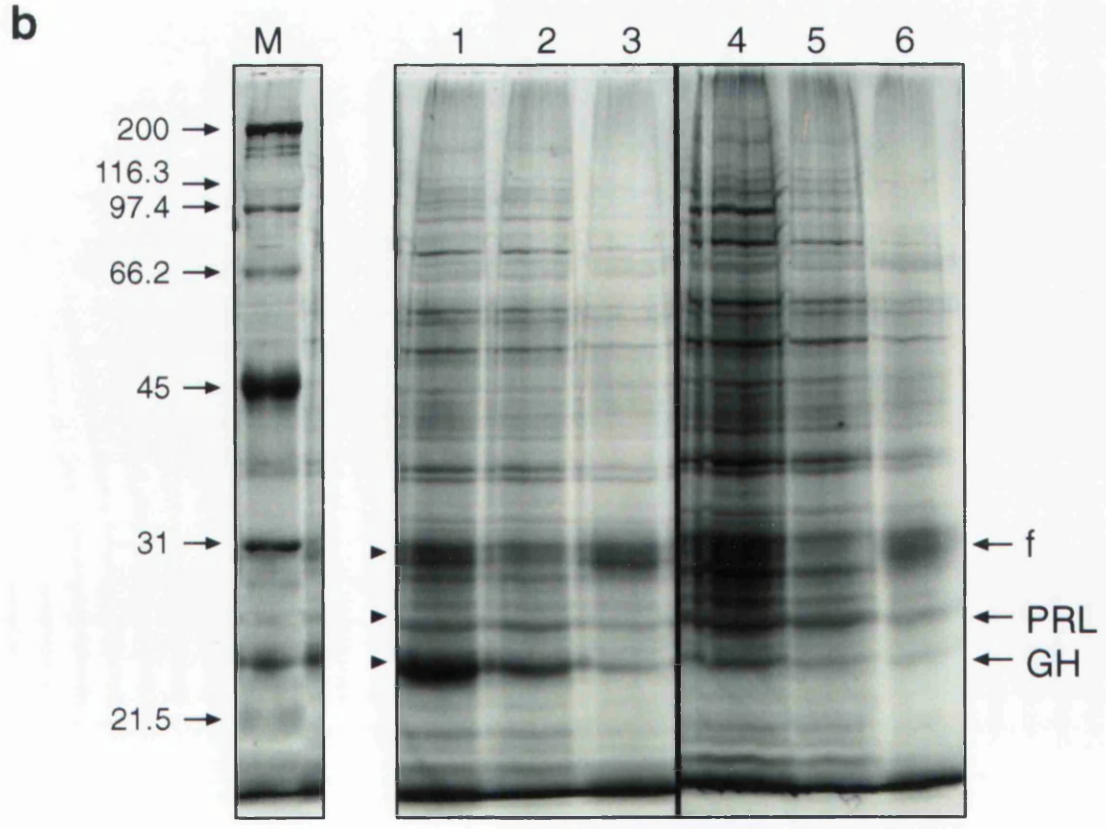
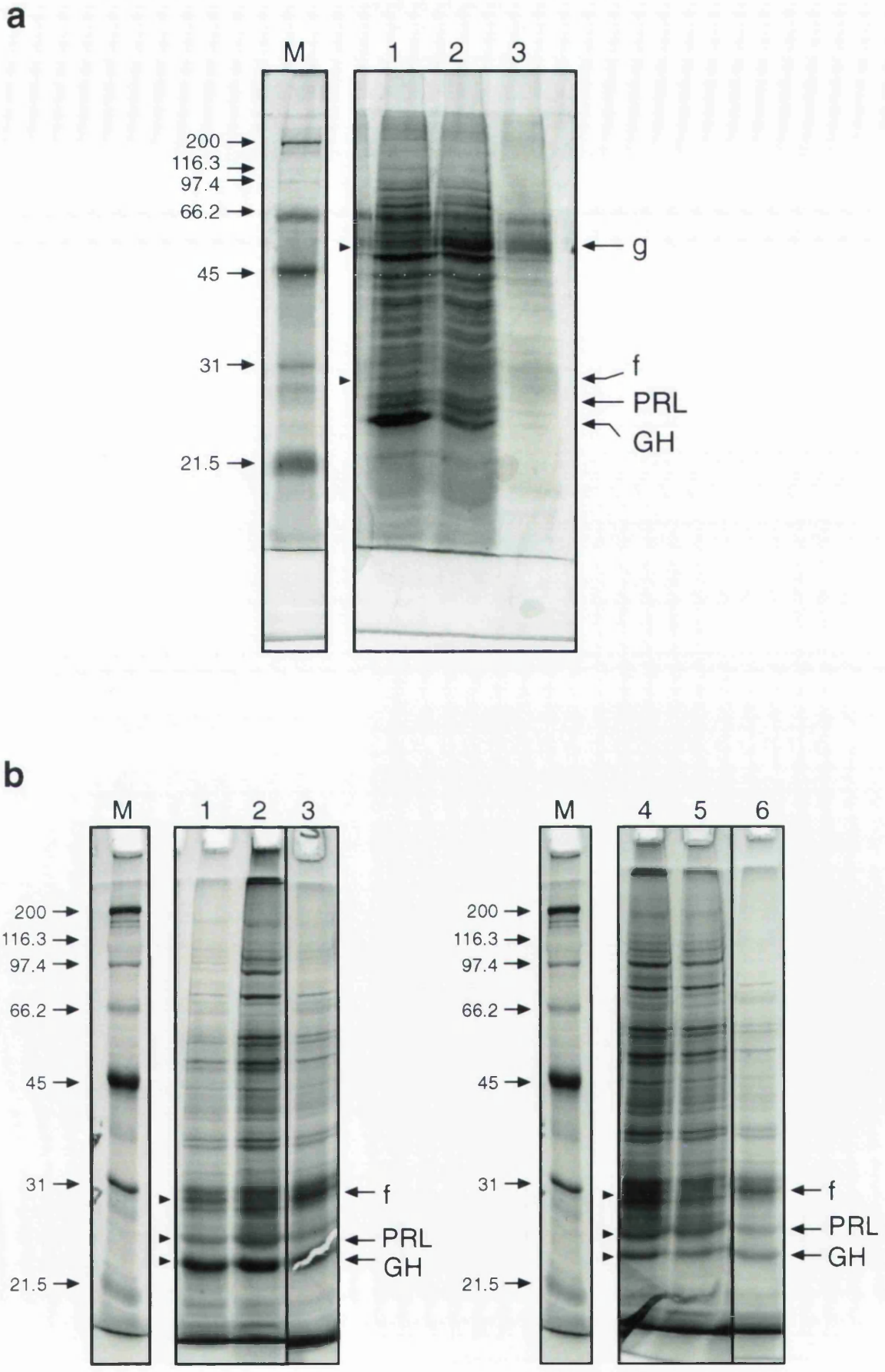

b
M
1
2
3
4
5
6
200
116.3
97.4
66.2
45
31
21.5
f
PRL
GH

**Figure 7.15: SDS-PAGE of sorted cells: PRL sorts.**

Unsorted, GH enriched and GH depleted cells were extracted in 200µl D-MEM + CPI and concentrated to 10µl. The extracts were separated by SDS-PAGE and silver stained to screen for enriched and depleted proteins.

a. AS PRL sort.
Lane 1: Unsorted
Lane 2: PRL enriched
Lane 3: PRL depleted.

b. AS and *dw/dw* PRL sorts.
Lane 1: AS unsorted
Lane 2: AS PRL enriched
Lane 3 AS PRL depleted.
Lane 4: *dw/dw* unsorted
Lane 5: *dw/dw* PRL enriched
Lane 6: *dw/dw* PRL depleted.


a
M
1
2
3
200
116.3
97.4
66.2
45
31
21.5
g
f
PRL
GH
b
M
1
2
3
M
4
5
6
200
116.3
97.4
66.2
45
31
21.5
f
PRL
GH

### 7.2.6 Experiment 6: Single Radiolabel of FACS Sorted Cells to Screen for GRF Stimulated Proteins

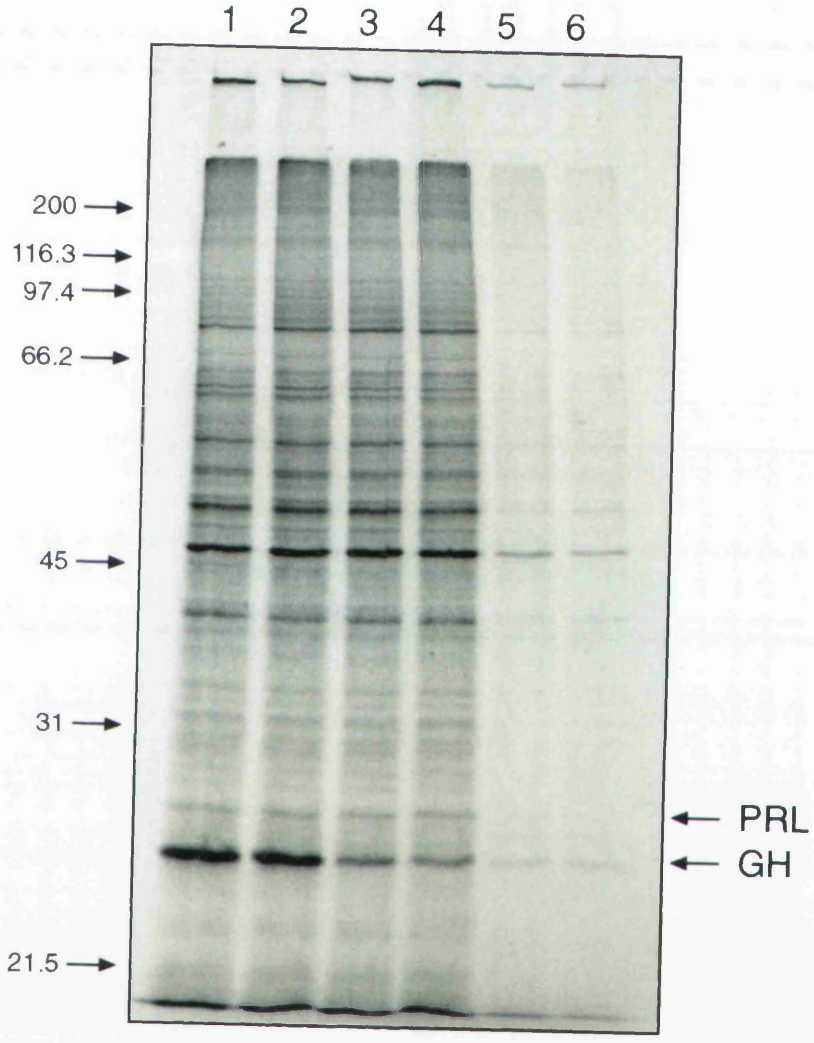
Like the previous experiment, the aim of this experiment was to screen for somatotroph marker proteins. This experiment was performed in the same way as experiment 3 in chapter 6, where [$^{35}$S]methionine incorporation was used to identify proteins up-regulated by GRF. 18 pituitaries from male AS rats were dispersed and sorted for GH. Four wells each of unsorted, GH enriched and GH depleted cells were plated in a 24 well plate and cultured for five days. At the end of this time, the culture was washed twice in treatment medium (methionine free D-MEM + 0.1% BSA). The wells were incubated in duplicate in treatment medium containing 0.74mBq/ml [$^{35}$S]methionine with or without 10nM GRF for three hours. At the end of the incubation, the cells were washed twice in ice cold PBS, extracted in RIPA + CPI and separated by SDS-PAGE. The gel was dried, exposed to film at room temperature for 3 weeks and developed.

Figure 7.15 shows an autoradiograph of SDS-PAGE separated extracts from the incorporation of $^{35}$S into sorted cells incubated with or without GRF for 3 hours. The GH depleted extracts show less incorporation than GH enriched or unsorted cell extracts. The GH band was unexpectedly less intense in the GH enriched cells. Unfortunately, there appeared to be no other differences in band intensities between unsorted and GH enriched cell extracts, indicating that no somatotroph specific proteins were identified in this experiment. In addition, GRF treatment did not result in differences in band intensity in any lane (even the unsorted), thus no GRF responsive proteins were identified.

**Figure 7.16: [$^{35}$S] methionine incorporation in sorted cells: AS GH sort.**

Unsorted, GH enriched and GH depleted cells were cultured for 5 days and treated with or without 10nM GRF in methionine free D-MEM + 0.1% BSA + 0.74 MBq/ml [$^{35}$S]methionine for 3 hours. The cells were washed and extracted in RIPA + CPI and separated by SDS-PAGE. The gel was dried and laid on film for 3 weeks.

Lane 1: Unsorted control
Lane 2: Unsorted GRF
Lane 3: GH enriched control
Lane 4: GH enriched GRF
Lane 5: GH depleted control
Lane 6: GH depleted GRF.


1
2
3
4
5
6
200
116.3
97.4
66.2
45
31
21.5
PRL
GH

## 7.3 DISCUSSION

The first aim of this chapter was to determine if FACS sorting was effective in the purification of somatotrophs and lactotrophs. The first experiment showed by SDS-PAGE that the relative hormone contents of extracts made from the sorted cells were enriched, although the band for the hormone not sorted for was still present. A more quantitative way of measuring the GH and PRL in the extracts would have been RIA, however the presence of antibody in the samples would have interfered with the assay by binding the tracer.

Immunocytochemistry of the cultured cells the day after sorting also indicated that although pure populations were not obtained, the cell types had been enriched. The unsorted AS population contained roughly equal numbers of GH and PRL staining cells. The GH enriched cells contained twice as many GH staining than PRL staining cells and PRL enriched cells contained 3.5 fold more PRL staining than GH staining cells. The *dw/dw* GH sort showed enrichment of somatotrophs from about 1/5 of the PRL count to almost double the PRL count. At about 40% somatotrophs, this is comparable to an AS culture. Some of the GH or PRL cells present in the enriched population of the other cell type may be attributed to the presence of mammosomatotrophs, particularly in the *dw/dw* where roughly half of the GH containing cells also contain PRL (see chapter 3).

Although the existence of mammosomatotrophs would contribute to the presence of GH in PRL enriched cells and *vice versa*, there could be several methodological explanations incomplete sorting;

Cells doublets that are small enough to pass as a single cell may consist of one cell sorted for with another cell type. This could only occur with smaller cells, as the suspension is strained through a 4μm cell strainer during dispersion. Furthermore, any aggregates remaining that pass through the FACS machine would be excluded by gating if they were large. Similarly, if the cell density of the unsorted population was too high, the droplets formed may contain more than one cell. The cells are sorted drop by drop, thus contamination could occur in this way. To avoid this, the cell density prior to sorting was kept below 1 million cells/ml.

If a blockage occurs during the sort, the stream may be diverted into one of the collecting tubes, contaminating it with unsorted cells. Mostly, blockages were not a problem although they did occur occasionally. During a sterile sort, blockages would also increase the risk of fungal or bacteriological contamination due to manually unblocking the nozzle.

Another possible source of contamination would be non-specific antibody binding. However, both GH and PRL primary antibodies have been found to be non cross-reactive with other pituitary hormones by NIDDK. Furthermore, the secondary antibodies were extensively tested for binding to pituitary cells or other primary antibodies than their target. No cross-reactivity was found, probably excluding this as the source of contaminating cells.

Specific hormone binding to GH or PRL on the surface of other cell types would introduce contaminating cell types. After sorting for lactotrophs, Wynick *et al* (1990b) found that the brightest stained cells in the PRL depleted cells were predominantly gonadotrophs. They suggested this may be due to PRL bound to receptors on the cell surface of these cells. However, the dispersion method used for this study includes the use of 0.125% trypsin for 30 mins followed by 0.5% for 15 mins (Wynick *et al*, 1990a). A much lower concentration of trypsin for a shorter time (0.05% for 10 mins) has been shown to abolish lipocortin 1 binding to pituitary cell surfaces due to proteolytic cleavage of receptor proteins (Christian *et al*, 1996). Furthermore, at least one day in culture was needed for cells to recover the ability to respond to dopamine after dispersion in 0.2% trypsin for 35 mins (Hoefer *et al*, 1984). It should be noted, however, that receptors may differ in their susceptibility to proteolytic cleavage during cell dispersion. In my study, trypsin was omitted from the dispersion medium as this tended to increase the percentage of stained cells, thus any cell surface receptors would not be cleaved off during dispersion. St. John *et al* (1986) found no evidence for PRL receptors by investigating the binding of iodinated PRL to anterior pituitary cells after 4 days of culture. However Giss and Walker (1985) found that cultured lactotrophs were able to internalize iodinated PRL, implying that PRL receptors may be present. This is supported by RHPA studies showing that ovine PRL can directly inhibit secretion from rat lactotrophs (Frawley and

Clark, 1986). The presence of PRL receptors on lactotrophs would not affect the purity of the sorted cell population.

The purity of the depleted cell populations can also be contaminated with unstained cells of the type sorted for. Cell surface staining relies on the antigen being present at the cell surface. Wynick and Bloom (1990) hypothesized that secretion of hormone at 4°C caused the hormone core to be transiently trapped within the lipid bilayer. Thus cells that are not secreting at the time would not be stained for. They suggested that co-incubation of the primary antibody with a releasing factor (GRF or TRH) could increase the yield of stained cells by promoting secretion (Wynick *at al*, 1990b). During optimization of the methods used in this study, attempts were made to increase the staining of GH by incubating the cells with 10nM GRF for 5-30 minutes prior to the primary antibody. This did not affect the percentage of cells or the intensity of staining, in agreement with St. John *et al* (1986).

The next aim of the work described in this chapter was to investigate the behaviour of the cells in culture. This was achieved by monitoring the daily basal release of GH and PRL, and also comparing the relative proportions of GH and PRL staining cells after 5 days of culture with the proportions on day 1.

The AS PRL enriched population (figure 7.4) had 3.5 fold more PRL staining cells on day 1. On day 5, the PRL staining cells were still more abundant than GH staining, but only by 1.8 fold. Although the enrichment in cell numbers appears to be not so good on day 5, the hormone release enrichment improved with time (figure 7.10). The hormone release did not show increased PRL initially, which could be attributed to the fact that actively secreting cells are selected for during the sort. Once these are cultured, they may not secrete if their stores had been depleted and may take time to recover, reflected by the increase in PRL secretion with time from the PRL enriched cells.

Although on day 1, the AS GH enriched population contained twice as many GH staining cells as PRL cells, by day 5, the proportions had returned to a similar ratio as the unsorted cells (unsorted: 1.25 fold as many PRL as GH staining; GH enriched: 1.4 fold) (figure 7.5). The amount of PRL release was much less than

GH for the entire culture time, although the ratio of GH:PRL release dropped on day 5. This change in ratio involved an increase in PRL release as well as a decrease in GH release. Therefore it is possible that transdifferentiation started to occur to return the equilibrium of GH and PRL cells. Both steroids (Kineman *et al*, 1992; Boockfor *et al*, 1986) and hypothalamic factors (Hoeffler and Frawley, 1987; Frawley and Hoeffler, 1988) have been shown to stimulate interconversion of somatotrophs and lactotrophs, and it is likely that the serum used in the culture medium contained some of these factors. This raises the possibility that even if a 100% pure lactotroph or somatotroph population could be obtained by sorting, the phenotype of the cells may change with time in culture.

The enrichment of GH staining cells compared to PRL staining in the GH enriched *dw/dw* population was actually better on day 5 than day 1 (figure 7.6). On day 1 there were 1.6 fold more GH staining cells than PRL, whereas this became 2.3 fold more on day 5. The unsorted population contained 5 fold more PRL staining than GH staining cells at both time points. The GH depleted population contained 40 fold more PRL staining than GH staining cells. However, on day 5 there were actually slightly more GH staining cells than PRL staining in the GH depleted population. In addition, the GH and PRL release showed a similar trend (figure 7.9) with the proportion of GH release compared to PRL release increasing during the culture period. Panel (e) revealed this to be due to a decline in PRL release while the GH release remained steady, suggesting that the somatotrophs survived better than the lactotrophs in this population.

The close relationship between somatotrophs and lactotrophs made it difficult to distinguish contamination by other cell types from mammosomatotroph staining. Likewise it was difficult to distinguish transdifferentiation from selective survival or proliferation over the five days. This could be investigated by re-trypsinizing the cells at stages during culture and staining for GH and PRL for FACS analysis to quantify the mammosomatotroph population. However, this was not possible due to the low cell numbers. In addition, the abundance of contaminating cell types could be better quantified by measuring immunoreactivity for another anterior pituitary hormones. Attempts were made

to measure TSH released into the medium by RIA, but the levels were below the sensitivity of the assay in all cases (data not shown).

The behaviour of the cells in culture differed from the normal primary cultures described in chapter 5, even in the unsorted population. The preparation of cells for sorting includes a step not used in the primary culture method. The centrifugation of the cells through 4% BSA removed not only red blood cells and cell debris, but also FS cells (H.C. Christian; personal communication) as they are less dense than secretory cells (Allearts and Denef, 1989). The importance of FS cells in modulating the functions of pituitary cells (Allearts *et al*, 1990) suggests that cultures lacking these cells behave differently. In even the unsorted cells the increase in PRL release during the early days of culture, described in chapter 5, was absent. The GH or PRL depleted cell populations in particular did not culture well. Fibroblast proliferation leading to confluence was not observed in these cells as often as the unsorted or GH or PRL enriched cells. This may also be due to the presence of any remaining blood cells or debris, as only the smaller debris was gated out during sorting. The GH or PRL depleted cells often didn't behave as expected for this reason. For instance, hormone release was very low in these cells, as were basal cAMP levels and $^{35}S$ incorporation. Due to the lack of FS cells (and also other endocrine cell types), it should be borne in mind that the behaviour of sorted cells is likely to differ from a primary culture of pituitary cells, which in turn differs from the *in vivo* pituitary.

The next aim was to investigate the responses of the sorted cells to GRF after 5 days in culture. The stimulation of GH release by GRF showed that the sorted cells were still viable and responsive after culture, in agreement with St John *et al* (1986) and Wynick *et al* (1990b). The AS and *dw/dw* GH sorts performed for this experiment showed a 2 fold and 4 fold enrichment in basal GH: basal PRL release in the GH enriched population compared to the unsorted population. However the basal release of GH and PRL from PRL enriched cells was not much different to the unsorted cells in the PRL sort. In experiment 2 the release and cell number did not always show the same degree of enrichment, thus although there was no enrichment in release on day 5, this does not necessarily mean that the PRL cells were not enriched in this experiment.

as healthy as normal primary cultures, possibly due to the lack of FS cells, however GRF did cause GH release, so the cells were still able to respond. The level of incorporation of $^{35}S$ was lower in this experiment than in normal primary cultures. This could be partly due to the lower cell number and partly due to the lower viability of the cells. The resulting gel was exposed to film for 3 weeks, longer than was necessary for previous experiments. This resulted in a higher background which may have masked any differentially expressed proteins.

The screening for marker proteins described in this chapter was mainly limited by the low yield of the sorting method. The extracts for the silver stained gels contained the entire extract obtained, concentrated to 10µl. The protein contents were not measured by Lowry assay, as the concentration was found to be too low in preliminary experiments. In addition this would use up too much of the sample. Instead, the extracts were made from a similar number of cells. The low levels of protein were also the reason for the use of miniature gels in this experiment, when perhaps large gels would have been more informative. In most cases, the yield was 1-3 million cells in the enriched and depleted populations from AS rats. Obviously the yield for *dw/dw* GH enriched cells was even lower (between 500,000 and 1 million). This was quite a low return, considering that the initial dispersion of 18 AS or *dw/dw* pituitaries would contain about 72 or 45 million cells, respectively. One of the main losses in cell number was due to extensive washing and aspirating. More recent experiments where the supernatants were tipped, followed by draining inverted on a tissue, appeared to increase the yield of cells. With increased cell yields, more wells of cells could be cultured and allow statistical analysis of the results. For practical and ethical reasons it is, of course, preferable to further optimize the method to increase the yield rather than use a larger number of animals.

In addition to the yield, another important variable in this method is the purity of the enriched and depleted cell populations. The main variation seen in purity appeared to relate to the cell density before sorting, suggesting that the presence of more than one cell per droplet may have been the cause of contaminating cell types. The cells that were resuspended at densities around 500,000 cells/ml yielded better purity than those at 1 million/ml.

Although the purity of the sorted cells are not as high as some previous reports of FACS sorted pituitary cells (Wynick *et al* 1990b, St. John *et al*, 1986), the enrichment achieved was still informative. The reduced GH content of the *dw/dw* somatotroph did not appear to prevent staining. Although the method still requires optimization, the work in this chapter has shown that FACS sorting is a viable method for comparing purified cell types from AS and *dw/dw* anterior pituitaries. Other methods, such as density gradient sedimentation, which relies on the fact that somatotrophs are more dense than other cell types, may not be so suitable for *dw/dw* somatotrophs as they are less granular. This means that AS and *dw/dw* somatotrophs will not separate in the same fraction, which could complicate the method.

Thus, to summarize the findings of this chapter;

1. Somatotrophs from *dw/dw* pituitary cells can be enriched by FACS sorting, in addition to somatotrophs from and lactotrophs from AS rats. GH enriched *dw/dw* pituitary cells contained a similar percentage of GH staining cells as a normal AS primary culture.
2. The cells obtained from GH and PRL sorts can be cultured, however the phenotype of these cells appears to change with time maybe due to interconversion of cell types.
3. The cells are still able to respond to GRF with a release of GH. It is possible that the different magnitudes of GH release indicate preferential staining and sorting for type II somatotrophs.
4. GH enriched *dw/dw* pituitary cells were still unable to respond to GRF with increased cAMP, despite showing a 5.7 fold increase in GH secretion. This again supports the selective nature of the deficiency in the somatotrophs of this model.
5. FACS sorting is an alternative strategy for screening for somatotroph specific marker proteins. Despite the limitations of cell number, bands differing in intensity were seen in GH and PRL enriched extracts. A band of about 40kDa, not detected in chapter 6, was shown to be enriched in GH enriched cells from AS pituitaries. In addition, there is evidence that one of the ~50kDa proteins described in chapter 6 could be a candidate lactotroph marker protein.

# GENERAL DISCUSSION

A main aim of this thesis was to validate the use of FACS for both sorting and analysis of pituitary cell types, as much of the data presented was obtained by these methods. In chapter 3, FACS analysis was shown to produce similar results to immunocytochemistry for GH and PRL in AS and *dw/dw* pituitaries. FACS has the advantage that large numbers of samples can be analysed in a short time, without the need for laborious cell counting by eye. In addition, it provides a convenient method to enumerate mammosomatotrophs.

While FACS analysis was carried out on fixed, permeablized cells, viable cells could also be stained using the same antibody systems. Thus FACS sorting and subsequent culture of pituitary cells was possible. While somatotroph and lactotroph sorts have been previously reported (Hatfield and Hymer, 1985; St. John *et al*, 1986; Wynick *et al*, 1990b), my work provides the first description of FACS sorting somatotrophs from *dw/dw* anterior pituitaries. Somatotrophs and lactotrophs from AS pituitaries, in addition to *dw/dw* somatotrophs, were shown by immunocytochemistry to be enriched in sorted cell populations. In addition, these cells were still able to respond to GRF by releasing GH. In both AS and *dw/dw* GH enriched cells, the magnitude of this response was more than in unsorted or GH depleted cells, suggesting preferential sorting for type II somatotrophs (as defined by Snyder *et al* (1977)).

Although only the hormone products were stained for during this study, previous reports have described FACS sorting by other cell markers. For instance, fluorescent ligands or antagonists can be used to mark receptors (eg. fluorescent CRF (Schwartz *et al*, 1986), rhodamine conjugated GnRH antagonist (Edwards *et al*, 1983)). Thus, somatotrophs conceivably could be sorted for using fluorescent ligands for the GRF or GHS receptors. The IL-1 receptor is expressed only on somatotrophs in the mouse anterior pituitary (French *et al*, 1996). Although I was unable to determine if it is also somatotroph specific in the rat due to technical problems (chapter 6), this possibility remains. This candidate protein could be sorted for by using a commercial fluorescent IL-1 (R&D systems). This was not attempted due to time and financial constraints, although would have been attempted had the cell specificity of the receptor been shown.

The primary role of the TGR rat in this thesis was as a control for the *dw/dw* rat. This rat has a pituitary exhibiting somatotroph hypoplasia without an inherent pituitary defect. Thus it could control for the reduced somatotroph number in the *dw/dw*, although the somatotroph deficiency was much more severe in the *dw/dw* rat.

This model, however, is also interesting in it's own right, in particular the behaviour of TGR pituitary cells in culture. The sensitivity of the cAMP response to GRF appeared to be similar to AS cells, while the amplitude was reduced only in parallel with the somatotroph number, suggesting that each somatotroph was normal in this respect. 5 day old cultures had similar cell populations to the intact pituitary (GH cells deficient, PRL cells similar to AS: Flavell *et al*, 1996). However, while both GH and PRL are deficient in the intact pituitary (Flavell *et al*, 1996), the GH content of culture extracts was no longer deficient (although the TGR culture data was only in duplicate). The secretion of GH in response to GRF appeared to follow a similar pattern to the cAMP accumulation, with the sensitivity to GRF the same as AS cultures and the amplitude of secretion deficient in line with somatotroph deficiency. The PRL content of the culture appeared to be double that of AS cultures (although this did not reach significance due to the low number of observations). Furthermore, the PRL release from male TGR cultures was three fold higher than the AS cultures. Thus the PRL phenotype of the TGR pituitary cells had recovered and apparently rebounded such that it was above normal. This would appear to reflect a continued effect of the different hypothalamic influence compared to AS rats, possibly due to the removal of higher inhibition of the PRL axis in the TGR. It would be interesting to study this further, but was beyond the scope of this thesis.

The majority of the work in this thesis is centred around the *dw/dw* rat, which was thought to possess a selective deficiency of somatotrophs due to the inability to respond to GRF by increasing intracellular cAMP (Downs and Frohman, 1991). Due to the severe deficiency in somatotroph number, it can be difficult to determine if there is also a deficiency in function per somatotroph. Downs and Frohman (1991) calculated a 6-10 fold deficiency in cAMP response per somatotroph, by measuring the responses of AS and *dw/dw* primary cultures to

GRF and correcting the responses for the somatotroph cell number. My results differed from those of Downs and Frohman (1991), as I detected no significant increase in cAMP in response to GRF (at doses up to 1000nM) in contrast to the small, but significant, response shown in their study.

I wished to further investigate the cAMP response by manipulating the somatotroph number in the cultures to control for the variable cell number. By mixing dispersed anterior pituitary cells from AS and *dw/dw* rats, cultures containing varying percentages of normal somatotrophs were obtained. At 5% AS cells, the equivalent of 2% normal somatotrophs, a significant cAMP response was still detectable. A 10 week old male *dw/dw* pituitary contains about 12% somatotrophs (chapters 3 and 4), 6-fold higher than in the diluted culture. This data therefore supports the theory that the *dw/dw* somatotrophs have an intrinsic defect.

In another approach, FACS sorting of *dw/dw* pituitary cells for GH resulted in a culture containing a comparable proportion of somatotrophs to the AS. Although these cells were only treated in duplicate, there appeared to be no cAMP accumulation in the GH enriched cells, again supporting the existence of a GRF signalling defect in these cells.

An important feature of the *dw/dw* signalling defect is it's selectivity. While the accumulation of cAMP in response to GRF is severely deficient, the secretory mechanisms of the somatotrophs is relatively intact, illustrated both *in vivo* (Carmignac and Robinson, 1990) and *in vitro* (Downs and Frohman, 1991; Kineman *et al*, 1989). This is also reflected by data in chapter 5, which showed that GRF stimulated release was only slightly less than AS cultures when expressed as fold over basal. This suggests that the deficiency in GH release reflects the reduced stores of GH due to the inability to synthesize GH, a function dependant on cAMP accumulation. The reduction in sensitivity of the secretory response to GRF may be due to the signalling defect indirectly, as the synthesis of GRF receptors is dependant on cAMP accumulation (Lin *et al*, 1992; Horikawa *et al*, 1996). In addition, while GH secretion does not depend on cAMP accumulation, addition of exogenous cAMP or stimulation of adenylate cyclase by forskolin does result in GH secretion (Down and Frohman, 1991). This is

consistent with communication between the intracellular pathways in the somatotroph (Sartin *et al*, 1996).

The current literature viewpoint is that the *dw/dw* phenotype is caused by the inability of somatotrophs to proliferate due to the signalling defect (Downs and Frohman, 1991). However, I do not believe that this explains the phenotype in this model. The data in chapter 4 show that the percentage of GH cells remains steady in the expanding AS pituitary. Similarly, the percentage of GH cells in the *dw/dw* declines only slightly during development, thus the somatotroph population is still able to expand as the pituitary grows. Thus the reduced somatotroph number at each age is due to the initial deficiency, present before birth (Carmignac *et al*, 1993b; Zeitler *et al*, 1994). As GRF signalling is not required for somatotroph proliferation at this time (Lin *et al*, 1993), I believe the deficiency GRF signal transduction to be a symptom of the phenotype, and not the cause.

The early reports concerning this rat found no difference to controls in the PRL content of the pituitary (Charlton *et al*, 1988; Bartlett *et al*, 1990). In addition, the increase in percentage of lactotrophs was thought to be merely compensating for the smaller pituitary size (Kineman *et al*, 1989). However, SDS-PAGE of anterior pituitary extracts revealed that the PRL band stained much more intensely in *dw/dw* extracts than AS. Although a larger proportion of the *dw/dw* pituitary was loaded, due to it's smaller size, the difference in the band intensity was large enough to suggest there was a real difference. I therefore decided to re-evaluate the PRL phenotype in this model. As NIMR houses the original colony of *dw/dw* rats, it seemed logical that this study should be carried out in our laboratory. Radioimmunoassay of adult pituitary extracts confirmed the elevated PRL contents. Both male and female *dw/dw* pituitaries contained more than double the amount of PRL compared to AS controls. Soon after my observations, others in our laboratory confirmed significantly higher PRL pituitary contents in *dw/dw* rats (Phelps *et al,* 1997).

In contrast to the adult, the number of PRL staining cells in *dw/dw* pups was found to be increased. At 9 days, the *dw/dw* pituitaries contained a 4 fold higher percentage of lactotrophs. Assuming the total cell number of the *dw/dw*

pituitary is 60% of controls, this translates to a 2.5 fold higher lactotroph number in the *dw/dw* pituitary. The increased lactotroph number in the *dw/dw* was maintained until at least 23 days.

Thus an important conclusion from this thesis is that the defect in the *dw/dw* is not restricted to the somatotroph. Due to the developmental relationship between somatotrophs and lactotrophs (Hoeffler *et al*, 1985), I propose that the *dw/dw* phenotype is caused by a developmental defect affecting the differentiation of both somatotrophs and lactotrophs. This will have important implications for further work in this laboratory as a comparative search for genetic differences should not necessarily be confined to somatotrophs, but also extended to lactotrophs of *dw/dw* and normal rats.

While an AS pituitary extract contained 2 orders of magnitude more GH than PRL (chapter 3), an AS culture extract contained only about twice as much GH as PRL due to the removal of the hypothalamic inhibition on the PRL axis. Despite this alteration in the ratio of the hormone contents, the *dw/dw* culture maintained the three fold higher PRL level than the AS, as seen in pituitary extracts. This provides strong evidence that the PRL phenotypes is inherent to the *dw/dw* pituitary and not a result of altered hypothalamic influences due to GH deficiency.

The mammosomatotroph cell line, $P_0$ (Kashio *et al*, 1990), is unusual in that it retains GRF receptors and does not form confluent monolayers suggesting it is less dedifferentiated than other cell lines (eg. $GH_3$, GC). This cell line may be an immortalized mammosomatotroph precursor and like the *dw/dw* shows reduced ability of GRF to increase cAMP. It is possible that the defect in the *dw/dw* rat is similar to that in this cell line, resulting in the lack of somatotroph commitment. It is therefore conceivable that the reduced cAMP response to GRF is a consequence of this developmental block. Perhaps the GRF receptor is not able to couple with adenylate cyclase in this early progenitor, resulting in the observed absence of a cyclase response to GRF. If the *dw/dw* phenotype was caused by such a defect, it would explain the early onset of the GH deficiency. As the target cell of the defect would be the mammosomatotroph progenitor, it is

also in-keeping with the presence of the lactotroph phenotype in this model, described in this thesis.

In support of this theory, a similar cell line (DP), derived from *dw/dw* pituitary cultures, showed a similar cAMP response to GRF as $P_0$ cells (Brain *et al*, 1991). The fact that the cell lines do not differ greatly in their response suggests that the $P_0$ cells have a similar defect to the *dw/dw* derived DP cells, even though they were derived from normal pituitary cells.

Estrogen is thought to affect the ratio of GH and PRL containing cells by recruiting cells producing GH only to express PRL. As the *dw/dw* phenotype appears to affect both somatotrophs and lactotrophs, I wanted to compare the effects of $E_2$ on *dw/dw* and AS rats. 10 days after receiving subcutaneous $E_2$ pellets, the GH-, PRL- and double staining cells in the pituitaries were quantified by FACS analysis. $E_2$ treatment of *dw/dw* males decreased the percentage of GH-only staining cells, becoming more similar to the female profile. This was in contrast to the AS, where no significant effects of $E_2$ were seen. If the somatotroph / lactotroph system is viewed as a dynamic equilibrium, in accordance with the theories of Frawley and Boockfor (1991), the *dw/dw* rat rests closer to the lactotroph side than the AS rat, and this result suggests that it can be pushed even closer by $E_2$ treatment. Thus, not only is the balance of the GH/PRL lineage altered during development, but it's sensitivity to $E_2$ is also altered. Furthermore, other studies in the lab suggest that the *dw/dw* rat treated with $E_2$ shows typical responses of mammosomatotroph excess, including large PRL responses to GHRP and TRH (Carmignac *et al*, 1998).

While the inheritance of the *dw/dw* phenotype is recessive, as defined by body weight, there is evidence that there maybe a gene dosage effect. Houston *et al* (1991) found that GH and Pit-1 mRNA levels were reduced in the *dw/dw*, and the heterozygote had an intermediate phenotype in these parameters. However, I found no differences between AS controls and AS/*dw* heterozygotes in GH or PRL pituitary content or cell number. Thus the reduction in GH mRNA does not extend to GH protein. GH and Pit-1 mRNA are synthesized downstream of the cAMP dependant signalling pathway, thus I also investigated the response of these cells in culture. As the deficiency in cAMP in the *dw/dw* is so severe, if

there were a gene dosage effect, it may be most likely to affect this parameter. However, the cAMP accumulation in response to GRF showed no evidence of deficiency in cultures of AS/*dw* pituitary cells.

Another aim of this thesis was to screen for somatotroph specific proteins. The purpose of this was three-fold;

1. To identify a marker protein that could be used to identify somatotrophs, other than GH. GH is an unreliable marker due to it's fluctuation in abundance within somatotrophs. In addition, the mammosomatotroph theory (Frawley and Boockfor, 1991) states that somatotrophs and lactotrophs are the same cell type exhibiting different phenotypes at different times and can interconvert. This highlights the need for other protein markers to define somatotroph function other than GH itself.
2. To identify proteins deficient in the *dw/dw* pituitary and thus further characterize the phenotype of this model.
3. To identify proteins involved in somatotroph function and thus further investigate the mechanisms of GRF signal transduction.

In addition to screening for new candidates, some work was carried out on the possibility of IL-1R being somatotroph specific within the pituitary of the rat, as it is in the mouse (French *et al*, 1996). While the investigation of IL-1R as a somatotroph specific protein did not yield much success, screening for new somatotroph specific proteins revealed several candidates by SDS-PAGE. Two basic strategies were used;

1. Comparison of control and GRF treated culture extracts to screen for proteins involved in somatotroph function, for instance GH synthesis of somatotroph proliferation. Although GRF stimulated, these proteins would not necessarily be somatotroph specific.
2. Comparison of pituitary extracts from AS and *dw/dw* rats or cultures and comparison of FACS sorted cells to screen for proteins more abundant in the somatotroph. These proteins would not necessarily be involved in somatotroph function.

An ideal cell specific protein marker would be present in the cell at a constant abundance, thus the second strategy would be preferable. However, both

strategies revealed differentially expressed proteins. These candidate proteins are classified by their approximate size, estimated by comparison to molecular weight markers on SDS-PAGE gels. No attempt was made to determine the exact size of the proteins. More accurate determination of the molecular weights of the proteins could have been obtained by the use of gradient acrylamide gels which show a more linear separation of molecular weight.

A protein running just ahead of the 97.4kDa marker (~95kDa) was shown to be up-regulated by GRF. The increased band intensity in GRF treated AS cultures was apparent by both [$^{35}$S]methionine incorporation studies and silver staining of SDS-PAGE acrylamide gels. However, this protein was also present in *dw/dw* pituitary extracts and was not enriched by FACS sorting for GH, indicating that it is probably not somatotroph specific. In addition, it was also up-regulated by GRF in *dw/dw* cultures (in [$^{35}$S]methionine incorporation studies), although this may have been a non-specific effect on other cell types. This protein, although probably involved in somatotroph function, is probably also present in other cell types and is therefore not an ideal specific somatotroph marker. It is also regulated by GRF, thus it's abundance in the somatotroph may not be stable.

Three bands were identified using the second strategy to screen for somatotroph proteins not regulated by GRF;

1. A band of about 40kDa was shown to be enriched in AS cells sorted for GH.
2. The larger of a doublet at about 50kDa was more abundant in AS pituitary extracts than *dw/dw* pituitary extracts.
3. A protein of about 35kDa was found to be more abundant in AS culture extracts than *dw/dw* culture extracts.

These proteins would make better marker proteins as they are more likely to be somatotroph specific. In addition, their abundance was not GRF stimulated although this does not rule out their regulation by other factors.

The smaller protein in the ~50kDa doublet was shown to be more abundant in the *dw/dw* than the AS pituitary extract. In addition, a protein of a similar size was shown to be enriched in AS cells sorted for PRL. If these two candidate bands represent the same protein, it may be a candidate lactotroph marker

protein. While the original aim of the work in this thesis was to use the *dw/dw* as a model to study somatotroph proteins, the subsequent discovery of the lactotroph phenotype altered the objectives. Therefore, this potential lactotroph marker protein may be valuable in studying the eitiology of the *dw/dw* phenotype.

Obviously, a future objective would be to identify these bands. To achieve this, amino acid sequencing could be carried out, however the proteins would first need to be isolated. Attempts to extract proteins out of acrylamide gel slices were unsuccessful. An alternative method would have been electro-elution, although this was not attempted to the lack of the necessary equipment and time constraints.

Whatever the method of used, it is unlikely that a single protein would be isolated. Even on the larger acrylamide gels, the bands were close together making it difficult to cut a single band out of the gel. Even a single band may contain several proteins, therefore in addition to making isolation difficult, some differentially expressed bands would be masked. Similar studies (Oh-Ishi *et al*, 1998; Yoyoka and Friesen, 1986) have employed 2D-gel electrophoresis, introducing a second method of purification.

In addition, a similar approach could be used focusing on RNA instead of protein. Differential display PCR analysis can been used to detect differences in the RNAs between samples (Liang and Pardee, 1992; Bauer *et al*, 1993). This method could be used to detect differences in mRNA species between AS and *dw/dw* pituitaries or to detect GRF stimulated RNA species. Due to the use of PCR to amplify the RNA, this method would be more sensitive than the protein based studies described in this thesis. However, differences in RNA may not necessarily translate to actual protein differences. Confirmation of differential protein abundance would need to be confirmed after the identification of the RNA species.

The use of these strategies to identify differences between AS and *dw/dw* pituitaries could provide further insight to the defect in the *dw/dw*, for which the mutation is still unknown. However, in light of the evidence for a

developmental defect in the *dw/dw*, presented in this thesis, there is a strong possibility that the gene defective in the *dw/dw* may be expressed transiently. If this is the case, screening for differences in protein or RNA in adult pituitaries may be futile. For example, genetically directed representational difference analysis (GDRDA), a positional cloning based method, has recently been used to identify a mutation in the *Prop-1* gene as the genetic basis of the Ames dwarf (Sornson *et al*, 1996). Prop-1 is expressed in normal mice between e10.5 and e15.5, and only trace amounts are detectable in the adult (Sornson *et al*, 1996). Thus, analysis of adult pituitaries would not have revealed the defect in this mouse, and the same my be so for the *dw/dw* rat.

In conclusion, the pituitary gland is often studied as a model of development and differentiation of cell lineages due it's possession of multiple cell types. While the *dw/dw* rat has been extensively used as a model of GH deficiency, this thesis has provided evidence that the defect affects both somatotroph and lactotroph phenotypes due to a developmental defect affecting the mammosomatotroph precursor cell lineage. As the defect is characterized further, the usefulness of this rat will continue to increase. Thus the data in this thesis has important implications in the future utilization of the *dw/dw* rat in research into pituitary function.

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# PUBLICATIONS

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## ANALYSIS OF SOMATOTROPHS AND LACTOTROPHS IN NORMAL AND DWARF (*dw/dw*) RATS BY FLUORESCENCE ACTIVATED CELL SORTING.

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The autosomal recessive dwarf (*dw/dw*) rat shows a 40% reduction in growth, a 95% reduction in pituitary GH and a markedly reduced GH cell number compared to normal rats of the same parent (AS) strain [1]. The nature of the mutation is still unknown, but involves a defect in the adenylate cyclase response to GHRH [2]. PRL concentrations in the *dw/dw* strain have previously been described as "not reduced". However, SDS-PAGE and RIA of *dw/dw* pituitary extracts shows that their PRL content is significantly increased despite profound pituitary hypoplasia. Since somatotrophs and lactotrophs may derive from a common precursor cell, the defect in the *dw/dw* rat may divert differentiation from somatotroph to the lactotroph phenotype. To study this we have developed methods for single and two-colour fluorescence activated cell sorting (FACS) of GH and PRL positive cells and compared these in *dw/dw* and normal AS rats. Adult *dw/dw* anterior pituitaries yielded about 2.5 million cells, compared to 4 million cells from AS pituitaries. GH positive cell numbers (% total) were reduced in dw/*dw* rats (males ~ 10%, females ~ 5%) compared with AS male and females (~30%), whilst PRL positive cells were higher (*dw/dw* male ~ 40%, *dw/dw* female ~ 50% vs AS male ~ 20%, AS female ~ 30%) such that the total of both cell types were similar in the two strains. The number of cells positive for both GH and PRL was similar in AS and *dw/dw* rats, suggesting that a higher proportion of GH cells in the *dw/dw* also contained PRL. Since interconversion of GH and PRL cell types is sensitive to estrogen, we also analyzed cells from AS and *dw/dw* male rats treated with oestradiol (25μg/d for 10d). The proportions of GH, PRL and double positive cells in AS males was unchanged by estrogen. The total percentage of GH positive cells in the *dw/dw* rats did not change, but the number also staining for PRL increased after estrogen treatment. The number of GH positive/PRL negative cells in the estrogen treated *dw/dw* male was similar that in the female *dw/dw*. The results suggest that the mutation in the *dw/dw* rat causes a switch from somatotroph to lactotroph differentiation whilst estrogen treatment of male *dw/dw* rats markedly stimulates PRL production in most of their GH-positive cells.

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