CHARACTERISATION OF PHENOTYPIC DETERMINANTS IN RAT MODELS OF GROWTH RETARDATION

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PUBLICATIONS

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El-Kasti MM, Christian HC, Huerta I, Hill N, Matthews DR, Wells T. (2004) Pregnancy-induced plasticity in rat pituitary: a morphological and endocrine study of somatotroph and lactotroph function. *The 23rd Joint Meeting of the British Endocrine Societies. Brighton-U.K. Endocrine Abstracts 7,* **P143.**

Huerta-Ocampo I, Wells T, ***El-Kasti*** MM, Christian HC. (2004) The role of growth hormone-releasing hormone (GHRH) on the somatotroph and lactotroph lineage in male and female dwarf (*dw/dw*) rats. *The 2004 Annual Meeting of the British Society for Neuroendocrinology. Glasgow-U.K.* **P13.**

El-Kasti MM, Houston PA, Wells T. (2004) Does placental ghrelin contribute to the elevation in baseline GH secretion in pregnant rats? *5th International Symposium on Growth Hormone Secretagogues. Ghrelin and its Analogues across the Lifespan. Camogli-Italy.* **Oct. 2004.**

El-Kasti MM, Huerta-Ocampo I, Houston PA, Christian HC, Carter DA, Wells T. Pregnancy-induced plasticity in the hypothalamo-pituitary-growth hormone (GH) and -prolactin axes: evidence for an extrapituitary GH-like substance. *Endocrinology* (in preparation).

Christian HC, Huerta-Ocampo I, ***El-Kasti*** MM, Thompson NM, Houston PA, Carmignac DF, Robinson ICAF, Wells T. The 'intermediate' lactotroph: a unique ghrelin-sensitive lactotroph in dwarf (*dw/dw*) rats. *Nature Cell Biology* (in preparation).

Stevenson AE, ***El-Kasti*** MM, Evans BAJ, Elford C, Evans SL, Perry M, Gregory JW, Wells T. Peripubertal development of adiposity and femoral strength in rat models of growth hormone deficiency. *Journal of Bone & Mineral Research* (in preparation).

ABSTRACT

In this thesis, I describe work on the physiological basis of dwarfism in both established (*dw/dw*) and novel (78N) rat models.
In the dwarf (*dw/dw*) rat, an unknown mutation causes a severe, sub-total growth hormone (GH) deficiency associated with somatotroph hypoplasia, and an increase in lactotroph numbers and prolactin (PRL) storage. It has, recently, been shown that the *dw/dw* pituitary contains a unique, morphologically distinct lactotroph, the 'intermediate' lactotroph, which has morphological features between those of the type I and II lactotroph subtypes. These 'intermediate' lactotrophs also show functional features of somatotrophic lineage, releasing PRL in response to ghrelin. This female-specific induction of PRL secretion is oestrogen-dependent. The present study reveals that in pregnancy the population of 'intermediate' lactotrophs increases in parallel with the lactotrophic lineage. We have taken two approaches to investigate whether elevated GH releasing factor (GRF) gives rise to the 'intermediate' lactotroph in the *dw/dw* pituitary. The absence of the 'intermediate' lactotroph in the GRF-insensitive little (*lit/lit*) mouse, and a possible reduction in 'intermediate' lactotrophs in monosodium glutamate (MSG)-treated *dw/dw* rats, suggest that the presence of this unique lactotroph subtype in *dw/dw* pituitary is regulated, in part by GRF.

Although small numbers of 'intermediate' lactotrophs are present in pregnant normal female rats, they do not appear to play a significant role. Pregnancy is also associated with an increase in type I lactotrophs, but no change in somatotrophs. The accompanying increase in baseline circulating GH does not elevate plasma insulin-like growth factor I (IGF-I) levels, or stimulate skeletal growth, and may be derived from a placental GH-like protein.

A novel transgenic rat line – 78N – was previously generated in our laboratory. It is a presumed insertional-mutant that exhibits a pleiotropic phenotype associated with neonatal male lethality. Females exhibit juvenile-onset growth retardation. Initial investigation of the cause of the growth retardation showed that the skeletal impairment is GH-independent. In the present study, parallel postmortem examination has revealed kidney abnormalities in both male and female mutants. Morphological analysis showed alteration of the normal cortico-medullary architecture. Molecular analysis of the neonatal male kidney transcriptome revealed numerous differentially expressed genes including kidney androgen-regulated protein (KAP) and neural precursor cells expressed developmentally down-regulated 4 (Nedd4) WW domain binding protein (N4WBP4). The 78N line may be a useful model of idiopathic short stature (ISS) associated with idiopathic nephritic syndrome – minimal change nephropathy (MCD) or disease.

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ABBREVIATIONS

A	Absorbance
ABL	Antibody blank
ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone/Vasopressin
AQP	Aquaporin
AUC	Area under the curve
BME	β-mercaptoethanol
BMP	Bone morphogenetic protein
BP	Binding protein
BSA	Bovine serum albumin
CD	Sprague Dawley (Charles River)
CNS	Central nervous system
cpm	counts per minute
D/A	Delayed addition
DA	Dopamine
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Disodium ethylenediaminetetraacetate
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ENaC	Epithelial sodium channel
EPW	Epiphyseal plate width
EST	Expressed sequence tags
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridisation
FS	Folliculostellate cells
FSH	Follicle-stimulating hormone
GA	Glutaraldehyde
GH	Growth hormone
GHD	Isolated GH deficiency
GH-R	GH receptor
GHS	GH secretagogue
GHS-R	GH secretagogue receptor
GRF/GHRH	Growth hormone releasing factor/hormone
GTC	Guanidium isothiocyanate
Hr	Hour
Hx	Hypophysectomised

IAV	Intra-assay variability
IDDM	Insulin-dependent diabetes mellitus
IGF	Insulin-like growth factor
IGFBP	IGF binding protein
ILT	'Intermediate' lactotroph
IMS	Industrial methylated spirit
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IR-SRIF	Immunoreactive somatostatin
ISH	In situ hybridisation
ISS	Idiopathic short stature
KAP	Kidney androgen-regulated protein
KO/Ko	Knockout
LH	Luteinizing hormone
Lhx	LIM homeobox
LT-I	Lactotroph type I
LT-II	Lactotroph type II
MCD	Minimal change nephropathy or disease
Min	Minute
MPHD	Multiple pituitary hormone deficiencies
MSG	Monosodium L-glutamate
MSH	Melanocyte-stimulating hormone
N4WBP4	Neural precursor cells expressed developmentally down- regulated 4(Nedd4) WW domain binding protein
Na_2HPO_4	Disodium hydrogen phosphate
NaAc	Sodium acetate
NaH_2PO_4	Sodium dihydrogen phosphate monohydrate
NaOH	Sodium hydroxide
NaPi	Sodium phosphate
NDI	Nephrogenic diabetes insipidus
NIDDK	National Institute of Diabetes & Digestive & Kidney Diseases
OC	Observed concentration
PAG	Protein A gold
PBS	Phosphate buffered saline solution
PEG	Polyethylene glycol
PeN	Periventricular nucleus
PG	Pregnant
PIF	PRL inhibiting factor
PL	Placental lactogen
POMC	Proopiomelanocortin
PRF	PRL releasing factor
PRL	Prolactin
PRLR	PRL receptor

PSP	Pseudopregnant
PVN	Paraventricular nucleus
RBC	Red blood cell
rGH	Rat GH
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rPRL	Rat PRL
RT	Room temperature
SAGE	Serial analysis of gene expression
SDS	Sodium dodecyl sulphate
Sec	Second
SL	Somatolactin
SMT	Somatomammotroph
SON	Supraoptic nucleus
SRIF	Somatostatin
SSTR	Somatostatin receptor subtype
ST	Somatotroph
TA	Tannic acid
TAE	Tris-acetic acid-EDTA
TE	Tris-EDTA
TG	Transgenic
TIDA	Tuberoinfundibular dopaminergic system
TRH	Thyroid releasing hormone
TSH	Thyroid-stimulating hormone
V-C	Virgin-control
VIP	Vasoactive intestinal peptide
WT	Wildtype

CHAPTER 1.
GENERAL INTRODUCTION – LITERATURE REVIEW

1.1 THE MASTER GLAND – THE PITUITARY

The pituitary gland is a vital component of neuroendocrine mechanisms for the regulation of development, homeostasis, and reproduction. It is controlled by the central nervous system (i.e. hypothalamic neuropeptides) and by positive/negative feedback loops from peripheral organs.

The posterior pituitary (neurohypophysis) contains the terminals of hypothalamic magnocellular neurones, whose cell bodies are located in the supraoptic (SON) and the paraventricular (PVN) nuclei. These terminals store and secrete antidiuretic hormone (ADH, vasopressin) and oxytocin. The principal physiological effect of ADH is the retention of water by the kidney, besides its additional action on the smooth muscle of the arterioles to elevate blood pressure. Oxytocin plays an important role in labour and milk ejection.

The anterior pituitary (adenohypophysis) appears to be a highly specialized tissue. Its parenchyma contains the agranular folliculostellate (FS) and the granular endocrine cells. FS cells are considered the functional units of a dynamically active cell network wiring the whole gland. They interact with neighbouring endocrine cells. The cell networking within the pituitary is believed to have a role in coordinating the activities of distant parenchymal cells. In culture, FS cells act as a 'pituitary buffer' modulating hormone secretion and cell proliferation (Fauquier et al., 2002). The endocrine cell types express growth hormone (GH) in somatotrophs, prolactin (PRL) in lactotrophs, thyroid-stimulating hormone (TSH) in thyrotrophs, and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in gonadotrophs, and proopiomelanocortin (POMC) – which is cleaved to adrenocorticotropic hormone (ACTH) in corticotrophs and melanocyte-stimulating hormone (αMSH) in melanotrophs. An embryonic cell type known as the rostral tip 'thyrotroph' also exists. These hormones regulate a diverse range of important biological processes in response to signals from the hypothalamus and peripheral organs. By and large, GH regulates linear growth and metabolism. PRL regulates milk production in females. TSH controls the secretion of thyroid hormones from the thyroid gland. FSH and LH regulate reproductive

development and function; FSH is involved in ovarian follicular and sperm maturation whereas LH triggers follicular rupture, ovulation, corpus luteal formation, and interstitial cells of Leydig to enhance testosterone production. ACTH regulates metabolic function by stimulating glucocorticoid synthesis in the adrenal cortex. MSH regulates melanin synthesis in the epidermis in some vertebrate species (Scully and Rosenfeld, 2002).

1.2 PITUITARY ORGANOGENESIS

The pituitary gland originates from two embryonic tissues. The neurohypophysis develops from neural ectoderm at the base of the developing diencephalon. In contrast, the adenohypophysis is derived from Rathke's pouch, an embryonic structure arising from oral ectoderm.

During embryogenesis, signalling molecules secreted from multiple organizing centers in the neural and oral ectoderm are required for initial formation of the pituitary gland, coordinating the commitment, early patterning, proliferation, and positional determination of the hormone-producing pituitary cell types. This process is summarised in figure 1.1. Extrinsic and intrinsic signalling mechanisms (i.e. transcriptional and non-transcriptional factors) govern the early (1) and late (2) aspects of pituitary development:

(1) The early steps of pituitary organogenesis are controlled by signals and transcription factors. The ventral diencephalon acts as the key organising center for the patterning and commitment of Rathke's pouch, the formation of which is a two-step process. From the diencephalon, the first signal, bone morphogenetic protein 4 (BMP4), triggers the formation of the pouch rudiment, the placode. A second signal, fibroblast growth factor (FGF8), initiates the expression of LIM homeobox 3 and/or 4 (Lhx3 and/or Lhx4) that induces the formation of the definitive pouch. The transition of Rathke's pouch into a pituitary gland with its proper cell types is controlled by the critical gene, Lhx3 (Dasen and Rosenfeld, 2001).

(2) Cell commitment and determination of pituitary cell types are multi-step events involving many signals and transcription factors. In particular, FGF8 promotes the proliferation of cell-type precursors while BMPs prompt initial proliferation. In the pituitary, transient signalling gradients, directing the development of specific cell types, stimulate nuclear mediators of cell type commitment. They comprise

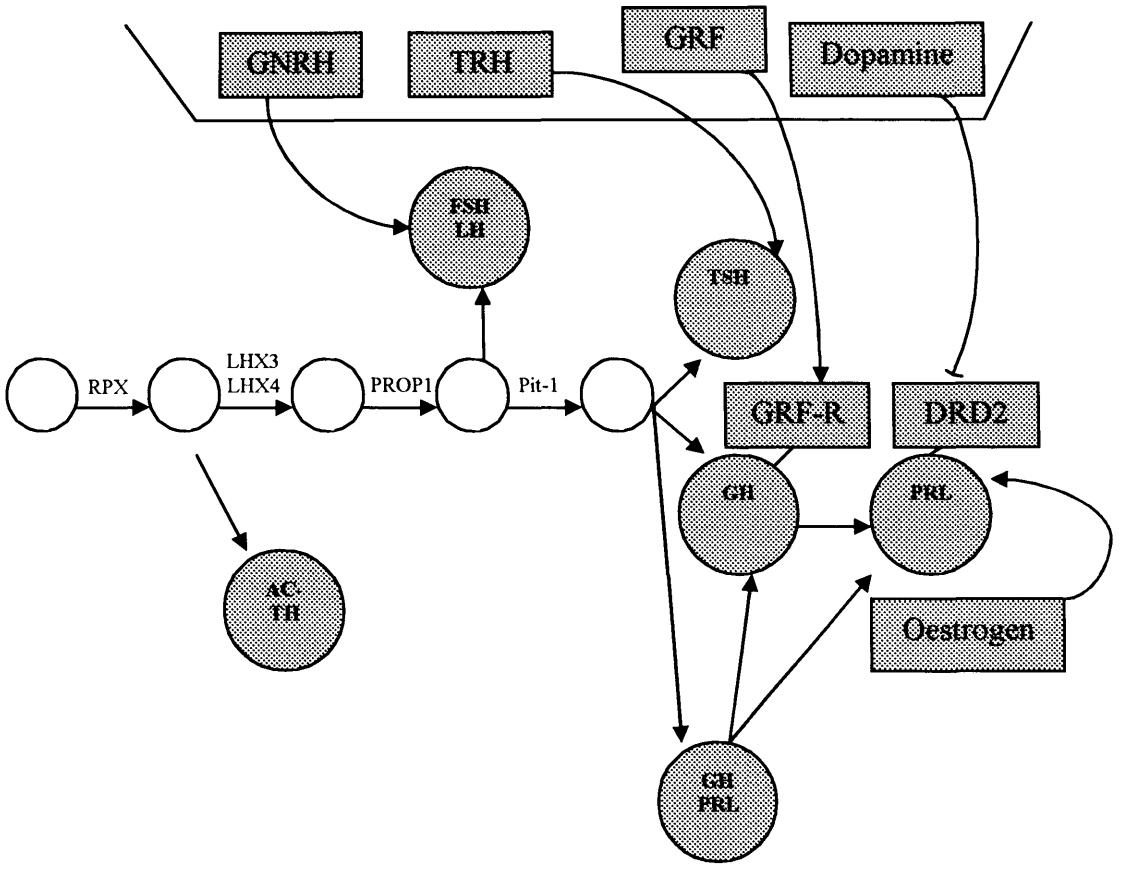


Figure 1.1 Proposed model for pituitary cell lineage and regulation of proliferation. The anterior pituitary cell types (grey circles) are labelled with the hormone they secrete. Steps in precursor-cell differentiation and the transcription factors that might regulate them are illustrated. Possible regulation of cell proliferation by secreted factors is designated (grey boxes) (adapted from Watkins-Chow and Camper, 1998). GRF-R, GH releasing factor receptor; DRD2, dopamine receptor 2.

transcription factors acting as activators or repressors, and their related coregulators (Olson and Rosenfeld, 2002). Five crucial homeodomain transcription factors are involved in the regulation of the anterior pituitary development and cell lineage determination. During early organogenesis, three homeobox genes, Lhx3, Lhx4, and Titf1, are required to control pituitary primordium formation. Two other homeobox genes, Prop-1 and Pit-1, appear to be critical for specialization and proliferation of subsets of the five differentiated cell types (Figure 1.1).

Therefore, from the nascent pituitary gland (Rathke's pouch), distinct cell types emerge in a precise spatial and temporal pattern according to their positions relative to the signalling sources. Corticotrophs and rostral tip thyrotrophs (Tr) emerge first ventrally, followed by melanotrophs dorsally. Somatotrophs and lactotrophs progress caudomedially, while thyrotrophs and gonadotrophs progress more rostroventrally (Kioussi et al., 1999; Sheng and Westphal, 1999).

Since this thesis addresses aspects of somatotroph and lactotroph development and function, this introduction will be restricted to the factors specifically regulating the hypothalamo-pituitary-GH and -PRL axes.

1.3 DEVELOPMENT OF THE NEUROENDOCRINE GROWTH HORMONE AND PROLACTIN AXES

1.3.1 Transcription Factors

1.3.1.1 Prop-1

The homeodomain factor Prop-1 (prophet of Pit-1) has been shown to be required for the ventral progression of anterior pituitary cell types. Prop-1 expression precedes Pit-1 expression (Gage et al., 1996), and seems to be required to permit asymmetrical cell division, by which the Pit-1-dependent lineage is generated (Kioussi et al., 1999). Prop-1 gene mutations are responsible for the phenotype of pituitary endocrine cell hypoplasia in the *Ames dwarf* mouse and some clinical cases of multiple pituitary hormone deficiency (Gage et al., 1996; Sornson et al., 1996). Loss of somatotrophs, lactotrophs, and thyrotrophs with a reduction in gonadotrophs are the result of this mutation (Sornson et al., 1996).

1.3.1.2 Pit-1 (GHF1, PUF-1)

The POU (Pit-1, Oct-1/2, and Unc-86) homeodomain factor, Pit-1, was initially identified as a transcriptional activator of the genes encoding GH and PRL. Evidence from mouse genetics (Camper et al., 1990; Li et al., 1990) revealed that this factor is essential for the determination and differentiation of three anterior pituitary cell types representing the somatotroph, lactotroph, and thyrotroph lineages. The *Snell* and *Jackson dwarves* occur due to spontaneous mutations in the Pit-1 gene (Li et al., 1990).

1.3.2 Non-Transcriptional Factors

1.3.2.1 GRF

GRF is synthesized in the arcuate nucleus of hypothalamus, released into the median eminence and transported to the anterior pituitary gland by a portal circulatory system. It regulates GH secretion and controls somatotroph proliferation and survival (Barinaga et al., 1983). The importance of this function is seen in the *little* (*lit/lit*) *mice* (Lin et al., 1993) which carry a mutation in the GRF receptor gene and exhibit profound somatotroph hypoplasia and dwarfism (Mayo, 1996). GRF-mediated somatotroph proliferation is mediated by activation of the cAMP signalling pathway (Lin et al., 1993).

1.3.2.2 The dopamine receptor (D2) and transporter (Dat1)

In contrast, lactotroph maintenance is negatively controlled by dopamine-mediated inhibition of the same pathway as GRF (Dasen and Rosenfeld, 2001). D2 receptors inhibit prolactin secretion and lactotroph differentiation (Spady et al., 1999). Dopaminergic action is terminated via the dopamine transporter situated on the membrane of the hypothalamic dopaminergic neurones. The *D2 receptor-deficient* (*Drd2-/-*) *mice* have progressive lactotroph hyperplasia and chronic hyperprolactinemia (Kelly et al., 1997), leading to tumours in aged female animals (Saiardi et al., 1997; Asa et al., 1999). D2R knockout mice also exhibit significant growth retardation accompanied with selective loss of somatotrophs, which is thought to be due to the absence of the effects of dopamine on GRF release in the arcuate nucleus (Bosse et al., 1997). In D2R-deficient mice, disruption of D2 receptors also alters the GH axis and causes dwarfism in males (Diaz-Torga et al., 2002). The *dopamine transporter knockout (Dat1-/-) mice* are hyperactive due to the protracted

duration of dopamine action in dopaminergic synapses. Female mutant mice are incapable of nursing their litters, which show significant growth retardation compared with their wildtype littermates (Giros et al., 1996). They show decreased GRF expression, which is under the inhibitory dopaminergic control. Over-stimulation of D2 receptors in the pituitary results in decrease in Pit-1 in the adult pituitary gland of these animals leading to hypoplastic gland due to selective loss of somatotrophs and lactotrophs (Bosse et al., 1997).

1.3.2.3 Oestrogen

Oestrogen plays an important role in the regulation of pituitary cell differentiation and sexual maturation. It induces lactotroph proliferation (Spady et al., 1999). In mature rats, chronic treatment with estradiol stimulates not only prolactin secretion (Lieberman et al., 1982), but also lactotroph proliferation (Takahashi et al., 1984). In pregnancy and lactation, lactotroph proliferation in the anterior pituitary occurs due to the high circulating oestrogen (Porter et al., 1991). When treating embryonic pituitary cells with estradiol, an increase in immature PRL cells occurs and is dose-dependent (Yamamuro et al., 2001). The *oestrogen receptor (ER) knockout mouse* exhibits a reduction in the number of lactotrophs and gonadotrophs, and an infertility phenotype in both males and females (Scully et al., 1997). In the pituitary, oestrogen is important in the control of both, the development and proliferation of PRL-cell lineages.

1.4 GH/PRL-SECRETING CELLS

1.4.1 Pituitary Plasticity and Proposed Mechanisms

The adenohypophysis appears to be a remarkably plastic organ. It has the ability to change its size (hypertrophy), and increase or decrease the number (hyper- or hypo-plasia) of particular cell populations in order to meet the exact requirements of the body. Cellular hypertrophy is characterised by development of cytoplasmic organelles and/or augmentation in the number of secretory granules. For the development of pituitary hyperplasia, three mechanisms are proposed.

(1) Stem cells differentiate into mature cells of the same cell type (Yoshimura et al., 1969).

(2) An increase in cell number may be due to proliferation of the stimulated cell type (Takahashi et al., 1984).

(3) Cells of one line may transform to another cell line. This process is termed 'transdifferentiation' in which a change in the morphologic features and secretory capacity is acquired (Horvath et al., 1990). This interconversion is not a direct process, and may occur through transitional cells displaying morphologic and functional components shared by both cell types (Vidal et al., 2001).

1.4.2 Somatotrophs, Lactotrophs, and Somatomammotrophs

Somatotrophs. In rats, somatotrophs are first detected at E18.5 (Frawley et al., 1985; Watanabe and Haraguchi, 1994; Taniguchi et al., 2001a). In the first two weeks after birth, a rapid increase in the number of somatotrophs is noticed, besides the lactotrophs (Taniguchi et al., 2001a) and thyrotrophs (Taniguchi et al., 2001b). Somatotrophs are considered to be the most abundant pituitary cell type.

Lactotrophs. The first detection of lactotrophs is at E18.5-19.5 (Watanabe and Haraguchi, 1994; Taniguchi et al., 2001a). Nevertheless, their number is small until after birth and shows slow increase postnatally compared to somatotrophs (Taniguchi et al., 2001a).

Somatomammotrophs. In primary cultures derived from the anterior pituitary of normal rats, the existence of secretory cells containing both GH and PRL in their secretory granules has been reported for the first time by Frawley and colleagues (Frawley et al., 1985; Hoeffler et al., 1985). These bi-hormonal cells are called somatomammotrophs (also called mammosomatotrophs or somatolactotrophs) and are considered by most of the researchers to be the common progenitor cell from which both somatotrophs and lactotrophs differentiate into their mono-hormonal cell types (Frawley and Boockfor, 1991). The presence of somatomammotrophs has been reported in several animal species under physiological conditions (Frawley and Boockfor, 1991). In dissociated rat pituitary cells, several groups have reported the presence of somatomammotrophs (Hoeffler et al., 1985; Boockfor et al., 1986; Chomczynski et al., 1988; Porter et al., 1991). In rat pituitary sections, some authors have reported the presence of these cells in the fetus (Chatelain et al., 1979), adult (Nikitovitch-winer et al., 1987; Pasolli et al., 1994) and pregnant females (Ishibashi and Shiino, 1989). Other reports deny the existence of such cells in the rat fetus

(Watanabe and Haraguchi, 1994; Taniguchi et al., 2001a) and adult rat (Baker et al., 1969), and/or their rare presence in other mammals (e.g. mouse, Sasaki and Iwama, 1988; sheep, Thorpe and Wallis, 1991). To date, the role of somatomammotrophs remains unknown. Many studies have shown that somatomammotrophs may act not only as progenitor cells but also as a mid-stage in the transdifferentiation between the somatotroph and lactotroph cells. The appearance of this cell depends on the demand of one hormone or the other (Vidal et al., 2001).

1.4.3 Transdifferentiation

1.4.3.1 Somatotroph-lactotroph conversion

Concerning somatotrophs and lactotrophs, the issue of 'transdifferentiation' (Rhodes et al., 1994) is raised in the context that some cells once committed to become somatotrophs transdifferentiate to become lactotrophs. In the human pituitary, the somatomammotroph serves as an obligatory transitional cell in the interconversion of somatotroph to lactotroph (Vidal et al., 2001). In human fetal pituitaries, GH-bearing cells could act as progenitors of PRL-bearing cells through an intermediate cell type, the somatomammotrophs (Asa et al., 1988; Mulchahey and Jaffe, 1988). As in humans, transdifferentiation involving somatomammotroph cells has been observed in neonatal rat (Hoeffler et al., 1985) and fetal bovine (Kineman et al., 1992a) pituitaries. In several studies, the conversion of somatotrophs to PRL-bearing cells has been reported to be bidirectional (Kineman et al., 1991; Porter et al., 1991).

1.4.3.2 Other cell-cell conversions

It was believed that transdifferentiation occurs between cells (somatotrophs, lactotrophs and thyrotrophs) belonging to a common cell lineage in which hormone expression is Pit-1 dependent (Rhodes et al., 1994). However, this issue appears to be more complicated since Pit-1-immunopositive gonadotrophs have also been reported in the normal female rat pituitary (Vidal et al., 1998).

Somatothyrotrophs. In humans, in cases of thyrotroph hyperplasia caused by protracted primary hypothyroidism, somatothyrotrophs were present (Vidal et al., 1997). In rats with chemically induced hypothyroidism, somatotrophs transform to stimulated thyrotrophs via bihormonal transitional cells – the somatothyrotrophs – that exhibit features common to both cell types. It has been reported that this transdifferentiation is reversible because thyrotrophs reverted to somatotrophs after

discontinuation of propylthiouracil administration (Horvath et al., 1990). Nevertheless, the subcellular events leading to the reversion of the bihormonal somatothyrotrophs into somatotrophs has not been documented. Moreover, the somatothyrotrophs may be also involved in the development of new thyrotrophs as observed in cases of pituitary thyrotroph hypoplasia (Vidal et al., 2000).

Somatogonadotrophs. Before ovulation, transdifferentiation of somatotrophs contributes to the increase in the number of gonadotrophs (Childs and Unabia, 1997). The transformation of somatotrophs into gonadotrophs is a complex process, the initial step of which could be GnRH receptor expression in somatotrophs.

1.5 THE HYPOTHALAMO-PITUITARY-GH AXIS

1.5.1 Growth Hormone (GH) and Regulation of the GH Axis

Mammalian GH is synthesised and released by somatotrophs of the anterior pituitary. GH secretion is regulated by two major hypothalamic peptides, GH releasing factor (GRF; stimulatory factor), produced by cell bodies in the arcuate nucleus at the base of the hypothalamus (Guillemin et al., 1982), and somatostatin (SS or SRIF; inhibitory factor), released from hypophysiotrophic neurones with cell bodies in the hypothalamic periventricular nucleus (PeN) (Brazeau et al., 1973) (Figure 1.2).

1.5.1.1 The growth hormone-releasing factor (GRF)

GRF was first isolated from patients with pancreatic tumours as a 40 and 44 amino acid peptide (Rivier et al., 1982; Guillemin et al., 1982). These two peptides are derived from one of two large polypeptide precursors, pre-pro GRF 107 and 108 (Mayo et al., 1983). GRF with a half-life of approximately 3-6min, is rapidly inactivated to a more stable and less potent metabolite (Frohman et al., 1989; Giustina and Veldhuis, 1998).

GRF is mainly produced in the cell bodies of the hypothalamic arcuate nucleus (Bloch et al., 1983; Merchenthaler et al., 1984) of which axons project to the median eminence from where GRF is released in the hypophyseal portal blood vessels and transported to the pituitary (Giustina and Veldhuis, 1998).

GRF-R and Signalling. GRF stimulates both GH synthesis (Barinaga et al., 1983) and release (Rivier et al., 1982) from somatotrophs. GRF regulates somatotroph function

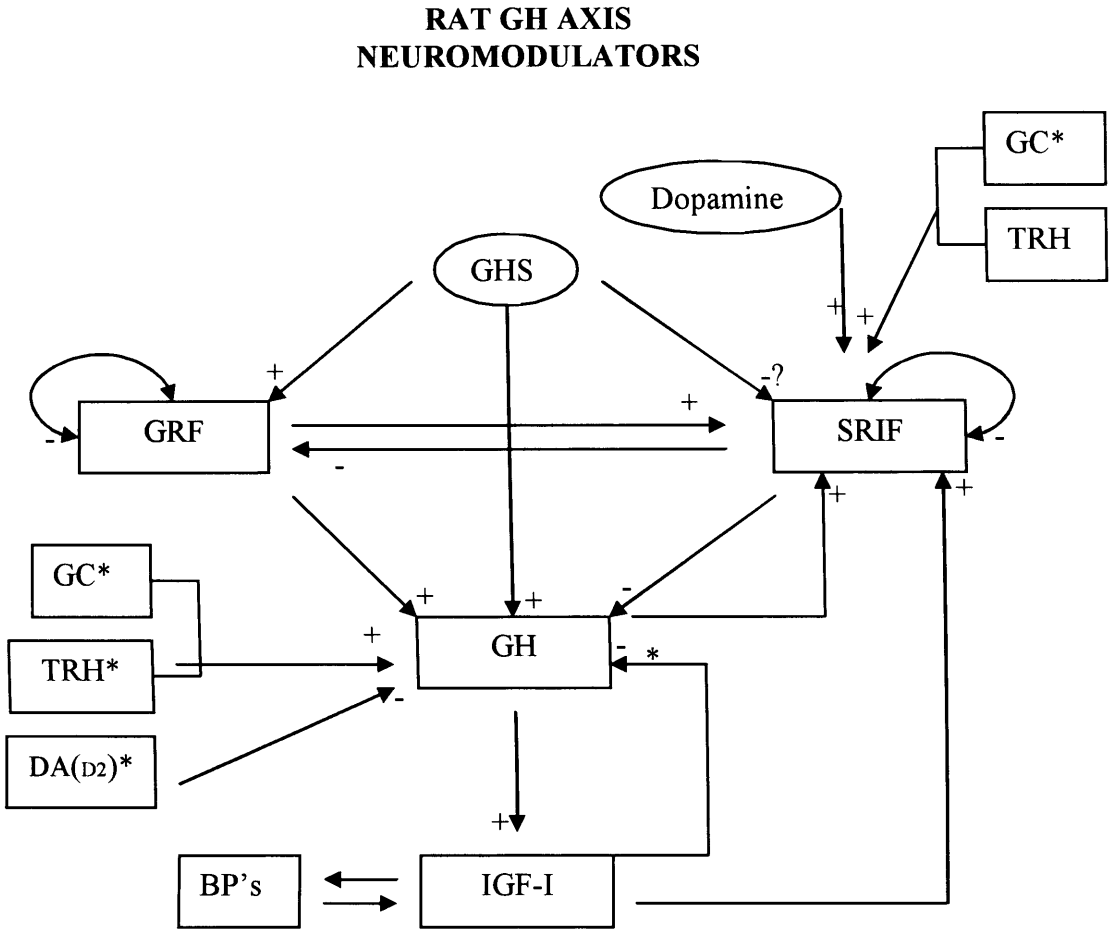


Figure 1.2 Summary representation of the documented functions of the principal neuropeptides and neurotransmitters that control GH secretion via GRF or SRIF or by acting directly on the adenohypophysis (GH, somatotroph) in the rat. Asterisks denote that two or more loci of action are identified. Other hormonal and metabolic effectors (e.g. glucocorticoids, IGF-I, etc.) that act via multiple pathways are not shown (adapted from Giustina and Veldhuis, 1998). GHS, GH secretagogue; GC, glucocorticoid; TRH, thyroid-releasing hormone; DA, dopamine; D2, dopamine receptor 2; IGF-I, insulin-like growth factor I; BP, binding protein.

via its receptor. GRF-R signals predominantly via cAMP-dependent pathways. GRF activates a stimulatory G protein, leading to activation of adenylyl cyclase and stimulation of cAMP/protein kinase A pathway. GRF-stimulated GH secretion is calcium dependent (Spence et al., 1980) (Figure 1.3). GRF-R is also required for the development of the somatotroph. A mutation in GRF-R gene (Godfrey et al., 1993) results in severe growth retardation, decreased pituitary size and GH (Cheng et al., 1983), and impaired somatotroph proliferation (Lin et al., 1993) as observed in *lit/lit* mouse. GRF-R defect in humans results in 'dwarfism of Sindh', an autosomal recessive disorder characterised by somatotroph hypoplasia (Baumann and Maheshwari, 1997).

1.5.1.2 Somatostatin (SRIF)

The inhibitory neuropeptide somatostatin was originally isolated from the hypothalamus (Brazeau et al., 1973). There are two major SRIF subtypes, the SRIF-14 and SRIF-28 amino acid peptides. SRIF is widely expressed in the central nervous system (CNS) and peripheral sites as well. It is mainly synthesised in the hypothalamic periventricular nucleus as a pre-pro-hormone. As GRF, SRIF neurones project to the median eminence and it is released into the portal vasculature to be transported to the anterior pituitary where it inhibits GH release from somatotrophs (Gillies et al., 1997).

SSTRs and Signalling. SRIF mediates its effect via five G-protein coupled receptors (1-5). All five somatostatin receptor subtypes (SSTRs) are expressed in the pituitary (Kumar et al., 1997) as well as in other tissues. The inhibitory effect of SRIF on GH secretion is mediated by SSTR-2 and SSTR-5 (Day et al., 1995). Activation of SSTRs results in a decrease in adenylate cyclase, cAMP, and PKA, therefore inhibiting GH release (Koch et al., 1984). GRF and SRIF use similar signal transduction pathways. However, the simultaneous exposure of somatotrophs to GRF and SRIF results in a decrease in intracellular Ca^{2+} (Figure 1.3).

1.5.2 GH Rhythmicity

In all species studied including man, GH is secreted in an episodic pattern, which is influenced by several factors including age, sex, and nutrition. In rats, a marked

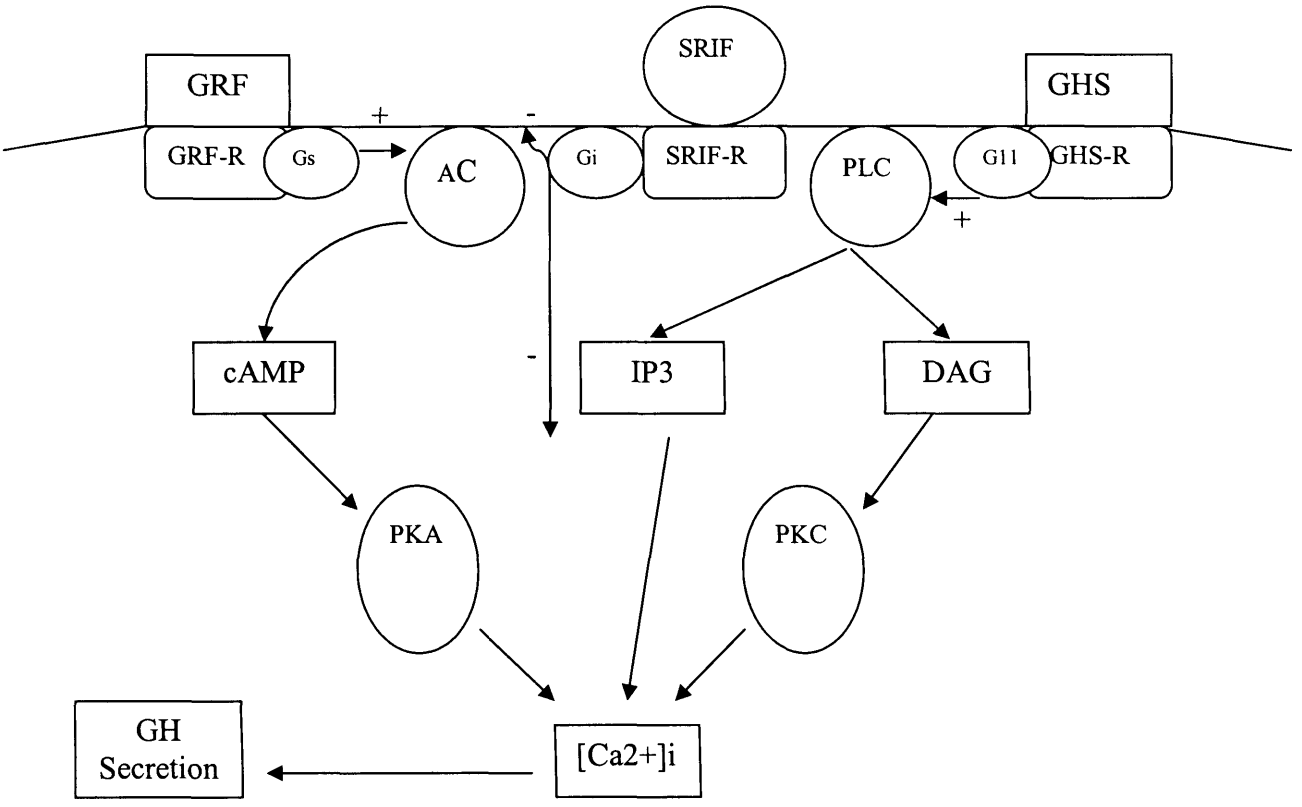


Figure 1.3 Model of the GRF, SRIF, and GHS signal pathways in somatotrophs. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; G, G protein; IP3, inositol triphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C (adapted from Frohman et al., 2000).

sexual dimorphism in the GH secretory pattern is well documented. The circulating rGH profile in male rats is characterised by high amplitude pulses (peak height >200ng/ml) every 3-3.5 hours and inter-pulse troughs that decrease to often undetectable levels (below detection limit <1ng/ml) (Tannenbaum and Martin, 1976). In female rats, the GH rhythm is characterised by more frequent, erratic pulses (peak height <150ng/ml) and GH inter-pulse values remain detectable (10-20ng/ml) (Eden 1979; Clark et al., 1987). Although the GH secretory output is higher in female rats, the pattern of GH release (Eden 1979) is not as effective in stimulating skeletal growth (Clark et al., 1985) as the regular episodes of GH release observed in males. In rats, the marked sexual dimorphism in the GH secretory pattern is believed to be due to oestrogen-mediated effects. In male rats treated with oestrogen, GH pulse height decreases with an increase in baseline GH levels (Carlsson et al., 1987). The pulses of GH release occur as the result of the temporal interaction between GRF and SRIF (Tannenbaum and Martin, 1976). The GH pulse is initiated when GRF release coincides with a periodic reduction in SRIF tone.

1.5.3 GH Receptor and Actions

GH plays a role in the regulation of many physiological processes including growth (e.g. skeletal growth) and metabolism (e.g. protein synthesis, plasma glucose levels, and mobilisation of fatty acids). In the regulation of skeletal growth, it acts directly, via binding to its receptor (GH-R) on target cells (Fuh et al., 1990), and indirectly via the insulin-like growth factor-1 (IGF-I) secreted from the liver and other tissues in response to GH (Daughady et al., 1989) (Figure 1.2).

1.5.4 GH Feedback

Within the hypothalamo-pituitary-GH axis, feedback loops occur at several levels. The release of GH from the adenohypophysis stimulates GH-Rs present on hypothalamic periventricular nucleus neurones. This enhances SRIF release, which subsequently inhibits further GH secretion by activating SSTRs on anterior pituitary somatotrophs (Bennett et al., 1995; Kamegai et al., 1996). In addition, GH may have a direct, autocrine inhibitory effect on its own secretion from the somatotroph. Moreover, circulating IGF-I shows regulatory feedback actions at the pituitary and hypothalamic levels (Berelowitz et al., 1981). Elevated serum IGF-I levels lead to a

reduction in GH secretion due to a direct suppression of the somatotrophs and a stimulation of SRIF from the hypothalamus.

1.5.5 GH Secretagogues (GHSs)

GH secretion is also stimulated by a group of synthetic molecules known as the GH secretagogues (GHS). In 1976, the first compounds studied were the synthetic enkephalin analogues. In 1981, the hexapeptide GHRP-6 was the first compound with *in vitro* and *in vivo* activity to be developed (see review, Bowers et al., 1984). The GHSs were known to act via a separate receptor, the GHS receptor (GHS-R), the cloning (Pong et al., 1996) of which led to the discovery of the endogenous ligand for GHS-R, ghrelin (Kojima et al., 1999).

1.5.5.1 The natural GHS – Ghrelin

Ghrelin was isolated and characterized from rat stomach (i.e. oxyntic gland) by Kojima and co-workers (Kojima et al., 1999). It is a 28 amino acid peptide with octanoyl side chain on the 3rd residue (serine). This octanoylation makes ghrelin the first known bioactive natural peptide to be modified post-translationally by an acyl acid (Kojima et al., 1999). In peripheral circulation, a non-acylated ghrelin has been detected. The des-acyl ghrelin has higher circulating levels than ghrelin, but does not bind to GHS-R type 1a (see below). Although initially considered biologically inactive, des-octanoyl ghrelin displays both cardiovascular (Bedendi et al., 2003) and adipogenic actions (Thompson et al., 2004). In rat stomach extracts, a third endogenous form of ghrelin with endocrine activity has been reported. In rat stomach, two isoforms of transcripts for pro-ghrelin are produced from the gene by alternative splicing mechanism. One mRNA encodes for the ghrelin precursor, whereas the other encodes for des-Gln^{14} ghrelin precursor. Des-Gln^{14} ghrelin activates GHS-R, increase the intracellular Ca^{2+} in cells expressing the GHS-R, and induces GH secretion with the same potency as ghrelin (Hosoda et al., 2000). However, the plasma des-Gln^{14} ghrelin levels are low and barely detectable, making ghrelin the major biologically active form (Hosoda et al., 2000).

Besides the stomach, ghrelin is expressed in the pancreas, liver, the arcuate nucleus of the hypothalamus, the pituitary (Kojima et al., 1999), the kidney (Mori et al., 2000), and the immune cells (Hattori et al., 2001). In humans, ghrelin is expressed in the placenta during the first trimester, whereas in rats, its expression peaks on day 16

(d16) of pregnancy (Gualillo et al., 2001). In the rat fetus, gastric ghrelin mRNA expression remains low or undetectable until after birth, but rises markedly during the second and third weeks of life (Wang et al., 2002). However, GHS-R mRNA is expressed in the fetal pituitary (Shimon et al., 1998), implicating a role for maternal ghrelin.

Ghrelin expression and secretion have been shown to increase during food deprivation and to decrease following food intake (Tschöp et al., 2000; Cummings et al., 2001). Besides fasting, gastric ghrelin expression can also be stimulated by insulin-induced hypoglycaemia and leptin administration (Toshinai et al., 2001). In rats, it has been reported that sugar ingestion powerfully suppresses ghrelin secretion (Tschöp et al., 2000). Ghrelin, therefore, represents a relationship between caloric intake and metabolic activity. More studies are required to elucidate the complete functional role of ghrelin.

1.5.5.2 The GHS receptor (GHS-R)

The GHS receptors (GHS-Rs) belong to G protein-coupled receptor family. GHS-R type 1a is the functionally active receptor (Pong et al., 1996), whereas activation of type 1b receptor to date has been reported to induce no biological response (Howard et al., 1996). GHS-R type 1a is expressed in many regions of the hypothalamus (Pong et al., 1996). In the arcuate nucleus, the expression of GHS-R type 1a is co-localised with 25% of the GRF neurones (Tannenbaum et al., 1998). GHS-R type 1a is also expressed in the anterior pituitary and has been localised to somatotrophs (Hreniuk et al., 1998). Similar distribution of GHS-R type 1a and type 1b expression has been reported. MK-0677, a non-peptide GHS, is a specific agonist for GHS-R type 1a (Pong et al., 1996). Other molecules can bind to GHS-R but without necessarily eliciting activation. Further investigation is needed to find whether ghrelin is the sole endogenous ligand or one of a number of ligands for the GHS-R (Muccioli et al., 2002).

GHS-R and Signalling. After GHS binds to its receptor, phospholipase C-signalling pathway is activated. This leads to increased inositol phosphate turnover and protein kinase C activation, followed by Ca^{2+} release from its intracellular stores, an increase in plasma membrane conductance, and subsequently GH secretion (Smith et al., 1997) from the somatotrophs (Figure 1.3).

It has been suggested that ghrelin and GHSs may be capable of inducing Pit-1 transcription via the GHS-R, and have a potential role in inducing somatotroph differentiation and function (Garcia et al., 2001).

In rats and under certain circumstances, systemic administration of ghrelin or GHS stimulates small but significant amounts of PRL, ACTH, glucocorticoids, and other hormones (Jacks et al., 1994). The mechanisms by which GHS-R activation leads to their release are still unknown.

In the severely GH deficient *dw/dw* rat, GHS-R activation elicits PRL secretion (Carmignac et al., 1998). This response can be abolished by ovariectomy, and induced in male *dw/dw* rats by oestrogen treatment (Carmignac et al., 1998). PRL releasing activity of the GHSs probably involves a direct action of the GHSs on the pituitary either via activation of GHS-R on lactotrophs or somatomammotrophs (Carmignac et al., 1998).

1.6 THE HYPOTHALAMO-PITUITARY-PRL AXIS

1.6.1 Prolactin

Prolactin (PRL) is structurally similar to two other hormones, GH, also of pituitary origin, and placental lactogen (PL), secreted by the mammalian placenta. These three hormones, along with two more recently identified, somatolactin (SL) and PRL-related proteins, belong to a unique family of proteins, the hematopoietic cytokines (Bole-Feysot et al., 1998; Goffin et al., 2002).

Somatolactin (SL): In fish, phylogenetic relationship is similar between GH and PRL, but not with SL. In adults, the SL-bearing cells are located mainly in the pars intermedia and are distinct from MSH-stimulating cells. They are organized in discontinuous cell cords bordering the neurohypophysis or surrounding the neurohypophyseal branches. Their processes are directed towards the neurohypophysis, blood vessels, or intermingled with the adenohypophyseal cells (Villaplana et al., 2001). Different SL cell populations can be classified according to secretory granular size. As with GH and PRL, SL is stored in the secretory granules and released by exocytosis (Villaplana et al., 2001). Although its biochemical and molecular features have become increasingly clear, its physiological significance is

still poorly understood. Some possible functions such as acid-base regulation, calcium regulation, background adaptation, stress response, and maturation are suggested (Kaneko, 1996).

Prolactin (PRL): PRL is produced not only by the lactotrophs of the anterior pituitary, but also by other cells and tissues. PRL gene expression was detected in various brain regions, other tissues (more details in review, Goffin et al., 2002), and fluid compartments (more details in review, Ben-Jonathan et al., 1996). It has been reported that hypophysectomised rats depend on residual prolactin for survival (Nagy and Berczi, 1991). 20-50% of biologically active PRL retained in the circulation of these rats suggests the importance of extrapituitary PRL and its compensation, under some circumstances, for pituitary PRL (Nagy and Berczi, 1991). PRL may act as a hormone via the classic endocrine pathway, and as a growth factor, neurotransmitter, or immunoregulator in an autocrine-paracrine fashion.

1.6.2 Regulation of PRL Secretion

PRL-secreting cells are affected directly by a large number of substances. The molecules are either PRL inhibiting factors (PIFs) such as dopamine (MacLeod, 1969), or as PRL releasing factors (PRFs) such as TRH (Tashjian et al., 1971; Gourdji et al., 1972). Pituitary PRL is primarily under negative hypothalamic control.

1.6.2.1 Dopamine

The prime inhibitor of PRL expression and secretion from the pituitary gland is the tuberoinfundibular dopamine. The tonic dopaminergic inhibition is mediated predominantly by the D2 receptors (D2R) localised on lactotrophs (Koga et al., 1987; Wood et al., 1991).

1.6.2.2 Somatostatin

In humans and rats, endogenous SRIF appears to exert a negative control over PRL secretion. SRIF inhibits the basal PRL secretion of the adenohypophyseal cells from female or oestrogen-primed male, but not from normal male rats (Vale et al., 1974). SSTRs are present on lactotroph cells. Compared to somatotrophs, lactotrophs show reduced responsiveness due to lower density of SSTR on PRL-secreting cells (Enjalbert et al., 1986).

1.6.2.3 TRH and other factors

Several neuropeptides are integrated in the regulation of PRL secretion (Samson et al., 2003). However, none of these PRL releasing factors (PRFs; such as TRH, VIP, oxytocin) appears to be a major determinant of PRL secretion *in vivo* or specific for lactotrophs. For example, TRH acts on both lactotrophs and thyrotrophs (Enjalbert et al., 1986). Other substances (neurotransmitters, neuromodulators, neuropeptides) (Freeman et al., 2000) modify the neuroendocrine regulation of PRL secretion at the hypothalamic level. Since their actions are not directly exerted at the lactotroph level, they might not be considered as true PRFs but as PRL releasing peptides (PRPs) (Samson et al., 2003).

1.6.3 PRL Rhythmicity

In humans and rats, PRL are secreted episodically. In male rat, PRL is secreted in more frequent episodes with a pattern distinct from that of GH. In females, PRL levels are low (<15ng/ml); it can reach 100-300ng/ml on the afternoon of the pro-estrous where PRL surges (Terry et al., 1977).

1.6.4 Prolactin Receptor (PRLR) and Actions

The action of PRL is mediated via its binding to a specific membrane receptor, the PRL receptor (PRLR). PRLR and GH-R are part of the larger class of receptors – the class 1 cytokine receptor superfamily (Kelly et al., 1991). PRLR or binding sites are broadly distributed throughout the body (Table 1, Bole-Feysot et al., 1998). PRLR mRNA and protein are widely expressed and this broad receptor distribution reflects the huge number of functions (>300 known functions) PRL fulfills. The actions of this multifaceted hormone include effects on water and electrolyte balance, growth and development, brain and behaviour, reproduction, and immunoregulation and protection (Goffin et al., 2002). The PRLR is expressed as short and long forms. These forms are differentially expressed or regulated during the estrous cycle and pregnancy suggesting that they may initiate distinct signalling pathways (Bole-Feysot et al., 1998).

Null mutation of PRLR gene. PRLR knockout mice show multiple reproductive failures. *Heterozygous (+/-) females* exhibit almost complete failure to lactate only after the first pregnancy; they showed impaired mammary gland development.

Homozygous (-/-) females are infertile due to multiple reproductive abnormalities such as ovulation of premeiotic oocytes, reduced fertilization of oocytes, reduced preimplantation oocyte development, lack of embryo implantation, and the absence of pseudopregnancy; their mammary glands demonstrated a great reduction in the ductal branching besides the absence of alveoli. In males, the action of PRL is less well understood, but *some homozygous* $PRLR^{-/-}$ *males* (~20%) demonstrated delayed fertility (Bole-Feysot et al., 1998; Goffin et al., 2002).

1.6.5 PRL Feedback

In the PRLR knockout mouse model, the presence of hyperprolactinemia effectively demonstrates that PRL feedbacks on its own secretion. This negative regulation may be direct on lactotrophs or indirect through an action on neuroendocrine dopaminergic neurones that have been demonstrated to express PRLRs (Goffin et al., 2002).

1.7 MODELS OF GROWTH RETARDATION

GH plays a major endocrine role in postnatal growth. GH deficiency manifested by a decrease in the somatotroph number and GH expression, results in two basic types of dwarfism, multiple pituitary hormone deficiencies (MPHD) and the isolated GH deficiency (GHD). In this section, the disorders are classified as hypothalamic-, pituitary-, and peripheral-derived dwarfism as mutations can occur in individual components of the hypothalamo-pituitary-GH and -PRL axes.

1.7.1 Hypothalamic-Derived dwarfism

In the *transgenic growth retarded (Tgr) rat*, dwarfism is due to the local feedback inhibition of GRF owing to the ectopic expression of an hGH transgene in the hypothalamus. Reduction in the number of somatotrophs is accompanied by a decrease in lactotrophs (Flavell et al., 1996).

The $Dat1^{-/-}$ *mouse* carries a targeted mutation in the dopamine transporter (Dat1), resulting in lactotroph hypoplasia. The elevated levels of dopamine repress lactotroph proliferation and PRL secretion. As stated earlier, somatotroph hypoplasia also occurs due to the decrease in the GRF levels (Saiardi et al., 1997).

Other dwarf models have been generated following knockout (KO) of one of the following genes [Otx-1 (Acampora et al., 1998); Krox-24/NGFI-A/Egr-1/zif268 (Lee et al., 1996; Topilko et al., 1997); Gsh-1 (Li et al., 1996)]. The only affected cell type for the latter KO is the somatotroph, compared to the other two KOs where both somatotroph and gonadotroph are affected. Mutation of the Gsh-1 homeobox gene in mice results in a loss of hypothalamic GRF expression; consequently, the mutant mice exhibit a severe pituitary hypoplasia and extreme dwarfism (Li et al., 1996).

1.7.2 Pituitary-Derived Dwarfism

In the *Lhx4$^{-/-}$ mouse*, a targeted disruption of Lhx4, a LIM class homeodomain family member, results in mild hypopituitarism. Reduced levels of GH, PRL, and LH, with a marked decrease in the somatotroph and lactotroph number and a slight reduction in the other cell types were observed (Li et al., 1994). In the hypothalamus, the loss of normal Lhx4 expression results in a hypothalamic deficiency in GRF synthesis, which might contribute to the somatotroph deficiency in Lhx4$^{-/-}$ mouse.

The *Snell mouse (dw, Pit1^{dw})* is the phenotype produced by mutations in the Pit-1 genes. It lacks GH, PRL, and TSH, and has a hypoplastic anterior pituitary. At birth, the anterior pituitary is of normal size, but in adulthood, its weight is only one third that of wildtype littermates. It uniformly lacks functional somatotrophs besides lactotrophs and thyrotrophs (Camper et al., 1990; Li et al., 1990).

The *Jackson dwarf mouse (dw^{J}, Pit1^{dw-J})* is a phenotypically similar strain to Snell mouse. The complex rearrangement of the Pit-1 gene precludes production of a functional Pit-1 protein, restricted to the nuclei of somatotropic, lactotropic, and thyrotropic cells (Li et al., 1990).

The *Ames mouse (df, Prop1^{df})* As the Snell and Jackson mice, the Ames mouse has GH, PRL, and TSH deficiencies. The adult size of this mouse is one third that of its wildtype littermate. Unlike the Snell mouse, the anterior pituitary of the Ames mouse has a very small number of functional somatotrophs (0.05% of the normal number), located in clusters. PRL-immunoreactive cells have been identified, although throughout development, the Ames as well as the Snell strains appears to have total deficiency of pituitary PRL production (Phelps et al., 1993). Pituitary content of TSH

is reduced in Ames mice, whereas it is minimal in Snell mice (Bartke, 1964; Roti et al., 1978). Normally, somatotrophs, lactotrophs, and thyrotrophs account for nearly 80% of anterior pituitary cell content. Thus, the small anterior pituitaries in Ames mice reflect nearly the complete loss of these three cell types (Gage et al., 1996; Sornson et al., 1996). The gonadotrophs are present, but reduced in number in both strains. Fertility is decreased in both models of dwarfism. But, injections of PRL or of PRL-producing homografts, restore fertility in male Ames mice and successful pregnancy and lactation can be produced in female Ames mice (Bartke, 1973; Bartke et al., 1977). The number of corticotrophs and adrenal function are normal in both types (Roux et al., 1982).

The *Little mouse (lit, Grfr^{lit})* As Snell and Ames mice provide models of recessive multiple pituitary hormone deficiencies, little mouse is a model of single isolated GH deficiency (Parks et al., 1999). The dwarf little mouse harbors a mutation in the extracellular domain of GRF-R that abolishes the receptor's hormone-binding and signalling properties (Lin et al., 1993; Godfrey et al., 1993) leading to severe somatotroph hypoplasia. (Mayo, 1996; Mayo et al., 2000).

The *spontaneous Japanese dwarf rat* (*SDR*) has a mutation of the GH gene, resulting in total absence of the hormone. This mutant rat demonstrates distinctive changes in the three regulatory hypothalamic hormones (GRF, SRIF, and GHS) due to absence of GH feedback and alterations in the expression of each of their pituitary receptors (Nogami et al., 1989).

The *dwarf (dw/dw) rat* is a spontaneous mutant in which an undefined genetic mutation leads to profound GH deficiency. The weight of the *dw/dw* rat is 40% less than normal rats. This dwarfism is associated with a reduction in pituitary GH and a decrease in the plasma GH levels to 6% of normal males and 10% of normal females (Charlton et al., 1988). Besides the impaired GH secretion, the pituitaries of the *dw/dw* rats have a marked reduction in somatotroph number; the population of somatotrophs is reduced to 80-90% in adults. In *dw/dw* rat, the deficiency for GH is selective, the pituitary-gonadal axis remaining intact (Charlton et al., 1988). Although the response is subnormal, the circulating GH levels are elevated by GRF (Kineman et al., 1989). ***This model is unique and different from other models of dwarfism, in***

that the reduction in the somatotroph number is accompanied by an increase in lactotroph population (Tierney and Robinson, 2002).

1.7.3 Peripheral-Derived Dwarfism

The *GH-R KO mouse (The Laron mouse)* has a small pituitary and decreased somatotrophs, despite elevated GRF secretion, and intact GRF-R signalling (Zhou et al., 1997; Chandrashekar et al., 1999).

The *78N rat model* – A novel transgenic rat line was generated by Slade et al. (2001) using a promoter-reporter (egr-1/NGFI-A-d4EGFP) construct. This line appears to be an insertional-mutant, exhibiting an overt phenotype. The initial characterisation of the 78N mutant rat was performed by Slade (2001). The overt phenotypes were evident after postnatal day 5 (P5). The main characteristics are premature death of the males (by P9) and retarded growth of the surviving females associated with patchy skin and delayed fur growth. Initial investigation of the cause of the growth retardation has been performed previously (Slade et al., 2001). In adult female mutants, the retardation in body weight is associated with reduced skeletal growth. However, anterior pituitary weight and GH content are normal, and episodic GH secretion is maintained. This novel transgenic rat appears to represent ***a model of postnatal growth retardation that is un-related to GH deficiency.***

1.8 REGULATION OF HYPOTHALAMO-PITUITARY AXIS DURING PREGNANCY

1.8.1 The Placental Hormones

The placenta is an important alternative source of a wide range of neuroendocrine factors including neuropeptides, neurosteroids, and monoamines. These substances control the secretion of other regulatory molecules, comprising the pituitary hormones of mother and fetus and their placental counterparts, via endocrine, paracrine, and autocrine mechanisms (Reis et al., 2001). The placental hormones contribute to all phases of gestation. They seem to play a role in the regulation of maternal and fetal physiology, ranging from the control of placenta anchoring to fetal growth and maturation, fine regulation of uterine blood flow and/or initiation of labor (Reis and

Petraglia, 2001). The placenta of several mammalian species synthesizes and secretes proteins with GH- and lactogenic-like properties.

1.8.1.1 GRF

The placenta expresses GRF. It is identical to the hypothalamic GRF, but it is regulated by distinct mechanisms. In human pregnancy, the role of the placental GRF is still unknown; the presence of the placental GRF-R suggests a possible paracrine role (Nogues et al., 1997; Gaylinn, 1999).

1.8.1.2 SRIF/DA

The presence of SRIF has been reported in the human placenta (Nishihira and Yagihashi, 1978). The placenta is also a source and target for dopamine and other catecholamines. Several studies have suggested local participation in the regulation of placental function (Vaillancourt et al., 1998).

1.8.1.3 Ghrelin

Ghrelin has been detected in human and rat placentae. Its expression shows a pregnancy-related time course (Gualillo et al., 2001). ***It is not yet known whether placental ghrelin is involved in the local modulation of GH release or it influences maternal and/or fetal pituitary GH secretion.***

1.8.1.4 GH and IGF-I

The human placental GH variant (hGH-V) is structurally distinguishable from the pituitary GH. It exhibits both somatogenic and lactogenic activities. It gradually replaces the pituitary GH from the second trimester of gestation. In contrast to the episodic release of GH pituitary, placental GH is released in a constant rather than pulsatile fashion. This continuous secretion seems to have important implications for physiologic adjustment to gestation and particularly in the control of maternal IGF-I levels (Owens, 1991; Alsat et al., 1998).

1.8.1.5 PRL and PRLR

The amniotic fluid PRL levels increase in parallel with decidual PRL secretion. They reach a peak by the end of the second trimester of pregnancy. Throughout gestation, PRL and its receptor are expressed by the placental trophoblast. In human pregnancy,

the decidual/placental PRL role remains unclear. Some hypotheses raised the issue of fluid homeostasis and fetal lung maturation (Wu et al., 1995; Ben-Jonathan et al., 1996; Frasor et al., 1999).

1.8.1.6 Placental lactogen (hPL)

The human placental lactogen (hPL, known also as chorionic somatotropin) is the classical placental hormone with GH-like activity. Due to the structural homology with GH and PRL, hPL possesses somatomammotropic characteristics. Although hPL is 96% homologous with GH structure, it shares no more than 3% with GH somatotropic activity. hPL is not maintained in the circulation because of its short-half life. hPL secretion is insensitive to the factors known to affect pituitary GH secretion. Like GH, hPL levels rise in hypoglycemia and fasting state.

During early pregnancy, hPL levels are very low and increase progressively, reflecting some correlation with placental weight. In pregnancy, hPL role is related to its metabolic properties, rather than somatotropic or lactogenic effects (Josimovich and MacLaren, 1962; Reis and Petraglia, 2001).

1.8.2 Comparison between Human and Other Species

During human and rat pregnancy, GH modulates maternal metabolism to induce nutrient repartitioning for fetal development. Placental GH stimulates placental IGF-I production (Caufriez et al., 1993). It may also alter the placental endocrine activity. *In vitro*, hGH stimulates the production of placental lactogens, oestradiol and progesterone (Barnea et al., 1989; Di Simone et al., 1995). It also increases DNA synthesis and the growth of rat and sheep fetal tissues during the latter stage of pregnancy (Botero-Ruiz et al., 1997; Jenkinson et al., 1999). As mentioned earlier, hGH-V is a biologically active somatogen and lactogen. However, lactogenic activity of placental GH (Frankenne et al., 1988) is reduced compared with pituitary GH, due to the small structural differences between the two molecules (MacLeod et al., 1991). Placental somatotropins may act in an endocrine fashion to regulate extraplacental GH action, as hepatic GH-R and GHBP mRNA are more numerous in mice with larger litters (Cramer et al., 1992), most probably due to higher levels of placental lactogens. Placental GH and/or lactogens may also act in an autocrine and/or paracrine way to modulate placental function, as binding activity for placental and pituitary GH is profusely present in the human placenta (Ray et al., 1990). In the

placenta, the GH-binding activity may reside in proteins identical to the cloned hepatic GH-R, as GH-R/GHBP expression and immunoreactivity are detected in human, bovine, ovine and rat (Barnard et al., 1994) placentae (Hull and Harvey, 2001).

1.9 HYPOTHESES TO BE TESTED

In *dw/dw* rat, the increase in the population of lactotrophs is associated with an increase in PRL secretion. In *dw/dw* rat pituitary, our group discovered the presence of a unique, morphologically distinct secretory cell, the 'intermediate' lactotroph. This novel cell was also observed in normal rats during pregnancy. In *dw/dw* female rat, ghrelin increases PRL secretion from these 'intermediate' lactotrophs. In ***chapter 2***, we have tested the hypothesis that placental ghrelin affects the plasticity of the anterior pituitary during pregnancy. This has been achieved by investigating the following aspects of hypothalamo-pituitary function:

1. The analysis of pituitary cell types and secretory profiles in pregnancy.
2. The effect of pseudopregnancy on pituitary cell types.
3. The endocrine responses to secretagogue treatment in 2-week pregnant rats.
4. The analysis of growth and adiposity consequences of modified GH secretion during pregnancy.

The 'intermediate' lactotroph shows a unique profile of responses to hypothalamic peptides and features of both lactotrophic and somatotrophic lineages. Therefore, ***Chapter 3*** tests the following hypotheses:

1. The population of 'intermediate' lactotrophs is regulated in parallel with the lactotrophic lineage.
2. The population of 'intermediate' lactotrophs is, like the somatotrophs, regulated by GRF.

This has been achieved in the following experiments:

1. The analysis of pituitary cell types and the endocrine responses to ghrelin treatment in 2-week pregnant *dw/dw* rats.
2. The analysis of pituitary cell types in male *lit/lit* mice.
3. The analysis of pituitary cell types, and growth and adiposity parameters in MSG-treated male *dw/dw* rats.

While investigating the aetiology of the growth retardation in the 78N rat model of GH-independent deficiency, young mutant female pituitary and other internal organ weights (i.e. heart, liver, spleen and kidney) were measured. All were normal except for the kidney. Further investigation has been carried out. Parallel postmortem examination has revealed kidney abnormalities in both male and female mutants. Since this finding indicates a correlation between the mutation and kidney function, the present study is aiming at a:

(1) Detailed morphological analysis of the kidney phenotype (***Chapter 4***).

(2) Molecular analysis of the mutant kidney transcriptome (***Chapter 4***).

CHAPTER 2.
ENDOCRINE REGULATION OF PLASTICITY IN HYPOTHALAMO – PITUITARY AXES DURING PREGNANCY

2.1 INTRODUCTION

The dwarf (*dw/dw*) rat is a model of dwarfism in which an unknown mutation causes severe, sub-total GH deficiency (Charlton et al, 1988). The profound decline in pituitary GH content (95%) is associated with somatotroph hypoplasia (80-85%) (Downs and Frohman, 1991), and a 90% reduction in GH secretory output (Charlton et al, 1988). Despite initial indications to the contrary, it is now known that the *dw/dw* mutation causes an increase in lactotroph numbers and PRL storage (Carmignac et al., 1998; Thomas et al., 1999; Tierney and Robinson, 2002).

In the rat adenohypophysis, the proportion of lactotrophs is approximately 30% of all pituitary cell types. This percentage is strain-, sex-, age-, and physiological state-dependent (Kineman et al., 1989; Kukstas et al., 1990; Tierney and Robinson, 2002). Lactotrophs are thought to be present in two morphologically distinct forms (Types I and II). Type I lactotrophs are distinguished from type II cells by their large, irregularly shaped secretory granules. Type I lactotroph is the dominant subtype [15-25% (LT-I) vs 1-2% (LT-II)] and is considered the mature form in the lactotroph lineage even though it remains relatively inactive compared to type II lactotroph. Thompson and co-workers showed that the increase in pituitary PRL content in the *dw/dw* pituitary is accompanied by the presence of a unique, morphologically distinct lactotroph – the 'intermediate' lactotroph, which has morphological features between those of the type I and II lactotrophs (Thompson et al., 2002). These unique lactotrophs also show a novel profile of responses to hypothalamic neuropeptides (see details in Chapter 3, section 3.1). A preliminary study indicated that this novel lactotroph subtype, not usually seen in normal animals, is present in the pituitary of normal female rats during pregnancy (Unpublished data, 2002). ***In study 1, we have quantified changes in lactotroph subpopulations and in the somatotroph population***

during pregnancy in normal Sprague-Dawley rats in order to determine the potential contribution of this novel lactotroph to the established pregnancy-induced modification of the hypothalamo-PRL axis.

In addition to these changes in the hypothalamo-PRL axis, the progression of pregnancy is also associated with significant modification of the pattern of circulating GH. In humans, baseline secretion is increased and pulse amplitude decreased. This is thought to be due to the production of the placental hormones hGH-V and placental lactogen (hPL) (Eriksson et al., 1989) which suppresses pituitary growth hormone (GH) secretion. A similar alteration in the pattern of circulating GH has been described in pregnant rats (Carlsson et al., 1990). This increase in circulating GH in rats is thought to be of pituitary origin, since a placental GH variant has not been identified in this species. However, due to the lack of availability of the appropriate mathematical tools, this group was only able to estimate the changes in baseline secretion. ***In this study (Study 1), we have used distribution and algorithmic analysis to quantify the parameters of baseline and episodic GH and PRL secretion during pregnancy in rats.***

In many species of rodents, including rats, the vaginal stimulation induced by mating with a vasectomised male, elicits pseudopregnancy. In normal rats, pseudopregnancy lasts for 13-15 days (Bradbury, 1937 cited in Anderson, 1968). In its initial stages, its regulation is identical to that of pregnancy; a rise in plasma PRL and progesterone concentrations occurring without alteration in oestrogen, LH or FSH levels (Terkel, 1986). Given the absence of a placenta in pseudopregnancy, ***we have used pseudopregnant rats to investigate the potential involvement of the placental hormones in the regulation of the pituitary cell populations in pregnancy (Study 2).***

The unique induction of PRL secretion by the GH-secretagogues (GHSs) and ghrelin in the *dw/dw* rat is female-specific and oestrogen-dependent (Carmignac et al., 1998; Thompson et al., 2002), being abolished by ovariectomy and induced in males by orchidectomy and oestrogen treatment (Carmignac et al., 1998). It is possible, therefore, that during pregnancy, ghrelin has an increasingly important role in the regulation of PRL secretion. ***We have therefore investigated the sensitivity of the GH and PRL axes to a range of secretagogues including ghrelin (Study 3).***

In addition to the steroid and somatogenic/lactogenic hormones, the placenta also expresses ghrelin mRNA and protein (Gualillo et al., 2001). In both humans and rats, ghrelin is produced during the second half of pregnancy with a peak in placental ghrelin mRNA expression at d16 of pregnancy in rats (Gualillo et al., 2001). ***In order to investigate whether this elevation in placental ghrelin expression contributes to ghrelin levels in the maternal circulation, we have measured both total- and octanoylated-ghrelin concentrations in the circulation and placentae of pregnant rats (Study 4).***

Exposure to ghrelin has profound metabolic consequences (Wu and Kral, 2004). This peptide has both orexigenic (Nakazato et al., 2001; Lawrence et al., 2002) and adipogenic (Tschöp et al., 2000; Thompson et al., 2004) actions, and could contribute to positive energy balance that results in the formation of significant maternal adipose reserves. We have, therefore, ***quantified the development of visceral adipose reserves during pregnancy and the potential effect upon circulating leptin (Study 4)***.

In this chapter, we have tested the hypothesis that placental ghrelin affects the plasticity of the anterior pituitary during pregnancy. This has been achieved by investigating the following aspects of hypothalamo-pituitary function:

1. The analysis of pituitary cell types and secretory profiles in pregnancy
2. The effect of pseudopregnancy on pituitary cell types
3. The endocrine responses to secretagogue treatment in 2-week pregnant rats
4. The analysis of the consequences of modified GH secretion on growth and adiposity during pregnancy

2.2 METHODS

2.2.1 Study 1 – Analysis of Pituitary Cell Types and Secretory Profiles in Pregnancy

Animal Maintenance: Sprague-Dawley (CD, Charles River UK Ltd., U.K.) rats were maintained under the following conditions: All animals were given tap water and standard laboratory diet (Teklad rat chow, Harlan Ltd., Oxfordshire, UK) *ad libitum* and maintained in a regulated environment [14:10 light/dark cycle (lights on 5:00h) in air-conditioned rooms (20-23^{0}C, 45-70% humidity)]. All procedures were conducted in compliance with both UK Home Office regulations [UK Animals (Scientific Procedures) Act, 1986], and local ethical review.

12-week old virgin female CD rats were mated overnight with normal male CD rats, successful mating being determined by the presence of a vaginal plug. The plugged females were separated from the males and housed in different cages until their use. The day on which the vaginal plug was detected was designated day 0 (d0) of pregnancy. The gestation period in rats lasts for 21-22 days. Virgin female littermates were kept as controls.

Three days prior to surgery, the prepared time-mated (1-, 2-, and 3-week pregnant) and age-matched virgin female CD rats were housed individually in metabolic cages for acclimatisation. Body and food weights were monitored daily throughout the experiment. On the day of surgery, general anaesthesia was induced with 4% Fluothane (Merial Animal Health Ltd., Essex, UK) propelled by a mixture of medical oxygen (1L/min), nitrous oxide (0.5L/min). During anaesthesia, a fluovac extraction system (Harvard Apparatus) was used to extract excess anaesthetic. After induction, the rat was transferred to the operating table and anaesthesia maintained with Fluothane (2.0-2.5%) through a face mask. The absence of tail, foot, or blink reflex was tested before starting the surgery. When the surgical procedure was completed, the flow rate of the anaesthetic was reduced to 0%, allowing the rat to recover. The rat was then transferred back to its metabolic cage. At least 48 hours were allowed before blood sampling.

Under anaesthesia, the right jugular vein was cannulated with a single bore cannula (see Appendix B for cannula construction). The cannula was then passed subcutaneously to the dorsal region of the head and exteriorised. The cannula was covered with 15cm stainless steel spring screwed to the skull; the two steel screws inserted into the drilled holes (Dremel multi-drill, Co.) in the skull were bonded in place by dental cement (Haumedica International Ltd., London, UK). PEP antibiotic powder (Park Vet, UK) was applied topically to the neck and head sutured lesions to prevent infection (Clark et al., 1985). After rat recovery, the spring was connected to a single channel infusion swivel (Instech Laboratories, Inc., PA, USA). The swivel allows the rat to move freely in its metabolic cage and permits a continuous intravenous (i.v.) access via the cannula. Cannula patency was maintained by an intermittent infusion of sterile heparinised saline (10U/ml; 20µl bolus every hour). The heparinised saline used in all experiments of the project consisted of sterile and non-pyrogenic saline [NaCl 0.9% (*w/v*); Macoflex 1000ml isotonic solution for i.v. infusion; Maco-Pharma Laboratories, London, UK], and heparin (10U/ml Multiparin; CP Pharmaceuticals Ltd., Wrexham, UK).

Growth hormone is secreted in a highly episodic/pulsatile fashion (Tannenbaum and Martin, 1976), in which pulse frequency (Clark et al., 1985), amplitude (Charlton et al, 1988) and interpulse baseline (Legraverend et al., 1992) are important biological determinants. These parameters are influenced by circadian rhythm, sex, age, and physiological conditions, including stress (Giustina and Veldhuis, 1998). In order to characterize these parameters, multiple serial blood samples are required, collected at 10-15min intervals. This is best achieved with fully automated microsampling system, in which samples are withdrawn without manual handling of the animals.

The automated blood sampling (ABS) system used in this study was developed at Bristol University based upon a system pioneered at the National Institute for Medical Research (Mill Hill, London; Clark et al., 1986), and illustrated in figure 2.1. In brief, the i.v. cannula was connected via a gas-tight swivel to a three-way solenoid-operated valves. The other two valve ports were connected to a saline reservoir, via a sample reservoir and a peristaltic pump, and the other to a fraction collector. The operation of the pump, valve and fraction collector was co-ordinated by a microcomputer and serial interface. Programming occurred at two levels. The sequence of steps required to produce the desired sample size and dilution is termed a 'cycle', and is illustrated in

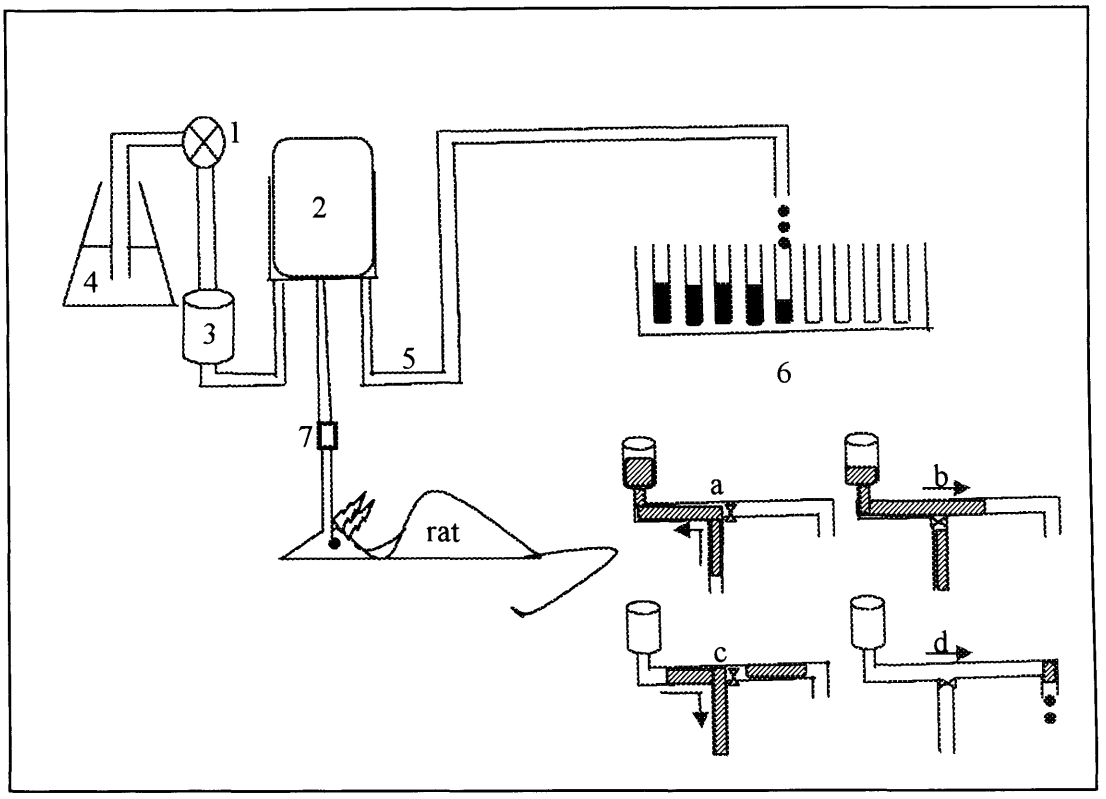


Figure 2.1: Schematic presentation of automatic blood sampling from a freely moving rat.
Blood sampling was carried out by microcomputer control of a peristaltic pump (1), pulling and pushing blood through a three-way solenoid-operated valve (2). All the lines were filled with heparinised saline from the reservoir (4). The blood reservoir (3) prevents blood from entering the distribution lines and collect microbubbles, preventing their infusion into the rats. The diagram illustrates one animal. In practice, up to 12 rats can be operated in parallel. The fluid-tight swivel (7) connects the cannula from the rat to the system, and allows the rat free movement in the cage.

In the right lower part of the diagram, blood (hatched area) flow (arrow indicates flow direction) is shown: (a) Blood (*whole blood*) is drawn past the valve into the reservoir (3); (b) a small portion of the sample (*whole blood*) was pushed into a side arm, the collector line (5); (c) the remainder of the sample (*all non-collected blood + the additional saline*) was returned to the rat; and (d) finally the delivery of the sample (*whole blood + the additional (following) saline*) from the side arm into the fraction collector (6).

1. Peristaltic pump; 2. Three-way solenoid-operated valve; 3. Blood reservoir; 4. Heparinised saline reservoir; 5. Collector line; 6. Fraction collector; 7. Swivel.
(Adapted from Clark et al., 1986)

table 2.1. Multiple similar (or different) cycles are then combined to generate a regular (or irregular) 'event sequence', for example a 300µl sample of 1:5 blood every 15min for 9hrs. Using this system, blood samples can be collected from up to 12 rats simultaneously.

On the sampling day, the ABS was commenced at 06:00h on day 3 (i.e. on d7, d14, d21 of pregnancy) post-surgery, a 300µl sample of 1:5 blood being collected every 15min for 9hrs. The total blood collected in any given sample sequence never exceeds 15% of total blood volume. It can also be seen from the 'Return' step in table 2.1 that the blood volume removed was compensated for by fluid replacement. As the collection method avoids major blood loss or volume depletion, blood transfusion is not required (Clark et al., 1986). After blood sampling, the blood samples were centrifuged at 2060xg (Eppendorf 4810R) for 10min at 4^0C; Plasma was subsampled, aliquotted for GH (25µl of 1:5 plasma) and PRL (200µl of 1:5 plasma) assays, and stored at -20^0C for subsequent determination of plasma GH and PRL concentrations by radioimmunoassay (RIA).

Following automated sampling, further blood samples were collected before, and at 5, 15, and 30min after a bolus i.v. injection of ghrelin [rat Ghrelin (Pharmacia-Upjohn, Stockholm, Sweden); 10µg/300µl sterile saline containing bovine serum albumin (BSA, 0.2mg/ml; Sigma), and heparin (10U/ml Multiparin; CP Pharmaceuticals Ltd., Wrexham, UK)]. The blood samples were subsampled and stored as above for subsequent determination of plasma GH and PRL concentrations by RIA.

Animals were killed by decapitation after being anaesthetised with Fluothane. The pregnant rats were checked for state of pregnancy. Any rat showing abnormal pregnancy or death/loss of its embryos was excluded from further analysis. Whole pituitaries were dissected and bisected, one half was frozen and stored at -20^0C for GH and PRL RIA, the other fixed by immersion in 2.5% glutaraldehyde [EM grade, (Agar Scientific Ltd., UK)] in phosphate buffered saline (PBS) for 2hr at room temperature. Fixed hemipituitaries were stored in 0.25% glutaraldehyde in PBS at 4^0C until being processed for electron microscopy (EM) analysis.

Table 2.1: The sampling cycle showing the sequence of steps in a single blood sampling event. The pump speed and duration illustrated are calculated to produce a 60µl blood sample in a 300µl final sample volume i.e. 1:5 blood.

Step	*Action*	*Duration*	*Pump*			*Valve*	*Collector*	*Time*
No	Name	(Sec.)	+/-	Speed(%)	I.D.*	State	Position	(Sec.)
1	Move to Drain	6		0	Main	Off	Drain	0
2	Clear Collecting Line	10	+	60	Main	Off		6
3	Flush Cannula	10	+	100	Main	On		16
4	Bleed	100	-	60	Main	On		26
5	Valves On	10		0	Main	On		126
6	Take Sample	15	+	83	Main	Off		136
7	Wait	5		0	Main	Off		151
8	Return	150	+	60	Main	On		156
9	Wait	5		0	Main	Off		306
10	Discard	13	+	90	Main	Off		311
11	Wait	5		0	Main	Off		324
12	Drop	3	-	90	Main	Off		329
13	Wait	5		0	Main	Off		332
14	Move to Sample Position	10		0	Main	Off	Sample	337
15	Collect	75	+	83	Main	Off		347
16	Wait	5		0	Main	Off		422
17	Drop	3	-	90	Main	Off		427
18	Wait	5		0	Main	Off		430
19	Move to Drain	6		0	Main	Off	Drain	435
20	Clear Collecting Line	15	+	60	Main	Off		441
21	Wait	5		0	Main	Off		456
22	Cycle End	0		0	Main	Off		461

*An additional auxiliary pump can be used for simultaneous i.v. infusion.

2.2.2 Study 2 – Effect of Pseudopregnancy on Pituitary Cell Types

12-week old virgin female CD rats were mated with vasectomised male CD rats (vasectomy was performed by Dr. Jeffrey Davies). The following day, the females were checked for the presence of a vaginal plug to confirm mating-induced pseudopregnancy. The plugged females were separated from the males and housed in different cages until their use. Virgin female littermates were kept as controls. After halothane anaesthesia and decapitation, the whole pituitaries of virgin female, and 12-day pregnant and pseudopregnant CD rats were fixed and processed for EM analysis as stated in study 1.

2.2.3 Study 3 – Endocrine Responses to Secretagogue Treatment in 2-week Pregnant Rats

2-week pregnant and age-matched virgin female CD rats were housed in metabolic cages 3-4 days prior to the insertion of single bore jugular vein cannula under halothane anaesthesia. The rats were permitted a minimum of 48hr recovery, during which body weight and food intake were monitored daily and cannula patency was maintained with an intermittent infusion of sterile heparinised saline (10U/ml; 20µl bolus every hour). On the day of sampling, 200µl blood samples were collected before, and at 5, 15 and 30mins after a bolus i.v. injection of either vehicle [100µl sterile saline containing BSA (0.2mg/ml), and heparin (10U/ml)], rat ghrelin (10µg/300µl), rat GRF [rat GRF (1-29) NH_2 (Novo Nordisk A/S, Bagsvaerd, Denmark); 1µg/100µl], or the D_2-receptor antagonist, sulpiride [SUL (Sigma-Aldrich Co. Ltd., Dorset, UK); 1µg/100µl]. 1µg/100µl of GRF is considered to stimulate GH release from the pituitary with the same magnitude as 10µg/300µl of Ghrelin (Wells and Houston, 2001). The collected blood samples were subsampled for subsequent determination of GH (10µl) and PRL (25µl; delayed addition (D/A), see below section 2.2.5.1.3) concentrations by RIA.

After 3hrs, rats were subjected to a second blood sampling session in conjunction with an alternative bolus i.v. treatment. Afterwards, animals were killed by decapitation after being anaesthetised with Fluothane. The pregnant rats were checked for state of pregnancy. Any rat showing abnormal pregnancy or death/loss of its embryos was removed from the study.

2.2.4 Study 4 – Consequences of Modified GH Secretion on Growth and Adiposity during Pregnancy

12-week old time-mated (1-wk, 2-wk, and 3-wk) and age-matched virgin CD rats were purchased from Charles River UK Ltd. On the day of sampling, the rats were stunned and decapitated. The following tissues were collected:

- Pituitaries were dissected and stored at -70^{0}C prior to determination of GH mRNA expression by northern blot (performed by Dr. D. Carter) (refer to RNA extraction protocol in section 4.2.5, and northern blotting in section 4.2.9.1).
- Trunk blood was collected in heparinised tubes on ice and centrifuged at 2060xg for 10min at 4^{0}C. Separated plasma subsamples were stored at -20^{0}C to assay for subsequent determination of plasma concentrations of *total-* and *octanoylated*-ghrelin by RIA (*total-* and *octanoylated*-rghrelin RIA Kits; Linco Research, Inc., USA), leptin by RIA (rLeptin RIA Kit; Linco Research, Inc., USA) and *total* IGF-I (i.e. free and bound) by enzyme-linked immunosorbent assay (ELISA) (IGF-I kit, sensitivity 82ng/ml (IDS); performed by Carol Elford, Department of Child Health, University of Wales College of Medicine).
- Placentae were dissected from 2- and 3-wk pregnant rats, frozen on dry ice and stored at -70^{0}C prior to determination of ghrelin protein content. Each placenta was homogenized in 1ml PBS using a polytron (Yellow Line Co.). 100µl of the homogenate was serially diluted (see section 2.2.5.2) and assessed for total- and octanoylated-ghrelin content by RIA.
- Left tibiae were dissected and fixed for 2 days in 10% buffered formal saline, and decalcified in 10% EDTA (in 0.3M NAOH) for 2-3 weeks. After embedding in paraffin wax, 8µm longitudinal/anterior-posterior sections were taken and stained with Masson's Trichrome (Masson, 1929). Epiphyseal plate width (EPW) was measured on Masson's Trichrome sections under light microscopy with an ocular graticule.
- Left visceral (retroperitoneal and perirenal) fat pads were dissected and weighed.

2.2.5 Tissue Analyses

2.2.5.1 Radioimmunoassay (RIA)

2.2.5.1.1 rGH RIA – 5µl subsampled plasma from automated sampling

RIA for rat GH (rGH) was conducted using reagents from the National Hormone and Peptide Program (NHPP) of the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK, USA) (Intra-assay variability (IAV) 7.7%; sensitivity 1.0-2000ng/ml). The GH antibody detects total (free and bound) GH in plasma. Binding protein interference with the assay is, therefore, not significant. GH antibody solution was made up to give a final tube dilution of 1:30,000. The iodinated labelled rGH (label) (see below rGH iodination protocol) was diluted in BSA/PBS buffer to reach 5000 counts per minute (cpm)/50µl. The 50µl solution in T-tubes (T= total radioactivity count) was counted on the gamma counter (Gamma Cobra II, Packard Instruments). For the standard curve, human plasma was diluted in BSA/PBS (1:10 & 1:5). The standard curve (in triplicate) was prepared by doubling dilutions, and the assay was constructed as shown below (***flow chart 2.2.5.1.1***). Hormone concentration in the sample was calculated by comparison with the binding of a known amount of radiolabelled rGH with the standard curve.

Flow Chart 2.2.5.1.1 (rGH RIA, 5µl plasma)

Tube #	Std/Sample	Volume (Standard)	Assay Buffer (B^0)	Antibody	Label*	PEG
1-3	T1-3				**50µl**	
4-6	ABL1-3		100µl (1:10)	***50µl*** (B^0)	50µl	400µl
7-9	BO1-3		100µl (1:10)	50µl	50µl	400µl
10-12	S1 (0.005ng)	100µl	100µl (1:10)	50µl	50µl	400µl
13-15	S2 (0.01ng)	100µl	100µl (1:10)	50µl	50µl	400µl
16-18	S3 (0.02ng)	100µl	100µl (1: 10)	50µl	50µl	400µl
19-21	S4 (0.039ng)	100µl	100µl (1: 10)	50µl	50µl	400µl
22-24	S5 (0.078ng)	100µl	100µl (1: 10)	50µl	50µl	400µl
25-27	S6 (0.156ng)	100µl	100µl (1: 10)	50µl	50µl	400µl
28-30	S7 (0.313ng)	100µl	100µl (1: 10)	50µl	50µl	400µl
31-33	S8 (0.625ng)	100µl	100µl (1: 10)	50µl	50µl	400µl
34-36	S9 (1.25ng)	100µl	100µl (1: 10)	50µl	50µl	400µl
37-39	S10 (2.5ng)	100µl	100µl (1: 10)	50µl	50µl	400µl
40-42	S11 (5.0ng)	100µl	100µl (1: 10)	50µl	50µl	400µl
43-45	S12 (10.0ng)	100µl	100µl (1: 5)	50µl	50µl	400µl
52....	Sample	25µl	75µl	50µl	50µl	400µl

(B^0) BSA/PBS (see Appendix A for composition of solutions).
With 100µl sample size, values read from the standard curve were multiplied by 200.

First, 100µl of 1:10 plasma:PBS was added to ABL_{1-3} tubes (ABL= antibody blank – measurement of non-specific binding), and BO_{1-3} tubes (BO= reference count – total binding tubes, maximum count for bound radiolabelled ^{125}I-rGH), and to the standard tubes (S1-11), whereas 100µl of 1:5 plasma:PBS was added to the standard (S12) tube. 100µl of the standard rGH was added to $S12_1$. After vortexing, 100µl was pipetted out of $S12_1$ and placed into $S11_1$ tube until reaching $S1_1$ where the removed 100µl was discarded. The double dilution was also carried out with the other two standard curve sets (S_2&S_3). The volume of the samples (#52 onwards) was adjusted by adding 75µl of buffer. Second, 50µl of the antibody was added from BO tubes onwards, whereas 50µl of the buffer was added to ABL instead. Third, 50µl of the label was added to all tubes. After the addition of the appropriate volumes of buffer, antibody and label, the tubes were carefully covered with nesco film, vortexed, and incubated overnight at room temperature. The following day (~ after 24hr), 400µl of 18% PEG (the volume of PEG should be double the assay volume) were added to all tubes, vortexed, and incubated for 30min at room temperature, except for T_1-T_3 which were capped and transferred to the gamma counter rack. After centrifugation at 2060xg for 10min at 4^0C, the supernatant was carefully aspirated from the tube. The pellet (bound fraction) was then counted for 3min on the gamma counter. rGH concentration (ng/ml) in the samples was calculated using automated data calculating procedures (the assay is a modified version of Clark et al., 1986).

** rGH RIA – 10µl subsampled plasma from manual sampling*

(IAV 3.10%; sensitivity 0.5-500ng/ml)

– 100µl diluted pituitary homogenates

(IAV 3.35%; sensitivity 0.5-500ng/per tube)

(refer to homogenisation and serial dilution in section 2.2.5.2)

RIA for rGH was conducted as mentioned earlier using NIDDK reagents. For these assays, the ^{125}I-rGH was purchased from Linco Research Inc. (Missouri, USA).

Iodination of rat growth hormone (rGH): The day before the iodination, a Sephadex column was prepared. 5g of G75 Sephadex was immersed in 100-150ml of 50mM RIA phosphate buffer saline (PBS). After de-gasing the gel with an aspirator, 0.6x25cm column was compactly loaded with the Sephadex gel. On the day of the iodination, the Sephadex column was equilibrated with 50mM RIA PBS containing 0.3% BSA (BSA/PBS). 2-5μg of rGH (NIDDK, USA) was dissolved in 20μl of 10mM $NaHCO_3$. ^{125}I (20MBq in 2-5μl) was diluted in 20μl of 0.2M phosphate buffer (PBS) and added to the rGH/$NaHCO_3$ solution. The mixture (40μl) was then added to a glass tube containing 5μl of iodogen (NIDDK, USA). After 5min incubation at room temperature, 200μl of PBS was added to the whole mixture, which was incubated for another 2min. After aspirating the excess of BSA/PBS solution from the top layer of the Sephadex column, its base was uncapped and aligned with the first fraction tube (15 previously labelled microcentrifuge tubes). ^{125}I labelled rGH solution was applied to the top of the column and allowed to penetrate the column. Throughout the sample collection period, the maintenance of the Sephadex column saturation was achieved by adding BSA/PBS solution to the top of the column when required. 12 fractions of 1ml were collected manually. The fractions were counted with a hand-held scintillation probe (Morgan, Series 900, mini-monitor scintillation probe), and those showing no activity were discarded. The labelled rGH eluted in fractions #5&6, followed by free ^{125}I (Salacinski et al., 1981). The standard curve was set up with 5000 cpm/tube (refer to rGH RIA protocol) (rGH iodination was carried out by Dr. Jeffrey Davies).

2.2.5.1.2 rPRL RIA – 40µl subsampled plasma from automated sampling

RIA for rat PRL (rPRL) was conducted using NIDDK reagents. The ^{125}I-rPRL was purchased from Perkin-Elmer life Sciences (Boston, USA) (IAV 6.49%; sensitivity 0.125-250ng/ml). Initially, the antibody solution was diluted from stock to a 1:500 dilution for a 300µl final tube volume. The iodinated labelled rPRL (label) was diluted in BSA/PBS to reach 5000cpm/50µl. The 50µl solution was counted on the gamma counter. For the standard curve, human plasma was diluted in BSA/PBS (1:5 & 1:2.5). The standard curve (in triplicate) and assay were constructed as shown below (refer to ***flow chart 2.2.5.1.2***).

Flow Chart 2.2.5.1.2 (rPRL RIA, 40µl plasma)

Tube #	Std/Sample	Volume (Standard)	BSA/PBS (B^o)	Antibody	Label*	PEG
1-3	T1-3				**50µl**	
4-6	ABL1-3		100µl (1:5)	***50µl*** (B^o)	50µl	600µl
7-9	BO1-3		100µl (1:5)	50µl	50µl	600µl
10-12	S1 (0.005ng)	100µl	100µl (1:5)	50µl	50µl	600µl
13-15	S2 (0.01ng)	100µl	100µl (1:5)	50µl	50µl	600µl
16-18	S3 (0.02ng)	100µl	100µl (1:5)	50µl	50µl	600µl
19-21	S4 (0.039ng)	100µl	100µl (1:5)	50µl	50µl	600µl
22-24	S5 (0.078ng)	100µl	100µl (1:5)	50µl	50µl	600µl
25-27	S6 (0.156ng)	100µl	100µl (1:5)	50µl	50µl	600µl
28-30	S7 (0.313ng)	100µl	100µl (1:5)	50µl	50µl	600µl
31-33	S8 (0.625ng)	100µl	100µl (1:5)	50µl	50µl	600µl
34-36	S9 (1.25ng)	100µl	100µl (1:5)	50µl	50µl	600µl
37-39	S10 (2.5ng)	100µl	100µl (1:5)	50µl	50µl	600µl
40-42	S11 (5.0ng)	100µl	100µl (1:5)	50µl	50µl	600µl
43-45	S12 (10.0ng)	100µl	100µl (1:2.5)	50µl	50µl	600µl
52....	Sample	200µl	-------	50µl	50µl	600µl

(B^o) BSA/PBS

N.B. With 100µl sample size, values read from the standard curve were multiplied by 25.

First, 100µl of 1:5 plasma:PBS was added to ABL_{1-3}, and BO_{1-3}, and to the standard (S1-11) tubes, whereas 100µl of 1:2.5 plasma:PBS was added to the standard (S12) tube. 100µl of the standard rPRL was added to $S12_1$. After vortexing, 100µl was pipetted out of S12 and placed into $S11_1$ tube and so on until reaching $S1_1$ where the removed 100µl was discarded. The double dilution was also carried out with the other two standard curve sets (S_2&S_3). 100µl of of 1:5 plasma:PBS was added again to make it up to 200µl total plasma. Second, 50µl of the antibody was added from BO tubes onwards, whereas 50µl of the buffer was added to ABL instead. Third, 50µl of the label was added to all tubes. After the addition of the appropriate volumes of buffer, antibody and label, the tubes were covered properly with nesco film, vortexed, and incubated overnight at RT. The following day (~ after 24hr), 600µl of 18% PEG were added to all tubes, vortexed, and incubated for 30min at room temperature, except for T_1-T_3 which were capped and transferred to the gamma counter rack. After centrifugation at 2060xg for 10min at 4^0C, the supernatant was carefully aspirated from the tube. The pellet was then counted for 3min on the gamma counter. rPRL concentration (ng/ml) in the unknown sample was calculated using automated data calculating procedures.

** rPRL RIA – 100µl diluted pituitary homogenates*

(IAV 3.90%; sensitivity 0.5-500ng/per tube)

RIA for rPRL was conducted as mentioned earlier. However, the samples were subjected to serial dilutions (refer to homogenisation and serial dilutions in section 2.2.5.2).

2.2.5.1.3 Delayed addition (D/A) rPRL RIA – 25µl subsampled plasma from manual sampling (Study 3)

Since the values in study 1 were near the detection limit, delayed addition RIA was adopted for the samples of study 3 to improve the sensitivity of the assay by lowering the detection limit. RIA for rPRL was conducted using NIDDK reagents. The ^{125}I-rPRL was purchased from Perkin-Elmer life Sciences (Boston, USA) (IAV 5.65%; sensitivity 0.048-100.0ng/ml). Initially, the antibody solution was made up to give a final tube dilution of 1:1000. The iodinated labelled rPRL (label) was diluted in BSA/PBS to reach 5000cpm/25µl. The 25µl solution was counted on the gamma counter. Delayed addition of rPRL label is performed 24 hours after the construction of the assay (details as mentioned previously) (refer to ***flow chart 2.2.5.1.3***), therefore, increasing the binding of the rPRL antibody to the rPRL in the sample.

Flow Chart 2.2.5.1.3 [(D/A) rPRL RIA, 25µl plasma]

Tube #	Std/Sample	Volume (Standard)	P + B°	Antibody	Label*	PEG
1-3	T1-3				**25µl**	
4-6	ABL1-3		25µl+25µl	***25µl*** (B°)	25µl	400µl
7-9	BO1-3		25µl+25µl	25µl	25µl	400µl
10-12	S1 (0.0012ng)	100µl	25µl+25µl	25µl	25µl	400µl
13-15	S2 (0.0025ng)	100µl	25µl+25µl	25µl	25µl	400µl
16-18	S3 (0.005ng)	100µl	25µl+25µl	25µl	25µl	400µl
19-21	S4 (0.01ng)	100µl	25µl+25µl	25µl	25µl	400µl
22-24	S5 (0.02ng)	100µl	25µl+25µl	25µl	25µl	400µl
25-27	S6 (0.039ng)	100µl	25µl+25µl	25µl	25µl	400µl
28-30	S7 (0.078ng)	100µl	25µl+25µl	25µl	25µl	400µl
31-33	S8 (0.156ng)	100µl	25µl+25µl	25µl	25µl	400µl
34-36	S9 (0.313ng)	100µl	25µl+25µl	25µl	25µl	400µl
37-39	S10 (0.625ng)	100µl	25µl+25µl	25µl	25µl	400µl
40-42	S11 (1.25ng)	100µl	25µl+25µl	25µl	25µl	400µl
43-45	S12 (2.5ng)	100µl	25µl+25µl	25µl	25µl	400µl
46-48	S13 (5.0ng)	100µl	100µl (B°)	Discard highest standard dilutions		
49-51	S14 (10.0ng)	100µl	100µl (B°)			
52....	Sample	25µl	125µl	25µl	25µl	400µl

(P) Human plasma
(B°) BSA/PBS
N.B. With 100µl sample size, values read from the standard curve were multiplied by 40.

2.2.5.1.4 Total-rGhrelin RIA – 100µl subsampled plasma

RIA for total-ghrelin was conducted using the total-rat ghrelin RIA *kit*, purchased from Linco Research Inc. (Missouri, USA) [Quality control values fell within the specified ranges: QC1: 0.239ng/ml (range: 0.198-0.411ng/ml); QC2: 0.792ng/ml (range: 0.584-1.213ng/ml)]. The duplicates for quality control samples were used to calculate IAV; (IAV 4.32%; sensitivity 0.081-6.0ng/ml). The tubes were initially labelled in accordance with the ***flow chart 2.2.5.1.4***. 300µl of assay buffer was pipetted into the non-specific binding (NSB) tubes 4-6, 200µl to the reference (BO) tubes 7-9, and 100µl to tubes 10 through to the end of the assay. 100µl of standards and quality controls (QCs) were pipetted in triplicate and duplicate respectively, followed by 100µl of each sample in duplicate. 100µl of total-ghrelin antibody was added to all tubes except total count (T) tubes 1-3 and NSB tubes 4-6. Tubes were vortexed, covered, and incubated overnight (20-24hrs) at 4^0C. The next day, 100µl of ^{125}I-ghrelin (label) was added to all tubes. Tubes were again vortexed, covered, and incubated overnight (22-24hrs) at 4^0C. On the third day, 1.0ml of cold (4^0C) precipitating reagent (PEG) was added to all tubes except total count tubes 1-3. Tubes were vortexed and incubated for 20min at 4^0C. After centrifugation at 3000xg for 20min at 4^0C, the supernatant was immediately decanted from all tubes, except the total count tubes 1-3. The tubes were drained for 30sec and excess liquid was blotted from the tips of tubes. The pellet was then counted for 3min on the gamma counter. Ghrelin concentration (ng/ml) in the unknown sample was calculated using automated data calculating procedures after programming the counter with respect to the specifications mentioned in the kit.

* *Total-rGhrelin RIA – 100µl diluted placental homogenates*

(IAV 4.32%; sensitivity 0.081-6.0ng/ml)

RIA for total-rGhrelin was conducted as mentioned earlier. However, the samples were subjected to homogenisation in 1ml PBS using a polytron (Yellow Line Co.). 100µl of the homogenate was serially diluted (refer to serial dilutions in section 2.2.5.2).

Flow Chart 2.2.5.1.4 (Total-rGhrelin RIA, 100µl plasma)

Tube #	Assay Buffer	Standard/QC sample	Antibody	Label	Precipitating reagent (PEG)
1-3	-	-	-	100µl	1.0ml
4-6	300µl	-	-	100µl	1.0ml
7-9	200µl	-	100µl	100µl	1.0ml
10-12	100µl	100µl of 0.081ng/ml	100µl	100µl	1.0ml
13-15	100µl	100µl of 0.162ng/ml	100µl	100µl	1.0ml
16-18	100µl	100µl of 0.325ng/ml	100µl	100µl	1.0ml
19-21	100µl	100µl of 0.750ng/ml	100µl	100µl	1.0ml
22-24	100µl	100µl of 0.150ng/ml	100µl	100µl	1.0ml
25-27	100µl	100µl of 3.0ng/ml	100µl	100µl	1.0ml
28-30	100µl	100µl of 6.0ng/ml	100µl	100µl	1.0ml
31-32	100µl	100µl of QC1	100µl	100µl	1.0ml
33-34	100µl	100µl of QC2	100µl	100µl	1.0ml
35-n	100µl	100µl of sample	100µl	100µl	1.0ml

Assay Buffer
0.01M phosphate, 0.01M EDTA, 0.08% sodium azide and 0.1% gelatin, pH 6.85

Antibody
Rabbit anti-ghrelin serum in assay buffer
(Information on final tube dilution not available from Linco)

Precipitating Reagent
Goat anti-rabbit IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05M phosphosaline, 0.025M EDTA, 0.08% sodium azide

2.2.5.1.5 Octanoylated-rGhrelin RIA – 100µl subsampled plasma

RIA for octanoylated-ghrelin was conducted using the octanoylated-rat ghrelin RIA *kit*, purchased from Linco Research Inc. (Missouri, USA) [Quality control values fell within the specified ranges: QC1: 0.031ng/ml (range: 0.027-0.055ng/ml); QC2: 0.408ng/ml (range: 0.279-0.580ng/ml)]; (IAV 5.52%; sensitivity 0.008-2.0ng/ml). The construction of the assay is as detailed earlier in section 2.2.3.4 and in accordance with the ***flow chart 2.2.5.1.5***.

** Octanoylated-rGhrelin RIA – 100µl diluted placental homogenates*

(IAV 5.52%; sensitivity 0.008-2.0ng/ml)

RIA for octanoylated-rGhrelin was conducted as mentioned earlier. However, the samples were subjected to homogenisation in 1ml PBS using a polytron (Yellow Line Co.). 100µl of the homogenate was serially diluted (refer to serial dilutions in 2.2.5.2).

Flow Chart 2.2.5.1.5 (Octanoylated-rGhrelin RIA, 100µl plasma)

Tube #	Assay Buffer	Standard/QC sample	Antibody	Label	Precipitating reagent
1-3	-	-	-	100µl	1.0ml
4-6	300µl	-	-	100µl	1.0ml
7-9	200µl	-	100µl	100µl	1.0ml
10-12	100µl	100µl of 0.008ng/ml	100µl	100µl	1.0ml
13-15	100µl	100µl of 0.015ng/ml	100µl	100µl	1.0ml
16-18	100µl	100µl of 0.031ng/ml	100µl	100µl	1.0ml
19-21	100µl	100µl of 0.062ng/ml	100µl	100µl	1.0ml
22-24	100µl	100µl of 0.125ng/ml	100µl	100µl	1.0ml
25-27	100µl	100µl of 0.25ng/ml	100µl	100µl	1.0ml
28-30	100µl	100µl of 0.5ng/ml	100µl	100µl	1.0ml
30-33	100µl	100µl of 1ng/ml	100µl	100µl	1.0ml
34-36	100µl	100µl of 2ng/ml	100µl	100µl	1.0ml
37-38	100µl	100µl of QC1	100µl	100µl	1.0ml
39-40	100µl	100µl of QC2	100µl	100µl	1.0ml
41-n	100µl	100µl of sample	100µl	100µl	1.0ml

Assay Buffer
0.01M phosphate, 0.01M EDTA, 0.08% sodium azide and 0.1% gelatin, pH 6.85

Antibody
Guinea pig anti-ghrelin serum in assay buffer
(Information on final tube dilution not available from Linco)

Precipitating Reagent
Goat anti-guinea pig IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05M phosphosaline, 0.025M EDTA, 0.08% sodium azide.

2.2.5.1.6 rLeptin RIA – 50µl subsampled plasma

RIA for leptin was conducted using the rat leptin RIA *kit*, purchased from Linco Research Inc. (Missouri, USA) [Quality control values fell within the specified ranges: QC1: 1.49ng/ml (range: 1.1-2.3ng/ml); QC2: 5.36ng/ml (range: 3.8-7.9ng/ml)]; (IAV 8.8%; sensitivity 0.5-50ng/ml). The construction of the assay is as detailed earlier in section 2.2.3.4 and in accordance with the ***flow chart 2.2.5.1.6***. However, the overnight incubation after the addition of the antibody (Day I) and the addition of the label (Day II) were carried out at room temperature.

Flow Chart 2.2.5.1.6 (rLeptin RIA, 50µl plasma)

Tube #	Assay Buffer	Standard/QC sample	Antibody	Label	Precipitating reagent
1-3	-	-	-	100µl	1.0ml
4-6	300µl	-	-	100µl	1.0ml
7-9	200µl	-	100µl	100µl	1.0ml
10-12	100µl	100µl of 0.5ng/ml	100µl	100µl	1.0ml
13-15	100µl	100µl of 1ng/ml	100µl	100µl	1.0ml
16-18	100µl	100µl of 2ng/ml	100µl	100µl	1.0ml
19-21	100µl	100µl of 5ng/ml	100µl	100µl	1.0ml
22-24	100µl	100µl of 10ng/ml	100µl	100µl	1.0ml
25-27	100µl	100µl of 20ng/ml	100µl	100µl	1.0ml
28-30	100µl	100µl of 50ng/ml	100µl	100µl	1.0ml
31-32	100µl	100µl of QC1	100µl	100µl	1.0ml
33-34	100µl	100µl of QC2	100µl	100µl	1.0ml
35-n	100µl	100µl of sample*	100µl	100µl	1.0ml

*Adjust with assay buffer (50µl sample +50µl assay buffer)

Assay Buffer

0.05M phosphosaline pH 7.4 containing 0.025M EDTA, 0.08% sodium azide, 1% RIA Grade BSA, and 0.05% Triton X-100.

Antibody

Guinea pig anti-rat leptin serum in assay buffer
(Information on final tube dilution not available from Linco)

Precipitating Reagent

Goat anti-guinea pig IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05M phosphosaline, 0.025M EDTA, 0.08% sodium azide.

2.2.5.2 Homogenisation and serial dilution for pituitary and placental samples

Before pituitary protein GH and PRL contents were assessed by RIA (see section 2.2.5.1.1 & 2.2.5.1.2), the pituitaries were subjected to homogenisation and serial dilution. 100µl diluted pituitary homogenates were used.

Homogenisation: Hemipituitaries were homogenised in 500µl PBS-RIA (assay buffer) using the homogeniser mini-boro glass (Fischer).

For *protein GH RIA*, two successive dilutions were prepared as follows:

1. 1:100 dilution (i.e. 10µl of pituitary homogenate was diluted into 990µl BSA/PBS)
2. 1:200 dilution (i.e. 10µl of 1:100 solution was diluted into 1990µl BSA/PBS)

For *protein PRL RIA*, two successive dilutions were prepared as follows:

1. 1:100 dilution (i.e. 10µl of pituitary homogenate was diluted into 990µl BSA/PBS)
2. 1:10 dilution (i.e. 10µl of 1:100 solution was diluted into 900µl BSA/PBS)

Serial dilution: For each hormone, five assay tubes per pituitary/placenta were prepared in duplicate. First, 100µl BSA/PBS was added to the 2nd, 3rd, 4th, and 5th tubes. Then, 100µl diluted solution (GH 1:20,000; PRL 1:1000) was pipetted in the 1st and 2nd tubes. After vortexing, 100µl was pipetted out of the 2nd tube and placed into the 3rd until reaching the 5th where the removed 100µl was discarded. The same approach was carried out with the duplicate set.

2.2.5.3 Electron microscopy (EM)

EM was adopted for our study since the different types of lactotrophs can only be distinguished by their features based upon the size and shape of the electron-dense secretory granules.

2.2.5.3.1 Tissue processing and immunogold labelling

Tissue processing into Spurr resin: After fixation in glutaraldehyde, the pituitaries were rinsed three times for 10min each with 0.1M phosphate buffer (PBS, pH 7.2), prior to incubation with 1% osmium tetroxide (BDH Chemicals Ltd., Dorset, UK) in 0.1M PBS for 1-2hr. The tissues were washed thrice for 10min each in distilled water followed by block staining in 2% uranyl acetate in distilled water for 1h. During the staining step, specimens were shielded from the light since uranyl acetate is sensitive to UV light. Following one wash for 5min with distilled water, the specimens were

dehydrated through a graded series of ethanol (70%-90%, 1x15min; 100%, 3x15min). The segments were then infiltrated overnight with 50% Spurr resin (Agar)-50% absolute ethanol. The following day, the tissues were transferred into pure Spurr resin pots and left for 24hr. Finally, the pituitaries were embedded in fresh Spurr resin in flat embedding trays and polymerised overnight at 60°C. The tissue segments were orientated such that the cut surfaces were running in parallel to the block face in order to facilitate cutting.

Microtomy: Resin blocks were trimmed and cut using a glass knife prepared on an LKB knifemaker and mounted on a Reichart-Jung Ultracut ultramicrotome. Semi-thin (50nm) sections were mounted on a glass slide, stained with 1% toluidine (with 1% borax) and examined to ensure having the proper anterior pituitary tissue. Ultrathin sections (50-80nm) were mounted on nickel grids (Agar) and processed as described below. Since it is difficult to keep track of the region in the pituitary, the approach was to cut the pituitary in half and section from the medial to lateral surface. A selection of transverse sections is taken from throughout. In order to avoid measuring from the same cells, every 4^{th} section was collected for later examination. 4 sections per animal were examined for quality of tissue.

Immunogold run and staining: Initially, grids were rinsed in distilled water or PBS followed by EA/PBS (PBS containing 1% chicken egg albumin) twice each for 5 min. Afterwards, the grids were incubated for 30min in EA/PBS to block non-specific binding sites. Grids were then incubated for 2hr with one of the primary polyclonal antibodies [National Hormone and Pituitary programme (NHPP); highly specific and very well established antibodies – NHPP data sheets, Dr. AF Parlow.], *monkey anti-rat GH antibody* (1:2000 in EA/PBS – Specificity/Cross-reactivity of the monkey anti-rGH-55 (rGH, 100%; rPRL, 0.158%; rTSH, 0.0087%; rLH, 0.0040%; rFSH, 0.0009%; AVP, 0.0003%) or *rabbit anti-rat PRL antibody* (1:2000 in EA/PBS – Specificity/Cross-reactivity of the rabbit anti-rPRL-59 (rPRL, 100%; rGH, 0.015%; rTSH, 0.0023%; rFSH, 0.0002%; rLH, 0.0002%). The grids were washed three times for 5min in EA/PBS prior to incubation in Protein A Gold [(PAG; 1:65 in EA/PBS); (goat anti-monkey 15nm PAG for GH; goat anti-rabbit 5nm PAG for PRL)] (British Biocell, Cardiff, UK) for 1hr. In control sections, the 1^0 antibody was replaced by nonimmune rabbit serum. Grids were rinsed in EA/PBS (1x15min & 2x5min)

followed by distilled water (3x5min). Finally the grids were double stained at room temperature with 2% uranyl acetate in 70% methanol followed by lead citrate. Lead citrate staining was done in carbon dioxide free environment to prevent the formation of lead precipitate. The sections were then rinsed in distilled water, dried carefully, then examined with a transmission electron microscope (JEM-1010, JEOL, Peabody, MA). However, for GH and PRL dual immunogold labelling, the sections were stained for PRL on day 1 and for GH on day 2.

2.2.5.3.2 Quantification of sections

Cells were identified according to the immunogold staining: Somatotrophs with 15nm gold particles in their granules and lactotrophs granules stained with 5nm gold particles. Lactotroph cells were subclassified into type I, type II, and intermediate cells according to their size and shape of granule populations (see below for details). Cells in which the secretory granules were stained with both sizes of immunogold labelling particles were classified as somatomammotrophs.

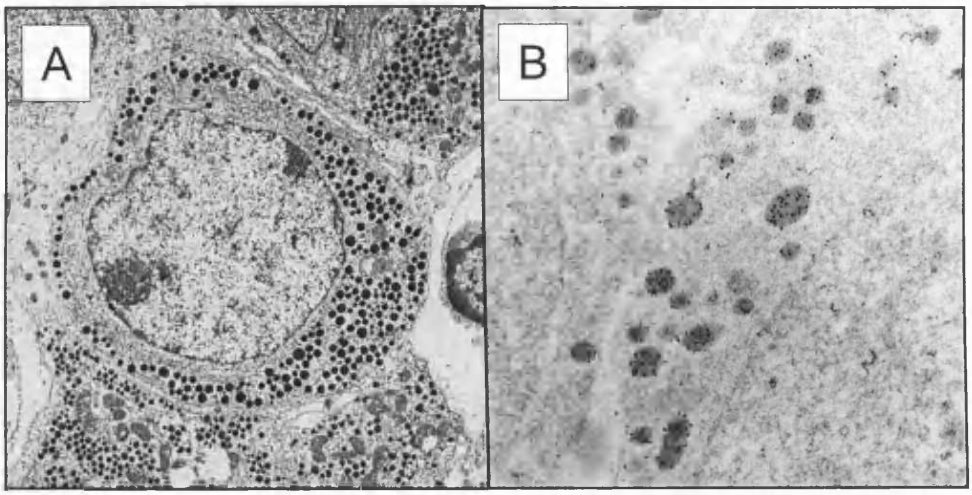
The sections and grid squares were selected at random for examination. Multiple grid squares per sections were examined from top left to bottom right in a random procedure. Only cells that included a visible nucleus and are whole or sectioned by the left and top edges of the grid square (but not right and bottom) were included in any of the cell counts. Cells with all their secretory granules immunogold labelled for GH or PRL hormones were included in the counts (except for *lit/lit* mice pituitaries in which some GH cells contained some granules without GH label, (Chapter3)).

For each animal, one grid was analysed for GH and another one for PRL. 6 squares per grid were counted unless great variation in the number of total cells was encountered per grid square.

Data are presented as a proportion of the total secretory cell population. For percentages of cell type, counting covers as many cells found on an intact section – approx 500 cells per section (pregnant and pseudopregnant CD rat pituitaries), 300-450 cells (2-week pregnant and MSG-treated *dw/dw* rat pituitaries), 500-900 cells (*lit/lit* mice pituitaries). For morphology, 8 cells per animal were measured; 30 granules were measured per cell, selected at random throughout the cell (pregnant and pseudopregnant CD rats). 3 animals per group were investigated (El-Kasti et al., 2004; Huerta-Ocampo et al., 2004).

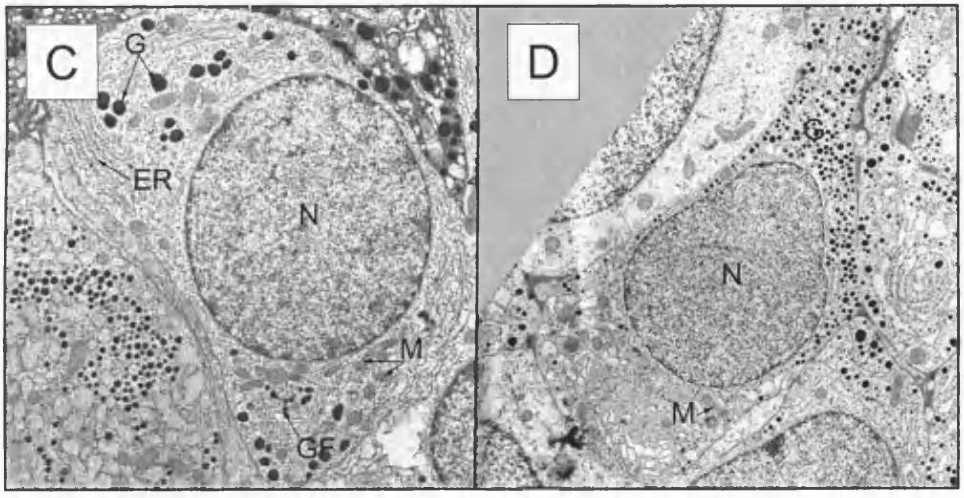
2.2.5.3.3 Classification of pituitary GH-/PRL-secreting cell types

Cells were identified under EM using established criteria (Farquhar and Rinehart, 1954; Nakane, 1975) (Figures 2.2A-D). They were classified by single (somatotrophs, lactotrophs) (Figures 2.2A, 2.2C-D) and double (somatomammotrophs) (Figure 2.2B) immunogold labelling. Somatotrophs were identified by their large number of regular shape, round granules. Lactotrophs were subclassified according to the size, shape, and density of their granular populations. Type I lactotrophs were distinguished by the small number of large irregularly-shaped (polymorphic) granules (>300nm) (Figure 2.2C), whereas type II cells were recognized by the abundant number of smaller (<200nm) and more regularly-shaped granules (Figure 2.2D). However, the novel 'intermediate lactotrophs' appeared similar to the somatotrophs with respect to the large number of regularly-shaped granules; they were distinguished from those of type I and type II lactotrophs on the basis of their unusual granular size (>250-300nm) (see Figure 3.19).



A. Somatotroph

B. Somatomammotroph
(PRL 5nm gold; GH 15 nm gold)



C. Type I Lactotroph

D. Type II Lactotroph

Figure 2.2: Representative electron micrographs of somatotroph (A), somatomammotroph (B) and types I (C) and II (D) lactotrophs from normal rats. Images shown are 6,000X (A,C,D) and 40,000X (B). ER, Endoplasmic reticulum; G, Granules; GF, Golgi fractions; M, Mitochondrion; N, Nucleus.

2.2.5.4 Light microscopy (LM) – Tibial bone analysis

As the epiphyseal plate width (EPW) is a marker of the rate of longitudinal skeletal growth (Geschwind and Li, 1955), we have measured EPW on Masson's Trichrome sections using light microscopy.

2.2.5.4.1 Tissue processing

Fixation and decalcification: Dissected left rat tibiae were fixed in 10% buffered formal saline for 2 days. They were decalcified in EDTA/NaOH solution (10% EDTA in 0.3M NaOH) for 2-3 weeks; the EDTA/NaOH solution being replaced every 2-3 days.

Tissue dehydration and clearing: This was performed using an automatic tissue processor (Leica TP-1050).

Container	Fluid	Time (min)
1	70% IMS*	60
2	95% IMS	60
3	100% IMS	60
4	100% IMS	90
5	100% IMS	120
6	Xylene	20
7	Xylene	20
8	Paraffin Wax (60^0C)	60
9	Paraffin Wax (60^0C)	90
10	Paraffin Wax (60^0C)	90

*IMS: Industrial methylated spirit

In the the last two steps, 'vaccum impregnation' was applied to the tissues. This treatment allows the removal of air bubbles and any small traces of the clearing agent. Afterwards, bone tissues were blocked out manually into paraffin wax using dispomoulds (RA Lamb, Eastbourne, UK). Once solidified (~20min), tissues were ready for sectioning.

Tissue sectioning: After embedding in paraffin wax, 8μm longitudinal/anterior-posterior tibial bone sections were cut on a rotatory microtome (Leica 5139), floated out on a waterbath (45^0C) for a few minutes to complete their flattening and expansion, and finally positioned on slides. Slides were drained for several minutes, dried on a hot plate (45^0C) for 30min, and then transferred to a 45^0C incubator overnight. Sections were cooled prior to staining.

2.2.5.4.2 Staining procedures and light microscopic examination

In order to visualize the tibial epiphyseal plates, sections were stained with Masson's Trichrome. As its name implies, the staining technique produces three colours: nuclei and other basophilic structures are stained blue-black; collagen, cartilage, and mucin are stained green or blue depending on which variant of the technique is used; and cytoplasm, muscle, erythrocytes and keratin are stained bright red (Masson, 1929; Asonova and Migalkin, 1996). Thus, the epiphyseal plates are seen as a pale band of cell columns across the epiphysis (Figure 2.3A).

Masson's Trichrome stain for paraffin sections: Bone sections were dewaxed twice in xylene and hydrated through graded alcohols [twice in 100%, once in 95%, and once in 70% - 2min each] to water. Afterwards, they were stained in each of *celestine blue B* (5min), *Mayer's haematoxylin* (5min), and *ponceau/acid fuschin* (5min) solutions – washing in running tap water in between stains. Following differentiation in 1% *phosphomolybdic acid* (5min), the sections were transferred directly to *light green* stain (3min), washed well in running tap water and then in 1% *acetic acid* (2min). After a quick wash in running tap water, the sections were dehydrated through alcohols [once in 95%, and twice in 100%], cleared twice in xylene, and mounted permanently in DPX. Sections were left at room temperature to dry overnight before measurement of the epiphyseal plate widths (EPW) by light microscopy.

Measurement of epiphyseal plate width: Epiphyseal plate width (EPW) was measured on Masson's Trichrome-stained sections under the light microscope (Leica DMLB, Germany) with an ocular graticule (at 20X magnification). 3 measurements at different locations were taken per section and 3 sections per animal (i.e. 9 measurements per animal). 3 animals per group were investigated. A micrograph of

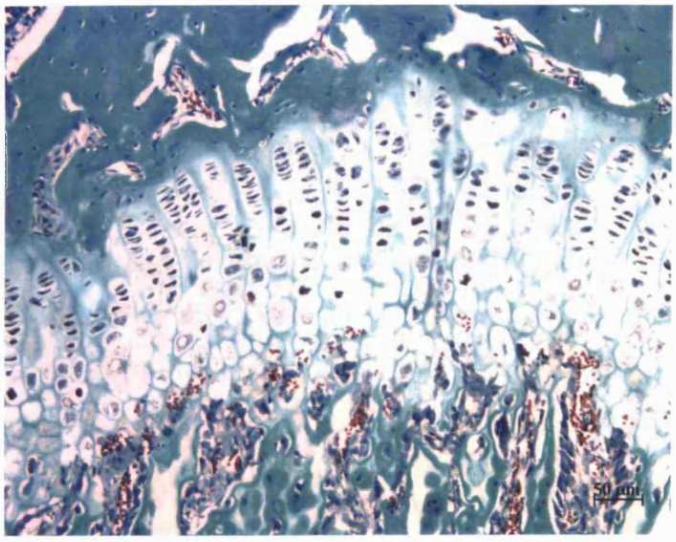
Masson's Trichrome-stained EPW is shown in figure 2.3A accompanied by a sketch detailing the different zones of the epiphyseal growth plate (Figures 2.3B&C).

2.2.6 Statistical Analysis

The secretory profiles of rGH were subjected to algorithmic (PULSAR) analysis [with the cut off parameters set (G1=3.98; G2=2.40; G3=1.68; G4=1.24; G5=0.93) to give a false positive error rate of 5% (Merriam and Wachter, 1982)] and distribution analysis (Matthews et al., 1991). The pulse amplitude was determined by calculating the mean peak heights within 9hr time period. The total secretory output was determined by the area under the curve (AUC) data, calculated by 'Graphpad Prism2'.

Results are expressed as the mean value ± SEM. Differences between groups were compared by Student's *t* test. When more than two groups were compared, differences were determined by one-way analysis of variance (ANOVA) followed by multiple comparisons using either the Dunnett's (vs. control) or Bonferroni's (for selected pairs) multiple comparison post hoc test. p-values<0.05 were considered significant.

A



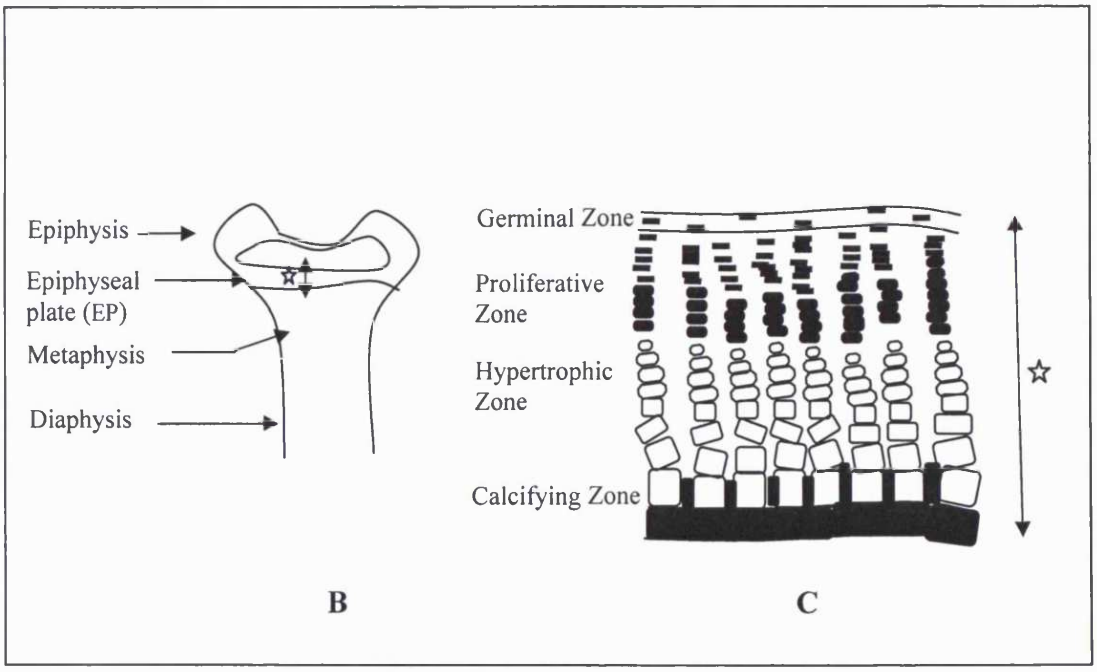


Figure 2.3: A. Micrograph of Masson's Trichrome stained epiphyseal plate width (EPW) from a virgin female rat tibia. **B.** Schematic representation of the structure of the bone epiphysis including EP. **C.** Zones encountered at the epiphyseal growth plate level (C, adapted from Ohlsson et al., 1993).

2.3 RESULTS

2.3.1 Study 1 – Analysis of Pituitary Cell Types and Secretory Profiles in Pregnancy

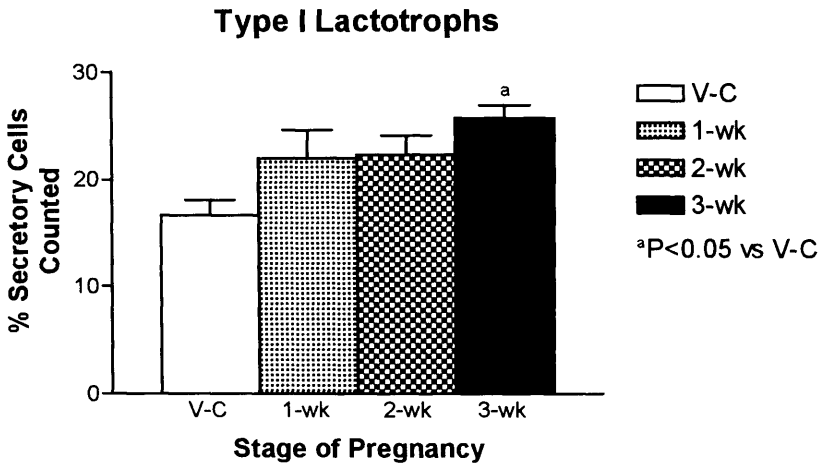
2.3.1.1 Pituitary cell types

In the first study, lactotroph, somatotroph and somatomammotroph populations were quantified in pituitaries of virgin (V-C) and 1-, 2-, and 3-wk pregnant (PG) CD rats, using electron microscopy in conjunction with immunogold labelling. During pregnancy, the proportion of type I lactotrophs (LT-I) increased progressively [from 16.0±1.5% in V-C (n=3) to 26.0±1.2% in 3-wk PG (n=3) ($p<0.05$)] (Figure 2.4A). In contrast, the proportions of type II lactotrophs (LT-II; Figure 2.4B) and somatotrophs (ST; Figure 2.4D) remained unchanged, but the proportion of somatomammotrophs (SMT) was reduced [Figure 2.4E; 1.0±0.0% (V-C; n=3) vs. 0.2±0.1% (3-wk PG; n=3); $p<0.01$] (see also cumulative graph, Figure 2.4F). The occasional lactotroph matching the 'intermediate' morphology seen in *dw/dw* rats was observed in normal virgin females (Figure 2.4C). These cells accounted for almost 0.2% of the total secretory cell population, and were unaltered by pregnancy (Figure 2.4C). The sub-cellular morphology of type I lactotrophs was examined in greater detail. Although cytoplasmic and nuclear areas were unaltered, secretory granule diameter in type I lactotrophs gradually decreased during pregnancy [401.0±19.0nm (V-C; n=3) vs. 326.0±16.0nm (3-wk PG; n=3); $p<0.05$]. This decrease may reflect an increase in the secretory activity of type I lactotrophs during pregnancy. Since type I lactotroph is an inactive cell, the increase in its secretory activity during pregnancy may have a crucial contribution in building-up maternal PRL reserves in preparation for lactation.

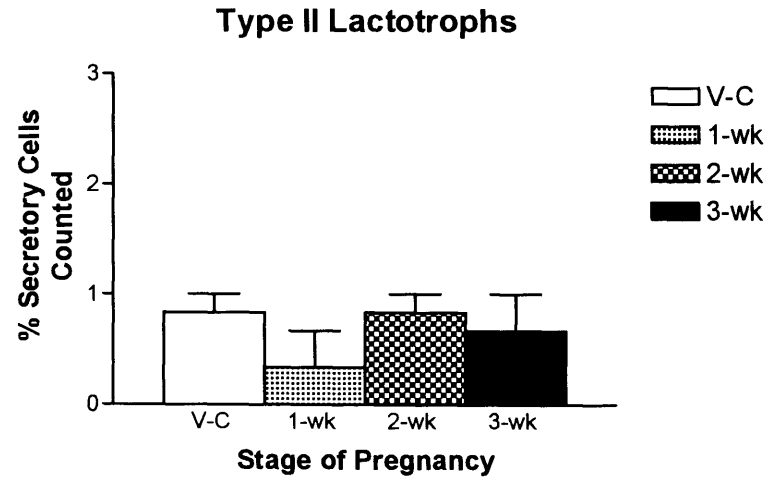
2.3.1.2 GH and PRL secretion

Spontaneous GH and PRL secretion was measured in conscious pregnant rats. Spontaneous GH secretory profiles were obtained from virgin, and 1-, 2-, and 3-wk pregnant rats. Representative examples are shown in Figure 2.5A and the complete set of GH profiles is shown in Figure 2.5B. The parameters of episodic secretion were determined by subjecting these profiles to algorithmic and distribution analysis. Total secretory output was progressively elevated during pregnancy [Figure 2.6A; area under the curve (AUC): 142.9±25.3ng/ml.min (V-C; n=6) vs. 339.3±39.2ng/ml.min

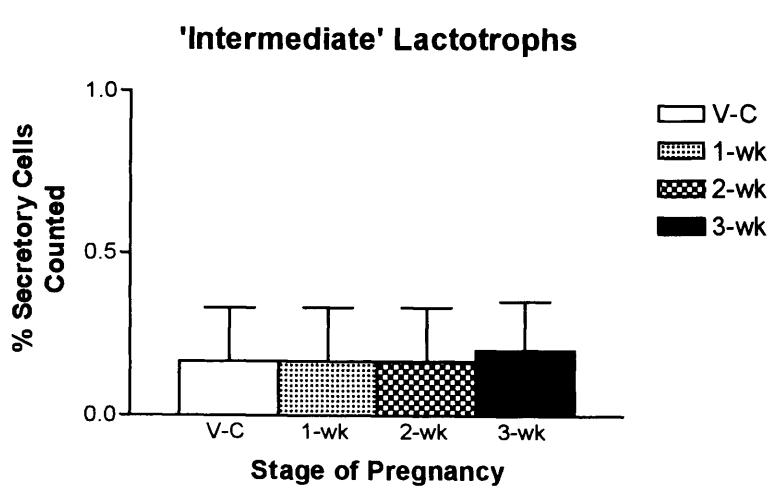
A


Type I Lactotrophs
% Secretory Cells Counted
30
20
10
0
a
V-C
1-wk
2-wk
3-wk
aP<0.05 vs V-C
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy

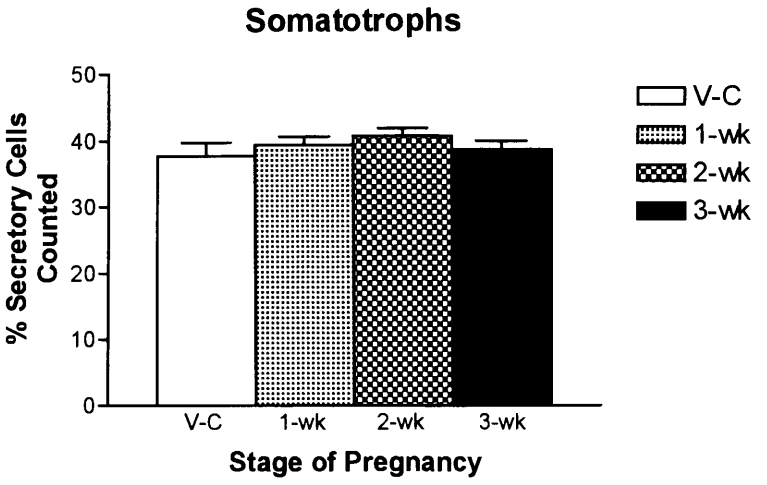
B


Type II Lactotrophs
% Secretory Cells Counted
3
2
1
0
V-C
1-wk
2-wk
3-wk
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy

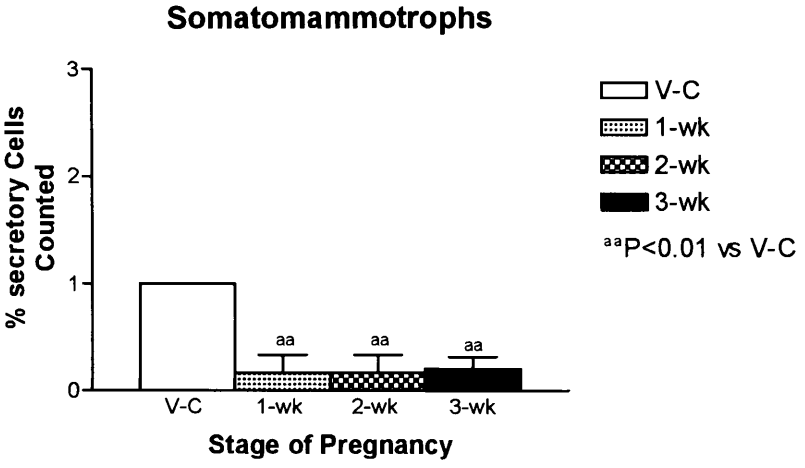
C


'Intermediate' Lactotrophs
% Secretory Cells Counted
1.0
0.5
0.0
V-C
1-wk
2-wk
3-wk
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy

D



E



F

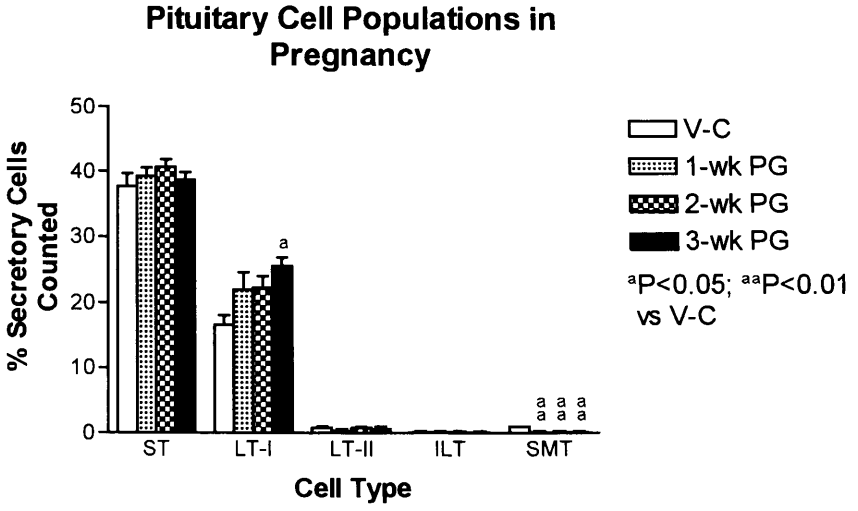


Figure 2.4: The proportion of type I lactotrophs (LT-I) (A), type II lactotrophs (LT-II) (B),'intermediate' lactotrophs (ILT) (C), somatotrophs (ST) (D), and somatomammotrophs (SMT) (E) in the total cell populations in pituitaries of virgin-control (V-C), and 1-, 2- and 3-wk pregnant rat. Cumulative graph shows the pituitary cell populations in pregnancy (F). Values shown are mean ± SEM (n=3).

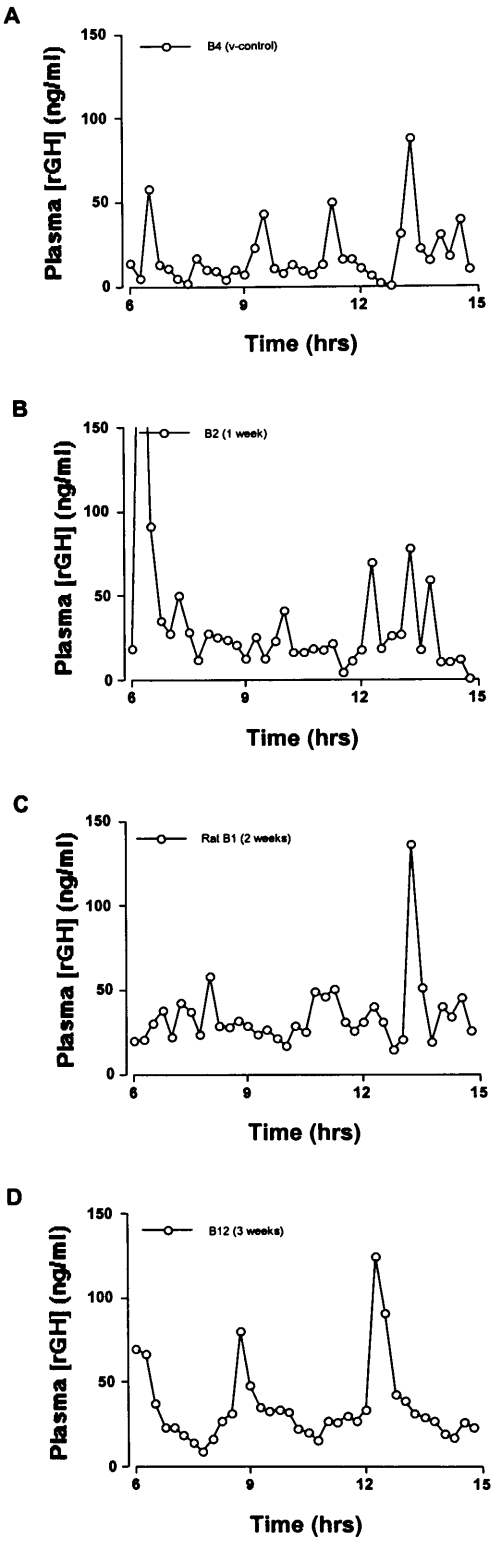


Figure 2.5A: Representative profiles of spontaneous GH secretion of virgin-control (A), 1-wk (B), 2-wk (C), and 3-wk (D) pregnant conscious normal rats.

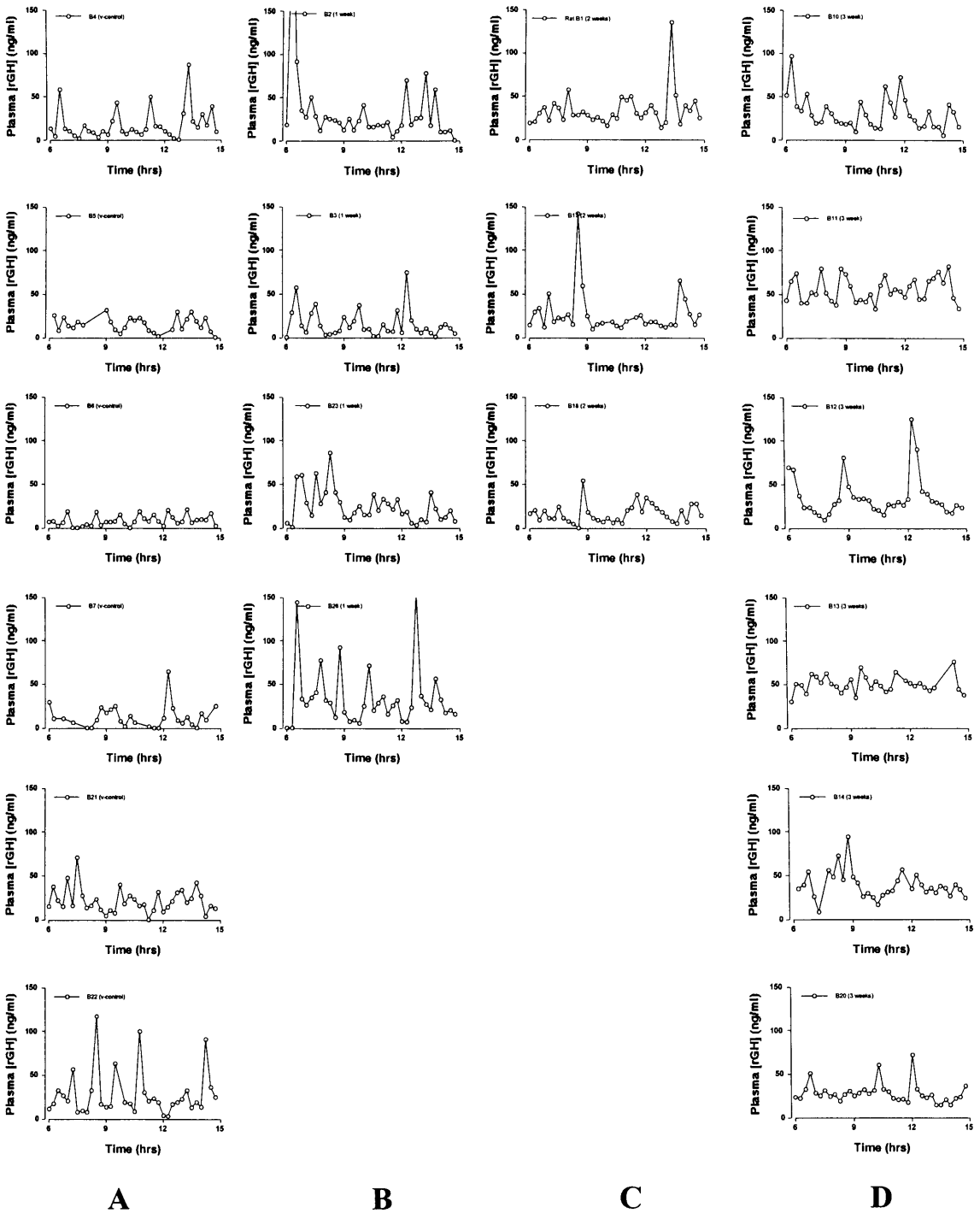
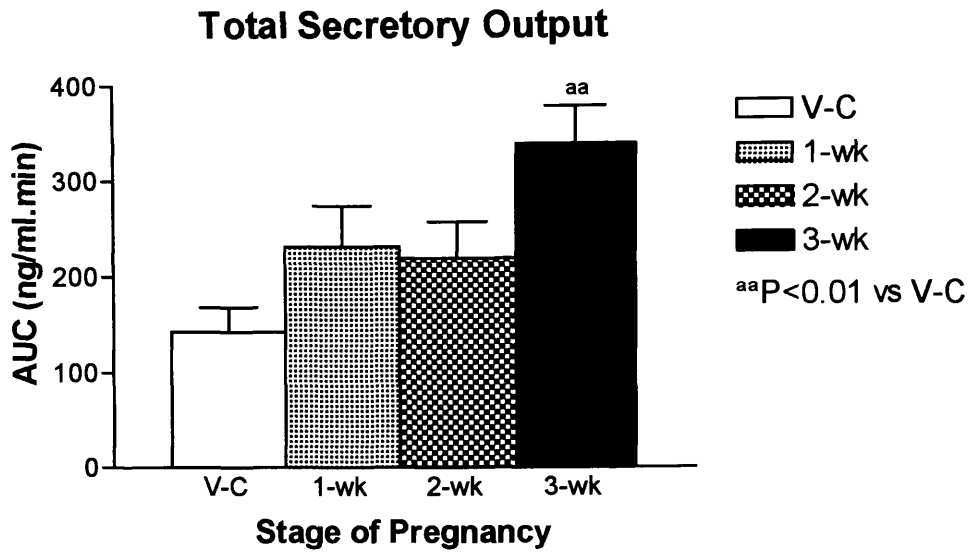


Figure 2.5B: The Complete Set of GH Profiles. Profiles of spontaneous GH secretion of virgin-control (A, n=6), 1-wk (B, n=4), 2-wk (C, n=3), and 3-wk (D, n=6) pregnant conscious normal rats.

A



B

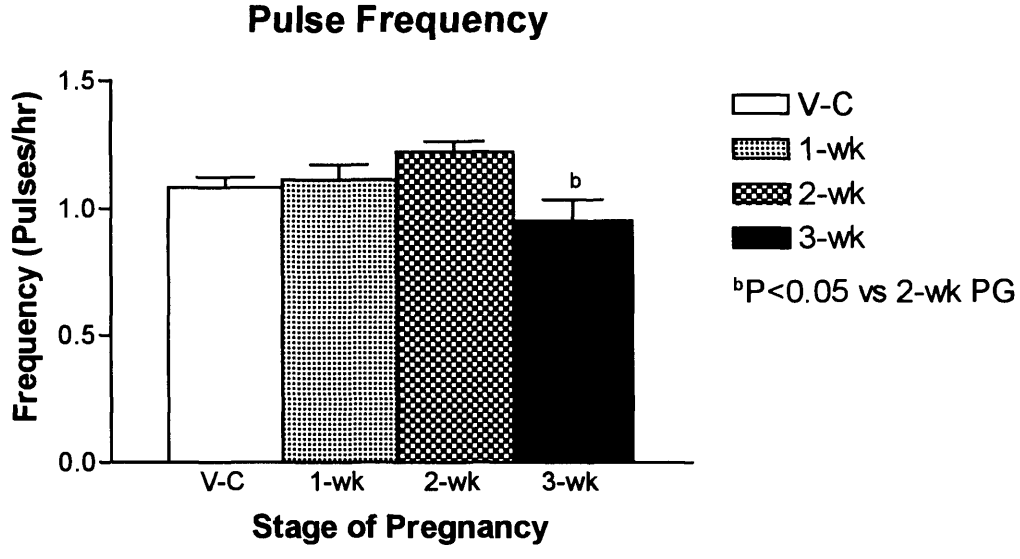
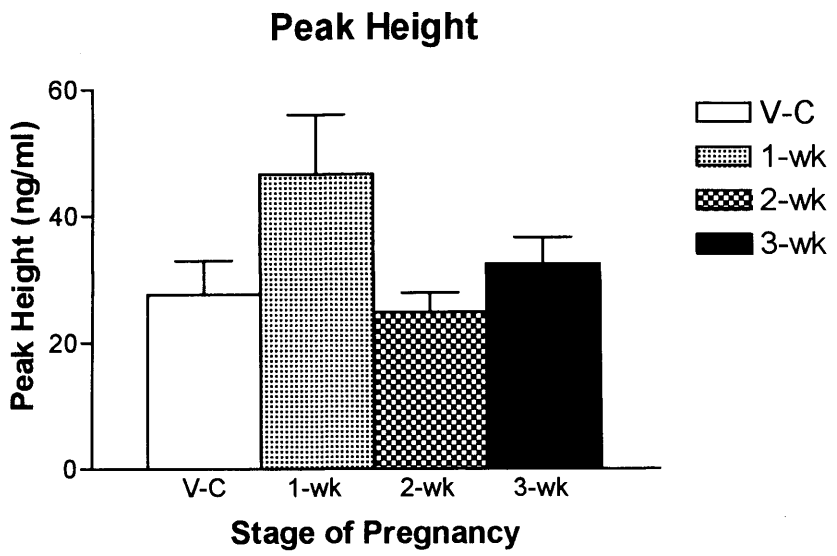
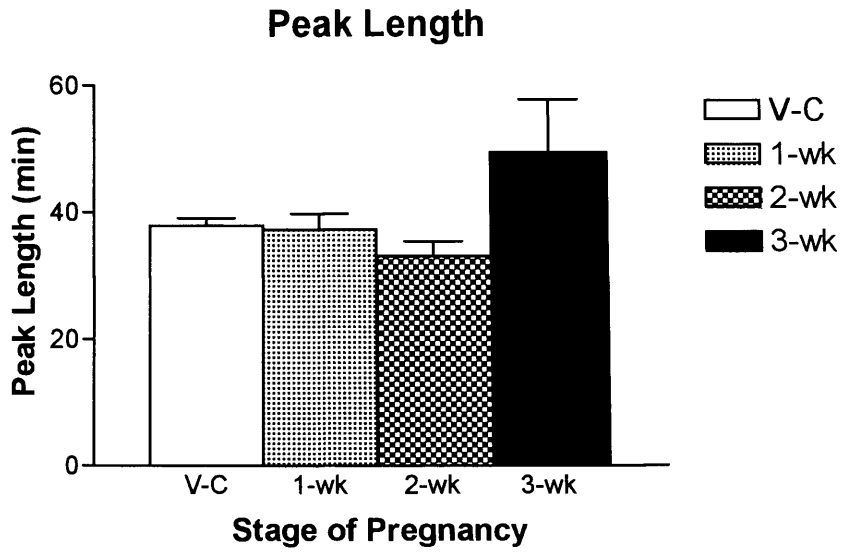


Figure 2.6: The characteristics of the GH secretory pattern in normal rat pregnancy. Data were obtained from ***PULSAR*** analysis of plasma GH profiles. The parameters shown are the total secretory output (AUC, A), the pulse frequency (B), the peak height (C), the peak length (D), and the inter-peak interval (E). Values shown are mean ± SEM (n=6 for V-C and 3-wk pregnant groups, n=4 for 1-wk pregnant group, and n=3 for 2-wk pregnant group).

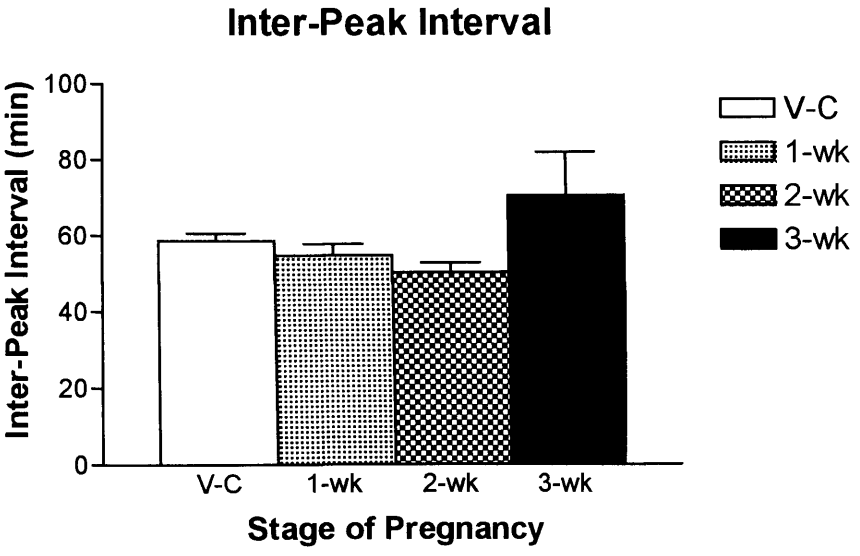
C


Peak Height
Peak Height (ng/ml)
60
40
20
0
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk

D


Peak Length
Peak Length (min)
60
40
20
0
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk

E


Inter-Peak Interval
Inter-Peak Interval (min)
100
80
60
40
20
0
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk

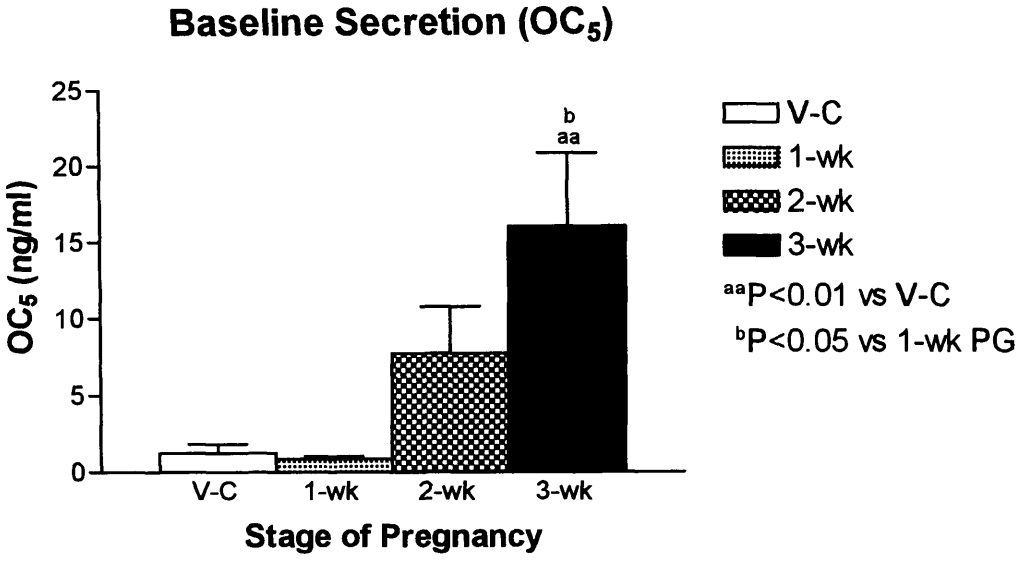
(3-wk PG; n=6); $p<0.01$]. There were no consistent changes in any of the parameters of episodic secretion [pulse frequency (Figure 2.6B); peak height (Figure 2.6C); peak length (Figure 2.6D); or inter-peak interval (Figure 2.6E)], although there was a significant decline in pulse frequency between the second and third week of pregnancy (Figure 2.6B). The absence of consistent changes in pulse frequency or pulse height was confirmed by Fourier analysis (Figure 2.7D) and distribution analysis [observed concentration 95 (OC_{95}); Figure 2.7B]. It should be noted that although the variation in spectral power was high at lower frequencies in 1-wk pregnant rats (Figure 2.7D), the mean was not significantly different to that in other groups. Distribution analysis revealed a progressive elevation in baseline secretion [Figure 2.7A; OC_5: 1.2±0.6ng/ml (V-C; n=6) vs. 16.0±4.8ng/ml (3-wk PG; n=6); $p<0.01$]. Despite this increase and the absence of any change in pulse height (OC_{95}) (Figure 2.7B), there was no significant decline in pulse amplitude [pulse height (OC_{95})-baseline (OC_5); Figure 2.7C].

Spontaneous PRL secretion was also measured in the same samples and representative examples are shown in Figure 2.8A. There were no consistent changes in the parameters of spontaneous PRL secretion (see Figure 2.8B for the complete set of PRL profiles). Due to the high inter- and intra-animal variation in measured plasma PRL concentration seen in 3-wk pregnant rats (Figure 2.9), PRL profiles were not subjected to either algorithmic or distribution analysis as applied to GH profiles. The dramatic increase in PRL secretion in profiles 2.9C and D was observed to coincide with the onset of parturition.

2.3.1.3 Sensitivity to ghrelin throughout pregnancy

As previously demonstrated (Kojima et al., 1999), ghrelin elicited a robust increase in circulating GH in virgin females [Figure 2.10A; 12.8±4.3ng/ml (at 0min) vs. 118.5±28.3ng/ml (at 5min); $p<0.01$]. Although the ghrelin-induced rise in circulating GH appeared more rapid in pregnant rats, the total secretory output was not significantly greater during pregnancy [Figure 2.10A; AUC, 4915±420ng/ml.min (V-C; n=5) vs.7973±1277ng/ml.min (3-wk PG; n=6); $p>0.05$]. Ghrelin failed to elicit PRL secretion in normal female rats at any stage of pregnancy (Figure 2.10B). Since this experiment was investigating the effect of pregnancy on the secretagogue activity

A



B

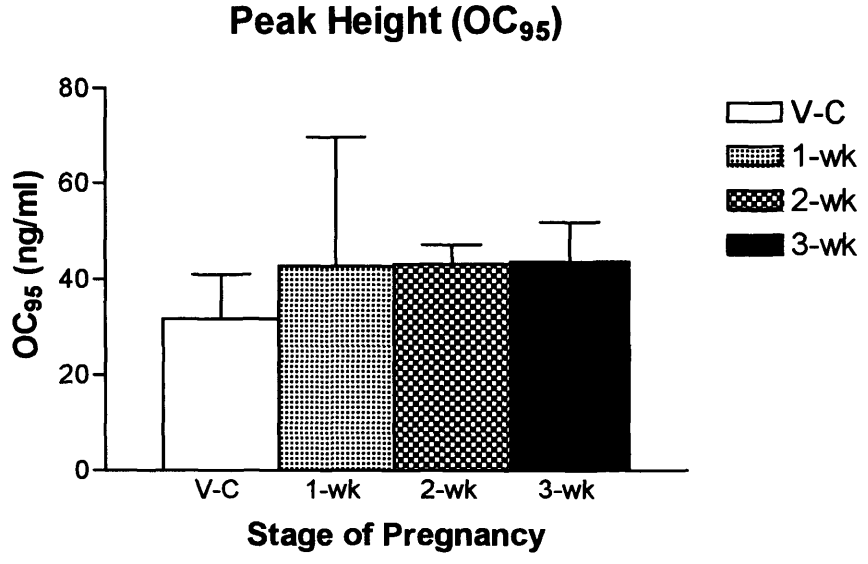
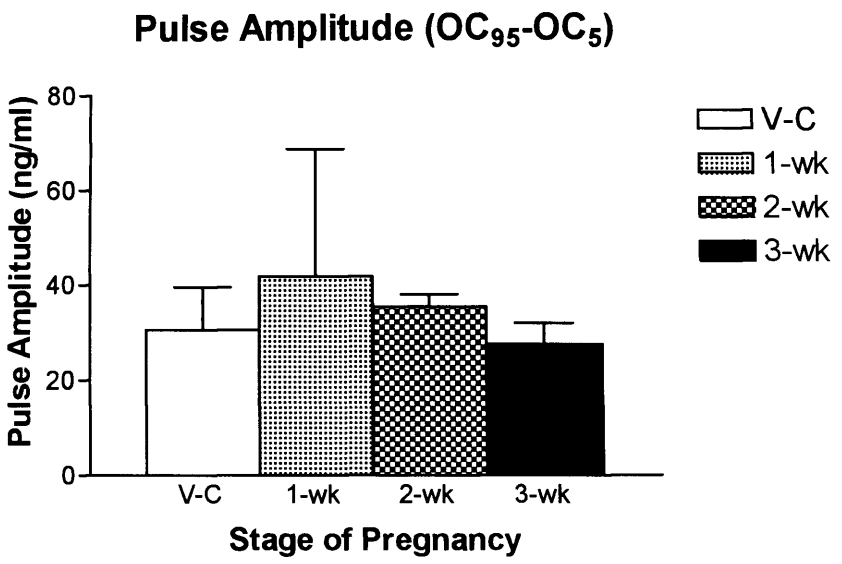
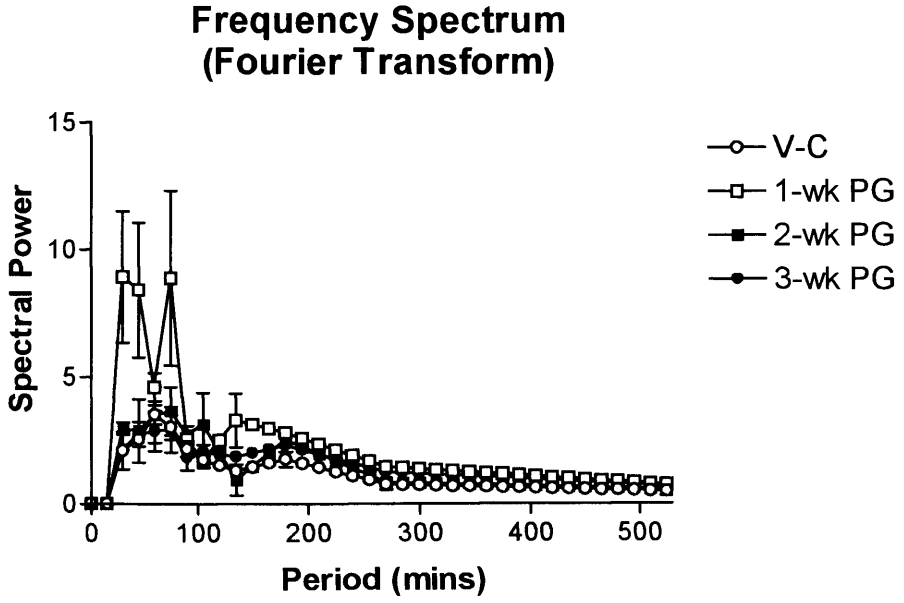


Figure 2.7: The characteristics of the GH secretory pattern in normal rat pregnancy. Data were obtained from the ***Distribution*** analysis of plasma GH profiles. The parameters shown are the baseline secretion (OC_5, A), the peak height (OC_{95}, B), the pulse amplitude [(OC_{95}-OC_5), C], and the frequency spectrum [Fourier transform, D]. Values shown are mean ± SEM (n=6 for V-C and 3-wk pregnant groups, n=4 for 1-wk pregnant group, and n=3 for 2-wk pregnant group).

C


Pulse Amplitude (OC_{95}-OC_5)
Pulse Amplitude (ng/ml)
80
60
40
20
0
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk

D


Frequency Spectrum
(Fourier Transform)
Spectral Power
15
10
5
0
0
100
200
300
400
500
Period (mins)
V-C
1-wk PG
2-wk PG
3-wk PG

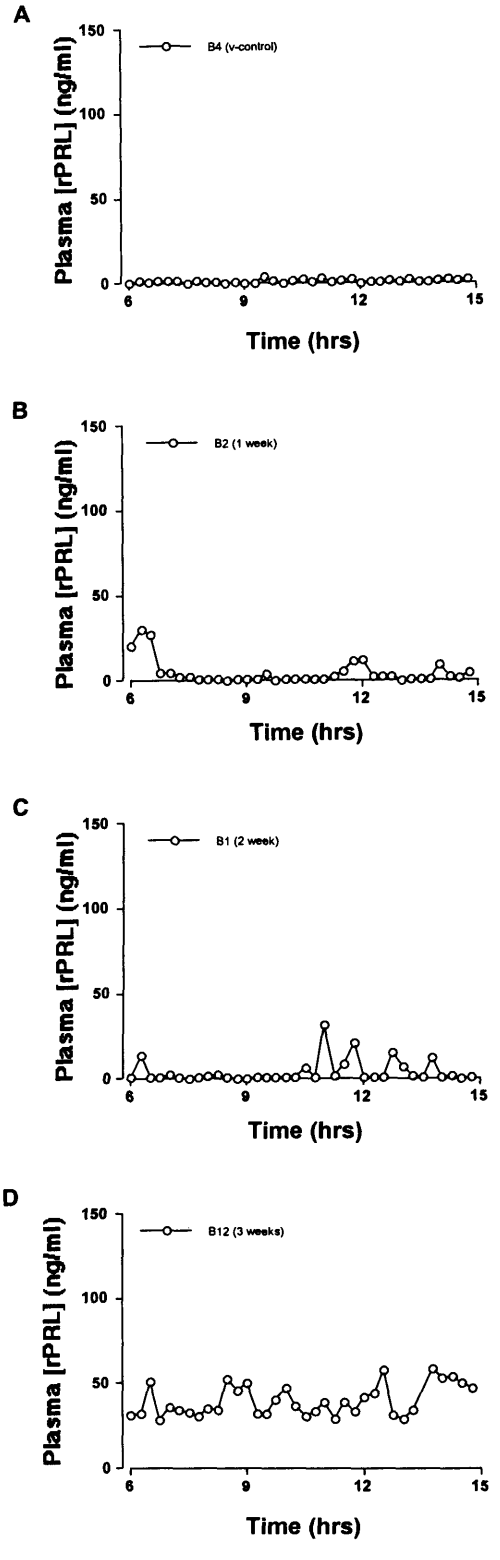


Figure 2.8A: Representative profiles of spontaneous PRL secretion of virgin-control (A), 1-wk (B), 2-wk (C), and 3-wk (D) pregnant conscious normal rats.

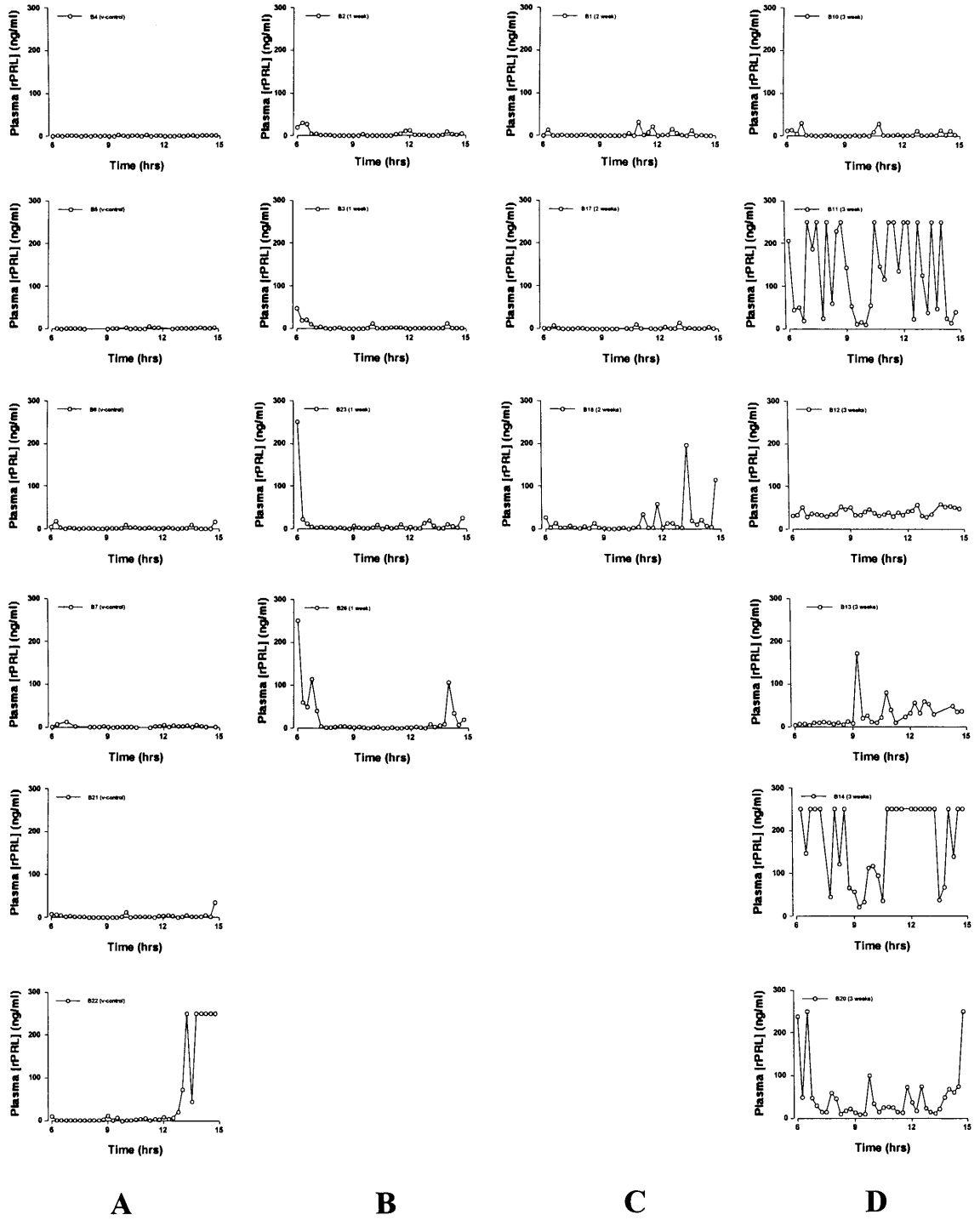


Figure 2.8B: The Complete Set of PRL Profiles. Profiles of spontaneous PRL secretion of virgin-control (A, n=6), 1-wk (B, n=4), 2-wk (C, n=3), and 3-wk (D, n=6) pregnant conscious normal rats.

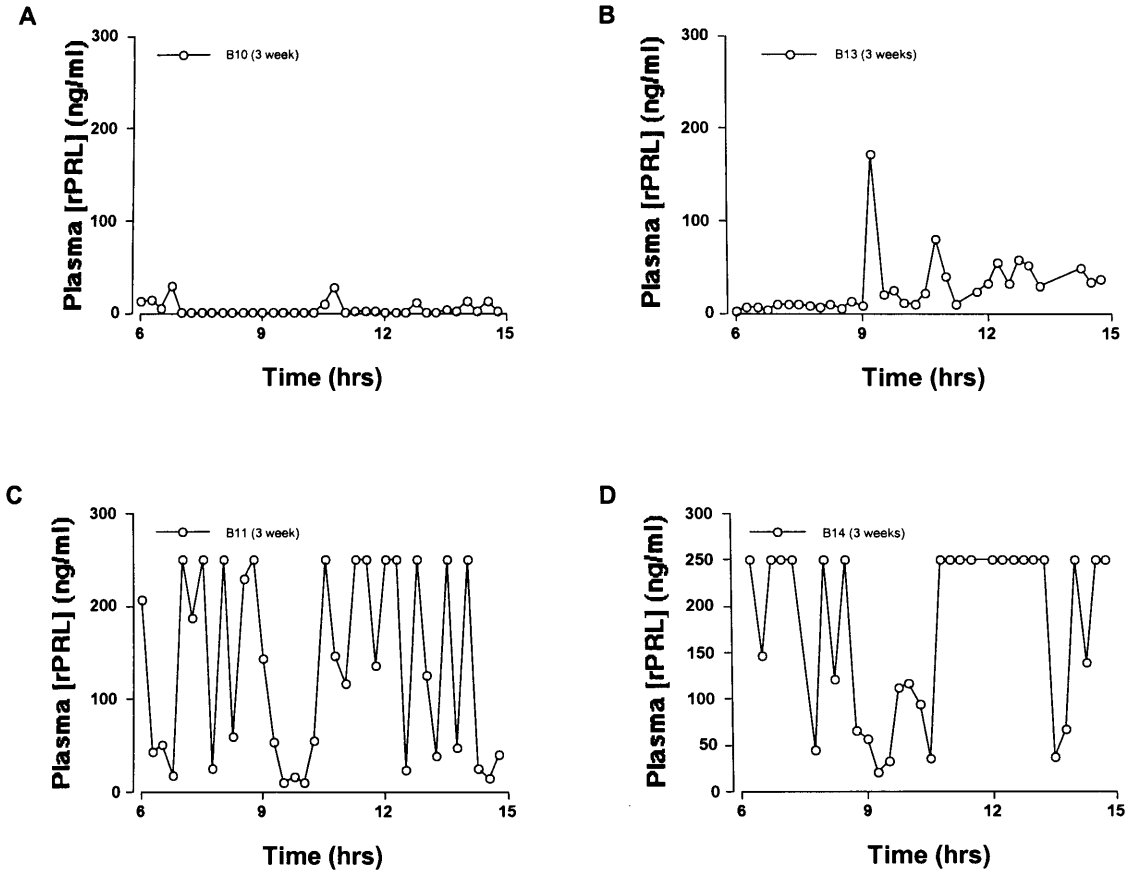
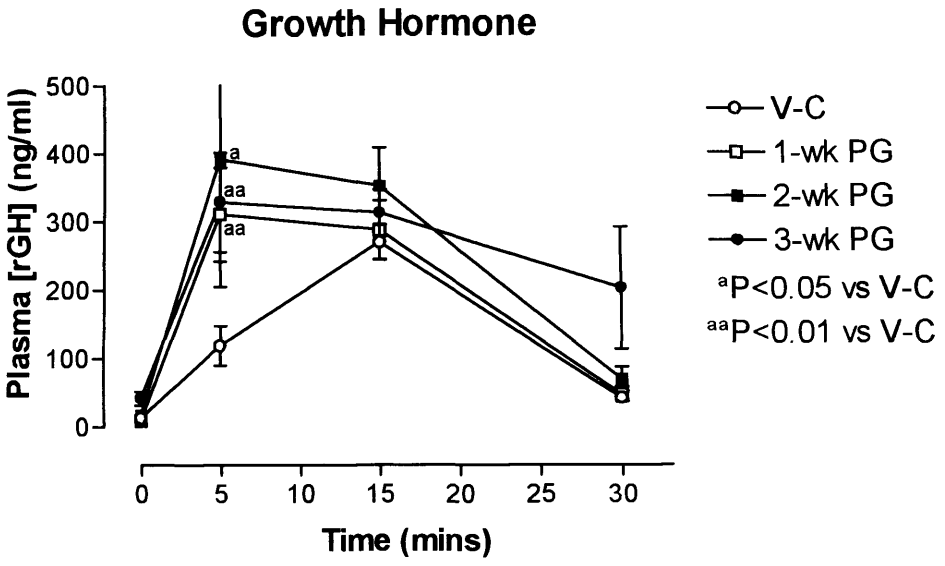


Figure 2.9: Additional examples of profiles of spontaneous PRL secretion of 3-wk pregnant conscious normal rats showing pattern variations (A-D). C and D represent plasma PRL profiles of 3-wk pregnant conscious normal rats entering parturition.

A



B

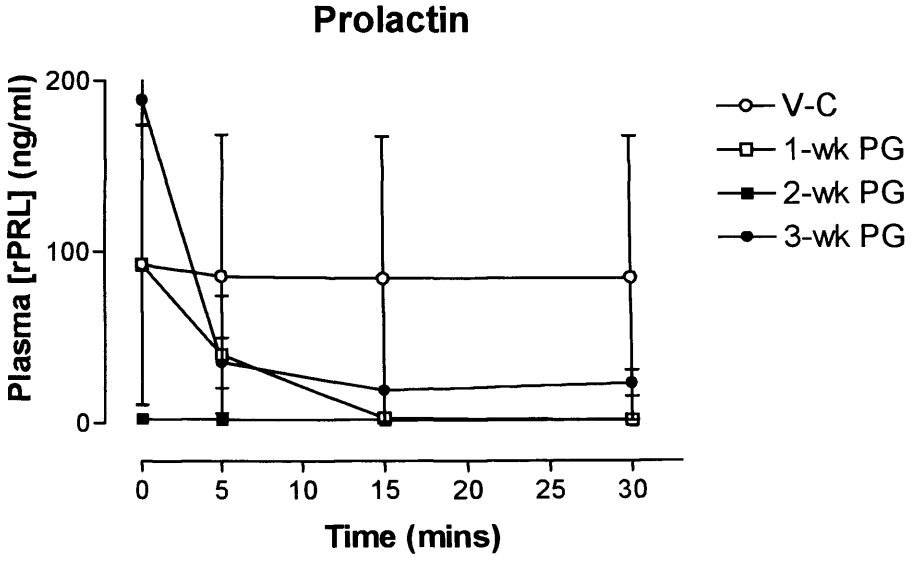


Figure 2.10: The rGH (A) and PRL (B) responses to a bolus i.v. injection of rat ghrelin (10μg) during normal rat pregnancy. Values shown are mean ± SEM (n=3 for 2-wk pregnant group and n=6 for all other groups).

of ghrelin, ghrelin-treated virgin females served as a control. Vehicle-treated females at each stage of pregnancy were unnecessary.

2.3.1.4 Pituitary GH and PRL contents

Over the time course of pregnancy, pituitary GH content was almost doubled [Figure 2.11A; 423.4ng/ml (V-C; n=6) vs. 796.5ng/ml (3-wk PG; n=6)], but this was not significantly different ($p>0.05$). Despite the observed changes in lactotroph subpopulation, there was no consistent change in pituitary PRL content (Figure 2.11B).

2.3.2 Study 2 – Effect of Pseudopregnancy on Pituitary Cell Types

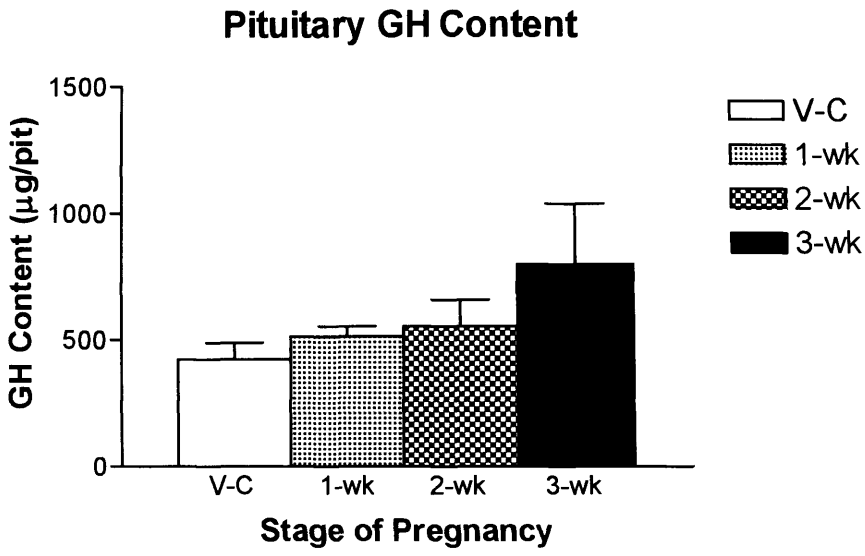
In order to determine if the pregnancy-induced changes in secretory cell populations in the pituitary are dependent upon the placenta, electron microscopy and immunogold labelling was used to quantify pituitary cell types in virgin, and 12-day pregnant and pseudopregnant rats. The increase in the type I lactotroph population reported in study 1 was also observed here in 12-d pregnant rats, but there was no significant reciprocal decline in somatomammotrophs ($p=0.0528$). However, the increase in type I lactotrophs (Figure 2.12A) and the changes in granular morphology in type I lactotrophs were not seen in pseudopregnant rats. Type II and 'intermediate' lactotrophs (Figures 2.12B & C), somatotrophs (Figure 2.12D), and somatomammotrophs (Figure 2.12E) remained unaltered (see also cumulative graph, Figure 2.12F)

2.3.3 Study 3 – Endocrine Responses to Secretagogue Treatment in 2-week Pregnant Rats

2.3.3.1 GH and PRL secretion

The GH and PRL responses to secretagogue treatment following an i.v. injection of vehicle, rat ghrelin (10μg), rat GRF (1μg), or the D_2-receptor antagonist, sulpiride (1μg) in 2-week pregnant rats is shown in Figure 2.13. An increase in GH plasma concentration was elicited by bolus injections of ghrelin and GRF, but not by injection of the D_2-receptor antagonist, sulpiride (Figure 2.13A). In contrast, PRL plasma concentration was stimulated by sulpiride, but not by ghrelin or GRF (Figure 2.13B).

A



B

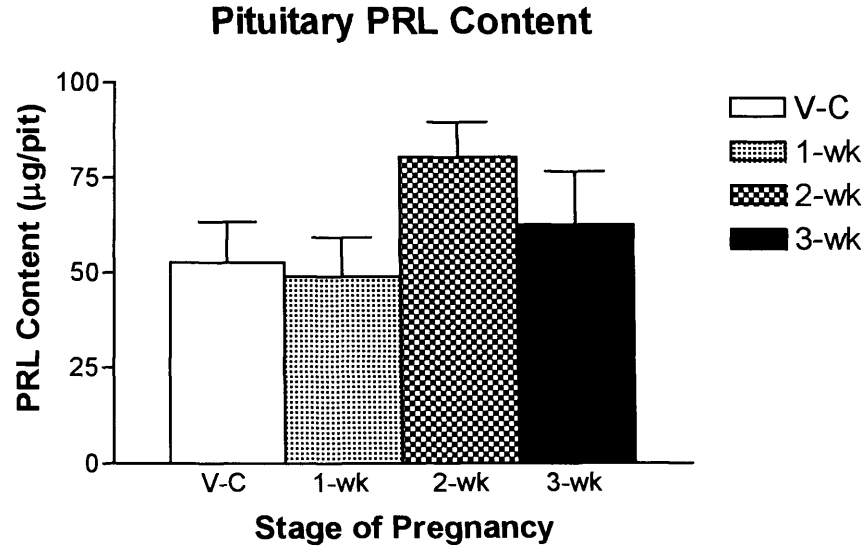
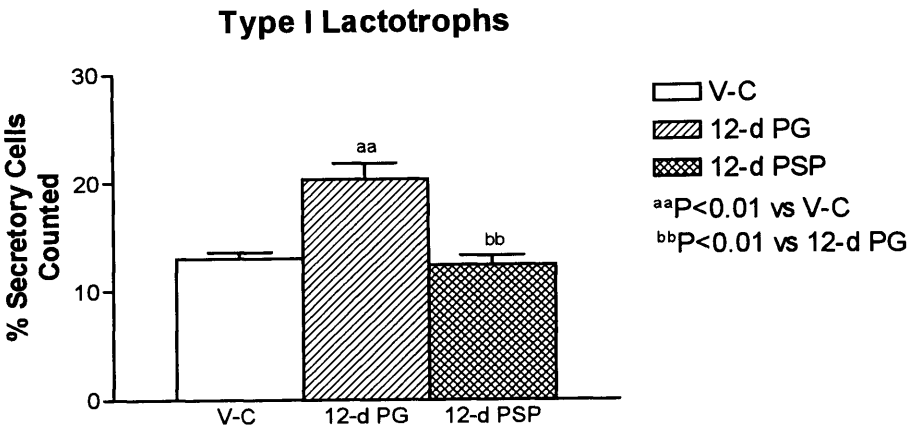
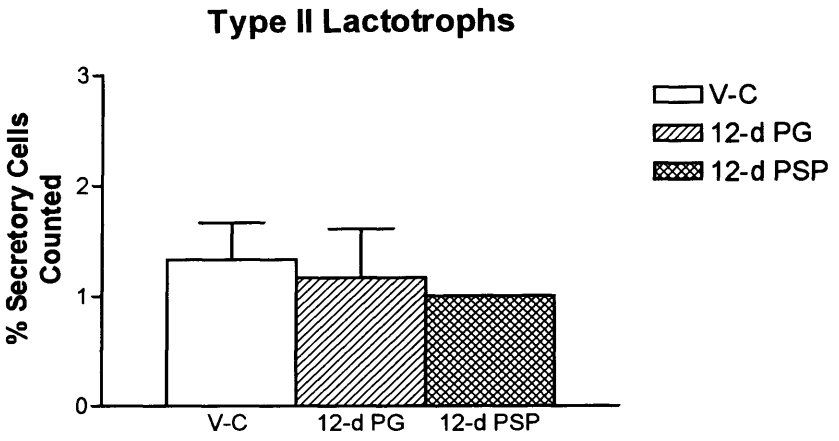


Figure 2.11: Pituitary GH (A) and PRL (B) contents in pregnant normal rats. Values shown are mean ± SEM (n=4 for 2-wk pregnant group and n=6 for all other groups).

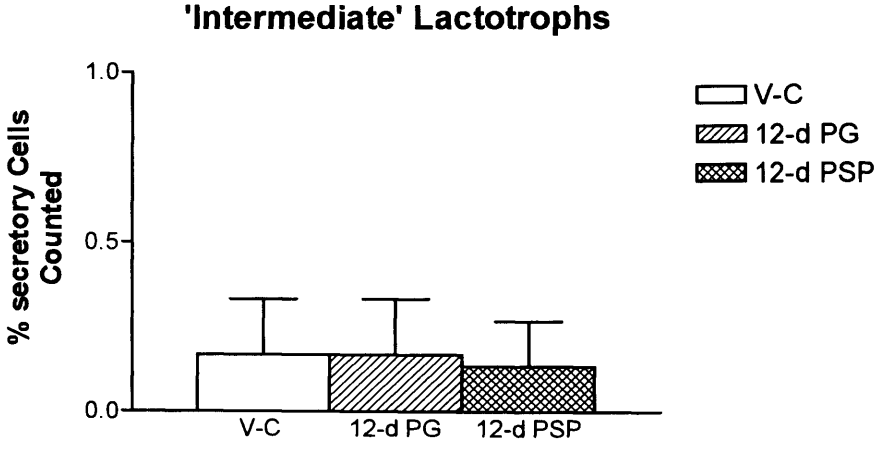
A


Type I Lactotrophs
% Secretory Cells Counted
30
20
10
0
aa
bb
V-C
12-d PG
12-d PSP
V-C
12-d PG
12-d PSP
aaP<0.01 vs V-C
bbP<0.01 vs 12-d PG

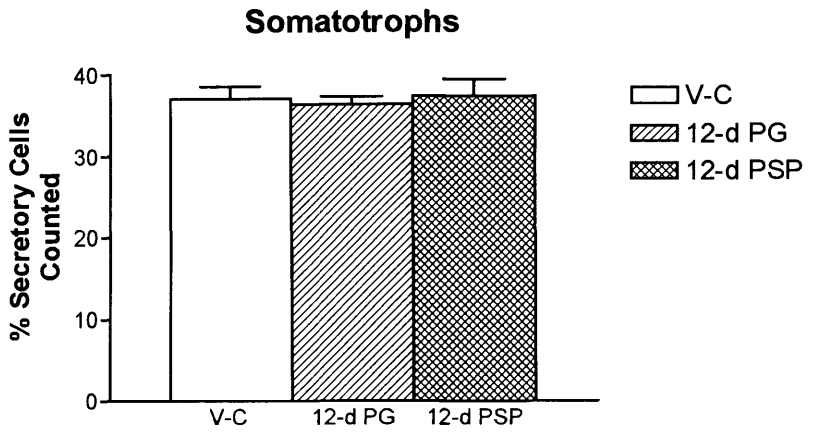
B


Type II Lactotrophs
% Secretory Cells Counted
3
2
1
0
V-C
12-d PG
12-d PSP
V-C
12-d PG
12-d PSP

C


'Intermediate' Lactotrophs
% secretory Cells Counted
1.0
0.5
0.0
V-C
12-d PG
12-d PSP
V-C
12-d PG
12-d PSP

D



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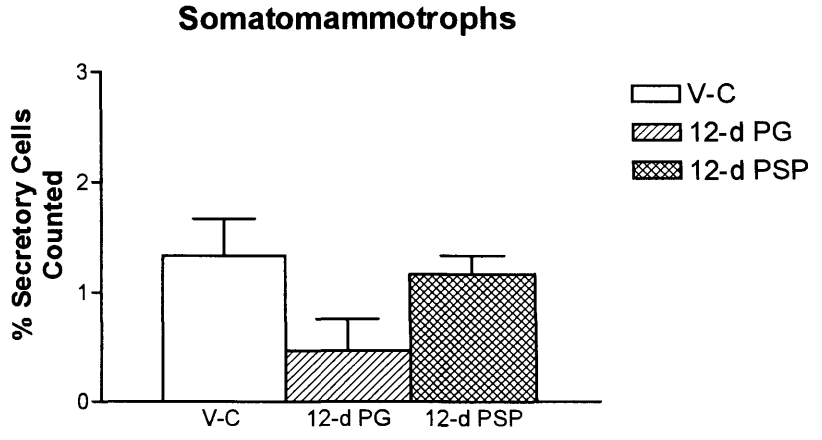


F

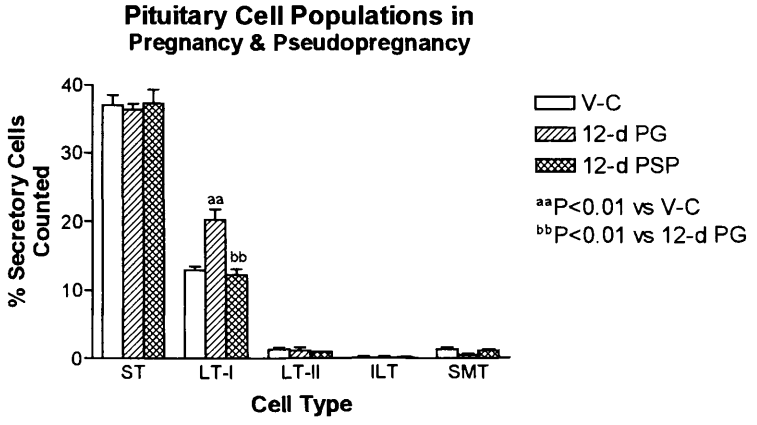
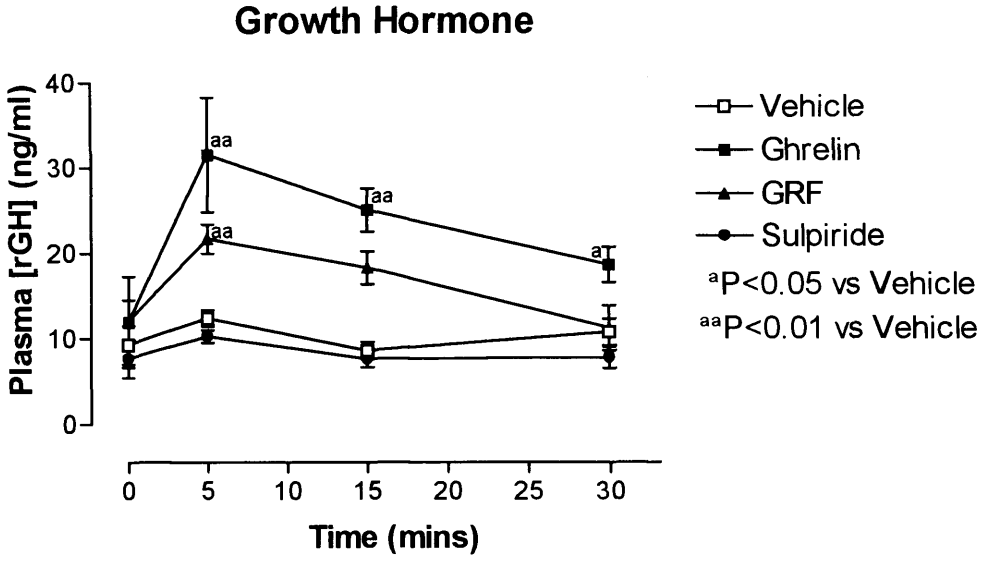


Figure 2.12: The proportion of type I lactotrophs (A), type II lactotrophs (B),'intermediate' lactotrophs (C), somatotrophs (D), and somatomammotrophs (E) in the total cell populations in pituitaries of virgin-control, and 12-d pregnant and pseudopregnant rats. Cumulative graph shows the pituitary cell populations in pregnancy and pseudopregnancy (F). Values shown are mean ± SEM (n=3).

A



B

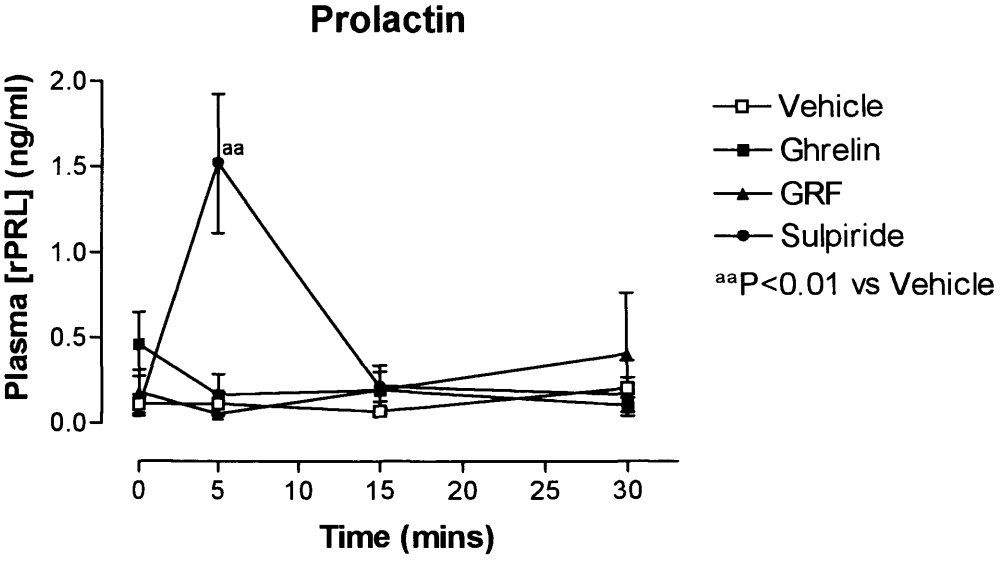


Figure 2.13: The effect of a bolus i.v. injection of vehicle, rat ghrelin (10μg), rat GRF (1μg), or the D_2-receptor antagonist, sulpiride (1μg) on GH (A) and PRL (B) plasma concentrations in 2-wk pregnant normal rats. Values shown are mean ± SEM (n=6 for vehicle treatment and n=5 for all other treatments).

2.3.4 Study 4 – Consequences of Modified GH Secretion on Growth and Adiposity during Pregnancy

2.3.4.1 Pituitary GH expression

In order to determine if the combination of the increased secretory output of GH, and the possible elevation in the pituitary content of GH is the result of increased GH production, pituitary GH mRNA expression was determined in pregnant rats by northern analysis (Figure 2.14A). GH mRNA levels were not increased during pregnancy, but if anything declined, mean GH mRNA levels in 3-wk pregnant rats being 84% of that in virgin-controls (Figure 2.14B). None of the means were significantly different.

2.3.4.2 Growth parameters

Given the observed changes in circulating GH during pregnancy, we investigated the potential effect on skeletal growth (section 2.3.4.2) and adiposity (section 2.3.4.3). Despite the observed increase in circulating GH during pregnancy (study 1), tibial length remained unaltered (Figure 2.15A). Surprisingly, tibial EPW, an index of the rate of skeletal growth, showed a progressive decline [Figure 2.15B; 264.8±4.0μm (V-C; n=6) vs. 196.6±7.0μm (3-wk PG; n=6); $p<0.01$].

Plasma IGF-I levels showed a marked decline with the progression of pregnancy (Figure 2.15C), being decreased in 3-wk pregnant rats to 25% of that seen in virgin-controls [1043.0±33.3ng/ml (V-C; n=6) vs. 264.9±25.2ng/ml (3-wk PG; n=3); $p<0.01$].

2.3.4.3 Metabolic parameters

2.3.4.3.1 Visceral fat pad weight and plasma leptin

As expected, body weight increased progressively during pregnancy [Figure 2.16A; 289.7±5.45g (V-C; n=6) vs. 373.4±13.5g (3-wk PG; n=3); $p<0.01$] but visceral adiposity (both in actual and proportionate terms) remained unaltered (Figures 2.16B and C). In contrast, plasma leptin levels were almost doubled by the 2nd week of pregnancy, hyperleptinaemia being retained until term (Figure 2.16D). However, due to the high degree of variation in parameters of adiposity, means of circulating leptin levels were not significantly different.

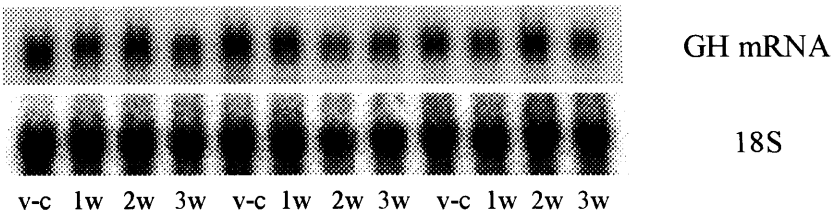


Figure 2.14A: Northern blot analysis of virgin-control (v-c; n=3), and 1-, 2-, and 3-wk (1w, 2w, and 3w; n=3 for each stage) pregnant rat pituitary RNA samples (10μg). $^{\alpha 32}$P-labelled cDNA's from GH and 18S RNA genes were used as probes.

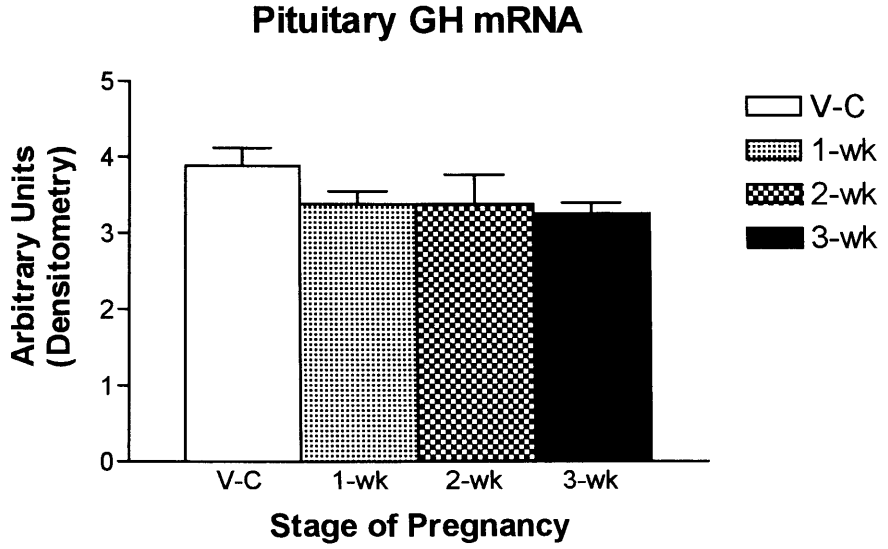
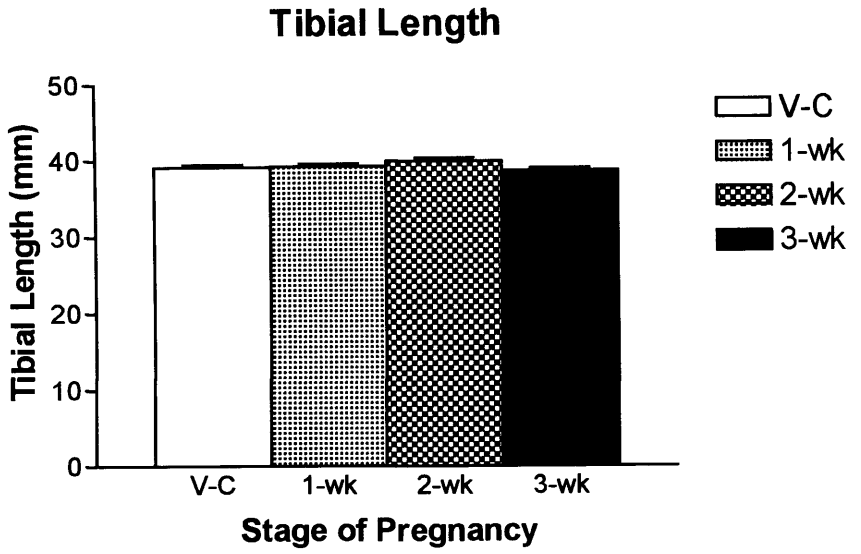
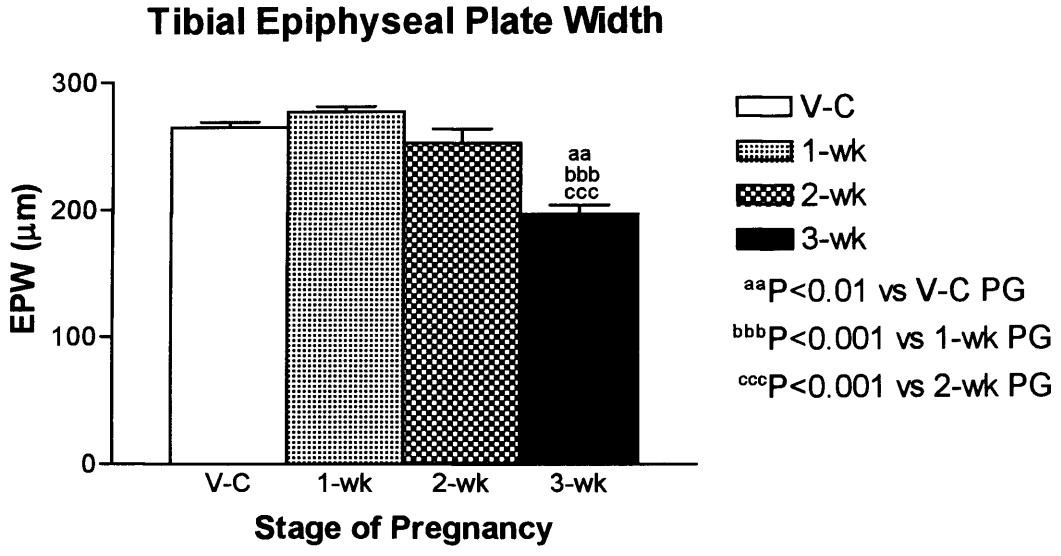


Figure 2.14B: Pituitary GH mRNA levels of virgin-control, and 1-, 2-, and 3-wk pregnant rats. The GH mRNA levels are expressed in arbitrary densitometric units after correction against level of 18S RNA. Values shown are mean ± SEM (n=3 for all groups).

A


Tibial Length
Tibial Length (mm)
50
40
30
20
10
0
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk

B


Tibial Epiphyseal Plate Width
EPW (µm)
300
200
100
0
aa
bbb
ccc
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk
aaP<0.01 vs V-C PG
bbbP<0.001 vs 1-wk PG
cccP<0.001 vs 2-wk PG

C

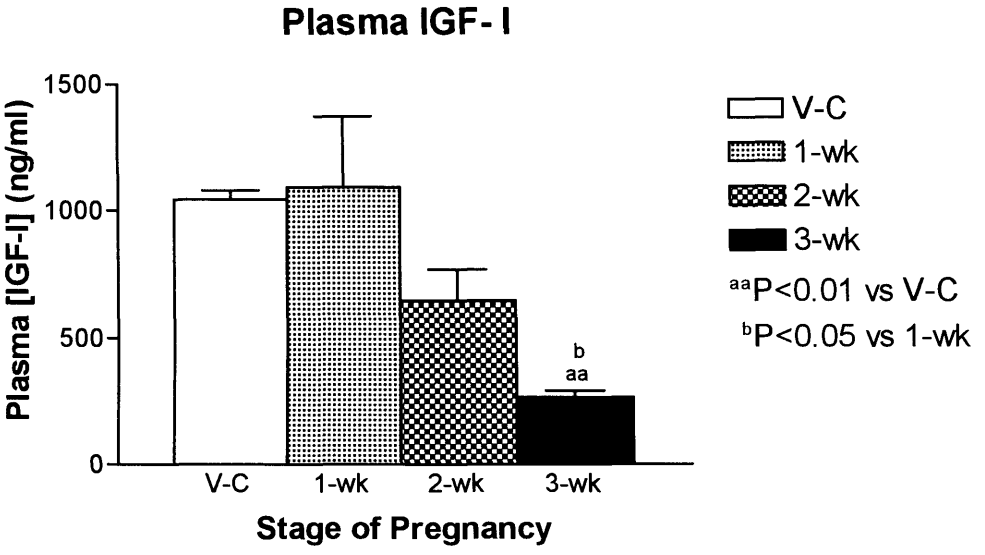
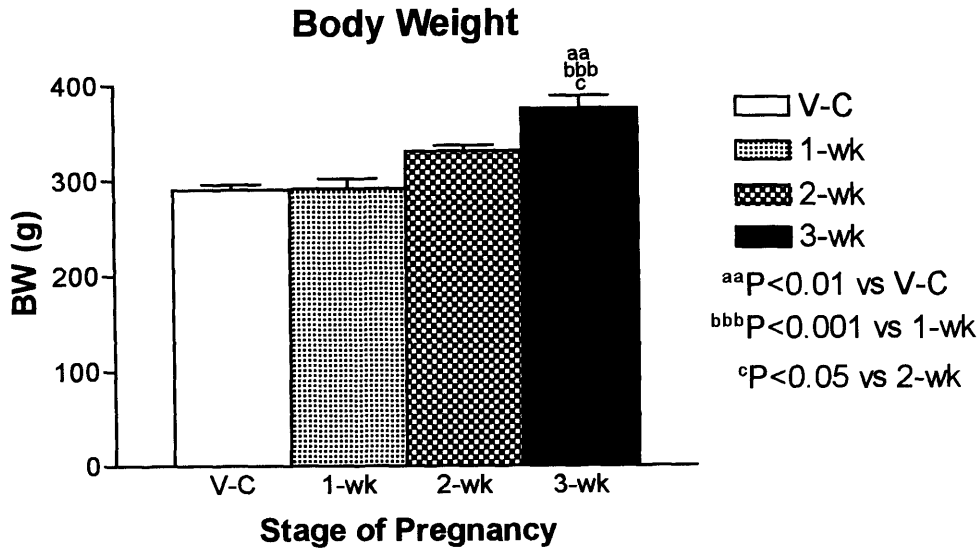
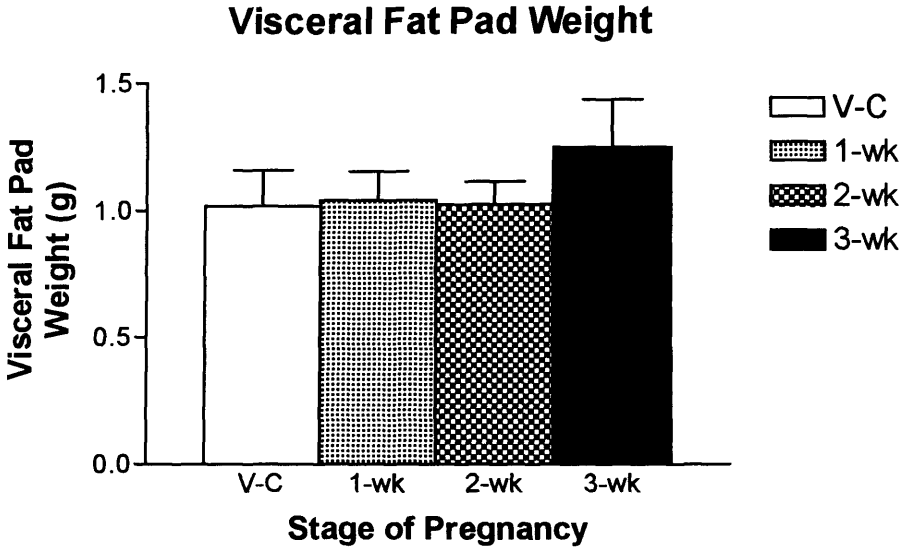


Figure 2.15: The effect of pregnancy on tibial bone length (A), tibial epiphyseal plate width (B), and plasma IGF-I concentrations (C). Values shown are mean ± SEM [for tibial measurements (n=3 for 2-wk pregnant group and n=6 for all other groups); for IGF-I assay (n=6 for virgin-control group, n=4 for 1-wk pregnant group, n=3 for each of 2- and 3-wk pregnant groups).

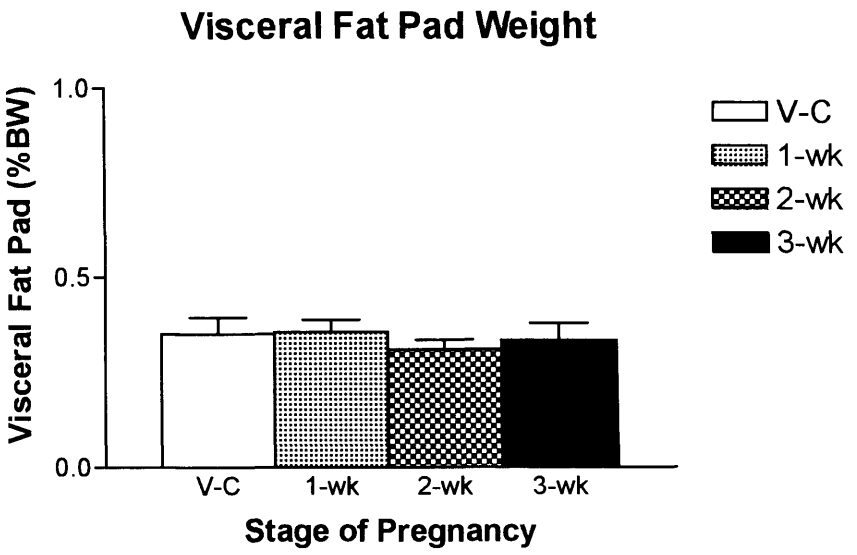
A


Body Weight
aa
bbb
c
400
300
200
100
0
BW (g)
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk
aaP<0.01 vs V-C
bbbP<0.001 vs 1-wk
cP<0.05 vs 2-wk

B


Visceral Fat Pad Weight
1.5
1.0
0.5
0.0
Visceral Fat Pad Weight (g)
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk

C



D

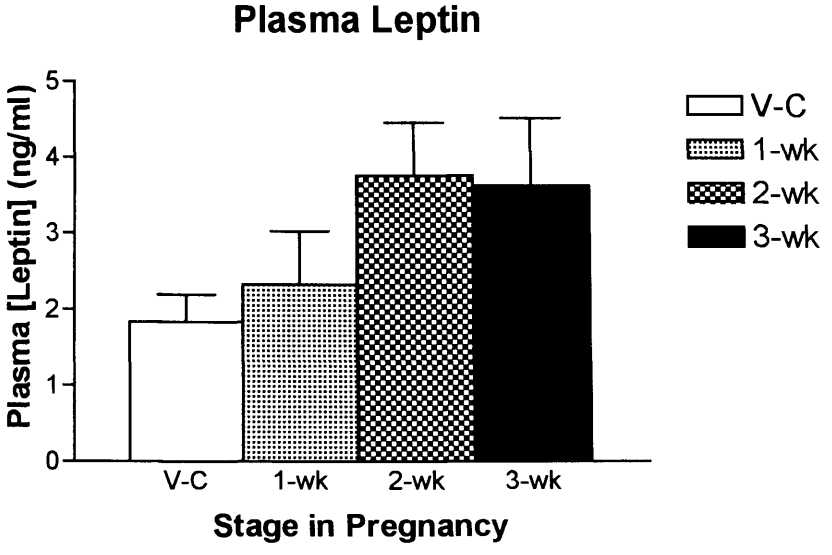


Figure 2.16: The effect of pregnancy on body weight (A), visceral (retroperitoneal and perirenal) fat pad weight (B), visceral fat pad weight [%BW, (C)], and plasma leptin concentrations (D). Values shown are mean ± SEM (n=3 for 2-wk pregnant group and n=6 for all other groups).

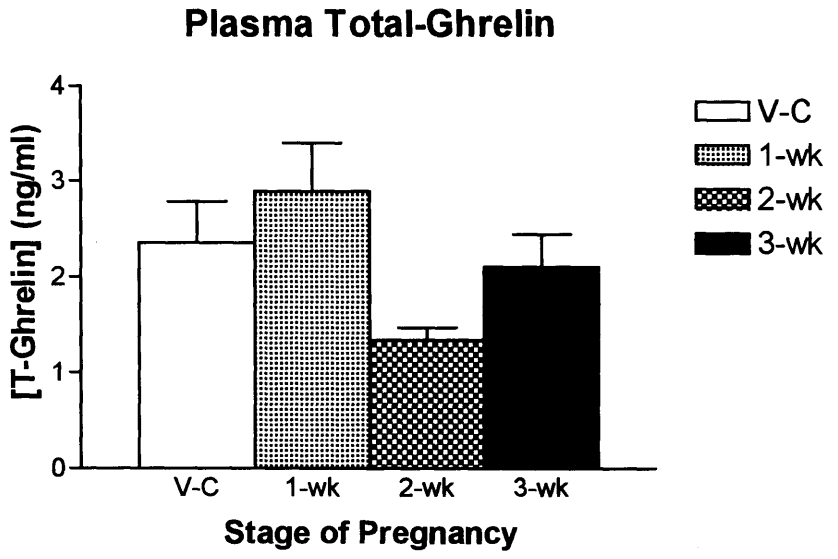
2.3.4.3.2 Plasma ghrelin

Circulating total-ghrelin levels were lowest during the 2nd week of pregnancy (Figure 2.17A), which coincided with the highest level of circulating octanoylated-ghrelin (Figure 2.17B). The proportion of octanoylated-ghrelin levels was significantly higher in the 2nd week of pregnancy, when compared to both the 1st and 3rd weeks [Figure 2.17D; 23.6±5.15% (2-wk PG; n=3) vs. 6.91±1.14% (1-wk PG; n=6); $p<0.05$] and [23.6±5.15% (2-wk PG; n=3) vs. 7.88±2.62% (3-wk PG; n=6); $p<0.05$]. Despite these changes in the proportion of octanoylated-ghrelin, there was no significant change in the proportion of non-acylated ghrelin (Figure 2.17C), this following the pattern of circulating total-ghrelin (Figure 2.17A).

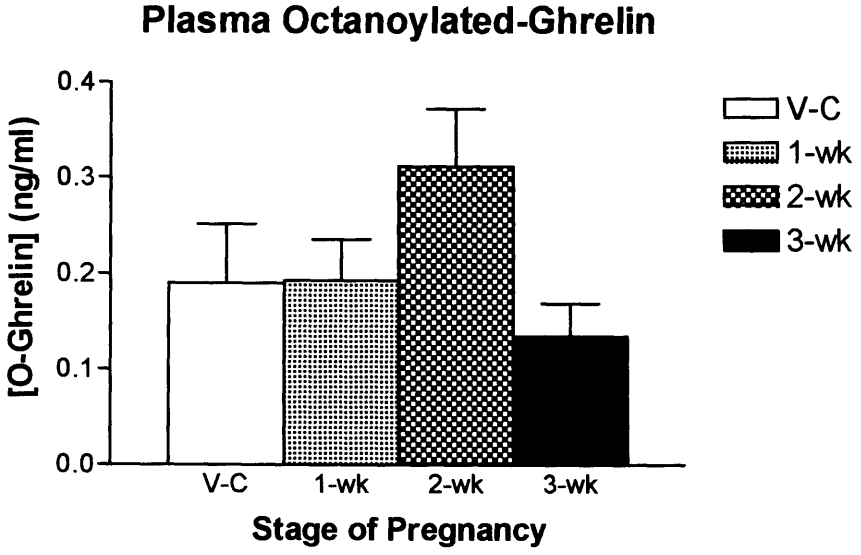
2.3.4.3.3 Placental ghrelin content

In an attempt to determine the ghrelin protein levels in placenta, placental homogenates were subjected to RIA for both total- and octanoylated-ghrelin. Since the serial dilution was not parallel [most of the RIA values (for 1:1, 1:2, 1:4, 1:8 dilutions) were off-curve/high except for the 1:16 dilution], the 1:16 dilution was only included in the results. Placental total-ghrelin was significantly higher in 3-wk than 2-wk placentae (Figure 2.18A), with a similar pattern seen in octanoylated-ghrelin (Figure 2.18B). However, these data need to be treated with caution, since the absolute levels of octanoylated-ghrelin appear to exceed that of total-ghrelin.

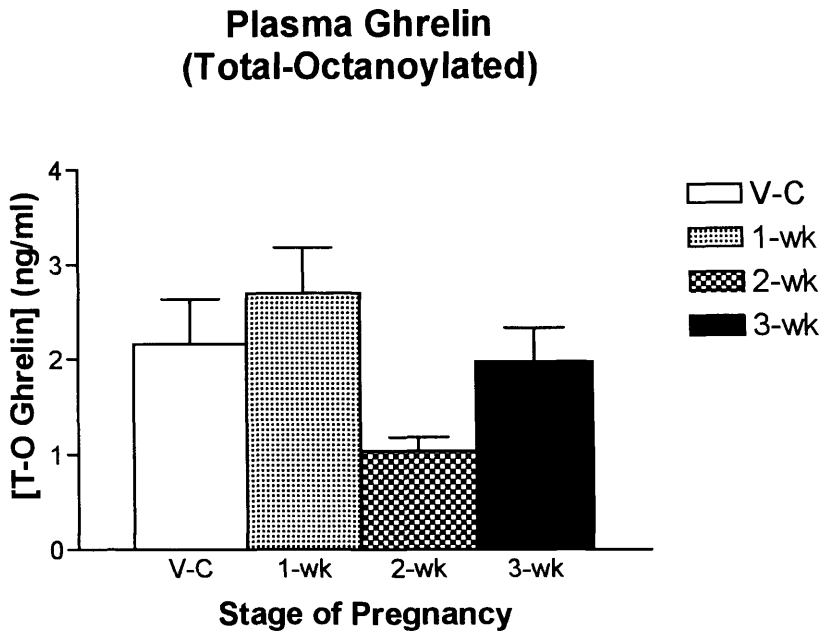
A


Plasma Total-Ghrelin
[T-Ghrelin] (ng/ml)
4
3
2
1
0
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk

B


Plasma Octanoylated-Ghrelin
[O-Ghrelin] (ng/ml)
0.4
0.3
0.2
0.1
0.0
V-C
1-wk
2-wk
3-wk
Stage of Pregnancy
V-C
1-wk
2-wk
3-wk

C



D

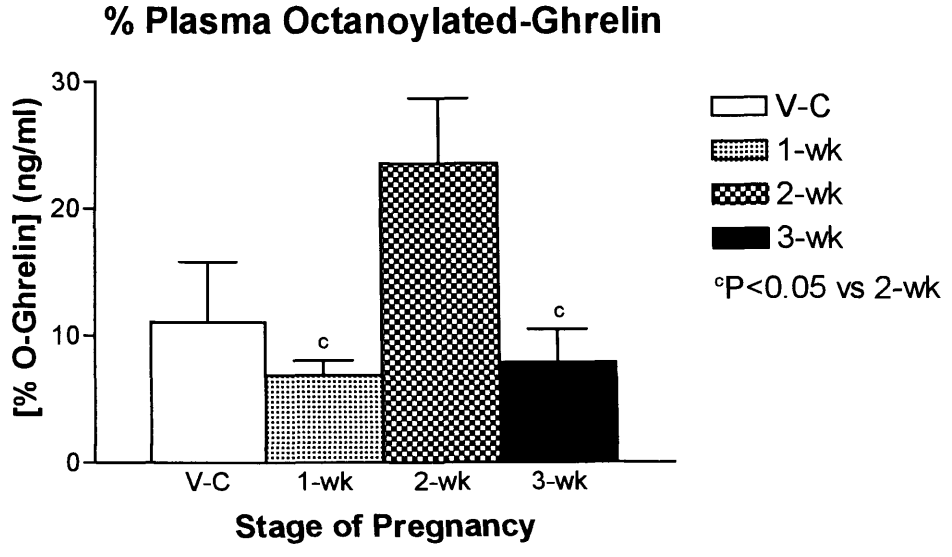
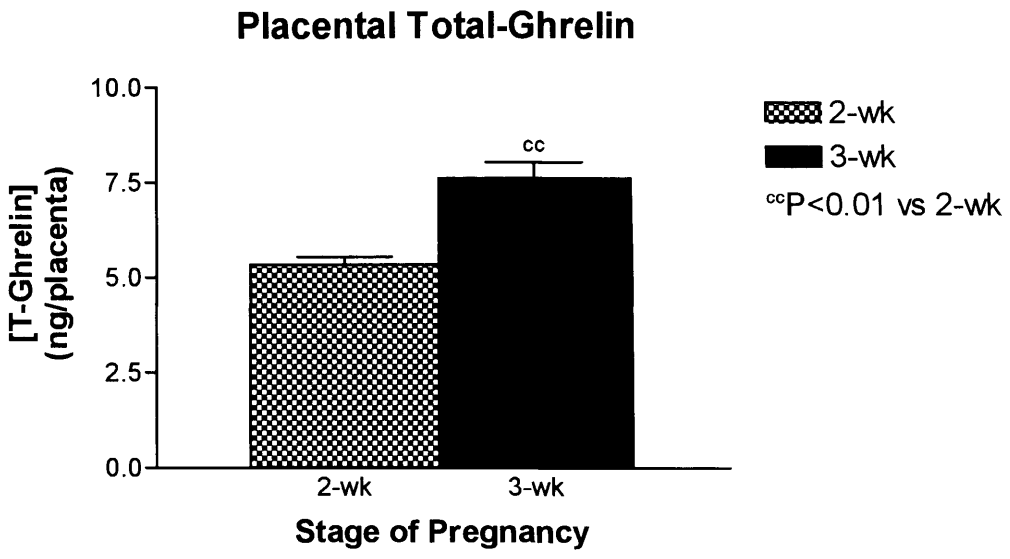


Figure 2.17: Plasma ghrelin concentrations during pregnancy in normal rats. A. Total ghrelin; B. Octanoylated-ghrelin; C. (Total-Octanoylated) ghrelin; D. Percent of octanoylated-ghrelin. Values shown are mean ± SEM (n=3 for 2-wk pregnant group and n=6 for all other groups).

A



B

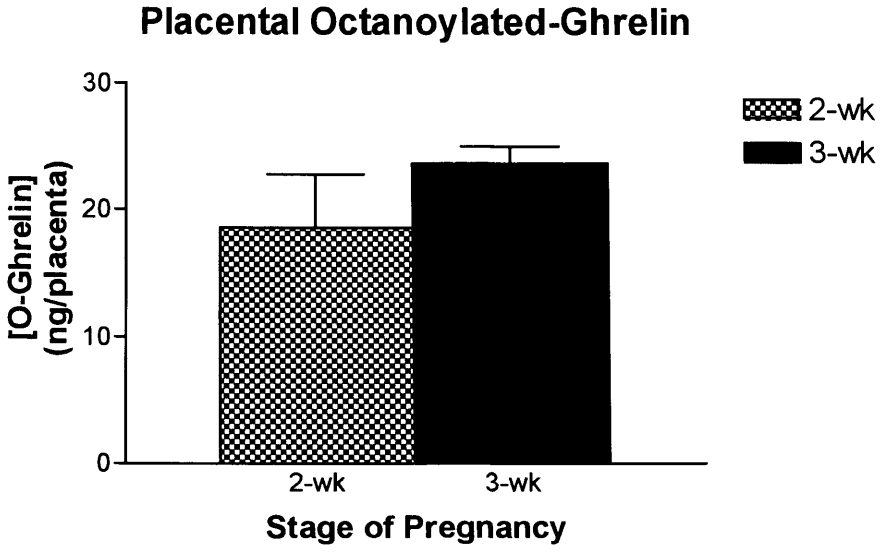


Figure 2.18: Placental total- (A) and octanoylated-ghrelin (B) concentrations in 2- and 3-wk pregnant normal rats. Values shown are mean ± SEM (n=3 for 2-wk pregnant group and n=6 for 3-wk pregnant group).

2.4 DISCUSSION

The progression of pregnancy is associated with profound changes in maternal endocrinology and metabolism. To ensure an adequate flow of substrates to the developing fetus and to prepare for lactation, maternal adaptation to pregnancy involves changes in body composition, food intake, energy consumption, and metabolism (Owens, 1991). These processes are influenced by the integrated function of the pituitary and placenta and their interacting hormones. This chapter reports changes in cellular plasticity in the pituitary during pregnancy and seeks to relate these to the activity of the hypothalamo-pituitary-GH and -PRL axes and the consequent impact on maternal metabolism.

2.4.1 Changes in GH Profile Parameters

In humans, pituitary GH output is higher in women than in men. This sexual dimorphism (Gevers et al., 1998) is manifested in differences in the pattern of pulses, characterized by intermittent high peaks with low or undetectable troughs in men and by more frequent, irregular episodes, with higher baseline secretion in women (Pincus et al., 1996). After the first trimester (12wk) of pregnancy, the 'normal' GH episodic peak activity is dramatically changed into a continuous, stable basal secretion (Eriksson et al., 1988a, 1989). During late pregnancy, the baseline GH secretion is increased and pulse amplitude decreased (Eriksson, 1989). In rats, a marked sex difference in GH secretion is also well recognized. In males, the plasma GH pattern consists of large GH pulses occurring at regular intervals of 3-3.3hr, separated by periods with low or undetectable GH levels (Tannenbaum and Martin, 1976). In females, the GH pulses are lower, more frequent and less regular than males. In addition, the basal GH secretion is higher than in males (Eden, 1979; Jansson et al., 1984). During late pregnancy, it has been reported that an increase in basal plasma GH levels (Terry et al., 1977; Carlsson et al., 1990) is accompanied with an increase in GH pulse amplitude with no change in pulse frequency (Carlsson et al., 1990). Our data confirm that, in female rats, the pulsatile pattern of GH secretion was maintained in pregnancy (Figure 2.5A) and as observed in the complete set of GH profiles (Figure 2.5B). In females, GH was secreted in frequent and irregular episodes as reported earlier (Eden, 1979; Clark et al., 1987). During pregnancy, the increase in

circulating GH levels (Figure 2.6A) is in agreement with other studies (Carlsson et al., 1990; Jahn et al., 1993). This rise in plasma GH levels is most likely to be due to an elevation in baseline secretion (Figure 2.7A). As shown previously (Carlsson et al., 1990), this progressive elevation in baseline GH secretion occurred without modification of GH pulses (Figures 2.7B & C). A transient elevation in pulse amplitude at day 20 (equivalent to day 19 in our study) has been reported (Carlsson et al., 1990). Moreover, further enhancement in the plasma GH levels was observed in pregnant rats entering parturition (the 3-wk pregnant set of GH profiles (Figure 2.5B; profiles 2D & 5D) confirming previous findings (Carlsson et al., 1990).

The mechanisms controlling GH secretion during pregnancy of the rat remain unknown. The possible factors that can influence the increase in basal GH secretion are numerous, acting at different levels of the hypothalamo-pituitary axis.

At the pituitary level, an increase in GH secretion usually accompanies an augmentation in the number of GH-secreting cells, the ***somatotrophs*** (Frohman et al., 2000). Although our data showed that there is no change in the proportion of these cells (Figure 2.4D), the well-established doubling of pituitary size in pregnancy, implies that an increase in the total somatotroph population may be significant. The ***somatomammotrophs***, common progenitors for both somatotrophs and lactotrophs, do not appear to contribute to the observed elevation in circulating GH since we observed a sustained decline in the proportion of these cells throughout pregnancy (Figure 2.4E).

In addition to a potential contribution of an increase in total somatotroph number, it is possible that the sensitivity of somatotrophs could also be increased during pregnancy. However, no alteration in the sensitivity of the hypothalamo-pituitary-GH axis to ghrelin was observed during pregnancy (Figure 2.10A). This could be further tested by quantifying somatotroph responses to GRF using the tannic acid/EM approach (see chapter 3, section 3.2.1 – Study 1). Quantification of granular fusion in vehicle-treated animals would also confirm the involvement of somatotrophs in the augmentation of baseline GH secretion seen in pregnancy.

At the hypothalamic level, two regulatory peptides, the excitatory peptide, GRF, and the inhibitory peptide, SRIF, act in concert to generate the pulsatile pattern of GH secretion (Tannenbaum and Ling, 1984). It is possible, therefore, that an increase in GH could be the result of either an increase in GRF secretion, or a decrease in SRIF

release. In addition to stimulation of GH secretion, ***GRF*** acts to stimulate both GH synthesis and somatotroph proliferation (Mayo et al., 2000). Therefore any increase in GRF output should be reflected in an increase in the number of somatotrophs and pituitary GH mRNA and protein content. Although the somatotroph population may be elevated, pituitary GH content [at either the mRNA (Figures 2.14A & B) or protein level (Figure 2.11A)] was not increased. Indeed, there was a trend towards decreasing GH expression and increasing GH protein storage in the pituitary. GRF is also known to be the primary determinant of GH pulse amplitude (Wehrenberg, 1986) in pregnant (Carlsson et al., 1990) as well as non-pregnant female rats (Clark and Robinson, 1985). Our algorithmic (Figure 2.6C) and distribution (Figure 2.7C) analysis confirmed previous reports that pulse amplitude is unaffected during pregnancy (Carlsson et al., 1990). Taken together, these results represent strong indirect evidence that GRF secretion is not elevated during pregnancy, corroborating more direct evidence that hypothalamic GRF mRNA expression is unchanged (Mizobuchi et al., 1991). Analysis of GRF secretion *in vivo* is difficult, but could be tested by application of 'SRIF rebounds' in pregnant rats. Using this method, the amplitude of GH release occurring in response to SRIF withdrawal is dependent on the level of endogenous GRF secretion (Clark et al., 1988; Tannenbaum et al., 1989). It should be noted that the placenta also produces significant quantities of GRF (Nogues et al., 1997; Gaylinn, 1999). But, the role of placental GRF (Baird et al., 1985) during gestation is still unknown.

On the other hand, a decrease in hypothalamic ***SRIF*** could give rise to the observed increase in the basal GH secretion, over which it is thought to be the primary neuroendocrine determinant. In humans and rats, SRIF is the major inhibitor of GH secretion (Patel and Srikant, 1986). Unlike GRF, it does not inhibit GH biosynthesis and has no effect on somatotroph proliferation (Giustina and Veldhuis, 1998). During pregnancy, it has been shown that immunoreactive (IR)-SRIF content increases progressively without any modification in SRIF mRNA expression (Escalada et al., 1997), suggesting increased SRIF storage or decreased secretion. Thus, it appears that SRIF does not cause the increase in the basal GH secretion.

The increase in GH secretion during pregnancy could also be the result of an increase in circulating ***ghrelin***. The gut-brain hormone, ghrelin, augments GH secretion (Kojima et al., 1999) by activating GHS secretagogue receptors (GHS-Rs) (Pong et al., 1996; Howard et al., 1996) primarily in the arcuate nucleus and also on the

somatotrophs. The central actions of octanoylated-ghrelin are mediated by the receptor subtype GHS-R_{1a} (Kojima et al., 1999). However, the activation of the hypothalamo-pituitary GH axis by ghrelin is dependent on the pattern of treatment (Wells and Houston, 2001; Thompson et al., 2003). Intermittent ghrelin infusion, which may mimic ghrelin release from hypothalamic ghrelinergic neurones (Kojima et al., 1999; Lu et al., 2002; Cowley et al., 2003), enhances GH secretion and accelerates skeletal growth (Thompson et al., 2003). In contrast, continuous ghrelin infusion, which is more likely to mimic gastric secretion in response to fasting (Tschöp et al., 2002) suppresses spontaneous GH secretion (Thompson et al., 2003). Despite the reported elevation in placental ghrelin mRNA expression during pregnancy (Gualillo et al., 2001), our data (Figure 2.17) suggest that this does not have a significant effect on ghrelin in the maternal circulation. Indeed, any continuous elevation in maternal circulating ghrelin is more likely to suppress GH secretion, rather than enhance baseline levels. This is further corroborated by our observation that the sensitivity of the hypothalamo-pituitary-GH axis to ghrelin is unaltered during pregnancy (Figure 2.10A), since it has previously been reported that continuous exposure to the synthetic ghrelin mimetic, GHRP-6, causes profound desensitization both *in vivo* (Wells and Houston, 2001) and *in vitro* (Blake and Smith, 1991).

Besides GRF and SRIF, GH itself and its tissue mediator IGF-I are regulated by a complex interplay of ***feedback*** signals (Giustina and Veldhuis, 1998). The sensitivity to GH feedback is dependent upon GH status since GH regulates its own receptor expression (Bennett et al., 1995). During pregnancy, the increase in circulating GH could result from down-regulation of hypothalamic ***GH-R*** expression. The effect of GH feedback is seen primarily in the suppression of GRF neurones (However, as already discussed, our data do not support any resultant elevation in GRF secretion.) (Frohman et al., 2000). In rats, oestrogen increases liver GH-R and GHBP expression (Carmignac et al., 1993). It has been reported that GHBP mRNA is up-regulated during pregnancy resulting in an increase in serum GHBP levels (Tiong and Herington, 1991). Since GHBP and GH-R are regulated in parallel in rats (Leung et al., 1987), this implies that GH-R expression is up-regulated during pregnancy. However, this should result in decreased GH secretion, which is clearly not the case in our study.

As previously reported (Sheppard and Bala, 1987; Gargosky et al., 1993; Travers et al., 1993), a sharp decline in circulating ***IGF-I*** levels occurred during pregnancy

(Figure 2.15C). This dramatic decrease in circulating IGF-I is remarkable, given the observed increase in detectable circulating GH. A prolonged reduction in IGF-I feedback could potentially cause the observed increase in circulating GH. This is most likely to be mediated by regulation of, or sensitivity to GRF and SRIF. It has been shown that an IGF-I administration decreases GRF and GRF-R mRNA expression (Aleppo et al., 1997), whereas it increases SRIF and SSTRs mRNA expression (Sato and Frohman, 1993). The actions of GRF and SRIF are mediated by their respective receptors GRF-R (Seifert et al., 1985) and SSTRs in the pituitary and in particular on the somatotroph cell membrane (Frohman et al., 2000). An increase in the expression of GRF and its receptor with a decrease in those of SRIF and its receptor could have occurred during pregnancy supporting the elevation seen in circulating GH secretion. In situ hybridisation will be carried out on brains taken in study 4 to assess the expression of hypothalamic peptides to delineate this issue.

It has previously been shown that, in humans, the late gestational increase in plasma GH is due to secretion of a placental GH variant, hGH-V, and this seems to suppress normal pituitary hGH gene expression and the amplitude of spontaneous episodes of hGH secretion in the pituitary (Eriksson et al., 1988a,b; 1989). Several lines of evidence suggest that this is not the case in rats. Firstly, there does not appear to be a counterpart to the hGH-V gene in rats (Chien and Thompson, 1980; Barta et al., 1981). Secondly, the amplitude of the spontaneous GH episodes is not suppressed (Figure 2.6C; Carlsson et al., 1990). Thirdly, and most convincingly, both basal and GRF analogue-stimulated plasma GH levels are undetectable in pregnant hypophysectomised (Hx) rats (Carlsson et al., 1990). But if the increase in circulating GH is of pituitary origin, why does plasma IGF-I concentration decline (Figure 2.15C), and why are there no increases in the parameters of skeletal growth (Figures 2.15A & B)?

The answer to this problem may lie in the specificity of the ***rGH antibody*** (details, NHPP, USA) used in the current study. According to the manufacturers' specifications, this has 0.16% cross-reactivity with rat PRL. But cross-reactivity with rPL, or one of other structurally placental somatolactogenic hormones has not been defined. If this cross-reactivity is significant, the detected elevation in baseline secretion may represent the addition of these placental hormones to the underlying rGH profile. However, Carlsson et al., (1990) appear to have used the same NHPP/NIDDK rGH RIA reagents. While the current explanation accounts for their

description of elevated baseline secretion, it does not explain why GH was undetectable in pregnant Hx rats. Thus, this question remains unresolved. ***Somatolactin*** (SL), the recently discovered member of the GH/PRL family identified in several teleost species, is not found in other classes of vertebrates (i.e. human or rodent) (Kaneko, 1996; Forsyth and Wallis, 2002; Fukamachi et al., 2004), and is therefore unlikely to contribute to the observed phenomena.

An analysis of the metabolic consequences of increased circulating GH/rPL is discussed below (section 2.4.3).

2.4.2 Changes in the PRL Axis

In humans, PRL is released in a pulsatile manner (Ben-Jonathan, 1985), but the episodes of pulsatility are abolished from early pregnancy (Scaglia et al., 1981). In pregnant and non-pregnant rats, PRL is released episodically. As shown previously (Blake et al., 1983), during the morning hours of early pregnancy (d7) (Figure 2.8B; profiles B), the time of onset of the rise was similar to that of the serum GH, but the increase in PRL levels was of a shorter duration than that of GH. From previously published findings (Jahn et al., 1993), as pregnancy advanced, maternal PRL was expected to increase gradually and peak on d21, in parallel with the rise in serum rGH concentrations. However, we didn't observe a consistent change in 3-wk pregnant rats. The dramatic rise in PRL levels seen in a couple of rats in labour is in accord with the elevation of such levels 4-6h before delivery (Terry et al., 1977). The time courses of the pregnancy-induced elevation GH and PRL secretion, therefore, appear independent.

As in humans, a remarkable lactotroph proliferation in the pituitary gland of pregnant rats occurs to meet the increased demands for PRL (Orgnero et al., 1993). This proliferation is seen in the current study as a progressive increase in the population of type I lactotrophs (Figure 2.4A). The type I lactotrophs are considered to be the more mature, inactive lactotroph subtype, and the end product of lactotroph differentiation (Figure 2.19). A number of potential mechanisms may give rise to this increase in type I lactotrophs, as illustrated in figure 2.19.

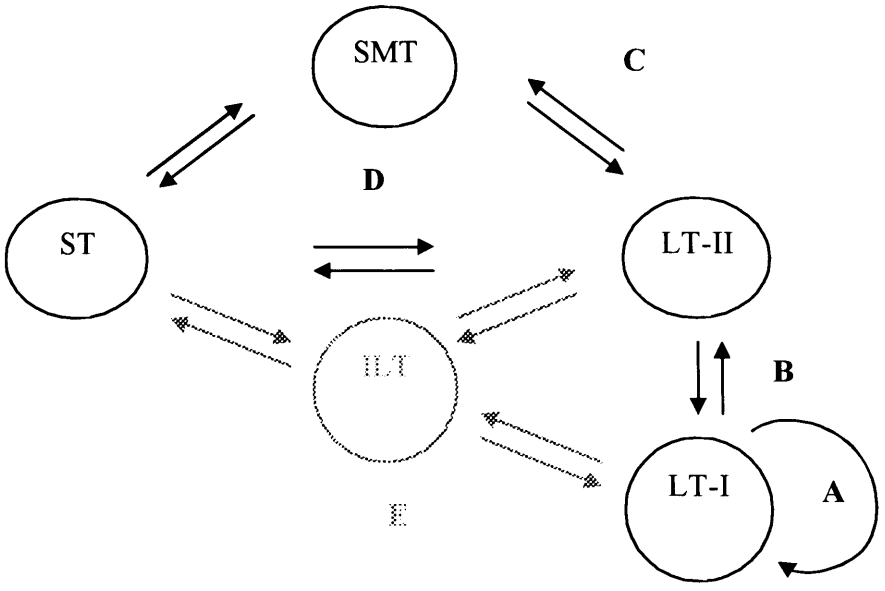


Figure 2.19: Proposed model for differentiation pathways of the somatotroph-lactotroph lineages during normal pregnancy, indicating a number of potential mechanisms causing an increase in type I lactotrophs.
The mechanisms may be proliferation of type I lactotroph (*Position A*), differentiation of type II lactotrophs into the type I lactotrophs (*Position B*), differentiation from somatomammotrophs to type I lactotrophs via type II lactotrophs (*Position C*), or transdifferentiation of somatotrophs into type I lactotrophs (*Position D*).
Any of these interconversions could include a potential intermediate stage, the 'intermediate' lactotroph (*Position E*).
Somatotroph (ST); somatomammotroph (SMT); type I lactotroph (LT-I); type II lactotroph (LT-II); 'intermediate' lactotroph (ILT).

Firstly, the most simple mechanism would be the proliferation of ***type I lactotrophs*** (Figure 2.19; position A). It is one of the proposed mechanisms in the development of pituitary hyperplasia (Takahashi et al., 1984). The elevated oestrogen level during pregnancy could have induced lactotroph proliferation. Previous studies have shown that lactotroph proliferation occurs in mature rats treated chronically with estradiol (Takahashi et al., 1984), and in pregnancy and lactation, due to high circulating oestrogen (Porter et al., 1991). Our pseudopregnancy experiment (Figures 2.12A-C) supports the involvement of placental hormones (probably oestrogen) in the regulation of pituitary cell types (i.e. lactotroph subpopulations). Double immunostaining of 5-bromo-2-deoxyuridine (BrdU; the use of BrdU incorporation to label proliferating cells) and PRL is needed to resolve the proliferation issue (Taniguchi et al., 2001a,b).

Secondly, the type I lactotrophs may arise from the ***type II lactotrophs*** (Figure 2.19; position B). However, the lack of change in the proportion of type II lactotrophs (Figure 2.4B) does not support the differentiation of this active type II into the mature type I lactotrophs, unless there is a corresponding differentiation of somatomammotrophs to type II lactotrophs. The type II lactotrophs are more responsive to secretagogue treatment than type I lactotrophs. In female *dw/dw* rats, type II lactotrophs demonstrate robust responsiveness to secretagogues (ghrelin and TRH), the most potent of which is TRH (Thompson et al., 2002). The investigation of the secretagogue response of the type II lactotrophs in pregnant rats is needed to determine the contribution of type II lactotrophs in the increase in the population of type I lactotroph during pregnancy.

Thirdly, the type I lactotrophs may arise from ***somatomammotrophs*** (Figure 2.19; position C). During pregnancy and lactation, variations in both single and dual hormone-secreting cells seem to contribute to the overall fluctuations in the relative abundance of somatotrophs and lactotrophs (Porter et al., 1990). As a common progenitor, the somatomammotrophs are thought to give rise to both somatotrophs and lactotrophs (Nikitovitch-Winer et al., 1987). The sharp decline in the proportion of somatomammotrophs (Figure 2.4E) could favour either a direct differentiation from somatomammotrophs to type I lactotrophs or an indirect differentiation from somatomammotrophs to type I lactotrophs via type II lactotrophs. A direct

differentiation into a mature cell is unlikely. The second option is possible if the transformation from somatomammotrophs to type II lactotrophs is matched by the maturation of type II lactotrophs to type I lactotrophs. This would lead to a stable population of type II lactotrophs, as seen.

Fourthly, transdifferentiation of ***somatotrophs*** into type I lactotrophs could have occurred (Figure 2.19; position D). Although the transdifferentiation of somatotrophs into lactotrophs have been reported to occur during pituitary hyperplasia in pregnancy and lactation (Vidal et al., 2001), our data did not show any decline in the proportion of somatotrophs (Figure 2.4D).

Fifthly, any of these interconversions could include a potential intermediate stage, the ***'intermediate' lactotroph*** (Figure 2.19; position E). However, the population of cells matching the 'intermediate' description accounted for less than 0.2% of the total secretory cell population, a proportion which remained unaltered during pregnancy (Figure 2.4C). More investigation is needed to determine if the 'intermediate' lactotroph seen during the pregnancy of normal rats corresponds to the same cell discovered in *dw/dw* rats. But it is unlikely that this very small population is able to account for a 10% increase in type I lactotrophs.

In summary, the most likely mechanism appears to be the differentiation of somatomammotrophs into type I lactotrophs via the type II lactotrophs (Figure 2.19; position C).

A number of placental hormones could have regulated this process. The modifications observed in the pituitaries of the pregnant rats are not seen in those of pseudopregnant rats. This supports a likely role for the placenta in the regulation of the pituitary function during pregnancy, by increasing type I lactotrophs to enhance pituitary PRL storage. PRL is known to stimulate milk production (Flint et al., 2003), and may contribute towards the build-up of fat reserves (Flint et al., 2003) in preparation for lactation.

2.4.3 Metabolic Consequences of Elevated Circulating GH

2.4.3.1 Pregnancy and growth

In contrast to humans, epiphyseal closure (i.e. fusion of the two centers of ossification on either side of the growth plate) does not occur in rats, and they continue to grow throughout their lifetime (Kacsoh, 2000). GH and IGF-I act in concert to promote body growth. GH induces the differentiation of progenitor cells in bone, whereas IGF-I stimulates their subsequent clonal expansion (Green et al., 1985). The skeletal growth parameters seem not to reflect GH pattern-dependent effects in our pregnancy study. The increase in GH levels did not affect the tibial length (Figure 2.15A), and a reduction in EPW, the marker of the rate of skeletal growth, was observed by late pregnancy (Figure 2.15B). It has been reported that many factors (i.e. genetic, nutritional, and hormonal) exert regulatory function on longitudinal bone growth (Nilsson et al., 1994). In particular, oestrogen could have played an important role during pregnancy. The decline in the circulating IGF-I levels observed in rats by the 2nd week of pregnancy (Figure 2.15C) is in agreement with the work of many groups (Davenport et al., 1990; Gargosky et al., 1990; Donovan et al., 1991; Escalada et al., 1997). Although the serum IGF-I levels have an inverse association with the circulating rGH levels throughout pregnancy (Terry et al., 1977; Carlsson et al., 1990; Kishi et al., 1991), maternal IGF-I seems not to be regulated by circulating GH, instead by the maternal nutritional status (e.g. changes in nitrogen balance) (Nagako et al., 1999). In pregnant rats, the reduction in circulating IGF-I might be attributable either to the suppression of hepatic IGF-I production (Davenport et al., 1990) or to the proteolysis of IGFBP3 in maternal serum (Davenport et al., 1992). Further investigation is needed here. During rat pregnancy, GH resistance may occur. As hepatic GH binding does not vary (Travers et al., 1993), and hepatic GH receptor and binding protein gene expression are not suppressed by maternal malnutrition (Woodall et al., 1998), a possible alteration in post-receptor mechanisms may contribute to GH resistance in pregnant rats (Travers et al., 1993). The high circulating oestrogen levels during pregnancy (Grow, 2002) could be one of the factors implicated in such state of GH resistance. Oestrogens are known to suppress circulating IGF-I and enhance GH secretion in humans, and to inhibit hepatic IGF-I mRNA generation in rats. In addition, rat placental lactogens could also be implicated in GH resistance state (Escalada et al., 1997). There is a possibility that during pregnancy, more IGF-I is extracted from the circulation and sequestered in the

pituitary and hypothalamus (Escalada et al., 1997). During late pregnancy in rats, the decline observed in circulating IGF-I levels may be essential for inhibiting maternal anabolic metabolism and redistributing maternal nutrients to support fetal growth.

Pregnancy is a physiological situation which shows complexity of neuroendocrine regulation – oestrogens and binding proteins (i.e. changes in GHBPs during pregnancy may contribute to the contrasting changes in apparent effects of GH and measured total plasma GH) – might play a role and make it difficult to propose a single regulatory pathway.

It is of note to mention that the rats used in the pregnancy study were going into their first pregnancy. It has been reported that the first pregnancy is not energy or metabolically efficient, partially due to the younger skeleton and absence of prior maternal experience (Kunkele and Kenagy, 1997).

2.4.3.2 Pregnancy and adiposity

Although the visceral adiposity (Figures 2.16B&C) was unaltered during pregnancy, the mean circulating leptin levels were almost doubled (Figure 2.16D). It is of note to mention that we only assessed the visceral fat pad. The excess in leptin could have originated from other adipose reserves (e.g. subcutaneous and inguinal fat pads). In adult rats, ***PRL*** stimulates leptin secretion (Gualillo et al., 1999) by a mechanism that may be dependent on insulin (Ling and Billig, 2001). It is not known whether similar effects are observed during pregnancy. Since the PRL secretion was not altered during pregnancy (Figures 2.8A) and since PRL administration doesn't increase maternal plasma leptin concentrations (Budge et al., 2002), PRL seems not to affect leptin levels. It has been reported that the rise in plasma insulin in pregnancy contributes to insulin resistance (Butte, 2000) and increases plasma leptin (Reitman et al., 2001).

Pituitary ***GH*** is known to be lipolytic. Although the structural similarity between pituitary GH, hGH-V, and hPL, a marked accumulation of maternal adipose reserves occurs by the 2nd half of the pregnancy and could be due to differential properties of GH, hGH-V, and hPL at the hGH receptor (Juarez-Aguilar et al., 1999). However, it has been reported that, under certain circumstances, the newly discovered gastric hormone, ***ghrelin***, promotes PRL secretion and adipogenesis (Thompson et al., 2004). In pregnancy, it is not known whether the adipogenic action of ghrelin contributes to the pregnancy-related accumulation in adipose reserves. In humans and rats, ghrelin is produced by the placenta during the second half of pregnancy (Gualillo et al., 2001).

By late pregnancy, the placental total-ghrelin (Figure 2.18A) increased with no alteration in the octanoylated-ghrelin (Figure 2.18B). Recent study has reported the involvement of the major circulating form of ghrelin, Des-octanoyl ghrelin, in promoting adipogenesis (Thompson et al., 2004). However, the proportion of the non-acylated ghrelin (Figure 2.17C) in this study was not significantly changed. Further investigation is required to delineate the contribution of ghrelin to the pregnancy-induced increase in adiposity.

2.4.4 Methodological Approaches

2.4.4.1 Automated serial blood sampling (ABS) and its importance

The use of the ABS system in this series of experiments has advantages as well as disadvantages.

The disadvantages of using ABS system are:

(1) The blood loss (i.e. red blood cells, RBCs) that may affect the circulating hormonal levels.

(2) Similarly, the saline replacement could have diluted the blood and thus the hormonal concentration. If this is significant, we would have expected declining peak height. However, the peak heights across the GH profiles (Figures 2.5A&B) were consistent [V-C (n=5), 15.6±3.7ng/ml (1^{st} sample) vs. 15.2±4.4ng/ml (last sample); $p>0.05$ – Paired t-test]. This suggests that the physiological adjustment of the body (i.e. the interference of ADH and an increase in water diuresis) is appropriate.

(3) The rat head is tethered (screws to skull) throughout the experiment. This would have put the rat in stressful situation which may influence the circulating hormonal levels. GH pulsatility should be suppressed. However, GH pulsatility across the profiles (Figures 2.5A&B) appeared to be well maintained.

(4) With such long-term sampling, samples accumulate and stand for several hours before the assay. However, hormones used in this project (GH, PRL) are stable for 1-2 days at room temperature. In case of labile substances, it is possible to cool the fraction collector tray or coat the collecting tubes with peptidase inhibitors to slow down degradation.

However, the advantages of using this system far outweigh its drawbacks:

(1) This system allows automatic collection of small samples of freshly circulating blood from conscious animals.

(2) Sampling including pattern, time, volume, frequency (intervals), etc. can be programmed according to the type of experiment/experimental requirements.

(3) This method is an automatic blood collection of minute samples diluted in saline. It allows the withdrawal of many consecutive samples without an excessive depletion of blood volume.

(4) All the rest of the sample as well as the leading edge which is mixed with saline are returned to the animal.

(5) The lines are regularly flushed with saline.

(6) The collection method avoids major blood loss so no blood transfusion is required to maintain rat blood volume.

(7) It allows frequent blood sampling over several hours to determine the secretory profile of a hormone.

(8) While blood sampling, there is a possibility to inject or infuse into the animals using double-bore cannulae.

(9) This method can be used for long-term patterned i.v. infusions in conscious rats.

(10) This system allows a uniform sampling and simultaneous blood sample collection from many rodents (rats, mice; max. n=12).

(11) Although not relevant to my study, this system can be applied to anesthetized animals as well.

(12) Using this sampling regime, the animal is undisturbed (and unstressed) compared to less frequent large samples taken manually; the access to the cannula is from outside the cage and its connection to the sampling system takes place hours before triggering the sampling procedure.

(13) This system avoids using extra volume of heparin as in manual sampling to maintain cannula patency.

(14) This system reduces the potential infection rate that manual sampling could face when returning the RBCs to the rat.

(15) The maintenance of GH pulsatility, including large differences in GH concentration in serial samples reflect the fidelity of the sampling system and lack of systematic serial contamination.

It is of note to mention that plasma concentration of a hormone is not only an indication of its secretion, but also of its removal (i.e. metabolism in liver) and its clearance (i.e. elimination by the kidney).

2.4.4.2 RIAs and confidence in results

2.4.4.2.1 GH/PRL/Ghrelin/Leptin RIAs

Inter-assay variability: represents the potential differences in equilibrium state arising from the incubation conditions in separate assays. In GH/PRL/Ghrelin/Leptin RIAs, all samples in each study were assayed together to avoid the inter-assay variability. However, large assays (for GH and PRL) increase the potential error caused by intra-assay variability. In order to diminish the potential 'drift' caused by the extension of the incubation time (between the first and last batches of samples centrifuged), samples were separated in the same order in which the reagents were added, and each batch was overlapped (PEG added and incubated, whilst previous batch centrifuged). This halves the potential for an effect of incubation time (see Table 2.2).

Table 2.2: The effect of overlapping centrifuge batches on total assay incubation time

	No overlap (distant runs: 40min)	% of 24hrs	Overlap (distant runs: 20min)	% of 24hrs
Std Curve +3 Blocks	24h (Incubation Time)	0.00%	24h (Incubation Time)	0.00%
4 Blocks	24:40	1.67	24:20	0.83
4 Blocks	25:20	3.34	24:40	1.67
4 Blocks	26:00	5.00	25:00	2.50
4 Blocks	26:40	6.67	25:20	3.34
4 Blocks	27:20	8.33	25:40	4.17
4 Blocks	28:00	10.0	26:00	5.00

Block: 96-well format/microtitre plates (serving as test-tube rack for assay)

2.4.4.2.2 PRL RIA and PEGylation

During pregnancy, the low circulating PRL levels observed in our study have been reported in other studies (Ochiai et al., 1983; Clark et al., 1987). The PRL RIA protocol used in this study is widely used. However, it is known that pregnancy is associated with hyperprolactinemia, and the presence of PRL multimers beside the monomers. The presence of such multimers may give rise to variability observed in our PRL data. A new approach to overcome this problem has recently been reported (Gibney et al., 2004; Smith et al., 2004). Pre-treatment of the samples with polyethylene glycol (PEG) is thought to precipitate bioinactive PRL IgG complexes (e.g. macroPRL) from plasma prior to immunoassay. This could be employed in future experiments to improve consistency.

2.4.4.2.3 Ghrelin RIA and placental protein content

The measurement of the placental ghrelin content was our first attempt and was not entirely successful. Previously published methods for quantifying ghrelin content in the stomach have been elaborate, including boiling the sample prior to homogenization (to inactivate intrinsic proteases), extraction with Sep-Pak cartridges, and purification with reverse phase HPLC (Shibata et al., 2004). It is difficult to compare our minimalistic homogenization and RIA approach with the published method, since we did not include stomach samples. However, it appears that we have been more successful in quantifying octanoylated- than total-ghrelin, the levels of the acylated form being three times greater than the total. Since the GHRA-88HK anti-octanoyl-ghrelin antibody binds to the N-terminal domain of the peptide, and the GHRT-89HK anti-ghrelin (total) antibody binds to the C-terminal portion, the lower absolute amount of total ghrelin measured implies significant C-terminal degradation in our method. Thus, the inactivation of proteases appears important, though this may be more reliably achieved with protease inhibition than boiling for several minutes.

Since all but the 1:16 dilutions fell outside the working range of the standard curve, further dilutions should be used when repeating this assay procedure. If such dilutions are not parallel with the standard curve, this implies significant interference in the process of antibody binding, suggesting that peptide extraction is required.

2.4.4.3 EM and the robustness of pituitary cell data

Pituitary cell number vs. cell proportion: The extensive analysis of pituitary cell populations in this study represents a small fraction of the pituitary tissue. To extend this quantification to the entire adenohypophysis, to generate absolute cell numbers, is impractical. Although determination of cell proportion may not always represent a true reflection of cell population, particularly in circumstances where pituitary size is altered, this is the standard method for the expression of this data.

Zone investigation: It is of note to mention that no apparent zonation of somatotrophs or lactotrophs was found either by light or electron microscopy although it might be expected for different lactotroph subtypes (Dr. Helen Christian, Personal communication). No differences were observed after trying to separate the different zones of the pituitary and to measure PRL responses to different secretagogues (Dr. Helen Christian, Personal communication).

Number of animals investigated: In most biological studies, a minimum n value of 5-6 is considered necessary for statistical comparison. However, in morphological studies employing EM such as those we performed on pituitary samples, the number of sections required for analysis makes this impractical. Thus, in these types of studies an n value of 2-3 is routinely used in publication (Taylor et al., 1997; Christian et al., 2000; Chapman et al., 2002; Lucocq et al., 2004; Mayhew and Desoye, 2004; Mayhew et al., 2004).

2.5 CONCLUDING REMARKS

In summary, pregnancy is associated with progressive increase in detectable baseline GH levels, a decrease in EPW with unaltered tibial length, and a decline in circulating IGF-I levels. This may reflect the involvement of a non-pituitary GH – probably of placental origin. In addition, a progressive rise in type I lactotroph population is likely to be due to differentiation of somatomammotrophs into the lactotroph lineage. These changes contribute to the maternal preparation for lactation.

It has been reported that GH plays an important role in metabolic changes taking place in late pregnancy (Leturque et al., 1984). The unaltered visceral adiposity accompanied by hyperleptinaemia would favour the possibility of the presence of placental GH-like activity which has a metabolic role during pregnancy. Nevertheless, the high levels of circulating oestrogens may also play a crucial role in these maternal metabolic adaptations (i.e. growth and adiposity). In addition, the changing proportion of octanoylated and des-octanoylated ghrelin may have a significant impact on the regulation of adipogenesis.

CHAPTER 3.
ONTOLOGICAL AND FUNCTIONAL REGULATION OF THE 'INTERMEDIATE' LACTOTROPHS IN *dw/dw* RATS

3.1 INTRODUCTION

The 'intermediate' lactotroph is a unique, morphologically distinct cell discovered recently in the pituitary of the dwarf (*dw/dw*) rat (Thompson et al., 2002). This newly identified lactotroph is distinguishable from type I and type II lactotrophs on the basis of the unusual granular size (>300nm; i.e. between those of type I and II lactotrophs). These electron dense secretory granules are numerous and regularly shaped. This novel lactotroph appears morphologically similar to the somatotroph, but expresses and secretes prolactin. These cells show a unique profile of responses to hypothalamic neuropeptides. These 'intermediate' lactotrophs are unresponsive to TRH, which activates type II lactotrophs, and unresponsive to GRF, which stimulates somatotrophs (Thompson et al., 2002). However, in females the 'intermediate' lactotrophs secrete PRL in response to the gastric neuropeptide, ghrelin (Thompson et al., 2002), which may account for the unique stimulation of PRL secretion induced by the synthetic GHSs in female *dw/dw* rats (Carmignac et al., 1998). This female-specific induction of PRL secretion is oestrogen-dependent (Carmignac et al., 1998). Thus, in relation to neuroendocrine regulation, the 'intermediate' lactotroph shows features of both the lactotrophic and somatotrophic lineages. ***In order to investigate whether the 'intermediate' lactotroph changes in parallel with the lactotrophic lineage, we quantified the populations of these cells during pregnancy in dw/dw rats. This study (Study 1) was also extended to determine whether the sensitivity of the secretory cell populations to ghrelin was modified during pregnancy.***

Among the rodent models of dwarfism, the *dw/dw* rat pituitary is unusual, in that a reduction in somatotrophs is accompanied by an increase in lactotroph numbers (Tierney and Robinson, 2002). It has been suggested that the 'intermediate' lactotroph could be a transitional stage in the progression from type II to type I lactotrophs or a

transitional stage in the transdifferentiation between type II lactotrophs and somatotrophs (Thompson, 2002). Postnatal expansion of the somatotroph population is thought to be largely regulated by GRF (Mayo et al., 2000). In the *dw/dw* model, the lack of GH feedback leads to a large over-expression of GRF (Carmignac et al., 1996), and it is possible that, although GRF does not elicit cAMP (Downs and Frohman, 1991) and GH expression (Charlton et al., 1988) in *dw/dw* somatotrophs, elevated GRF may provoke transdifferentiation of somatotrophs into 'intermediate' lactotrophs. We have taken two approaches to investigate whether elevated GRF gives rise to the 'intermediate' lactotroph in the *dw/dw* pituitary. Firstly, ***we have quantified the lactotroph and somatotroph populations in the little (lit/lit) mouse, a model in which elevated hypothalamic GRF (Lin et al., 1993) accompanies an inactivating point mutation in the GRF receptor (GRF-R; Godfrey et al., 1993) (Study 2).*** Secondly, we have investigated the effect of reducing the influence of GRF in the *dw/dw* model by neonatal monosodium glutamate (MSG) treatment, a procedure that causes a 70-90% destruction of the neuronal cell bodies within the hypothalamic arcuate nucleus (Bloch et al., 1984) including most of the GRF-producing neurones, thereby providing a model of GRF-deficiency (Kovacs et al., 1995). ***Pituitaries from vehicle- and MSG-treated dw/dw rats were subjected to quantification of the secretory cell populations by EM (Study 3).*** In studies 2 and 3 we used ***male*** rodents as it had previously been shown that the 'intermediate' lactotrophs were not only present in male *dw/dw* rats, but were possibly more abundant (Thompson et al., 2002). Since, in normal rats, MSG treatment causes a profound reduction in circulating GH (Maiter et al., 1991), ***we also investigated the effect of removing residual GH secretion in dw/dw rats on skeletal growth and adiposity (Study 3).***

This chapter tests the following hypotheses:

1. The population of 'intermediate' lactotrophs is regulated in parallel with the lactotrophic lineage.
2. The population of 'intermediate' lactotrophs is, like the somatotrophs, regulated by GRF.

This has been achieved in the following experiments:

1. The analysis of pituitary cell types and the endocrine responses to ghrelin treatment in 2-week pregnant *dw/dw* rats.
2. The analysis of pituitary cell types in male *lit/lit* mice.
3. The analysis of pituitary cell types, and growth and adiposity parameters in MSG-treated male *dw/dw* rats.

3.2 METHODS

3.2.1 Study 1 – Effect of Pregnancy on 'Intermediate' Lactotroph in *dw/dw* Rats

12-15-week old virgin female dwarf (*dw/dw*) rats from our colony (School of Biosciences, Cardiff University) were mated overnight with male *dw/dw* rats, successful mating being determined by the presence of a vaginal plug. The plugged females were separated from the males and housed in different cages until their use. Virgin *dw/dw* females from each litter were kept as controls.

2-week pregnant and age-matched virgin female *dw/dw* rats were housed in metabolic cages 3-4 days prior to the insertion of single bore jugular vein cannula under halothane anaesthesia. The rats were permitted a minimum of 48hr recovery, during which body weight and food intake were monitored daily and cannula patency was maintained with an intermittent infusion of sterile heparinised saline (10U/ml; 20µl bolus every hour). On the day of sampling, 200µl blood samples were collected before, and at 5, 15 and 30mins after a bolus i.v. injection of either vehicle [100µl sterile saline containing BSA (0.2mg/ml), and heparin (10U/ml)] or rat ghrelin (Tocris Cookson Ltd., Avonmouth, UK; 10µg/300µl). The collected blood samples were centrifuged at 2060xg (Eppendorf 4810R) for 10min at 4^0C. Plasma was subsampled, aliquotted for GH (25µl) and PRL (50µl) assays, and stored at -20^0C for subsequent determination of plasma GH and PRL concentrations by radioimmunoassay (RIA).

After 3hrs, rats were subjected to a second blood sampling session in conjunction with an alternative bolus i.v. treatment. After further 3hrs, rats received a third i.v. injection of either vehicle or ghrelin, and immediately anaesthetized with halothane prior to transcardial perfusion of tannic acid, with heparinised saline at a rate of 5ml/min for 3min at 37°C, followed by 0.2% tannic acid (BDH Chemicals Ltd., Dorset, UK) in PBS for 5min at 37°C, and then 2.5% glutaraldehyde in PBS for 5min at 4°C. After decapitation, the pituitary was carefully removed, postfixed for 60-90 min in 2.5% glutaraldehyde at room temperature, and stored in 0.25% glutaraldehyde at 4^0C until being processed for EM analysis as described in chapter 2.

3.2.2 Study 2 – Analysis of Pituitary Cell Types in Male *lit/lit* Mice

Pituitaries from 8-week old male (C57BL/6J) wildtype and *lit/lit* [C57BL/6J-Ghrhr; (homozygous)] mice were purchased from The Jackson Laboratory (Maine, USA). Whole pituitaries were fixed on collection and processed for EM analysis as described in chapter 2.

3.3.3 Study 3 – Analysis of Pituitary Cell Types, and Growth and Adiposity Parameters in MSG-Treated *dw/dw* Male Rats

MSG Treatment: Newborn *dw/dw* male rat pups were injected intraperitoneally (i.p.) with 4mg/gBW/50µl monosodium-L-glutamate (MSG, Sigma), five times, on alternate days for the first ten days of life. The treatment started the following day after birth [i.e. the day of birth was termed postnatal day 0 (P0)]. Littermate controls received an i.p. injection of 50µl saline. Following treatment, care was taken to check for continued maternal behaviour. The pups were inspected daily. Any animal showing signs of poor health other than the expected consequences of endocrine deficit (e.g. dwarfism, obesity, hypogonadism) induced by the treatment, were removed from the study and killed. The treated pups were regularly monitored until their use.

On the day of sampling, the 8-week MSG-treated and age-matched vehicle-treated male *dw/dw* rats were stunned and decapitated. The whole pituitaries of vehicle-treated and MSG-treated male *dw/dw* rats were fixed and processed for EM analysis as described in chapter 2.

The following tissues were also collected from the 8-week MSG-treated and age-matched vehicle-treated male *dw/dw* rats:

- Left tibiae were dissected for subsequent determination of EPW.
- Trunk blood was collected in heparinised tubes on ice and centrifuged at 2060xg for 10min at 4^0C. Separated plasma subsamples were stored at -20^0C for subsequent determination of plasma concentrations of leptin by RIA (Chapter 2, section 2.2.5.1.6).
- Left visceral (retroperitoneal and perirenal) fat pads were dissected and weighed.

3.2.4 Tissue Analyses

3.2.4.1 Delayed addition radioimmunoassay (D/A RIA)

** (D/A) rGH RIA – 25µl subsampled plasma from manual sampling*

(IAV 5.45%; sensitivity 0.12-250.0ng/ml)

**(D/A) rPRL RIA – 50µl subsampled plasma from manual sampling*

(IAV 8.01%; sensitivity 0.0480-100.0ng/ml)

(D/A) RIA for rGH and rPRL was conducted as described in chapter 2, section 2.2.5.1.3.

3.2.4.2 Electron microscopy (EM)

Tannic acid and granular counting: Pituitary cell types were identified as in study 1 (Chapter 2, sections 2.2.1 and 2.2.5.3). The secretory response was determined by counting the number of exocytosed granules fused with the plasma membrane of each cell type (i.e. the exocytosis in 6 cells of each subtype per animal was counted).

Quantification of sections: is as described in chapter 2, sections 2.2.1 and 2.2.5.3, except for the following refinement: Two grids (9 squares in total) per animal were analysed for *lit/lit* mice pituitaries.

3.2.4.3 Light microscopy (LM) – Tibial bone analysis

The tibiae were fixed and stained with Masson's Trichrome as described in chapter 2, section 2.2.5.4. Epiphyseal plate width (EPW) was measured on Masson's Trichrome sections under light microscopy with an ocular graticule.

3.2.5 Statistical Analysis

Results are expressed as the mean value ± SEM. Differences between groups were compared by Student's *t* test. When more than two groups were compared, differences were determined by one-way analysis of variance (ANOVA) followed by multiple comparisons using either the Dunnett's (vs. control) or Bonferroni's (for selected pairs) multiple comparison post hoc test. p-values<0.05 were considered significant.

3.3 RESULTS

3.3.1 Study 1 – Effect of Pregnancy on 'Intermediate' Lactotroph in *dw/dw* Rats

3.3.1.1 Pituitary cell types

Lactotroph and somatotroph populations were quantified in pituitaries of virgin (V-C) and 2-wk pregnant *dw/dw* rats, using electron microscopy in conjunction with immunogold labelling. During pregnancy, the proportion of intermediate lactotrophs (IL) increased [Figure 3.1; 2.1±0.2% (V-C; n=5) vs. 3.8±0.5% (2-wk PG; n=4); $p<0.01$]. However, the proportions of somatotrophs, and type I and type II lactotrophs were unaltered at the 2nd week of pregnancy in *dw/dw* rats.

3.3.1.2 GH and PRL secretion

The GH and PRL responses to ghrelin treatment following an i.v. injection of either vehicle or rat ghrelin (10μg) in 2-wk pregnant *dw/dw* rats is shown in Figure 3.2. A small, but robust stimulation of GH secretion was seen in virgin females, which remained unaltered during pregnancy (Figure 3.2A). PRL secretion was not stimulated by ghrelin during pregnancy compared to the response seen in virgin-controls (Figure 3.2B).

3.3.1.3 Neuroendocrine responses of individual secretory cells

The endocrine responses of individual secretory cell types in pituitaries of virgin (V-C) and 2-wk pregnant *dw/dw* rats were examined by electron microscopy (Figure 3.3). A bolus injection of ghrelin failed to elicit GH secretion from somatotrophs in virgin-control and pregnant rats. Lactotroph subtypes showed no significant responsiveness to ghrelin treatment. However, a significant increase in secretory response of type II lactotrophs to vehicle treatment was observed during pregnancy.

Representative electron micrographs of lactotroph subtypes from non-pregnant and pregnant female *dw/dw* rats are shown in figures (3.4-3.6).

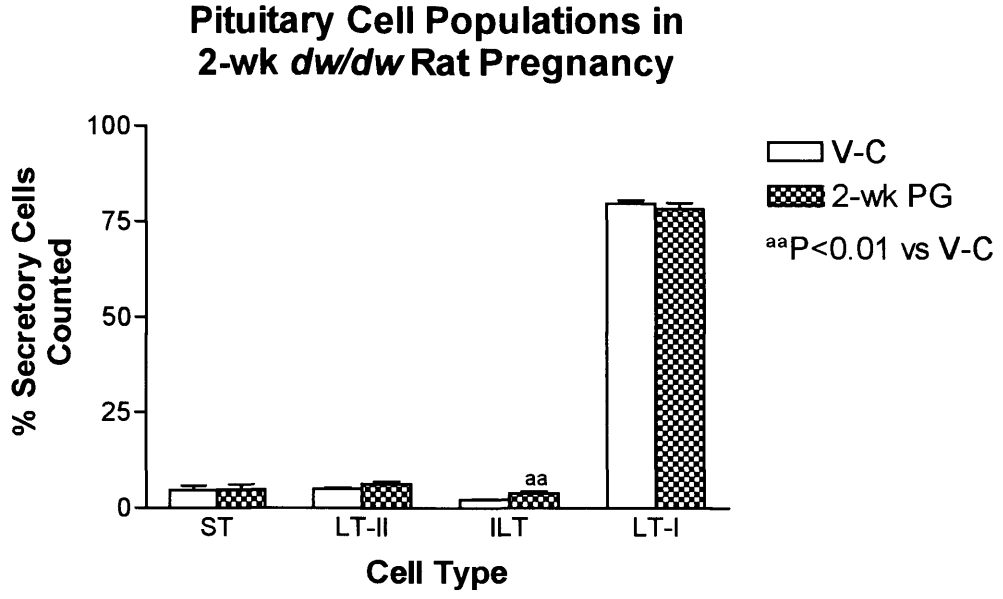
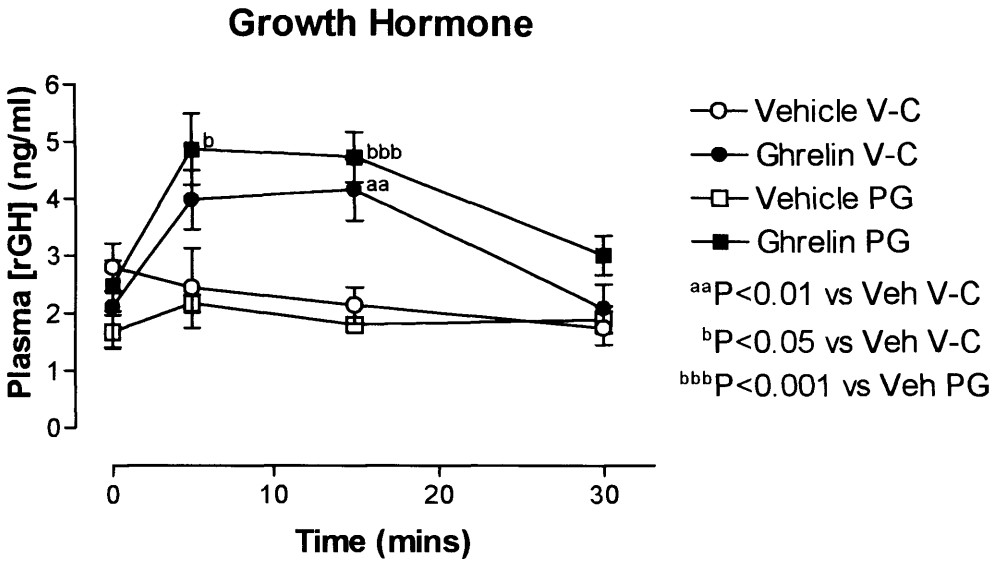


Figure 3.1: The proportion of lactotroph and somatotroph populations in the total cell populations in pituitaries of virgin-control (n=5) and 2-wk pregnant (n=4) *dw/dw* rats. Values shown are mean ± SEM. Virgin-Control (V-C); pregnant (PG); Somatotroph (ST); type I lactotroph (LT-I); type II lactotroph (LT-II); 'intermediate' lactotroph (ILT).

A



B

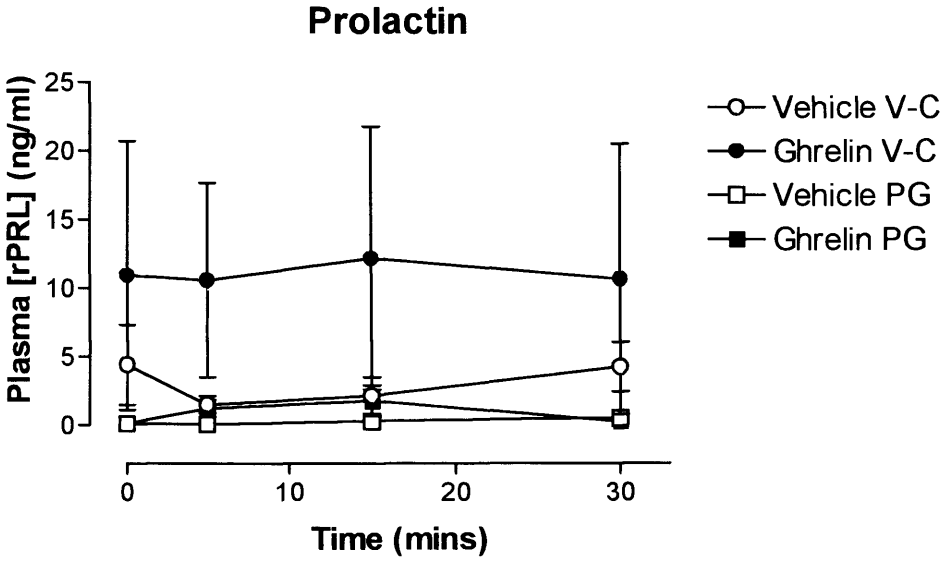


Figure 3.2: The effect of a bolus i.v. injection of vehicle or rat ghrelin (10μg) on GH (A) and PRL (B) secretions in virgin and 2-wk pregnant *dw/dw* rats. Values shown are mean ±SEM (n=5 for each group and each treatment).

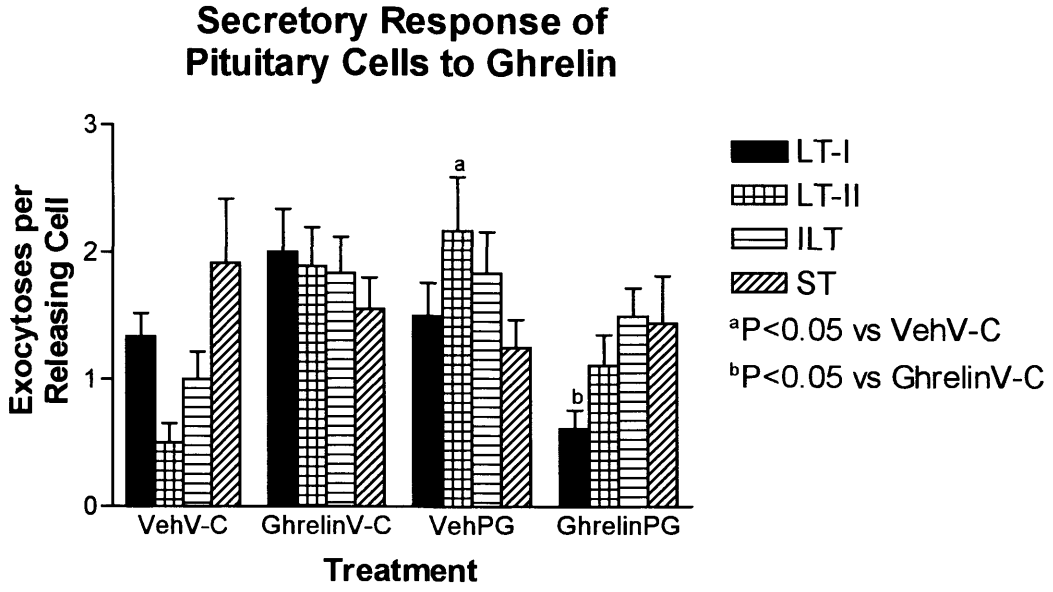


Figure 3.3: Secretory response of lactotroph and somatotroph populations in *dw/dw* rat pituitaries to vehicle (n=2) and ghrelin (n=3) treatments. Values shown are mean ± SEM. Virgin-Control (V-C); pregnant (PG); vehicle (Veh); somatotroph (ST); type I lactotroph (LT-I); type II lactotroph (LT-II); 'intermediate' lactotroph (ILT).

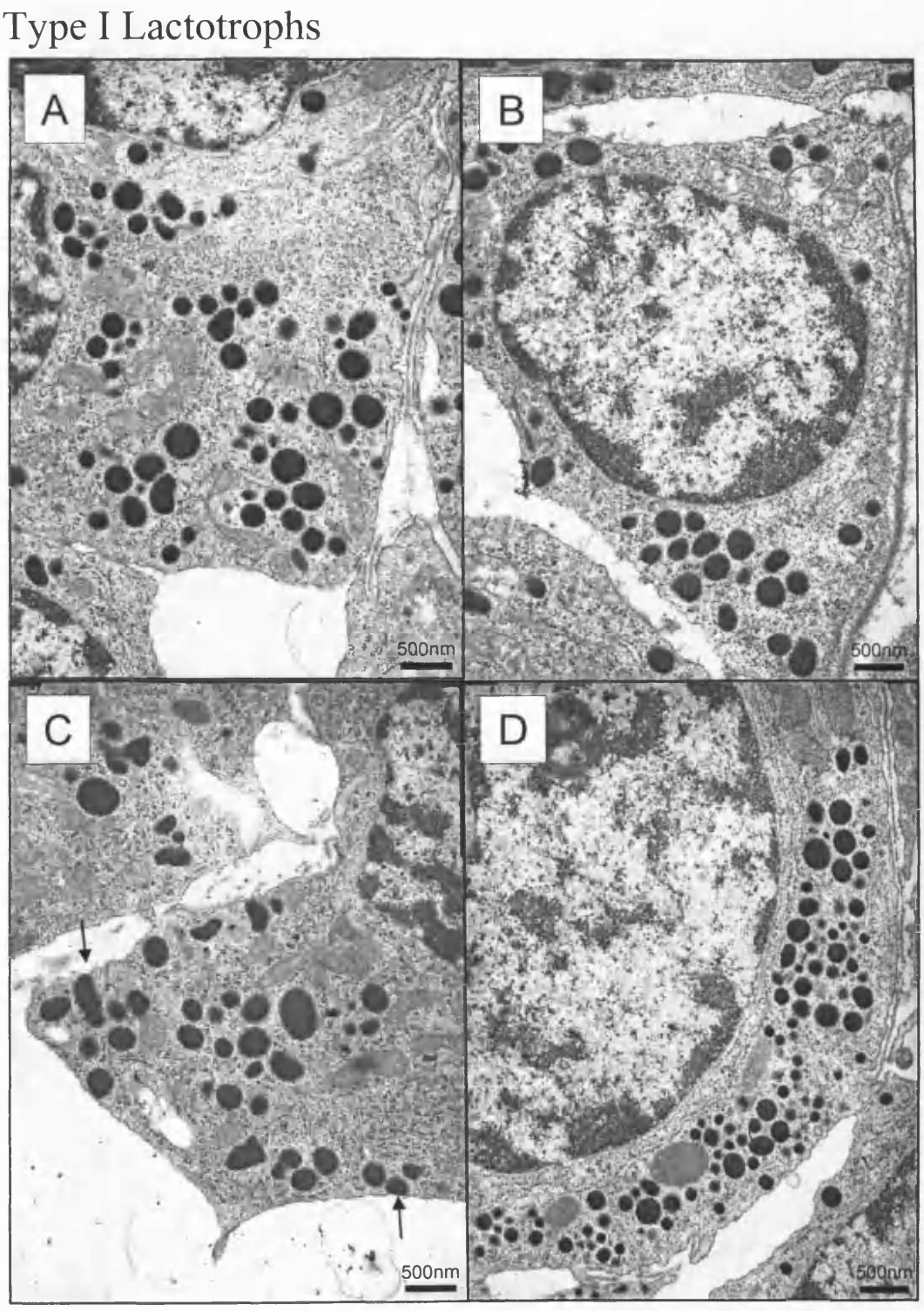


Figure 3.4: Representative electron micrographs of Type I lactotrophs (PRL 5nm gold) from non-pregnant (A,B) and pregnant (C,D) female *dw/dw* rats following a bolus i.v. injection of either vehicle (A,C) or rat ghrelin (B,D). Images shown are 12,000X; black arrows indicate exocytosed secretory granules.

Type II Lactotrophs

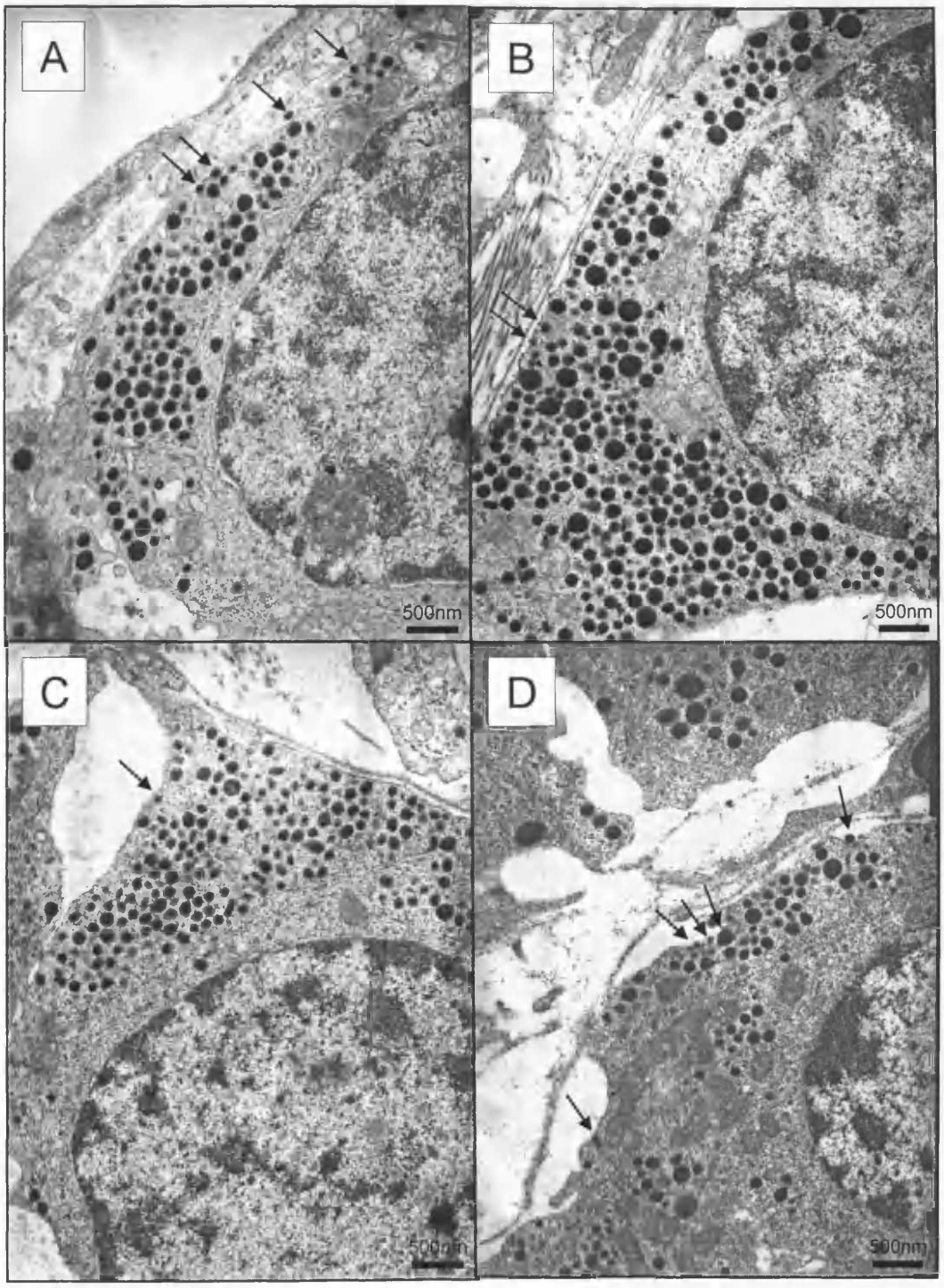


Figure 3.5: Representative electron micrographs of Type II lactotrophs (PRL 5nm gold) from non-pregnant (A,B) and pregnant (C,D) female *dw/dw* rats following a bolus i.v. injection of either vehicle (A,C) or rat ghrelin (B,D). Images shown are 12,000X; black arrows indicate exocytosed secretory granules.

'Intermediate' Lactotrophs

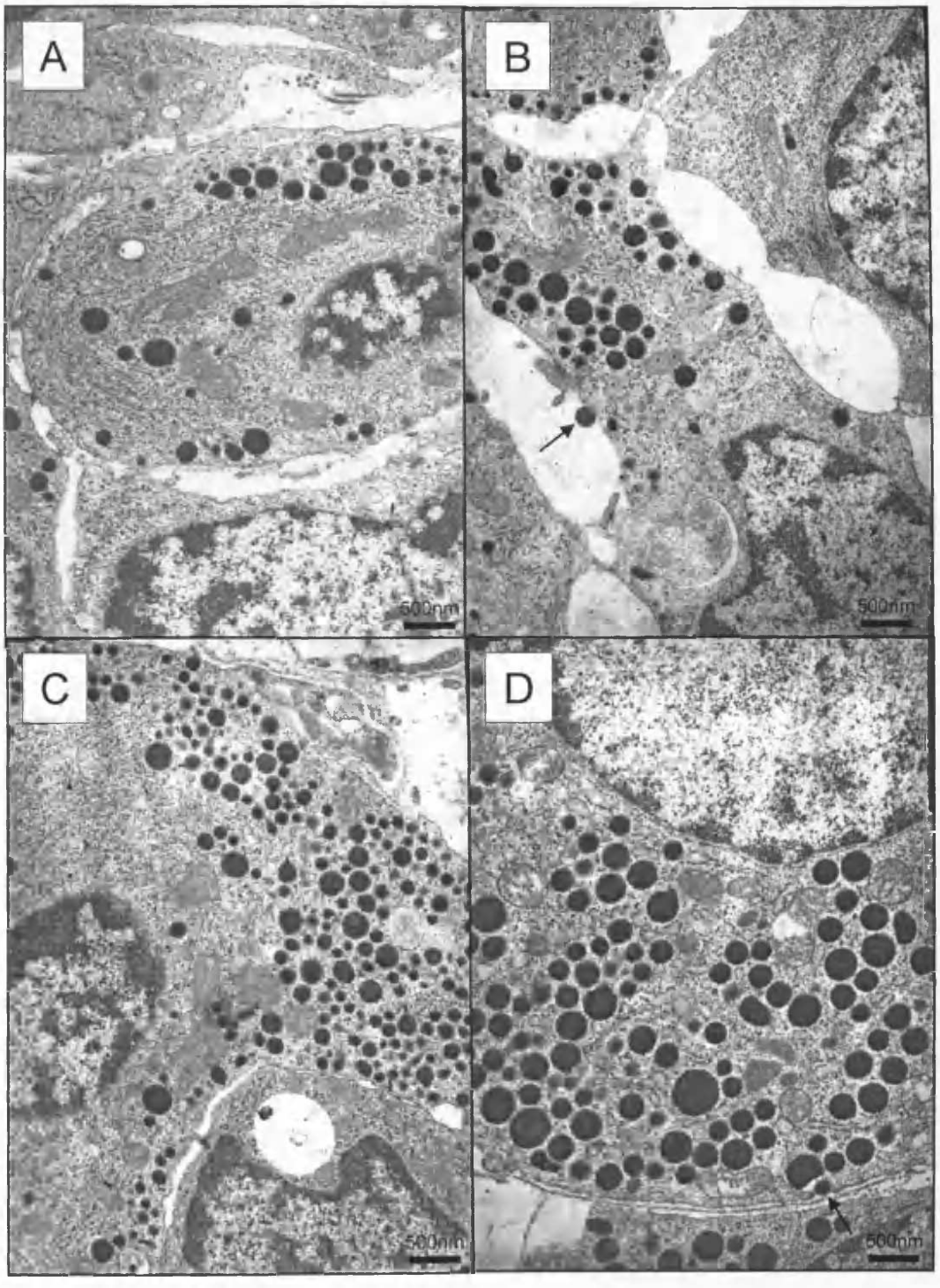


Figure 3.6: Representative electron micrographs of 'Intermediate' lactotrophs (PRL 5nm gold) from non-pregnant (A,B) and pregnant (C,D) female *dw/dw* rats following a bolus i.v. injection of either vehicle (A,C) or rat ghrelin (B,D). Images shown are 12,000X; black arrows indicate exocytosed secretory granules.

3.3.2 Study 2 – Analysis of Pituitary Cell Types in Male *lit/lit* Mice

Lactotroph and somatotroph populations were quantified in pituitaries of male wildtype (WT) and *lit/lit* mice, using electron microscopy in conjunction with immunogold labelling (Figures 3.7-3.11). An increase in the proportions of type I (LT-I) [(Figures 3.7; 3.11); 17.3±1.5% (WT; n=3) vs. 20.9±0.5% (*lit/lit*; n=3); $p<0.05$], and type II (LT-II) [(Figures 3.7; 3.10); 3.5±0.5% (WT; n=3) vs. 6.0±0.2% (*lit/lit*; n=3); $p<0.01$] lactotrophs was observed in *lit/lit* mice compared to normals. In contrast, the proportions of somatotrophs (ST) [(Figures 3.7; 3.8); 30.7±1.3% (WT; n=3) vs. 10.0±2.6% (*lit/lit*; n=3); $p<0.01$], and somatomammotrophs (SMT) [(Figures 3.7; 3.9); 22.4±1.2% (WT; n=3) vs. 8.3±2.4% (*lit/lit*; n=3); $p<0.01$] decreased dramatically. No lactotrophs corresponding to the 'intermediate' morphology were observed in either the wildtype or the *lit/lit* mouse pituitaries. It should be noted that the use of anti-rat primary antibodies in this study may have resulted in the higher than expected non-specific background staining seen in EM micrographs (Figures 3.8-3.11).

3.3.3 Study 3 – Analysis of Pituitary Cell Types, and Growth and Adiposity Parameters in MSG-Treated *dw/dw* Male Rats

3.3.3.1 Pituitary cell types in MSG-treated male dw/dw rats

Lactotroph and somatotroph populations were quantified in pituitaries of vehicle-treated (V-*dw*) and MSG-treated (MSG-*dw*) male *dw/dw* rats, using electron microscopy in conjunction with immunogold labelling (Figures 3.12-3.15). The proportions of type I (LT-I) [(Figures 3.12; 3.14A-B); 41.0±2.2% (V-*dw*; n=3) vs. 30.4±1.4% (MSG-*dw*; n=3); $p<0.01$], and type II (LT-II) lactotrophs [(Figures 3.12; 3.14C-D); 12.6±0.7% (V-*dw*; n=3) vs. 6.9±0.1% (MSG-*dw*; n=3); $p<0.001$] were reduced in MSG-treated *dw/dw* rats. Although the proportion of 'intermediate' lactotrophs in MSG-treated rats was 50% of that in vehicle-treated rats, this was not statistically significant ($P>0.05$) (Figures 3.12; 3.15). The proportion of somatotrophs was unaltered by MSG treatment (Figures 3.12; 3.13).

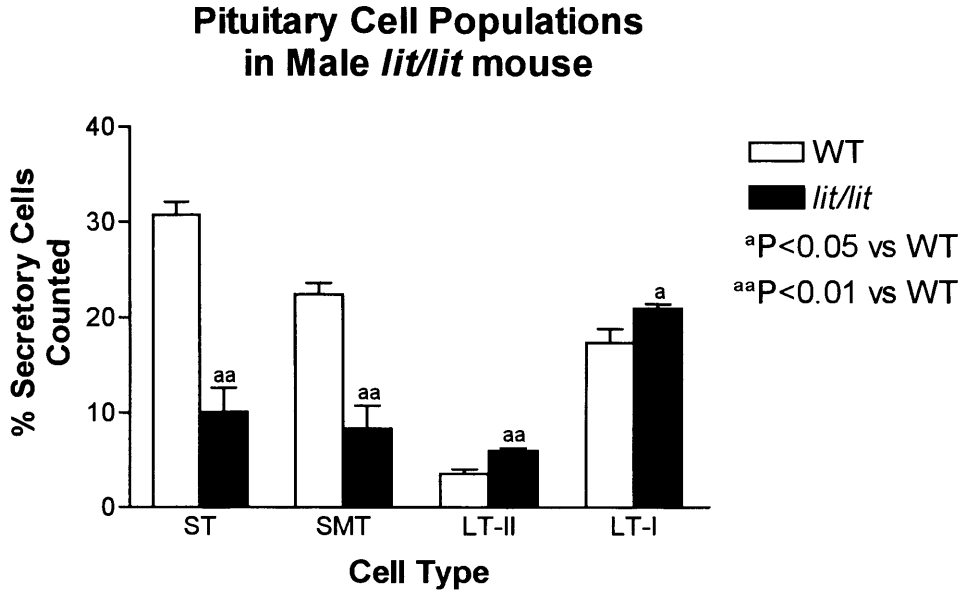
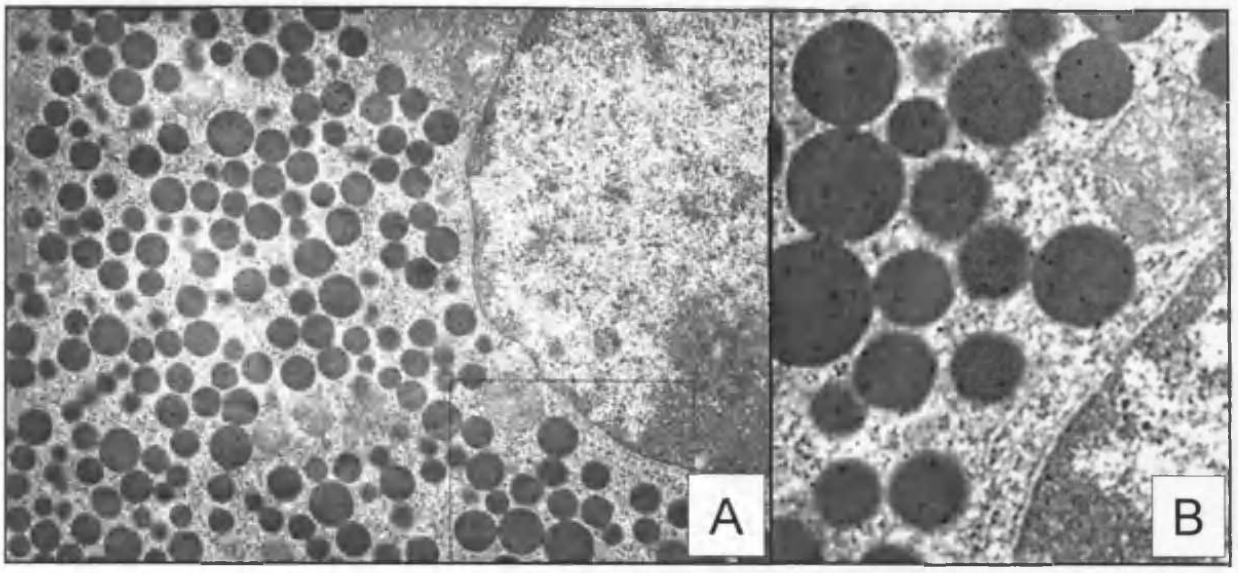


Figure 3.7: The proportion of somatotroph (ST), somatomammotrophs (SMT), and types I (LT-I) and II (LT-II) lactotroph populations in pituitaries of wildtype (WT) and *lit/lit* male mice. Values shown are mean ± SEM (n=3 for each group).

Wild-type (C57BL/6J +/+) mice



Lit/lit (C57BL/6J-Ghrh-r -/-) mice

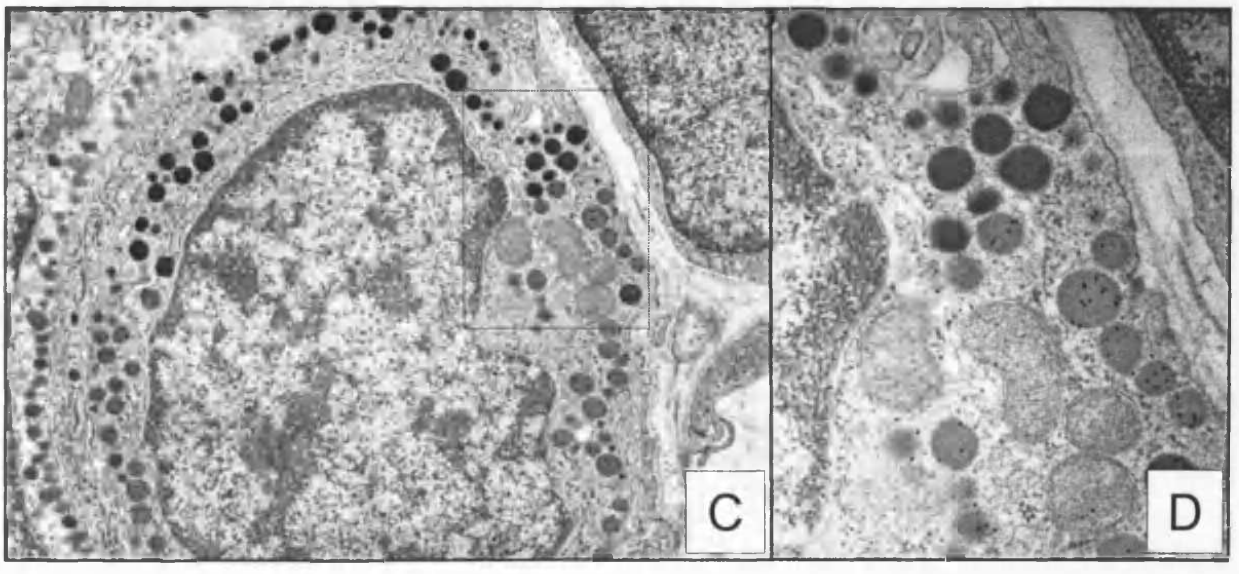
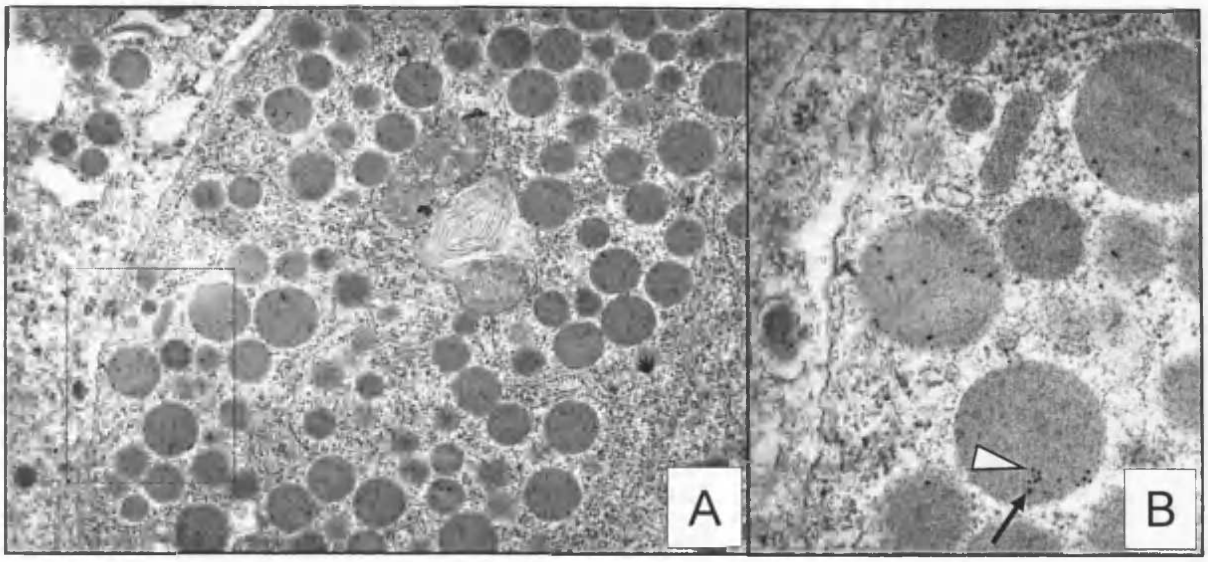


Figure 3.8: Electron micrographs of somatotrophs (GH 15nm gold) from male wild-type (C57BL/6J +/+; A-B) and lit/lit (C57BL/6J-Ghrh-r -/-; C-D) mouse pituitaries. Images shown are 10,000X (A & C) and 25,000X (B & D).

Wild-type (C57BL/6J +/+) mice



Lit/lit (C57BL/6J-Ghrh-r -/-) mice

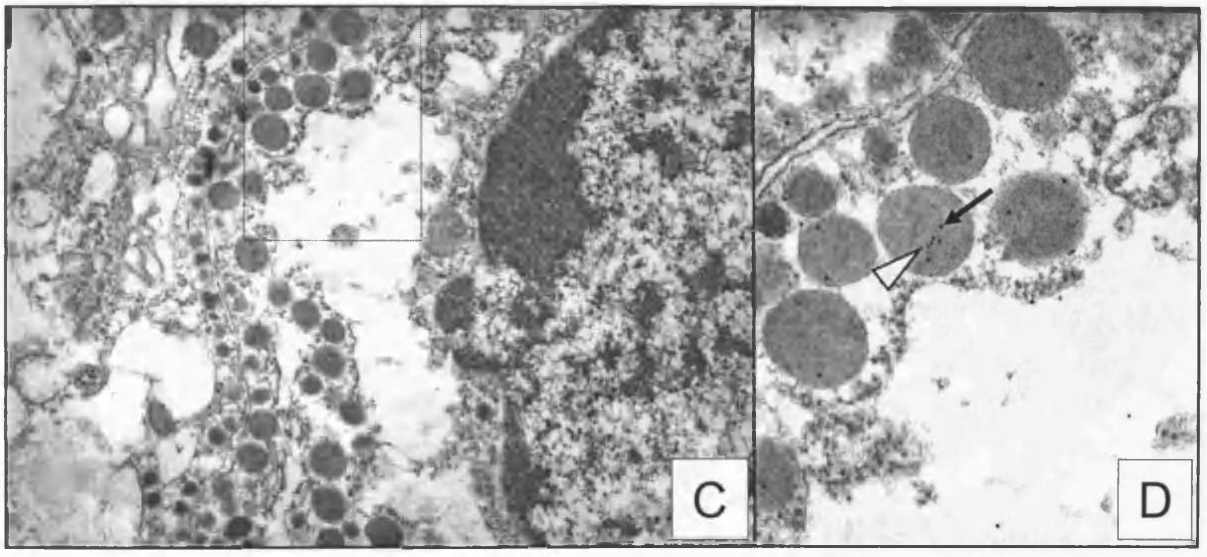
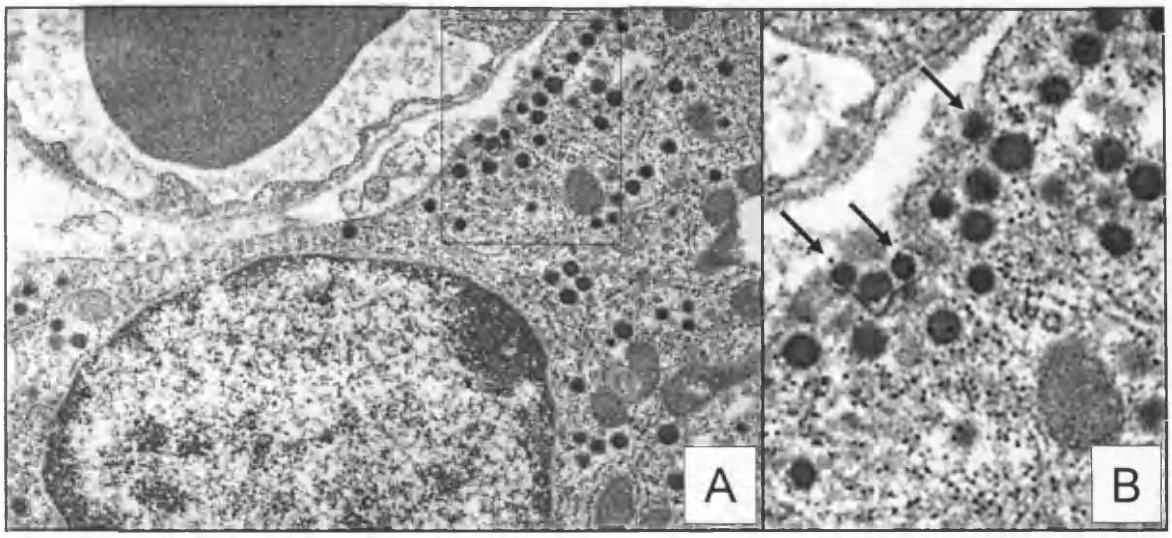


Figure 3.9: Electron micrographs of somatomammotrophs from male wild-type (C57BL/6J +/+; A-B) and lit/lit (C57BL/6J-Ghrh-r -/-; C-D) mouse pituitaries. Images shown are 15,000X (A & C) and 40,000X (B & D). Black arrows: 15nm gold-conjugated anti-GH; White arrowheads 5nm gold-conjugated anti-PRL.

Wild-type (C57BL/6J +/+) mice



Lit/lit (C57BL/6J-Ghrh-r -/-) mice

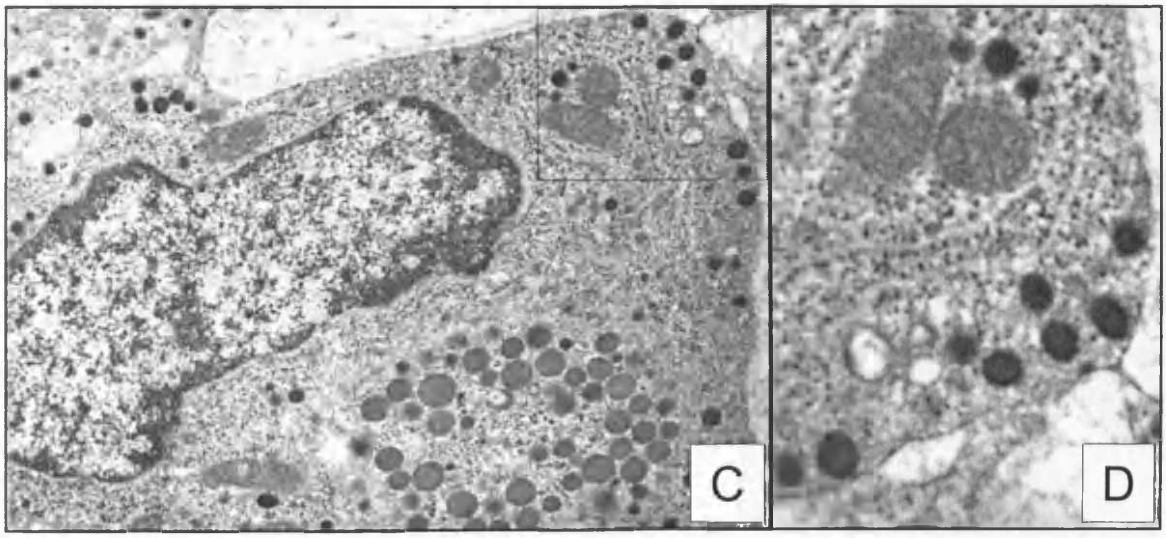
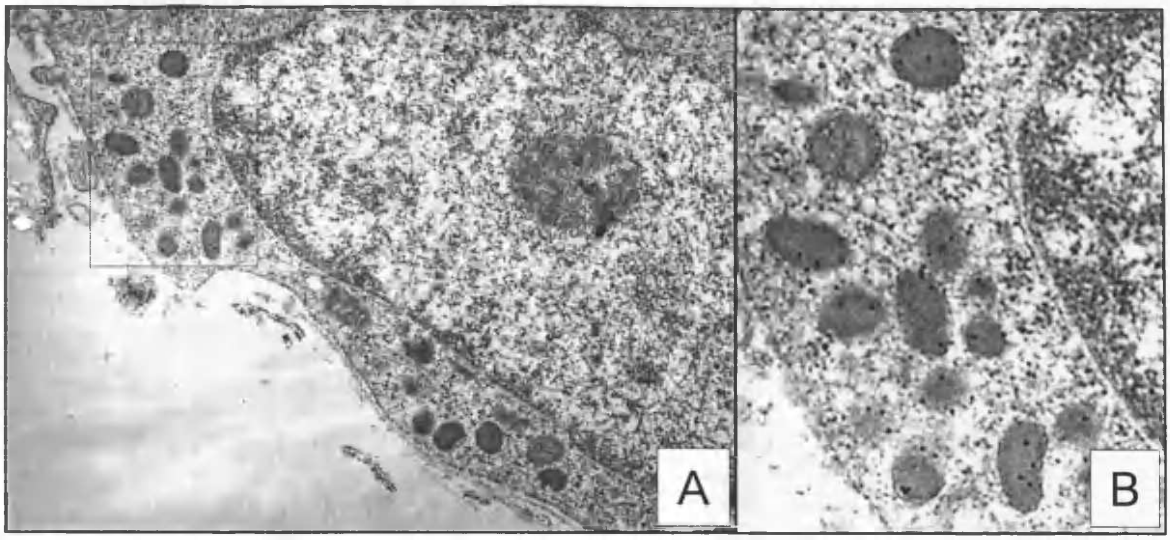


Figure 3.10: Electron micrographs of type II lactotrophs (PRL 5nm gold) from male wild-type (C57BL/6J +/+; A-B) and lit/lit (C57BL/6J-Ghrh-r -/-; C-D) mouse pituitaries. Images shown are 15,000X (A & C) and 40,000X (B & D). Arrows show exocytosed granules.

Wild-type (C57BL/6J +/+) mice



Lit/lit (C57BL/6J-Ghrh-r -/-) mice

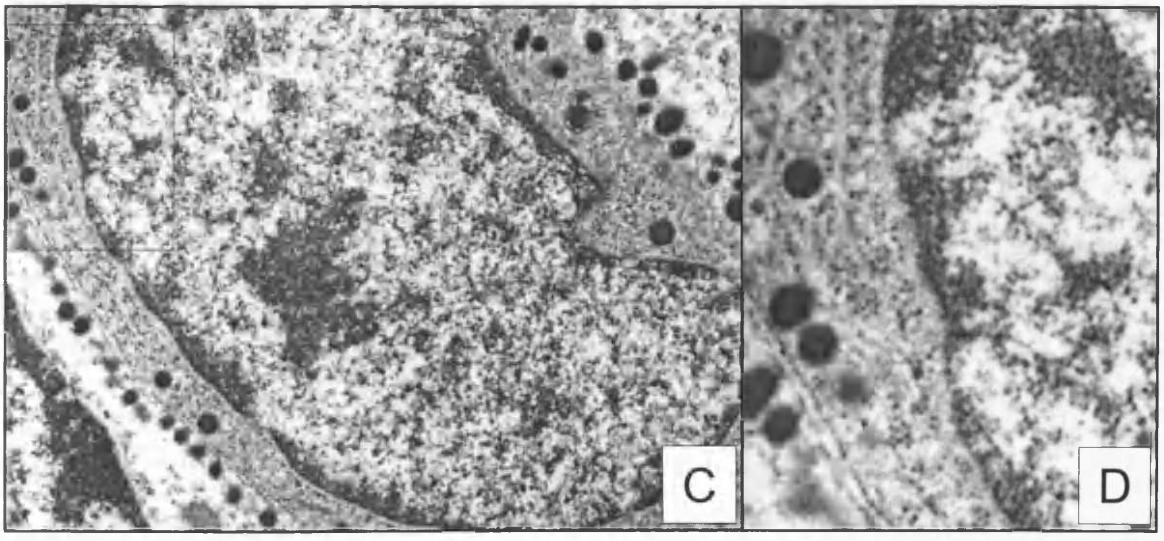


Figure 3.11: Electron micrographs of Type I lactotrophs (PRL 5nm gold) from male wild-type (C57BL/6J +/+; A-B) and lit/lit (C57BL/6J-Ghrh-r -/-; C-D) mouse pituitaries. Images shown are 15,000X (A & C) and 40,000X (B & D).

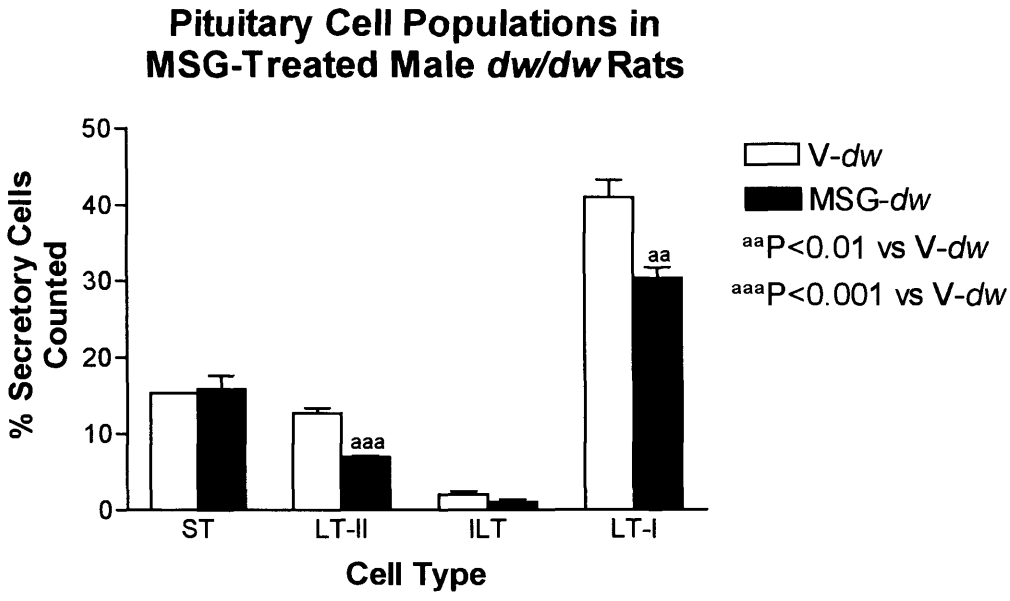


Figure 3.12: The proportion of somatotroph (ST), and types I (LT-I), II (LT-II), and 'intermediate' (ILT) lactotroph populations in the total cell populations in pituitaries of vehicle-treated (V-*dw*) and MSG-treated male *dw/dw* (MSG-*dw*) rats. Values shown are mean ± SEM (n=2 for somatotroph population of V-*dw* group; n=3 for all other groups).

Somatotrophs

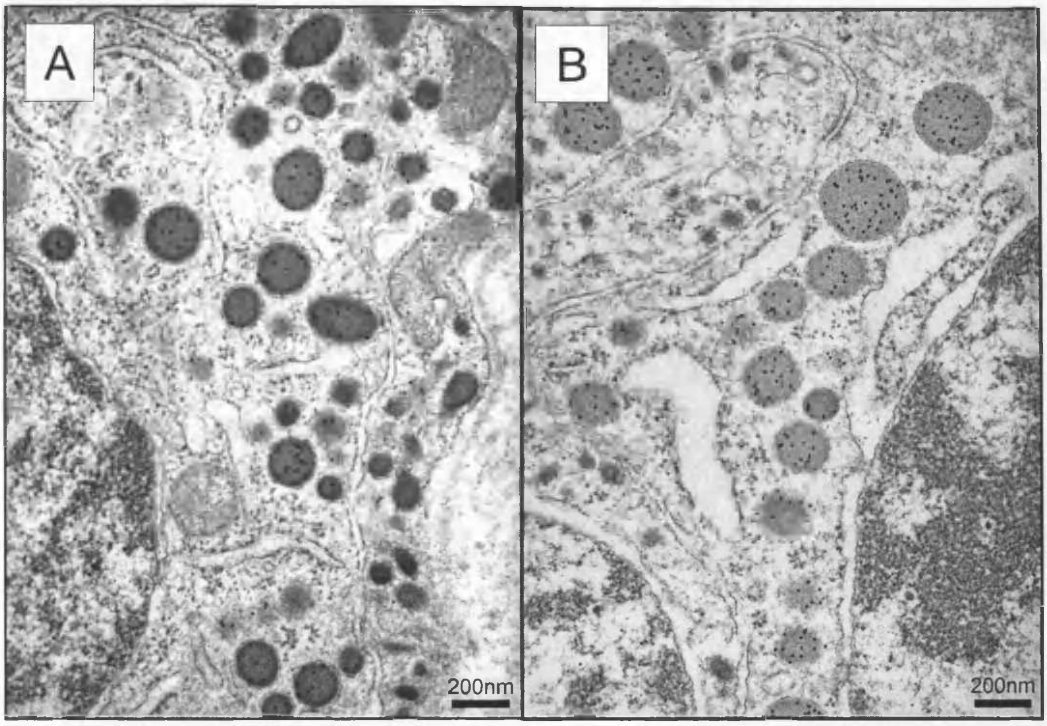
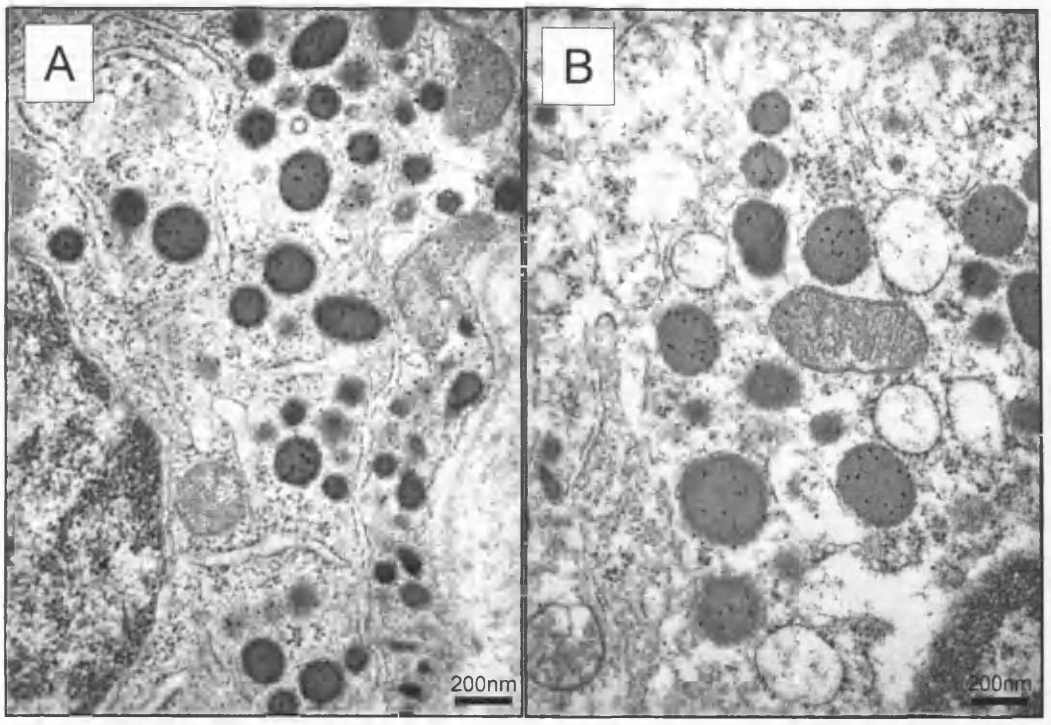


Figure 3.13: Electron micrographs of Somatotrophs (GH 15nm gold) from male *dw/dw* rats after neonatal treatment with either vehicle (A) or monosodium glutamate (B). Images shown are 25,000X.

Type I Lactotrophs



Type II Lactotrophs

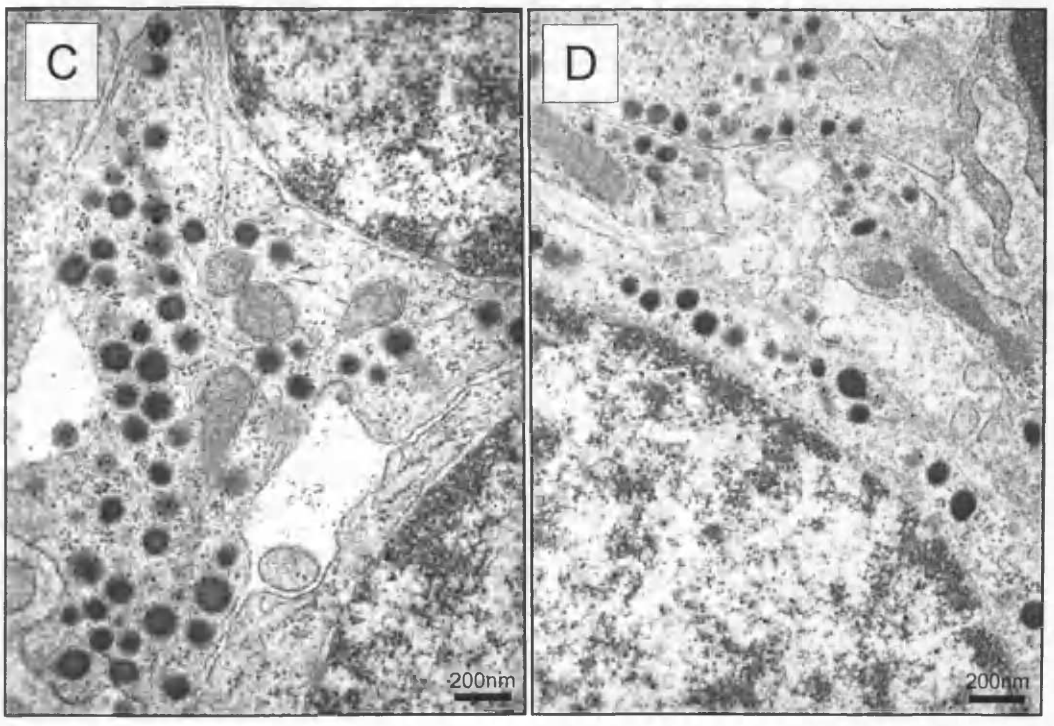


Figure 3.14: Electron micrographs of Type I (A,B) and Type II (C,D) lactotrophs (PRL 5nm gold) from male *dw/dw* rats after neonatal treatment with either vehicle (A,C) or monosodium glutamate (B,D). Images shown are 25,000X.

'Intermediate' Lactotrophs

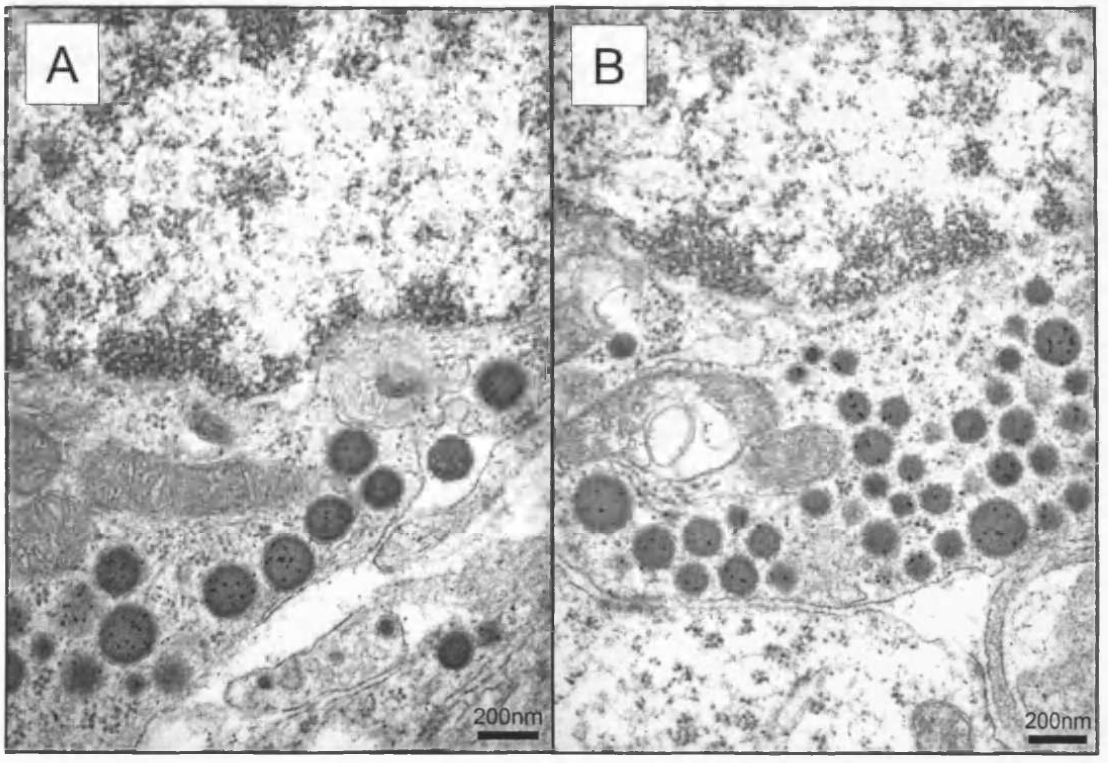


Figure 3.15: Electron micrographs of 'Intermediate' lactotrophs (PRL 5nm gold) from male *dw/dw* rats after neonatal treatment with either vehicle (A) or monosodium glutamate (B). Images shown are 25,000X.

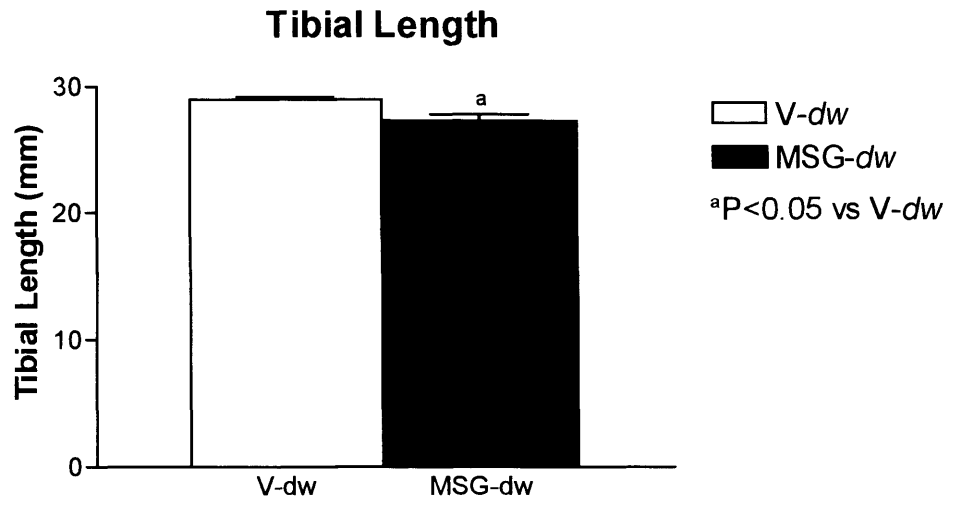
3.3.3.2 Assessment of skeletal growth

Tibial bone length [Figure 3.16A; 28.97±0.20mm (V-*dw*; n=5) vs. 27.32±0.48mm (MSG-*dw*; n=6); $p<0.05$], and EPW [Figure 3.16B; 329.7±9.2μm (V-*dw*; n=5) vs. 291.3±7.3μm (MSG-*dw*; n=6); $p<0.01$] were significantly decreased in MSG-treated male *dw/dw* rats compared to vehicle-treated controls.

3.3.3.3 Assessment of adiposity

Body weight of *dw/dw* rats was not significantly affected by MSG treatment (Figure 3.17A). However, the visceral fat pad weight increased significantly in the MSG-treated group when expressed as either raw or proportionate data [Figure 3.17B; 0.21±0.03g (V-*dw*; n=5) vs. 0.45±0.01g (MSG-*dw*; n=6); $p<0.001$] [Figure 3.17C; 0.21±0.02% (V-*dw*; n=5) vs. 0.48±0.01% (MSG-*dw*; n=6); $p<0.001$]. Moreover, plasma leptin levels showed a dramatic increase in the MSG-treated group compared to control [Figure 3.17D; 0.97±0.16ng/ml (V-*dw*; n=5) vs. 11.4±1.5ng/ml (MSG-*dw*; n=6); $p<0.001$].

A



B

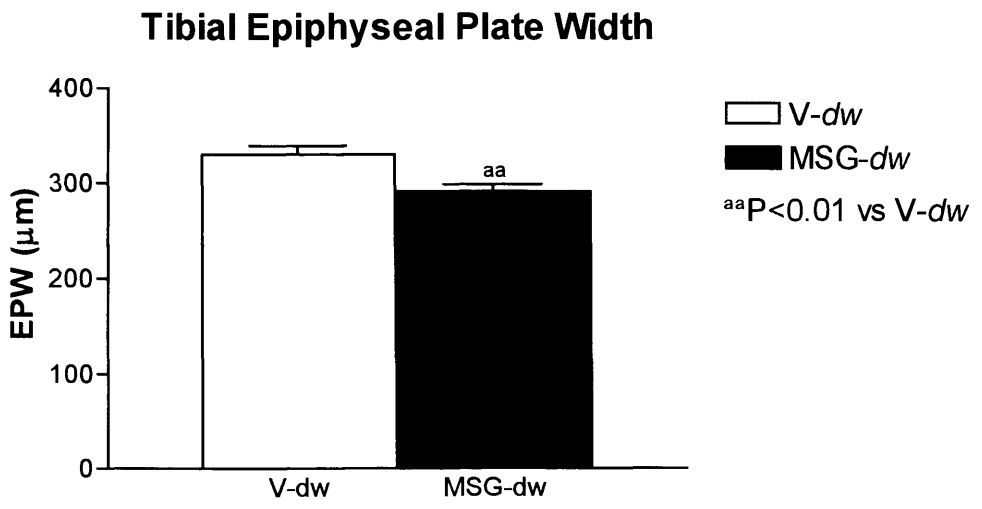
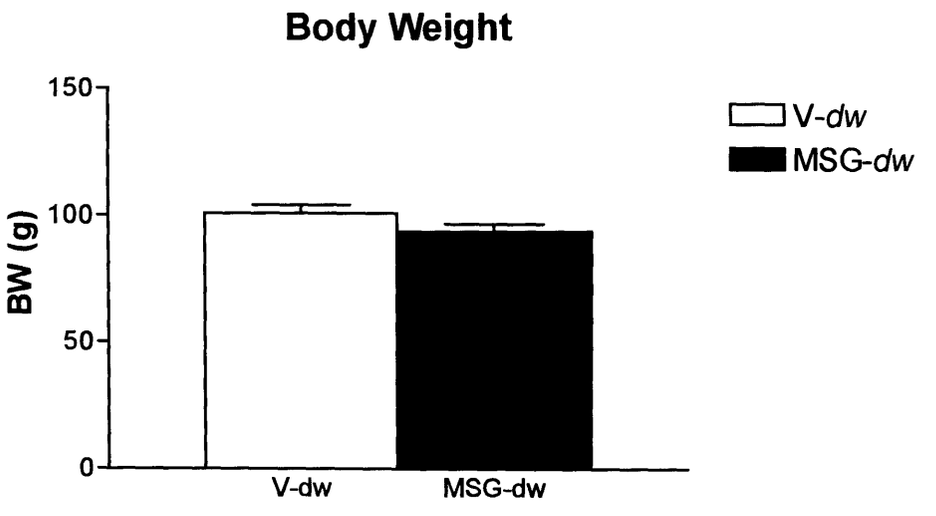
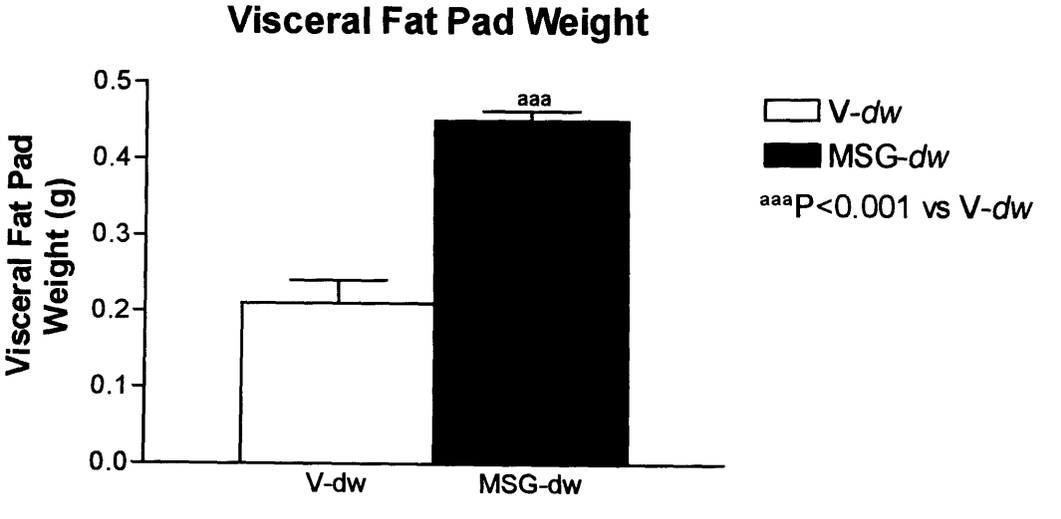


Figure 3.16: The effect of MSG treatment on tibial bone length (A) and tibial epiphyseal plate width (B) in male *dw/dw* rats. Values shown are mean ± SEM [n=5 for vehicle-treated (V-*dw*) groups and n=6 for MSG-treated (MSG-*dw*) groups].

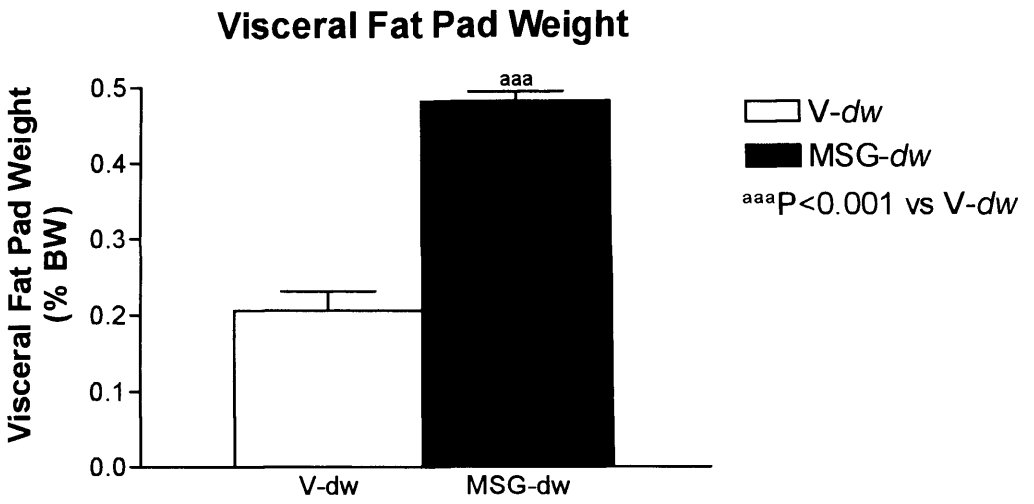
A


Body Weight
150
100
50
0
BW (g)
V-dw
MSG-dw
V-dw
MSG-dw

B


Visceral Fat Pad Weight
0.5
0.4
0.3
0.2
0.1
0.0
Visceral Fat Pad Weight (g)
aaa
V-dw
MSG-dw
aaaP<0.001 vs V-dw
V-dw
MSG-dw

C



D

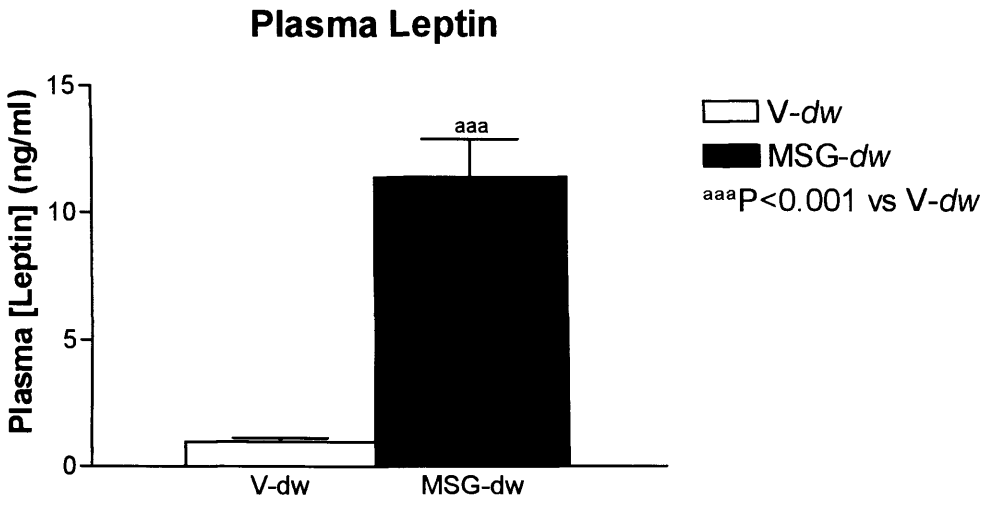


Figure 3.17: The effect of MSG treatment on body weight (A), visceral (retroperitoneal and perirenal) fat pad weight (B), visceral fat pad weight [%BW, (C)], and plasma leptin concentrations (D) in male *dw/dw* rats. Values shown are mean ± SEM [n=5 for vehicle-treated (V-*dw*) groups and n=6 for MSG-treated (MSG-*dw*) groups].

3.4 DISCUSSION

3.4.1 Classification of Lactotrophs

Hedinger and Farquhar were the first to describe the lactotroph as the pituitary cell with the largest secretory granules (Hedinger and Farquhar; 1957). Later, PRL was demonstrated to be produced in the adenohypophysis by heterogeneous subpopulations of lactotrophs. For the identification of lactotrophs, specific techniques such as immunocytochemistry (Nogami and Yoshimura, 1980; Boockfor and Frawley, 1987; Takahashi and Miyatake, 1991), reverse haemolytic plaque assay (Luque et al., 1986), and gravity sedimentation in discontinuous Percoll gradients (Velkeniers et al., 1988) have been used. Classically, two morphologically distinct forms of lactotrophs exist in the rat pituitary: type I lactotrophs are the mature, inactive cells with large polymorphic secretory granules (>300nm) whereas type II lactotrophs are the active, secretagogue-sensitive cells with smaller regularly shaped secretory granules (<200nm). However, morphological and functional lactotroph heterogeneity in rat pituitary has been described in the literature (Nogami, 1984; St John et al., 1986; Kukstas et al., 1990; Maldonado and Aoki, 1994; De Paul et al., 1997; Huang et al., 2002). The two lactotroph subpopulations have been further sudivided into three functional subtypes. In the pituitary of adult female and oestrogen-treated male rats, typical (type I) lactotrophs are the predominant type with large secretory granules (500-900nm), whereas atypical lactotrophs (type II and type III) with smaller secretory granules (150-300nm) are found in male and ovariectomised rats (Takahashi and Miyatake, 1991; Maldonado and Aoki, 1994). Four lactotroph subtypes have been also reported in a lactotroph response study in relation to changes of PRL secretion and intracellular Ca^{2+} to TRH stimulation (Huang et al., 2002). The different functional lactotroph subtypes can be related to the fact that the main morphologically-based subtypes of lactotrophs are not distributed uniformly in the pituitary gland (Papka et al., 1986) and that different regions exhibit a variable response to secretagogues (Boockfor and Frawley, 1987).

The novel lactotroph discovered in *dw/dw* rats (Thompson et al., 2002) is only detectable with electron microscopy as its distinguishing features are based upon the size and shape of the electron-dense secretory granules. Since it shares morphological characteristics (granule size and shape) between those of type I and type II

lactotrophs, it has been designated the 'intermediate'. Its absence, in significant numbers, in the normal pituitary, even in the context of pregnancy-induced lactotroph hyperplasia, confirms that this cell is unique to the *dw/dw* model.

3.4.2 Origin of 'Intermediate' Lactotrophs

In conjunction with previous findings, these data also shed further light on the cellular origin of the 'intermediate' lactotroph. In this regard, there are a number of potential sources, as indicated in figure 3.18.

Firstly, the 'intermediate' lactotroph may originate from the ***type I-type II lactotroph lineage*** (Figure 3.18; position A). In the adult pituitary, it is now known that each somatotroph is physically associated with another somatotroph (Magoulas et al., 2000; Fauquier et al., 2002) probably to form a functional syncitium. Our observation of the 'intermediate' lactotroph reveals that these cells are in direct juxtaposition with other lactotrophs (Figure 3.19). In addition to this physical association, our data reveal that the proportion of the 'intermediate' lactotrophs is regulated in parallel with changes in the proportion of type II lactotrophs and that this is not related to the proportion of somatotrophs. The up-regulation of 'intermediate' lactotrophs occurs in pregnancy without any change in the somatotroph lineage (Figure 3.1). Following MSG-treatment, the proportion of 'intermediate' lactotrophs appeared to change in parallel with changes in the proportions of type I and type II lactotrophs, also without any change in the proportion of somatotrophs (Figure 3.12). However, despite these similarities, the 'intermediate' lactotrophs are functionally distinct. Unlike type II lactotrophs, they are insensitive to TRH (Thompson et al., 2002), and in females, at least, their sensitivity to ghrelin is high compared to type I and type II lactotrophs. Although these data suggest that the 'intermediate' lactotroph could be an intermediate form between type I and type II lactotrophs (Figure 3.18; position A), further double immunogold labelling is required to exclude the second possible origin, namely the somatomammotroph.

The ***somatomammotroph*** is considered to be the common progenitor of both somatotroph and lactotroph lineages (Frawley and Boockfor, 1991). Although not quantified in study 1, we have shown in chapter 2 that in normal rats the proportion of somatomammotrophs decreases during pregnancy (Figure 2.4E), probably as a result of differentiation into type II lactotrophs. In male and female *dw/dw* rats, the proportion of the somatomammotrophs is low (Thompson et al., 2002; Tierney and

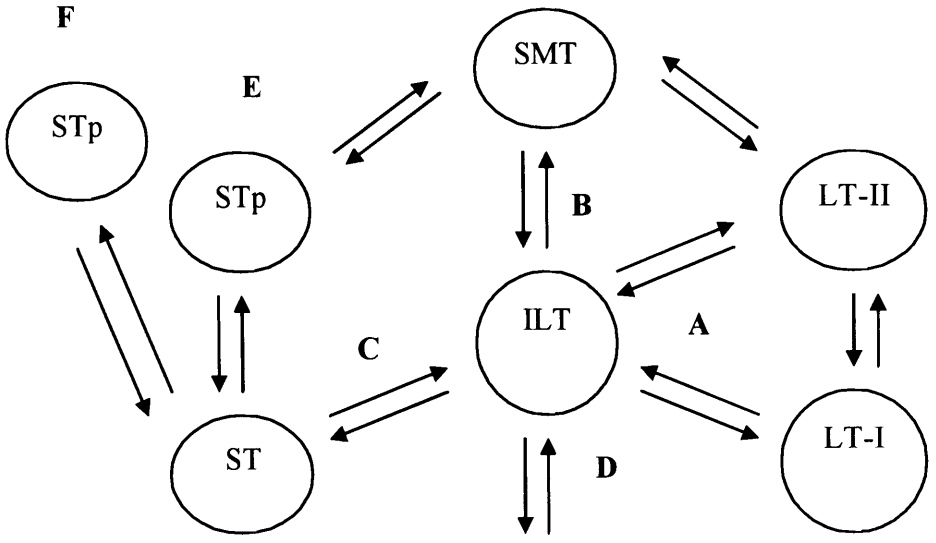


Figure 3.18: Proposed model for differentiation pathways of the somatotroph-lactotroph lineages in the *dw/dw* rat, indicating possible ontological positions for the 'intermediate' lactotroph (ILT).
The 'intermediate' lactotroph may represent a transitional stage in the progression from type II to type I lactotrophs (*position A*), a transitional stage in the differentiation from somatomammotrophs to type II lactotrophs (*position B*), a transitional stage in the transdifferentiation of somatotrophs into type II lactotrophs (*position C*), or a transitional cell in the transdifferentiation from a separate bi- or mono-hormonal cell type (*position D*).
GRF-independent somatotroph (STp) may represent an intermediate from the somatomammotroph (*position E*) or somatomammotroph-independent progenitor (*position F*).
Progenitor Somatotroph (STp); Somatotroph (ST); somatomammotroph (SMT); type I lactotroph (LT-I); type II lactotroph (LT-II); 'intermediate' lactotroph (ILT).

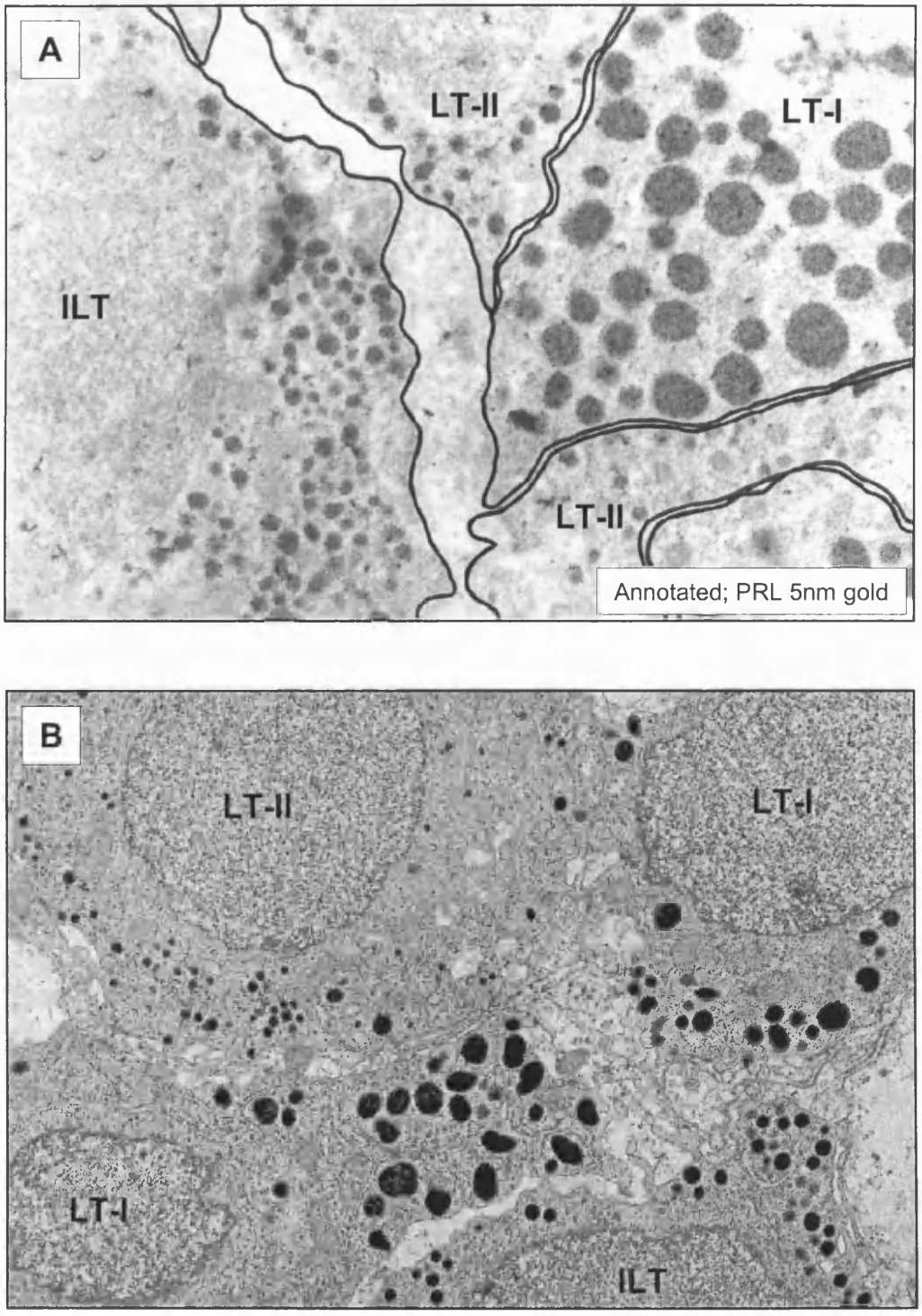


Figure 3.19: Electron micrographs of 'Intermediate' lactotrophs (ILT) from male (A) and female (B) *dw/dw* rats, showing juxtaposition with type I (LT-I) and type II (LT-II) lactotrophs. Images shown are 25,000X (A) and 12,000X (B).

Robinson, 2002), possibly due to differentiation into 'intermediate' lactotrophs. The 'intermediate' lactotroph may be a transitional cell type between the somatomammotroph and type II lactotroph (Figure 3.18; position B). Further experimentation, such as the investigation of the secretagogue (i.e. ghrelin) response of the somatomammotrophs in pregnant *dw/dw* rats, and double immunogold labelling on pregnant and MSG-treated *dw/dw* rats, are needed to determine the involvement of somatomammotrophs in the occurrence of the 'intermediate' lactotroph.

A third possibility is that the 'intermediate' lactotroph may be derived from the ***somatotroph lineage*** (Figure 3.18; position C). In *dw/dw* rats, the 'intermediate' lactotrophs share several morphological features with the somatotroph. For example, the electron dense secretory granules are the same size and are regularly shaped. In addition, these two pituitary cell types demonstrate robust responsiveness to ghrelin (Thompson et al., 2002). Since there is now considerable evidence of transdifferentiation between the somatotrophic and lactotrophic lineages (Vidal et al., 2001), the 'intermediate' lactotroph may represent a transitional stage in this process. This is supported by the sensitivity of 'intermediate' lactotrophs to ghrelin, but it is not supported by the insensitivity of these cells to GRF in males (Thompson et al., 2002), and their differential regulation following pregnancy (Figure 3.1) and MSG treatment (Figure 3.12). We were surprised that the somatotroph population was unaffected in *dw/dw* rats by MSG treatment. This implies that the small number of somatotrophs present in the *dw/dw* pituitary is not dependent upon postnatal GRF, and gives further support to the proposed presence of GRF-independent somatotroph progenitors (Lin et al., 1993). In the developing adenohypophysis, a zone of stem cells is maintained, although somatotroph proliferation continues across the pituitary; somatotrophs divide sequentially from the anterolateral to the caudomedial region. The anterior pituitary is arranged in two functional zones of proliferation; the periphery is populated with GRF- and cAMP-independent somatotroph stem cells, whereas, the centre is populated with GRF- and cAMP-dependent mature somatotroph (Lin et al., 1993). Our data indicate that the somatotrophs present in the *dw/dw* pituitary are comprised of these GRF-independent somatotroph progenitors (Figure 3.18; positions E/F). In addition, our data suggest that, given the lack of parallel regulation of the 'intermediate' lactotroph and somatotroph populations, the 'intermediate' lactotrophs are unlikely to be derived from the somatotrophic lineage (Figure 3.18; position C).

A fourth possibility is that the 'intermediate' lactotroph represents a transitional stage in the transdifferentiation from another secretory cell type to the lactotroph lineage (Figure 3.18; position D). The existence of bihormonal thyrosomatotroph cells has been reported in both rats (Horvath et al., 1990) and humans (Vidal et al., 2000). However, there is no evidence in the literature concerning the presence of thyrolactotrophs even though it has been noticed the concomitant occurrence of thyrotroph and lactotroph hyperplasia with hyperprolactinemia in humans (Pioro et al., 1988; Horvath et al., 1990). Double immunogold labelling could be performed to determine whether the 'intermediate' lactotrophs either express one of the anterior pituitary hormones, or are physically associated with such cells.

A number of possible regulatory mechanisms may affect the occurrence of the 'intermediate' lactotroph in the *dw/dw* rat.

The first regulator may be ***GRF***. GRF is known to regulate GH secretion and controls somatotroph proliferation and survival (Barinaga et al., 1983). Transdifferentiation between the somatotrophic and lactotrophic lineages is well documented (Vidal et al., 2001). A marked elevation in hypothalamic GRF mRNA expression is produced in both the *dw/dw* rat and *lit/lit* mouse by profound reduction in circulating GH (Frohman et al., 2000). Despite this elevation in hypothalamic GRF expression and a significant elevation in type I and type II lactotrophs (Figure 3.7), no corresponding lactotrophs to the 'intermediate' phenotype were present in the *lit/lit* mouse pituitary (Figure 3.7). The sub-total reduction in GRF following MSG treatment in the *dw/dw* rat produced 27% and 45% reductions in type I and type II lactotrophs respectively (Figure 3.12). Although not significant, the 'intermediate' lactotrophs appeared to be regulated in parallel with the more traditional lactotroph subtypes. From the models we have used, it is possible that GRF might interfere with the occurrence of the 'intermediate' lactotroph, but this is not conclusive. In M2-GRF transgenic mice, ablation of GRF neurones leads to a fall in pituitary GH content accompanied by a reduction in PRL content (Le Tissier et al., 2002). This suggests a direct dependence of lactotroph differentiation/proliferation on a secretory product of the GRF neurones. In a GRF knockout mouse, in which a portion of the GRF gene that encodes for the initial 14 amino acids of GRF 1-42 is disrupted, while the rest of GRF gene (GRF precursor and other cell products) remains intact (Alba and Salvatori, 2004), a reduction in the GH mRNA and protein content occurs without alteration in PRL

mRNA and content. A GRF-related peptide, ***GRF-RP***, has been reported (Berry and Pescovitz, 1988), which is also expressed in the testes. There is a high possibility that GRF-RP influences the occurrence of 'intermediate' lactotrophs in *dw/dw* rats. More investigation is needed to delineate this issue.

The second regulator may be ***dopamine***. Lactotroph maintenance is negatively controlled by dopamine-mediated inhibition of the same pathway as GRF (Dasen and Rosenfeld, 2001). In *dw/dw* rats, tuberoinfundibular dopaminergic (TIDA) neurones increase following endogenous PRL stimulation with an increase in GRF cell complement due to GH deficiency (Thomas et al., 1999). MSG treatment in mice, provokes the necrosis of most dopaminergic perikarya in the arcuate nucleus (Stoeckel et al., 1985), but spares the tuberohypophyseal dopamine system (Dawson et al., 1985). Dopamine seems not to influence the occurrence of the 'intermediate' lactotroph.

The third regulator may be oestrogen. ***Oestrogen*** and progesterone play important roles in pregnancy (Rosenblatt et al., 1988). Oestrogen stimulates the proliferation of lactotrophs and enhances their survival (Spady et al., 1999). The increase in oestrogen levels during pregnancy could have enhanced the occurrence of the 'intermediate' lactotrophs as seen in *dw/dw* pregnant females (Figure 3.1). In the *dw/dw* rats, GHS-mediated prolactin release is oestrogen-dependent (Carmignac et al., 1998) and the expression of GHS-R_{1a}, which mediates the neuroendocrine action of ghrelin and the GHSs, is also regulated by oestrogen (Bennett et al., 1997). However, the 'intermediate' lactotrophs are present and possibly more abundant in male than female *dw/dw* rats (Thompson et al., 2002). Moreover, our data in normal pregnancy showed that these cells were unaltered (Figure 2.4C). Upon treatment with oestrogen, somatotrophs shift to lactotrophs (Boockfor et al., 1986), which is not the case in the pregnant and MSG-treated *dw/dw* rats where the proportion of somatotrophs was unaltered. Therefore, the occurrence of the 'intermediate' lactotroph seems to be oestrogen-independent.

The fourth regulator may be the ***Glucocorticoids***. Glucocorticoids function to induce the final stages in the differentiation of fully functional somatotrophs from cells previously committed to this lineage (Porter and Dean; 2001). In cell culture, they can stimulate transdifferentiation from a lactotroph to somatotroph phenotype (Kineman et al., 1992b). Knowing that transdifferentiation could be reversible (Vidal et al.,

2001), glucocorticoids may have some effect on the occurrence of the 'intermediate' lactotroph.

3.4.3 'Intermediate' Lactotroph and Plasticity (i.e. Transdifferentiation)

Cellular plasticity in the adenohypophysis is remarkable. Modifications in cell morphology (size, number) occur in adults. The increase in the pituitary cell number can take place via three different mechanisms: (1) proliferation or multiplication of specific cell types (Takahashi et al., 1984), (2) differentiation of stem cells/ progenitors (e.g. somatomammotroph) (Severinghaus, 1973), or (3) transdifferentiation (i.e. conversion of one monohormonal cell type into another; Kineman et al., 1992b).

The morphological characteristics of the newly identified 'intermediate' lactotroph suggest that it represents an intermediate form in the progression of type II lactotroph to type I lactotrophs. However, the profile of its secretory regulation does not correspond with either type I or type II lactotrophs. In combination with the fact that this novel cell is not detected in normal rats, it could be more than just a marker of progression (Figure 2.19; position E) (Thompson, 2002).

The bihormonal somatomammotroph is a common progenitor of both somatotrophs and lactotrophs (Frawley et al., 1985). In cases of pituitary hyperplasia (e.g. during pregnancy), it has been reported that transdifferentiation occurs between somatotrophs and lactotrophs via the somatomammotrophs (Frawley and Boockfor, 1991; Vidal et al., 2001). The morphological resemblance and the responsiveness to ghrelin with the somatotroph suggest that this new lactotroph is not just a classical lactotroph but may be a marker of transdifferentiation between type II lactotrophs and somatotrophs (Figure 2.19; position D) (Thompson, 2002).

3.4.4 Consequences of MSG Treatment

3.4.4.1 MSG dw/dw rat model and growth

Despite the absence of a reduction in the proportion of the somatotrophs following neonatal MSG treatment, the growth in the *dw/dw* rats was significantly impaired. This was seen by the decrease in the tibial length and the EPW (Figure 3.16). The neonatal administration of MSG destroys 70-90% of the neuronal cell bodies within the hypothalamic arcuate nucleus (Bloch et al., 1984) including most of the GRF-producing neurones, thereby providing a model of GRF-deficiency (Kovacs et al.,

1995). GRF is essential in the control of GH secretion and the regulation of linear growth (Clark and Robinson, 1985; Mayo et al., 1995). GH is the most affected pituitary hormone by MSG treatment (Wakabayashi et al., 1986). It has been reported that neonatal MSG treatment in normal rats decreases circulating GH concentrations in adults, reducing peak height and frequency, and suppressing baseline GH levels (Kovacs et al., 2000). The reduction in pituitary size and GH content and the consequent decline in IGF-I (Millard et al., 1982, Hu et al., 1993) results in the severe impairment of growth rates. The absence of a reduction in pituitary weight following MSG treatment in *dw/dw* rats (data not shown) may reflect the failure of this treatment to reduce the somatotroph population, which in normal rats accounts for 50% of the secretory cell population in the adenohypophysis. Similarities between the MSG treatment and the aging-related decrease in hypothalamic GRF content and GH regulation with respect to bone pathology have been documented in rats (Thompson et al., 1994; 1996). The MSG-treated *dw/dw* rat may represent a model of non GH-dependent skeletal growth. Since sex difference has been reported to exist in the effect of MSG on GH secretion elicited by GRF (Wakabayashi et al., 1986), further investigation will be carried out to study growth in MSG-treated female *dw/dw* rats.

3.4.4.2 MSG dw/dw rat model and adiposity

In male MSG-treated rats, an increase in fat mass, accompanied by hyperleptinaemia, was observed (Figure 3.17D). GH-deficient humans and *dw/dw* rats show increased relative adiposity (Snel et al., 1995). In rats, the neonatal MSG administration induces not only severe neuroendocrine but also metabolic disruptions. It results in enhanced adiposity and chronic hyperleptinaemia (Perello et al., 2003). In MSG-treated rodents, the obesity is common, hyperinsulinaemia accompanying hyperleptinaemia in mice (Cameron et al., 1978). There is a tendency of female MSG-lesioned rats to become more obese than males. However, the development of obesity, in turn, may antagonize the parameters that tend to slow linear growth (Maiter et al., 1991). Leptin regulates pituitary hormone secretion. Leptin promotes GRF secretion thereby stimulating GH release (Tannenbaum et al., 1998; Sone and Osamura, 2001). Hyperleptinaemia may contribute to the observed rise in GH secretion. Since there is a tendency of female MSG-lesioned rats to become more obese than males (Maiter et al., 1991), further investigation will be carried out to study adiposity in MSG-treated female *dw/dw* rats.

3.4.5 Methodological Approaches

3.4.5.1 Ghrelin treatment

It is worthy to note that, in study 1, i.v. injection of ghrelin did not cause the expected release of PRL (Figure 3.2B) and did not result in the significant increase in the number of exocytosed granules (Figure 3.3). Whilst it may be possible that GHS-R_{1a} expression is down-regulated in pregnancy, this does not appear to be the case in chapter 2, study 1 (Figure 2.10). The most likely explanation is that the batch of ghrelin used in this study (from different supplier) was less potent than that previously used. This is corroborated by the fact that the peak circulating GH levels in figure 3.2A are 10 times less than that previously obtained in our laboratory (Thompson et al., 2002).

3.4.5.2 EM and selection of 'Tannic acid' fixative

Tannic acid (TA) is a naturally occurring polyphenolic compound that aggregates membranes and neutral phospholipids vesicles and precipitates many proteins (Simon et al., 1994). In 1971, TA was originally introduced in electron microscopy as a supplementary fixative. Chemicals such as glutaraldehyde (GA) and TA are used in situ- or immersion-fixation to preserve and visualize proteins (Guerra et al., 1996); TA in particular protects the oxidation of several of the polyunsaturated fatty acids (Ramanathan et al., 1994). So, TA is known to stabilise tissue components against extraction that could have occurred during dehydration, and subsequent processing for EM (see Kalina and Pease, 1977). Besides improving preservation, TA fixation has been known for its ability to enhance visualization and contrast of various intra- and extra-cellular structures including plasma membranes (Asami, 1986). A specific lipid-fixing technique of tissue preparation for EM (TA-GA) is used to preserve and enhance the visibility of storage granules. The fixation with GA and OsO_4 showed vesicles limited by a single membrane which appears triple-layered following staining with TA (Skriver et al., 1980). Following secretagogue administration, the membrane events (i.e. membrane interaction and subsequent lipid processing) are visualised more easily following TA cell membrane highlighting (Reger et al. 1989). Thus, the TA is applied to arrest the release of the hormone-containing, electron-dense secretory granules during exocytosis.

3.5 CONCLUDING REMARKS

Mice transgenic for hGRF develop somatotroph, lactotroph, and somatomammotroph hyperplasia (Asa et al., 1992) due to the chronic exposure to GRF (Stefaneanu et al., 1989). Over-expression of GRF or a co-expressed neuropeptide appears to play an important role in stimulating the lactotroph lineage in *dw/dw* strain, and therefore may contribute to the occurrence of the 'intermediate' lactotroph. More investigation is needed to elucidate the origin and the factors behind the occurrence of this novel lactotroph cell. In addition, the generation of MSG-treated *dw/dw* rats may represent a useful model of GH-independent skeletal growth, the elevated adiposity being similar to that seen in profound GH-deficiency. The significance of the *dw/dw* rat model in the context of growth retardation will be discussed in the final chapter.

CHAPTER 4.

PHENOTYPIC & GENOTYPIC CHARACTERISATION OF A NOVEL RAT MUTANT – THE 78N TRANSGENIC LINE.

4.1 INTRODUCTION

A novel transgenic rat line – 78N – was generated by Slade et al. (2001) using a promoter-reporter (egr-1/d4EGFP) construct. Of five transgenic lines, only 78N exhibited an overt phenotype, indicating that this line may be an insertional mutant.

The initial characterisation of the 78N mutant rat was done by Slade et al. (2001). The overt phenotype was evident after postnatal day 5 (P5). The main characteristics were premature death of the males (by P9) and retarded growth of the surviving females associated with patchy skin and delayed fur growth. Premature eyelid opening and variable tail kinking/curling were also observed. Maternal behaviour was impaired. Nevertheless, the three major criteria (i.e. feeding, grooming, and locomotor behaviour) for humane maintenance of the line were satisfied.

In the present study, further analyses were carried out in order to understand the pathological basis of this interesting phenotype. In particular, we were interested to determine the basis of retarded body growth. Postmortem analysis has revealed kidney abnormalities in both male and female mutants. ***Therefore, a detailed morphological analysis of the kidney phenotype was performed.***

To reveal the presumed disrupted gene(s) causing the kidney phenotype, molecular analysis of the mutant kidney transcriptome is required. For this purpose, oligonucleotide microarray analysis was used. Among the genetic profiling techniques, DNA microarrays are considered one of the most powerful, being capable of genome-wide profiles of mRNA expression. This technology evaluates expression levels of thousands of genes simultaneously, by means of miniaturisation and parallel

processing (Lockhart and Winzeler, 2000; Lockhart and Barlow, 2001; Mirnics et al., 2001; Zapala et al., 2002).

4.2 METHODS

4.2.1 Animal Maintenance

The transgenic rat (Sprague-Dawley, CD) line – 78N – was maintained under standard conditions. All animals were given tap water and standard laboratory diet (Teklad rat chow, Harlan Ltd., Oxfordshire, UK) *ad libitum* and maintained in a regulated environment [14:10 light/dark cycle (lights on 5:00h) in air-conditioned rooms (20-23^{0}C, 45-70% humidity)]. Care was taken to use the lowest possible number of animals and to minimise suffering. Animals were killed by cervical dislocation or decapitation, or anesthetising deeply with Fluothane (Halothane) before being decapitated. All procedures were conducted in compliance with both UK Home Office regulations [UK Animals (Scientific Procedures) Act, 1986] under a licence held by Dr. David Carter, and with local ethical review. The mutant males were culled by P6-P8 before showing aggravated signs of health deterioration.

4.2.1.1 Animal weighing

The 78N mutant females and their wildtype littermates were weighed for 12 weeks, starting from week 1 after birth.

4.2.2 Postmortem Examination

Parallel postmortem examination was performed on neonatal mutant males and females, and on adult females. Control non-transgenic siblings of similar age and gender were killed at identical time points. Tissue (i.e. kidney) with apparent abnormalities was weighed and sampled for histological investigation.

4.2.3 Tissue Preparation for Histological Analysis

4.2.3.1 Tissue fixation

Wildtype and mutant, neonatal male and adult female left kidneys were excised, weighed, and then fixed by immersion in 10% buffered formalin for a few hours for neonatal tissue, to two days for adult tissue, before being processed.

4.2.3.2 Tissue dehydration and clearing

This was performed using an automatic tissue processor (see section 2.2.5.4.1).

4.2.3.3 Tissue sectioning

Kidney sections of 5µm thickness were cut longitudinally on a rotatory microtome (Leica 5139), floated out on a waterbath (45^0C) for a few minutes to complete their flattening and expansion, and finally positioned on slides. Slides were drained for several minutes, dried on a hot plate (45^0C) for 30min, and then transferred to a 45^0C incubator overnight. Staining of cooled sections was carried out subsequently using the appropriate staining procedure relevant to the present investigation.

4.2.3.4 Staining procedures and light microscopic examination

4.2.3.4.1 Standard haematoxylin and eosin (H&E) stain for paraffin sections

Kidney sections were dewaxed in xylene, hydrated through graded alcohols [twice in 100%, once in 95%, and once in 70%] to water, and stained in an alum haematoxylin (Mayer's) for 2min. After washing well in running tap water until the sections become 'blue', they were stained in 1% eosinY for 5min. After a quick wash in running tap water, the sections were dehydrated through alcohols [once in 70%, once in 95%, and twice in 100%], cleared in xylene, and mounted permanently in DPX.

4.2.3.4.2 Glomeruli counts

Glomeruli were counted on H&E-stained sections under the light microscope. From each group of animals (neonatal P6 males and adult 20wk females), 3 mutant and their 3 wildtype littermate kidneys were studied. 6 longitudinal kidney sections per animal were investigated. Since it was impossible to select equivalent sampling areas in wildtype and mutant kidney sections due to alterations in the cortico-medullary architecture in mutants (see results), it was decided to count all visible glomeruli in each section. Care was taken to avoid double counting. The counting of glomeruli was performed at 20X magnification for neonatal kidney and 10X magnification for adult kidney.

4.2.4 Tissue Sampling for Molecular Analysis

Wildtype and mutant males were killed at postnatal day 6 (P6) by decapitation. Following laparotomy, the right kidney was removed, frozen on dry ice, and stored at -70^0C until used for RNA preparation.

4.2.5 RNA Extraction

Kidney tissue samples were disrupted mechanically in 400µl of GTC-A denaturation solution (Appendix A3). 40µl of NaAc (3M, pH 5.5) and 400µl of phenol (pH 4.3) were added and mixed by shaking. 80µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed by shaking for 10sec, incubated on ice for 10min, and centrifuged in a pre-chilled (4^0C) centrifuge (Micromax RF; IEC) at 21,004xg for 10min. The upper aqueous layer was transferred to a fresh 1.5ml microcentrifuge tube carefully to avoid contact with the interphase preventing protein carryover. The RNA in the aqueous layer was precipitated by adding 2 volumes (~800µl) of ice-cold absolute ethanol. The sample was frozen at -70^0C for 1-2hr, and then centrifuged at 21,004xg for 10min at 4^0C to pellet the total RNA. The supernatant was removed and discarded by gentle pipetting. The pellet was resuspended in 120µl ice-cold GTC-A solution by pipetting up and down. The sample was vortexed briefly after the addition of 2 volumes (240µl) of absolute ethanol and then frozen at -70^0C for 30min. Following centrifugation at 21,004xg for 10min at 4^0C, the supernatant was removed and discarded. The pellet was washed by adding 120µl of 95% ethanol and centrifuged for 2min at RT. After removal of the supernatant, the RNA pellet was allowed to air-dry for 5min before resuspending it in an appropriate volume (40-60µl) of DEPC-treated H_2O. The sample was briefly vortexed, centrifuged for 2min, kept on ice until checking for RNA integrity (gel electrophoresis) and quantity, then stored at -70^0C (Chomczynski and Sacchi, 1987). [RNA extraction for the microarray analysis was performed by Dr. D. Carter.]

4.2.5.1 RNA quantification

1-2µl of RNA sample was diluted in DEPC-treated H_2O in a total volume of 500µl. After pipetting the sample into a 1ml quartz cuvette (Pharmacia), absorbance at the wavelength of 260nm (A_{260}) was measured using the GeneQuant spectrophotometer (Pharmacia) using DEPC-treated H_2O as reference. RNA concentration was calculated knowing that 1 A_{260} unit of single-stranded RNA is equivalent to 40µg/ml.

4.2.6 Oligonucleotide Microarrays

Affymetrix GeneChip microarray analysis was conduced as a service by the Wales Gene Park Affymetrix GeneChip Expression Profiling Service, Department of Pathology, University of Wales College of Medicine.

4.2.6.1 Choice of experimental material (Design)

Wildtype and mutant littermate kidneys were selected from two different litters. This approach permits a *blocked design* in which mutant gene expression was compared to a littermate wildtype in two independent microarray analyses. These were coded as Set D (1wildtype vs 1mutant) and Set E (1wildtype vs 1mutant).

4.2.6.2 Sample quality control

Before any processing, every RNA sample was quality controlled using *Agilent RNA6000 chips*. This quality control replaces conventional RNA quality control that uses agarose gel electrophoresis and/or absorbance (A_{260}/A_{280}) measurements, and is more suitable for array analysis because of the standardization of the Agilent chips.

4.2.6.3 Sample labelling

10μg of each RNA sample was used to generate biotinylated cRNA using Affymetrix GeneChip protocols.

4.2.6.4 Hybridization

The biotinylated cRNA (*target*) was probed with rat genome U34A Genechips. Hybridization and washing were performed using a GeneChip fluidics station 400 (Affymetrix).

4.2.6.5 Image acquisition/Image analysis

After scanning the microarrays, initial analysis was conducted with Microarray Suite 5.0 (Affymetrix). For comparison between arrays, the average signal intensity of each array was scaled to 100. This procedure normalizes the raw data and corrects for technical variation between the arrays (e.g. differences in hybridization conditions). Further analysis of the microarray data was conducted using ***GeneSpring 5.0*** (Silicon Genetics) [see 4.3.4.1 below and Figure 4.7].

4.2.7 PCR Amplification and Cloning of DNA Fragments

KAP (Kidney androgen-regulated protein) & N4WBP4 (Nedd4 WW domain binding protein) probes were produced by cloning PCR amplified DNA fragments into the pGEM-T vector (Promega).

4.2.7.1 RT-PCR

RT-PCR was conducted in 2 steps:

First, Superscript™ II reverse transcriptase was employed for first-strand cDNA synthesis (Superscript II protocol, Invitrogen). Second, standard PCR amplification using gene-specific primers was employed.

4.2.7.2 Standard PCR amplification method

The 25µl PCR reaction contained 1x *Taq* PCR buffer, 200µM of each dNTP, 1.5mM magnesium chloride, 100-400nM of each specific primer, 1-3µl of cDNA, 1.25 units *Taq* polymerase, and an appropriate volume of sterile water. A master mix of all these reagents was prepared and aliquoted into 0.5ml PCR tubes prior to the addition of DNA template. The pipetting was performed on ice and the reactions were loaded on a pre-heated (95^0C, for 2min) block of the PCR machine [PTC-100, MJ Instruments, Inc. ***or*** PCR Express, Hybaid]. Thermal cycling conditions varied between experiments: 94^0C for 2.5min, 30-35 PCR cycles (94^0C for 45sec, * for 45sec and 72^0C for 45sec), and 72^0C for 10min (*53^0C for KAP probe, 56^0C for N4WBP4 probe.

4.2.7.3 Extraction and purification of DNA fragments from agarose gel (QIAEX II Purification)

After electrophoresing PCR products on 1% agarose TAE gels, DNA fragments were isolated from the gel using a scalpel blade and extracted using the QIAEX II Gel Extraction Kit (Qiagen). The extraction and purification process involved the solubilisation of the bands excised from the agarose gel in a sodium iodide solution, and selective DNA adsorption to the QIAEX II silica-gel particles in the presence of high salt. The adsorbed DNA was washed prior to elution in low salt buffer (10mM Tris-HCl, pH 8.5) or H_2O.

4.2.7.4 Ligation of PCR products into the pGEM-T vector

Ligation was performed by mixing 5µl of 2x ligation buffer, 1µl of pGEM-T vector (50ng, Promega), 1-3 µl of the purified PCR fragment, 1µl of T4 DNA ligase, and water to a total volume of 10µl. With each batch of reactions, a negative control reaction containing only vector was prepared. Ligation reactions were incubated for 1-2hr at RT or overnight at 4^0C, prior to bacterial transformation.

4.2.7.5 Preparation of agar plate cultures

Agar plates, containing antibiotic relevant to the plasmid vector, were prepared. Using a sterile loop, bacterial colonies were removed from glycerol stocks or agar plates. In a cross-hatched manner, the loop was streaked across the agar plate. The agar plates were inverted and incubated overnight at 37^0C.

4.2.7.6 Preparation of bacterial glycerol stocks

For long-term storage, glycerol stocks for all recombinant bacterial cultures or bacterial strains were prepared. Colonies, picked from agar plates were inoculated into LB broth containing an appropriate antibiotic. Cultures were incubated in a shaking incubator overnight at 37^0C. The next day, 850µl of liquid culture was mixed with 150µl of sterile glycerol in a 1.5ml eppendorf tube. The glycerol stocks were stored at -70^0C. The recovery of bacterial glycerol stocks was done by streaking onto freshly prepared LB agar plates (containing the suitable antibiotic) using an inoculation loop (see below). The agar plates were inverted and incubated overnight at 37^0C.

4.2.7.7 Transformation of Escherichia coli

After thawing competent cells on ice, a 50µl aliquot was placed in an Eppendorf tube. 1-5µl of plasmid DNA or ligation reaction (pGEM-T/PCRproduct) was added to the cells, mixed gently and placed on ice for twenty minutes. The bacterial suspension was heat-shocked for 90sec at 42^0C, and then returned briefly on ice. 100µl of LB broth was added to the bacteria and incubated for 30min at 37^0C. The manipulated cells were plated out onto the appropriate medium [LB agar containing the appropriate antibiotic (e.g. ampicillin, 50µg/ml)]. Then, the plates were incubated overnight at 37^0C to allow bacterial growth.

4.2.7.8 Expansion of transformed bacteria

5ml of LB broth containing the appropriate antibiotic was prepared in a 10ml Universal tube. An individual colony of bacteria was picked from an agar plate using a sterile loop, and mixed thoroughly in the LB medium. This culture was then incubated in a shaking incubator overnight at 37^0C.

4.2.7.9 Isolation of plasmid DNA

Plasmid was purified from the remaining bacterial culture (after preparation of glycerol) using the Wizard Plus SV Miniprep Kit (Promega) according to the manufacturer's instructions, for small-scale plasmid DNA isolation and purification. To verify if the plasmid contained the correct PCR insert, DNA sequencing and restriction digests were conducted.

4.2.7.10 DNA sequencing

The work was performed by the research support group, School of Biosciences, Cardiff University. Sequencing on both strands was performed (ABI-PRISM 3100). Sequence files were analysed using the Chromas software package (version 1.43). Sequencher, software package (Gene Codes), was used for subsequent alignment of DNA sequences.

4.2.7.11 Restriction endonuclease digestion

A 50-60µl restriction digest reaction was prepared as follows: 40-50µl of the specific pGEMT-plasmid together with the appropriate restriction buffer (Promega), and restriction enzymes (NcoI and SalI for KAP and N4WBP4 plasmids). Digests were incubated for 1-4hr at 37^0C. The extraction and purification of the DNA probes was conducted according to the QIAEX II Gel Extraction Kit protocol.

4.2.7.12 Agarose gel electrophoresis

Linearised DNA samples were quantified by using a 2.5-5.0µl aliquot of HyperladderI (Bioline) as quantitative marker. Quantitative analysis was performed by importing the gel image into the GeneTools^{TM} software (Syngene), where the HyperladderI marker was used as a calibration standard to estimate the concentration of the DNA sample.

4.2.8 Radiolabelling of DNA Probe – The OLB Method

4.2.8.1 ^{32}P-Labelled DNA probes – Generation and Purification

The DNA probe generation was done by random primer labelling of a QIAEX II purified restriction fragment. The random primer labelling technique involved the generation of a dsDNA probe where random hexadeoxynucleotides (Boehringer Mannhein), [α^{32}P]-dCTP (Amersham) and dNTPs [dATP, dGTP, dTTP (Promega)]

were incorporated into the probe by the catalytic activity of DNA polymerase I – Klenow fragment. DNA (20-50ng) was made up to 32µl with sterile distilled water. The DNA was boiled for 10min and directly quenched on ice for 1min. After quick centrifugation, 10µl of OLB 5x buffer and 2µl of BSA (10mg/ml) were added and incubated for 45min at 37^0C to allow random priming to occur. After a brief centrifugation, 5µl of α^{32}P dCTP (~3000Ci/mmol) and 1µl Klenow large fragment (5U/µl) were added. After gentle mixing and overnight incubation at RT, 50µl OLB-C Stop solution was added. DNA probes were purified using Sephadex G-50 (see section 4.2.8.2) purification. The labelled probe was centrifuged through a Sephadex G-50 column at 2040xg for 3min and collected in a fresh tube. The purified labelled probe was stored in a lead pot at -20^0C until use. Prior to use, the probe was boiled for 10min, quenched on ice and added to 10-15ml of hybridisation buffer.

4.2.8.2 Preparation of Sephadex G-50 column

To prepare a Sephadex G-50 purification column, autoclaved glass wool (BDH) was placed at the bottom of a 1ml syringe barrel and tightly packed down to approximately 0.1 ml level using the syringe plunger. The column was placed in a 15ml tube (Becton Dickinson) and filled to the top with a cold (4^0C) well-shaken Sephadex G-50 solution. The column was centrifuged at 2040xg (CR312; Jouan) for 3min at RT. At this stage, the column was ready for nucleic acid purification.

4.2.9 Nucleic Acid Hybridisation

4.2.9.1 Northern blotting

Right kidney total cellular RNA was extracted from tissue sampled from neonatal wildtype and mutant male (P6) and female (P9), and adult (5-6mo) female animals. 10 or 15µg total RNA samples were resolved on a 1XMAE formaldehyde-agarose gel, transferred to Hybond-N membrane (Amersham) overnight, and probed with a ^{32}P-labelled cDNA probe (section 4.2.8) specific for KAP mRNA. Following washing, the northern blots were exposed to a phosphor screen (Kodak-K) or X-Ray film for time interval ranging from 6hr to 3 days. 18S ribosomal RNA was visualized as ethidium bromide fluorescence in the northern gel, captured using GeneSnap software (Syngene), inverted, and exported to Adobe Photoshop for montage. Reprobing the northern blots with a ^{32}P-labelled cDNA probe specific for N4WBP4 mRNA was also

performed. Following washing, the northern blots were exposed to an X-Ray film for 2 days.

4.2.9.2 Southern blotting

To investigate localization of the transgene integration site, fluorescent in situ hybridisation (FISH) analysis has been conducted (Slade, 2001). This showed that the integration event might be in chromosome 3. N4WBP4, a chromosome 3 gene, was therefore investigated by Southern analysis of genomic DNA.

4.2.9.2.1 Genomic DNA preparation

4.2.9.2.1.1 Tail/Ear biopsy

To extract genomic DNA for analysis, small piece of tissue were obtained from either the tail or ear. Pups were restrained with one hand, holding the tail between thumb and forefinger. After anaesthetising the tail-tip with ethyl chloride (Roche Consumer Health, Welwyn Garden City, UK), a 2-5mm piece of the tail was cut off using sharp dissecting scissors. A silver nitrite pencil (Bray Health & Leisure, Faringdon, UK) was used to cauterise the tip of the tail. Alternatively, a 2-5mm ear biopsy was taken from the rat by either cutting or punching.

4.2.9.2.1.2 Extraction of genomic DNA from tail/ear biopsies

Tail/ear biopsies were placed into 200µl of STE buffer containing 500µg/ml proteinase K (Melford Labs). The tissue was incubated at 55^{0}C until degraded (4-6h) or overnight. 200µl of phenol (equilibrated with Tris-HCl, pH 8.0) was then added and mixed vigorously (without vortexing) for 3min. After centrifuging at 21,004xg (Micromax RF; IEC) for 3min, the upper aqueous phase and white interphase were transferred to a fresh 1.5ml Eppendorf tube. Subsequently, 200µl of phenol:chloroform:isoamyl alcohol [25:24:1 (*v/v*)] was added and mixed vigorously (without vortexing) for 2min. After centrifugation at 21,004xg for 2min, 50µl of 3M NaAc (pH 6.0) and 500µl of 100% ethanol were added to the transferred upper aqueous phase to a fresh 1.5ml tube. The tube was inverted several times until the DNA precipitated. The tube was then centrifuged at 21,004xg for 10min and the supernatant was decanted. After air-drying, the pellet was washed in 1ml of 100% ethanol. Following mixing by inversion and centrifugation at 21,004xg for 10min, the supernatant was carefully pipetted off and the pellet was air-dried for 15-30min. DNA

was resuspended in 50-100µl of TE buffer (pH 8.0) overnight at 4^0C. DNA concentration was quantified by spectrophotometry.

4.2.9.2.2 Southern blot analysis

The technique involved the transfer and immobilisation of DNA fractionated by agarose gel electrophoresis onto a Hybond-N membrane (Amersham). Hybridisation was performed using a ^{32}P-labelled DNA probe (N4WBP4) to identify homologous DNA sequences present on the membrane. Restriction enzyme analysis was carried out using the restriction endonucleases EcoRI, HindIII, and XhoI since these enzymes produce average fragment sizes of 5, 4 and 7 kb respectively in genomic DNA. 20µg of genomic DNA was restricted overnight at 37^0C. The reaction mixture contained 20µg DNA (1µg/µl), 1:10 of the enzyme buffer and restriction endonuclease, and water to a total volume of 20µl. Digested samples were resolved on a 1% agarose gel. After photographing, the agarose gel was then submerged in depurination buffer for 15-30min, followed by denaturation buffer for 30min. The gel was then equilibrated in transfer buffer for 10-15min and the digested DNA bands from the gel were transferred to Hybond-N membrane (Amersham) by capillary blotting. The following day, the membrane was air-dried for 10min followed by 30min baking at 80^0C. Then, the DNA was cross-linked by UV treatment (Stratalinker, Stratagene). The membrane was briefly rinsed with wash buffer and pre-hybridised in 15ml pre-warmed (65^0C) hybridisation buffer for 30min at 65^0C. The membrane was hybridised overnight at 65^0C in 10ml ^{32}P-labelled-specific probe (N4WBP4). Following overnight hybridisation, the membrane was washed in pre-warmed (65^0C) wash buffer for 20min at RT followed by 2-4 washes for 30min each at 65^0C, until background radiation levels became minimal. The membrane was finally sealed in a plastic bag and exposed to a phosphor screen for three days.

4.2.10 Statistical Analysis

Results are expressed as the mean value ± SEM. Differences between groups were compared by Student's *t* test. When more than two groups were compared, differences were determined by one-way analysis of variance (ANOVA) followed by multiple comparisons using either the Dunnett's (vs. control) or Bonferroni's (for selected pairs) multiple comparison test. *p*-values less than 0.05 were considered significant.

4.3 RESULTS

4.3.1 Body Weight

The body weight of the mutant female rats was observed to be significantly lower than their wildtype littermates throughout most of the 1-12 week period of life (Figure 4.1). A difference in weight between the wildtype and the mutant females was evident throughout this period, but significant differences were found at P9-P14, P29, P36, and P42-P84. Mutant females thus exhibit juvenile-onset growth retardation.

4.3.2 Postmortem Analysis

The postmortem examination performed in mutant male and female neonates, and adult females, demonstrated apparently normal internal organs with the exception of the kidney. On gross inspection, a clear distinction between the wildtype and mutant kidney with respect to its colour, texture, and size was observed. The mutant kidneys were paler, non-smooth and even granular in appearance. When compensated for body weight, mutant kidney weight was higher compared with wildtype littermates (Figure 4.2). This increase was statistically significant in adult (11wk) females ($P<0.05$), as observed previously by Slade (2001). Some variation in the penetrance of this phenotype was noticed. It is of note that the mutant neonatal male bladders were devoid of urine.

4.3.3 Kidney Histology

Microscopically, the mutant kidney exhibited some alteration of the normal cortico-medullary architecture. A decrease in the number of glomeruli was observed in neonatal mutant males (17.0% ± 8.5) and adult females (37.0% ± 3.0) compared with their wildtype counterparts (Figure 4.3). This decrease was statistically significant in adult (20wk) females ($P<0.001$). The tubule length was not subjected to any measurement. Visual inspection of H&E-stained sections under the light microscope revealed short mutant tubules compared to the wildtype ones (Figure 4.4). Other dysmorphologies such as glomerular enlargement, tubular ectasia (with some cystic changes), tubular atrophy, fibrosis with mononuclear cell infiltration, and colloid-filled tubules were also detected (Figures 4.4-4.6). These features were more

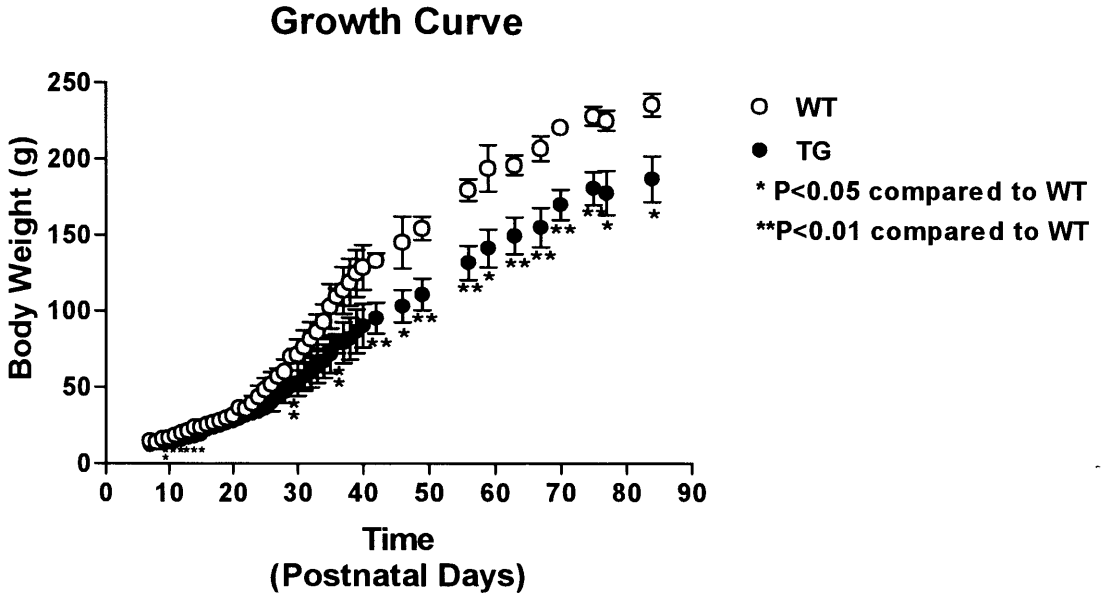


Figure 4.1: Growth curve showing the weight difference between wildtype and mutant females from week1-week12 (WT, n=2-17; TG, n=3-11).

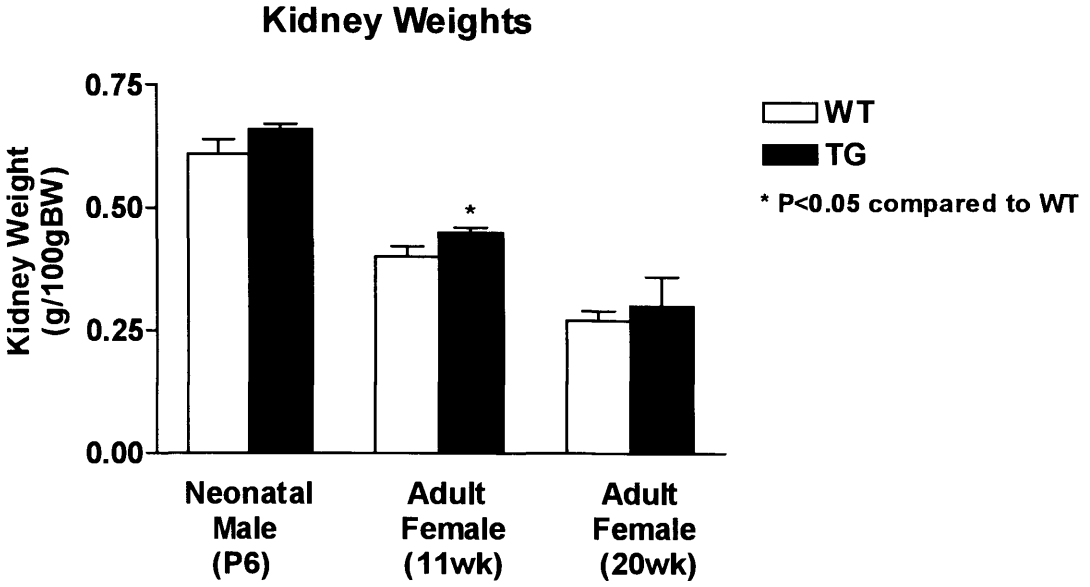


Figure 4.2: Weight differences between wildtype and mutant kidneys [P6 male group, n=3; 11wk female group, n=4; 20wk female group, n=2].

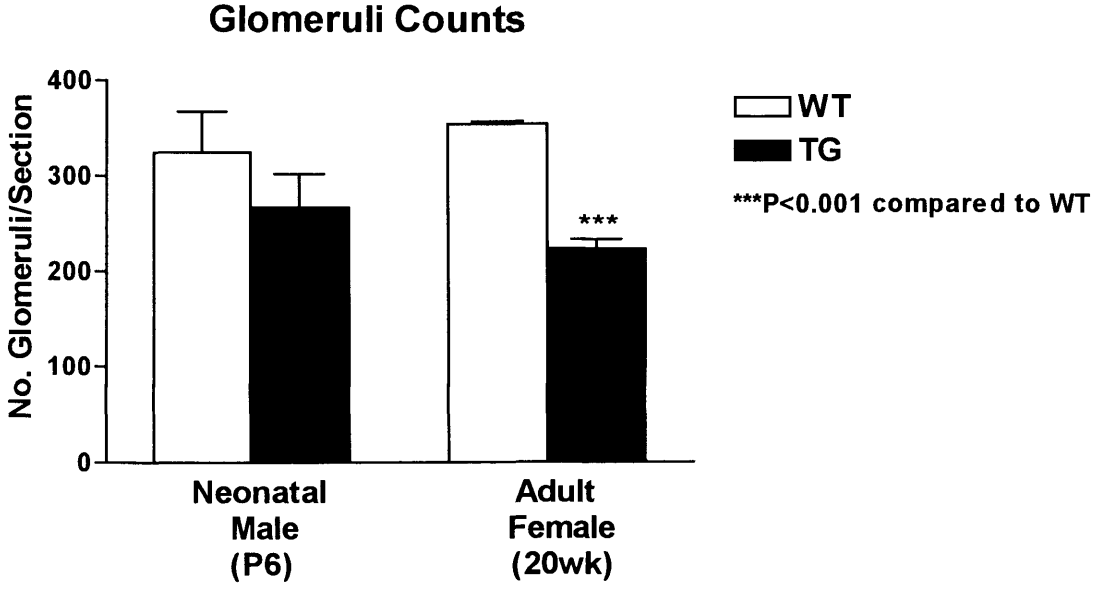


Figure 4.3: Number of glomeruli in wildtype and mutant rats (In each group: WT, n=3; TG, n=3).

Figure 4.4: Light microscopic analysis demonstrates an alteration of the normal cortico-medullary architecture in 78N neonatal male rat kidney [(a, wt ***vs*** b, tg); cortex (arrow), medulla (block arrow)] (*25X*) (Scale bar = 639μm). Reduction in cellular density is observed in mutant [(a ***vs*** b; cortex (arrow) and medulla (block arrow)]; [(e ***vs*** f, block arrow)]. Note various periglomerular changes [shorter cortical proximal tubule in mutant (c ***vs*** d arrow); wider capsular space (e ***vs*** f, arrow)], tubular ectasia accompanied with distortion (arrow) or atrophy (block arrow) (g ***vs*** h) (*200X*) (Scale bar = 79μm).

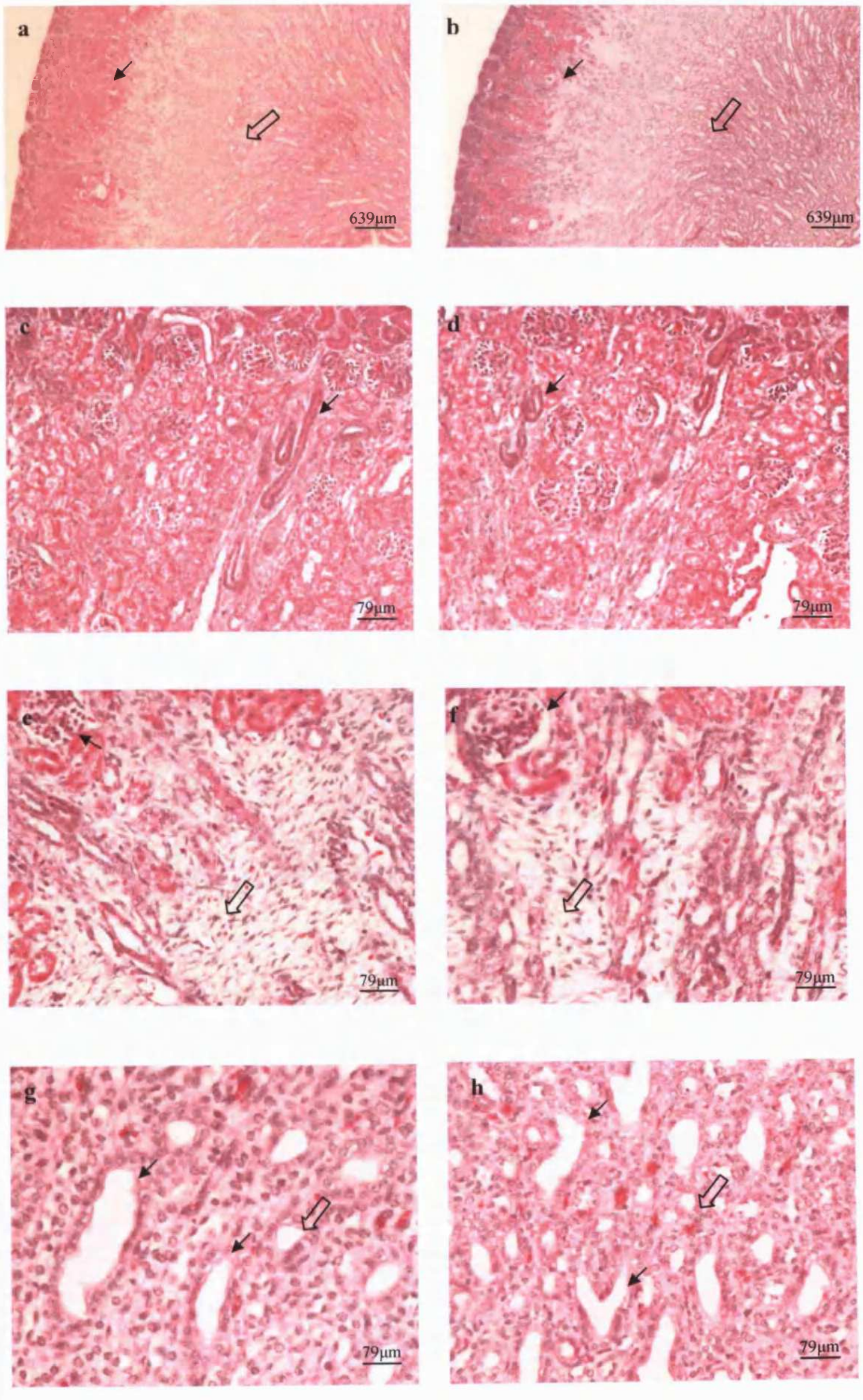
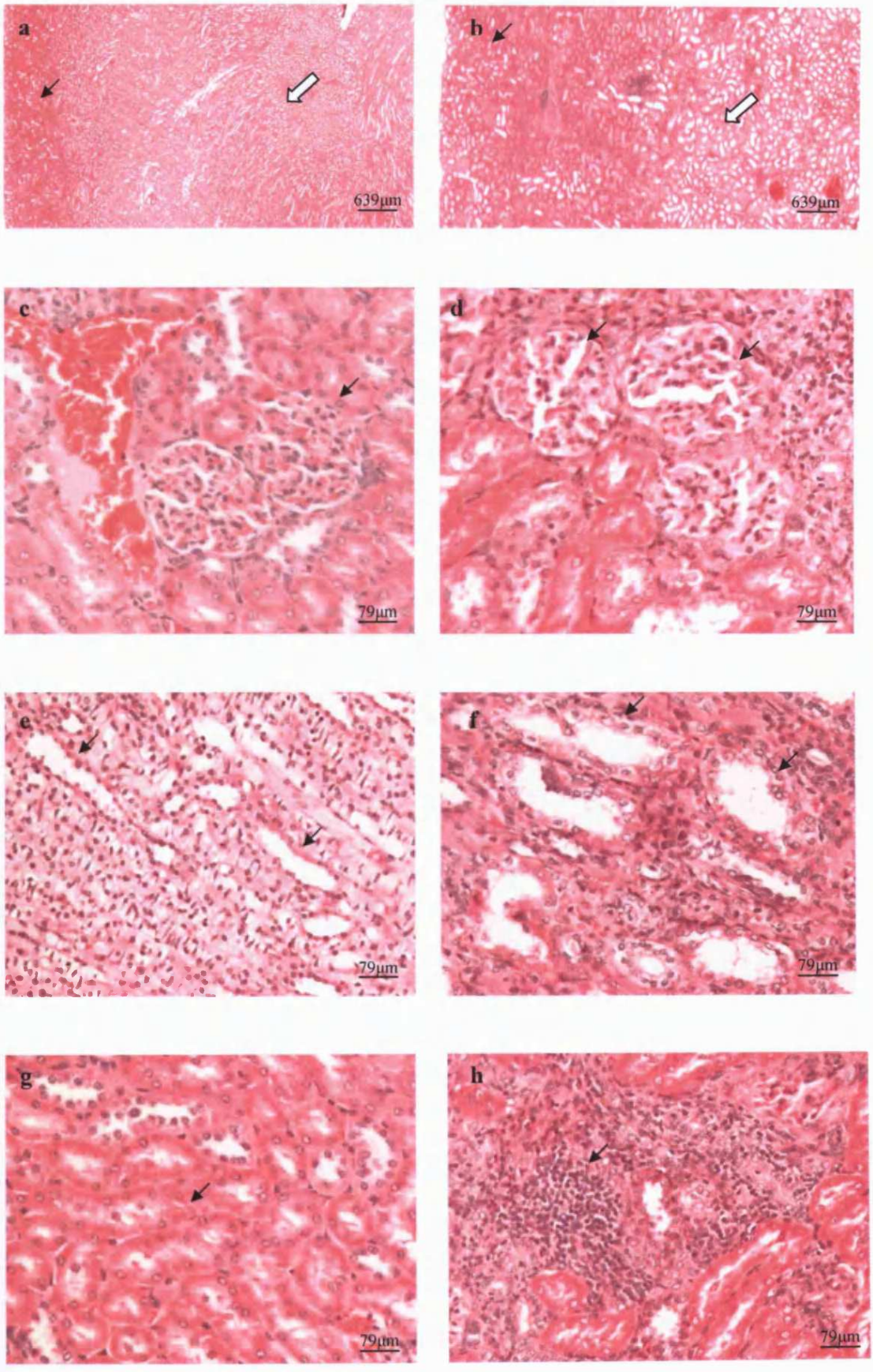
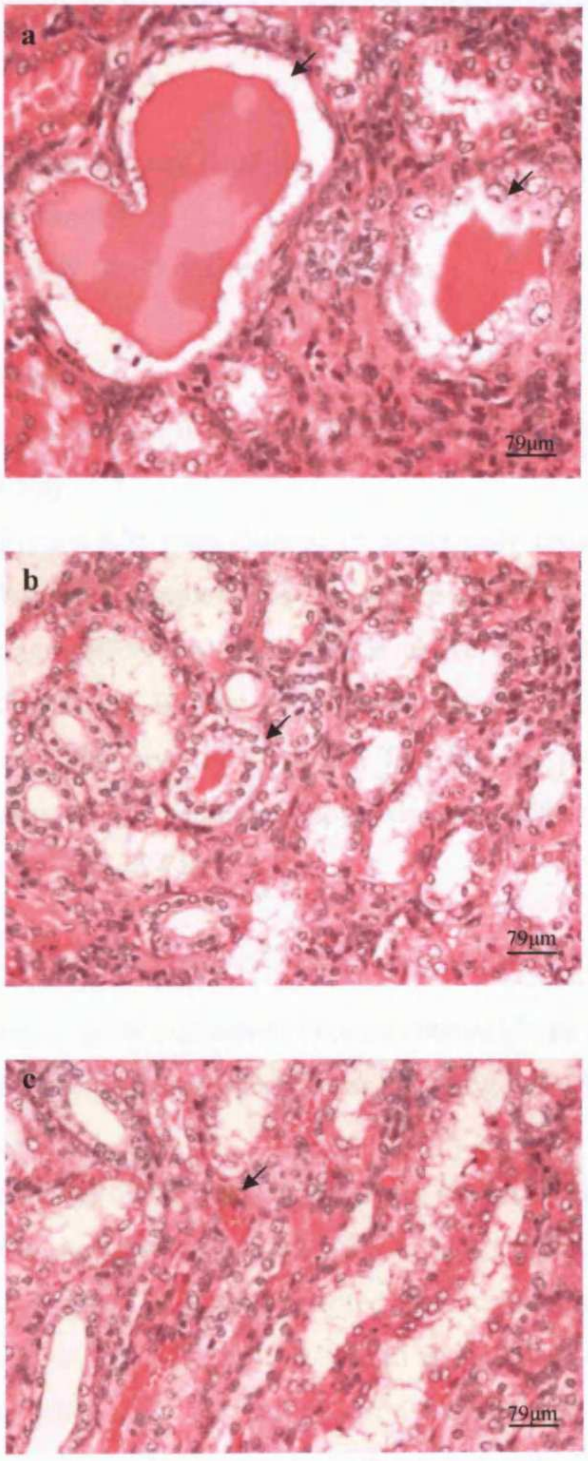

a
639µm
b
639µm
c
79µm
d
79µm
e
79µm
f
79µm
g
79µm
h
79µm
WT
TG

Figure 4.5: Light microscopic analysis demonstrates an alteration of the normal cortico-medullary architecture in 78N adult female rat kidney [(a ***vs*** b); cortex (arrow), medulla (block arrow)] (*25X*) (Scale bar = 639μm). Reduction in cellular density is observed in mutant [a ***vs*** b; cortex (arrow) and medulla (block arrow)]. Note various glomerular changes [enlargement of mutant glomeruli (c ***vs*** d, arrow)], tubular ectasia (e ***vs*** f, arrow), and the dense mononuclear cell infiltrate (g ***vs*** h, arrow) (*200X*) (Scale bar = 79μm).



WT TG

Figure 4.6: Light microscopic analysis demonstrates more pronounced features in 78N adult female rat kidney compared to neonatal male kidney such as the colloid-filled tubules (a & b, arrow). There is some evidence of mineral deposits in adult female mutant kidney (c, arrow) (*200X*) (Scale bar = 79μm).



TG

pronounced in the mutant adult females (Figures 4.5-4.6) compared with the mutant neonatal males (Figure 4.4).

4.3.4 Oligonucleotide Microarray Analysis

4.3.4.1 Filtering procedures

Filtering was conducted by Dr. D. Carter in two stages:

4.3.4.1.1 Primary filtration

In this context, the word 'genes' is used to denote individual probes, or features, of the GeneChip microarray.

Initially, all genes (Figure 4.7) were filtered to select only those genes with a raw value of >100 in at least two samples. This procedure selects those genes with a raw value (so-called average difference value) that is above an arbitrary minimal value (typically 100; Lockhart and Barlow, 2001) and thereby selects genes that are expressed at a relatively high level in the tissue. This filtering procedure generated a gene list of 2302 genes, which represents approximately 25% of the total of 8799 genes on the microarray (Figure 4.8).

4.3.4.1.2 Secondary/Manual filtration

Subsequently, differential gene expression between the wildtype and mutant samples was investigated using two independent secondary filtering procedures:

1. Data were normalised (to 1.0) to control (wildtype) values by dividing each gene by the median of that genes measurements in the two control samples. Using the Filtering tools of GeneSpring 5.0, genes were then selected that exhibited normalised values of either greater than 1.8, or less than 0.56 (1/1.8) in at least two of the samples. The value of 1.8 was selected from the criteria of Lockhart and Barlow (2001) for differentially expressed genes. In the mutants, this generated candidate gene lists of 18 up- and 20 down-regulated genes. To remove either inconsistent or replicate genes, the application of manual filtering resulted in a revised list of 16 up-regulated genes (Figure 4.9), and 9 down-regulated genes (Figure 4.10).

2. Each individual (blocked) data set was normalised to the individual control (wildtype) samples. Using the Filtering tools of GeneSpring 5.0, genes were then

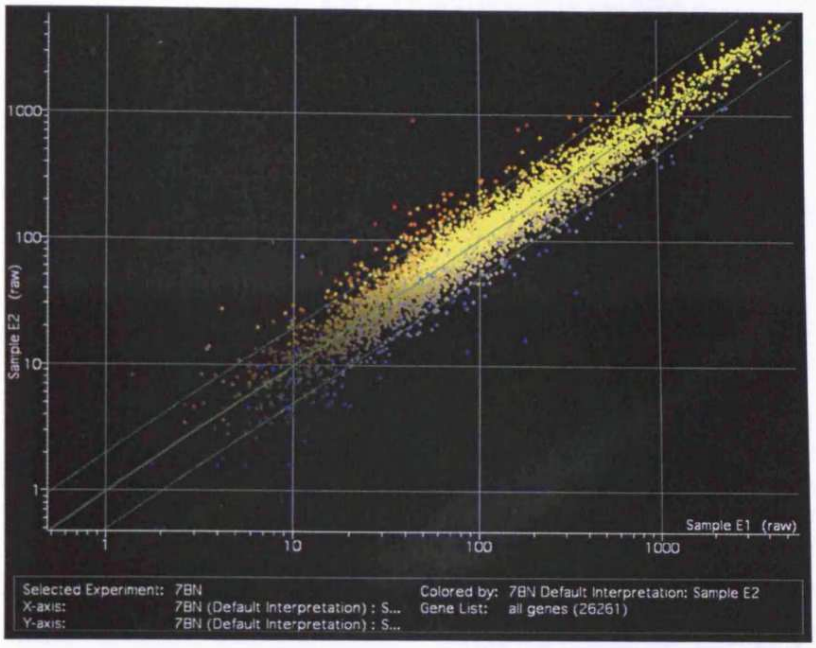


Figure 4.7: Gene signal intensities of mutant (E2, Y-axis) versus wildtype (E1, X-axis) plotted on a Log/Log scale. Comparison between E1 and E2 after scaling the average signal intensity of each array to 100. All genes (8799) from the U34A chip are represented. The colour representation is as follows: grey (no expression), yellow (E1=E2), red (E2>E1), and blue (E2<E1).

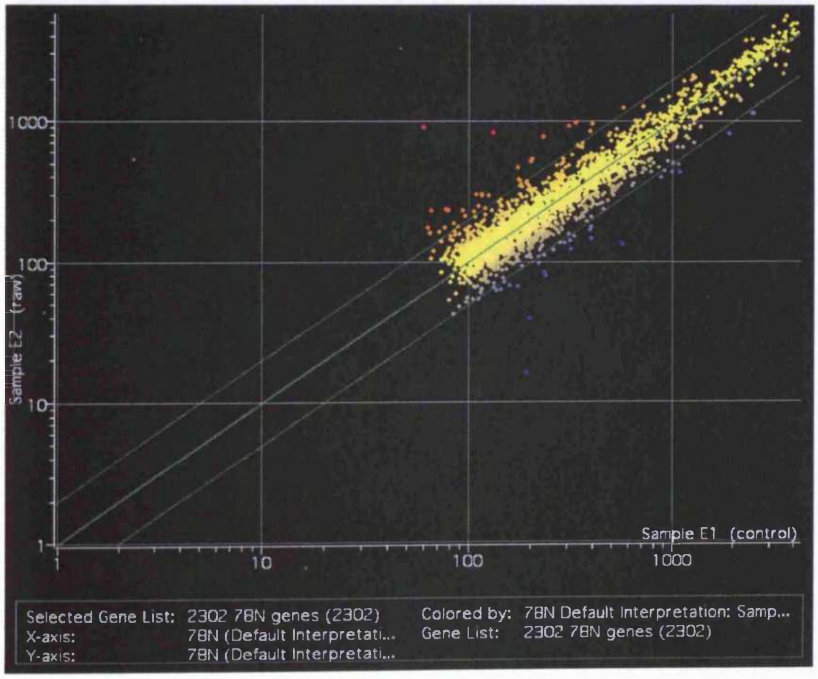


Figure 4.8: The same data as in Fig.4.7 after primary filtration; genes with a raw value >100 have been selected. This selection creates 2302 genes, equivalent to 25% of the total gene set (8799). Colour code is the same as Fig.4.7.

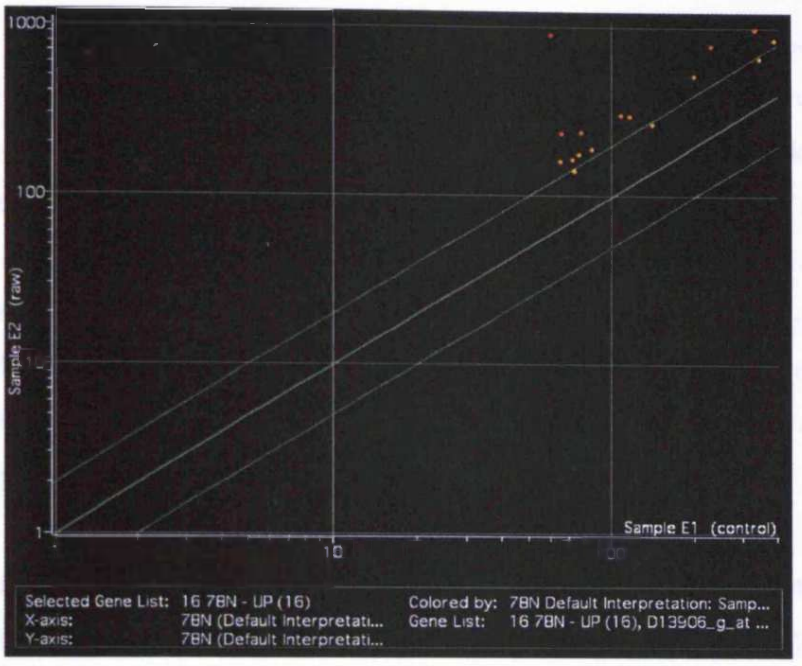


Figure 4.9: The same data as in Fig.4.8 after normalisation, and secondary and manual filtration. This approach leads to the generation of a list of 16 up-regulated genes in the mutant rat kidney.

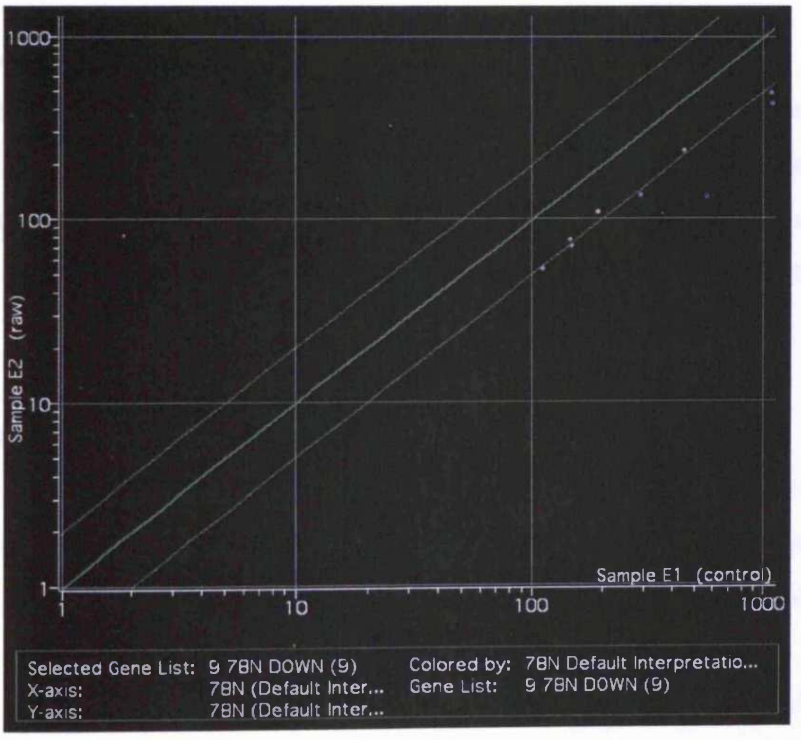


Figure 4.10: The same data as in Fig.4.8 after normalisation, and secondary and manual filtration. This approach leads to the generation of a list of 9 down-regulated genes in the mutant rat kidney.

selected that exhibit normalised values of either greater than 1.5, or less than 0.67 (1/1.5) in at least two of the samples. The value of 1.5 (rather than 1.8) was selected because a less conservative value was considered appropriate for the blocked samples. In the mutants, this procedure generated candidate gene lists of 112 (D) and 191 (E) up-regulated genes, and 115 (D) and 196 (E) down-regulated genes. Afterwards, the Venn diagram tool of GeneSpring 5.0 was used to merge the (D) and (E) gene lists. In the mutants, this produced lists of 32 up-regulated genes (Figure 4.11), and 15 down-regulated genes (Figure 4.12), respectively. Once more, manual filtering resulted in a revised up-regulated gene list of 28 up-regulated genes.

Using the Venn diagram tool, these lists were then compared to the respective lists generated in (1) above. A large degree of overlap in the up- and down-regulated genes lists was observed in each case. However, two non-overlapping genes were added to each of the lists generated in (2), providing final candidate gene lists of 30 up-regulated (Tables 4.1&4.2), and 17 down-regulated (Tables 4.3&4.4) genes [see the combined gene list in Table 4.5].

4.3.4.2 Data analysis

Candidate differentially expressed genes were derived using simple criteria of 'fold-difference', coupled with consistency between the two sets of blocked samples. The omission of statistical procedures is appropriate given the small number of biological replicates (2 groups of 2 animals), and our use of northern blots for candidate gene validation.

4.3.5 Candidate Gene Validation – Northern Analysis

To validate which genes in the lists are reliable candidate genes, northern blot analyses were performed on two of the most interesting down-regulated genes:

(1) The kidney androgen-regulated protein [KAP (U25808); average fold-decrease (>3.0) in mutant compared with wildtype]

(2) The neural precursor cells expressed developmentally down-regulated (Nedd) WW domain binding protein [N4WBP4 (AI639058); average fold-decrease (>2.0) in mutant compared with wildtype]

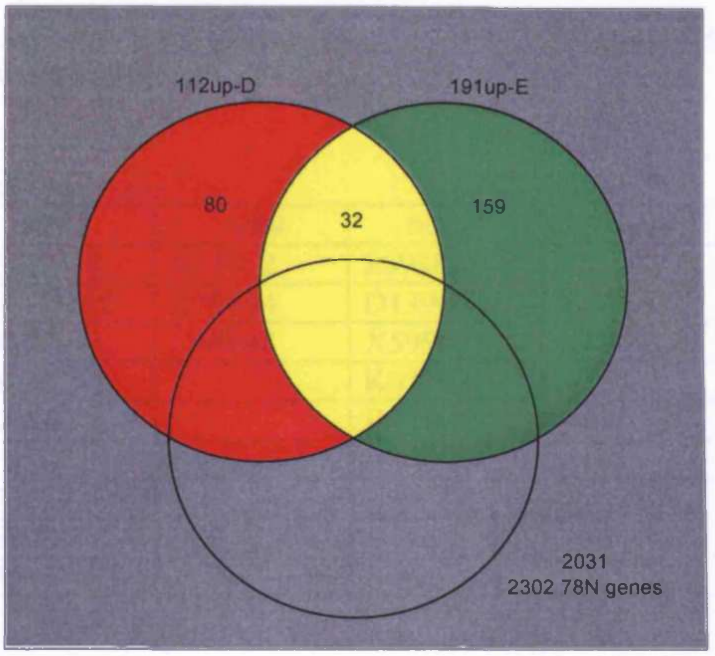


Figure 4.11: Venn diagram showing the up-regulated gene list (n=32) shared among three sets (Total Set, Set D, and Set E). The colour representation is as follows: grey (Total gene Set), red (Set D genes), green (Set E genes), and yellow (Shared gene Set).

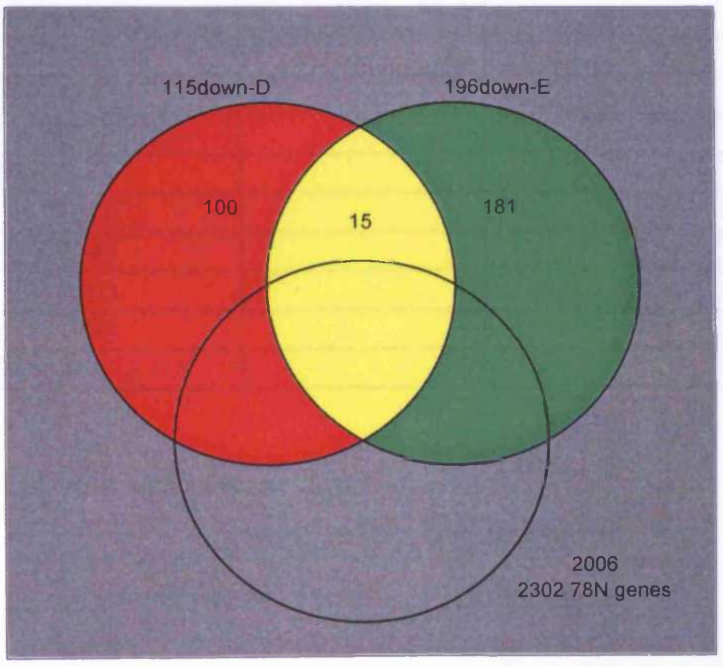


Figure 4.12: Venn diagram showing the down-regulated gene list (n=15) shared among three sets (Total Set, Set D, and Set E). Colour code is the same as Fig.4.11.

Table 4.1: The candidate gene (Accession no.) list of up-regulated genes (n=30) in 78N neonatal male rat kidney

*A. Wildtype signal level**

>50	*>100*	*>200*	*>300*	*>400*	*>1000*
M58634	AI179610	X17012	S69383	L23148	AA891785
AA891054	AA875620	X59864	D13906	M57664	
AJ224879	AI178971	U44948	X59961	AI008638	
L19998	AI230354		K03243		
M74067	X75207		D17695		
X96437	J02612				
AI229637	Z75029				
AA800613	D86297				
X94185	AI014169				

*B. Fold increase**

>1.5	*>2.0*	*>3.0*	*>5.0*	*>10.0*
AI229637	AA891054	AI178971		M58634
AA800613	AJ224879	AI014169		
AA875620	L19998			
X75207	M74067			
J02612	X96437			
Z75029	AI179610			
D86297	X94185			
X17012	AI230354			
X59864	S69383			
U44948	D13906			
X59961	K03243			
M57664	D17695			
AI008638	L23148			
AA891785				

*Values observed in both sets of array data.

Table 4.2: The candidate gene list of up-regulated (n=30) genes in the *78N* neonatal male rat kidney

Accession no.	***Gene Name***	***Wildtype signal level**** *(Average difference value)*	***Fold-Change****
AA800613	TIS11/ Zfp36	>50	>1.5
AA875620	Hsp70.3	>100	>1.5
AA891054	4.5 S RNA	>50	>2.0
AA891785	NADP+-Specific Isocitrate Dehydrogenase-Like	>1000	>1.5
AI008638	O-acetyl GD3 ganglioside synthase/Mfge8	>400	>1.5
AI014169	Thioredoxin Interacting Factor (*Vdup1*)	>100	>3.0
AI178971	Alpha-Globin	>100	>3.0
AI179610	Heme Oxygenase (p32 Protein)	>100	>2.0
AI229637	MYB Binding Protein 1a (*Mybbp1a*)	>50	>1.5
AI230354	Phosphatidate Phosphohydrolase Type 2 (*Ppap2a*)	>100	>2.0
AJ224879	Collagen Alpha 1 Type II	>50	>2.0
D13906	ADH Water Channel (AQP2)	>300	>2.0
D17695	Aquaporin 3 (AQP3)	>300	>2.0
D86297	Erythroid-Specific Delta-Aminolevulinate Synthase	>100	>1.5
J02612	UDP-Glucuronosyltransferase	>100	>1.5
K03243	Phosphoenolpyruvate Carboxykinase (GTP)	>300	>2.0
L19998	Minoxidil Sulfotransferase	>50	>2.0
L23148	Inhibitor of DNA-Binding (*Id1*)	>400	>2.0
M57664	Creatine Kinase-B (*CKB*)	>400	>1.5
M58634	IGFBP-1	>50	>10
M74067	EST	>50	>2.0
S69383	12-Lipoxygenase	>300	>2.0
U44948	Smooth Muscle LIM Protein	>200	>1.5
X17012	IGFII	>200	>1.5
X59864	Adult Skeletal Muscle Gene (*ASM15*)	>200	>1.5
X59961	H2A and H2B Histones	>300	>1.5
X75207	Cyclin D1	>100	>1.5
X94185	MAP Kinase Phosphatase (*MKP-3*)	>50	>2.0
X96437	PACAP-Responsive Gene 1 (*PRG1*)	>50	>2.0
Z75029	Hsp70.2	>100	>1.5

*Values observed in both sets of array data.

Table 4.3: The candidate gene (Accession no.) list of down-regulated genes (n=17) in 78N neonatal male rat kidney

*A. Wildtype signal level**

>100	*>200*	*>400*	*>500*	*>600*	*>900*	*>1000*
Y09945	M83107	M83740	U25808	U07971	U22424	AI639058
AJ010828	S72594	AI172064			S75960	AI232087
AA86649	AB01048					
AA89320	AI639012					
AI639097						

*B. Fold decrease**

>1.5	*>2.0*	*>3.0*	*>5.0*
AJ010828	Y09945	U25808	
AA866419	AA893230		
AI639097	AB010428		
M83107	AI639058		
S72594	AI232087		
AI639012			
M83740			
AI172064			
U07971			
U22424			
S75960			

*Values observed in both sets of array data.

Table 4.4: The candidate gene list of down-regulated (n=17) genes in the *78N* neonatal male rat kidney

Accession no.	*Gene Name*	*Wildtype signal level** *(Average difference value)*	*Fold-Change**
AA866419	PDGF-α	>100	>1.5
AA893230	Calmodulin-Like	>100	>2.0
AB010428	Acyl-CoA Hydrolase	>200	>2.0
AI172064	Lectin, Galactose Binding, Soluble 1 (*Lgals1*)	>400	>1.5
AI232087	(S)-2-Hydroxyacid Oxidase (Glycolate Oxidase)	>1000	>2.0
AI639012	EST	>200	>1.5
AI639058	Nedd4 WW domain-binding protein 4	>1000	>2.0
AI639097	EST	>100	>1.5
AJ010828	Chemokine Orphan Receptor 1 (*RDC-1*)	>100	>1.5
M83107	Smooth Muscle 22 Alpha (*SM22*)	>200	>1.5
M83740	Dimerisation Cofactor-HNF-1 Alpha (*DCoH*)	>400	>1.5
S72594	Tissue Inhibitor of Metalloproteinase Type 2	>200	>1.5
S75960	Tamm-Horsfall Protein	>900	>1.5
U07971	L-Arginine:Glycine Amidinotransferase	>600	>1.5
U22424	11-Beta-Hydroxysteroid Dehydrogenase Type 2	>900	>1.5
U25808	KAP	>500	>3.0
Y09945	Putative Integral Membrane Transport Protein (UST1r)	>100	>2.0

*Values observed in both sets of array data.
- Highlighted items were subjected to northern blot analyses

Table 4.5: Summary list of up-regulated (n=30) and down-regulated (n=17) candidate genes in the *78N* neonatal male rat kidney

Accession no.	*Gene Name*	*Wildtype signal level** *(Average difference value)*	*Fold-Change**
AA800613	TIS11/ Zfp36	>50	>1.5
AA866419	PDGF-α	>100	>1.5
AA875620	Hsp70.3	>100	>1.5
AA891054	4.5 S RNA	>50	>2.0
AA891785	NADP+-Specific Isocitrate Dehydrogenase-Like	>1000	>1.5
AA893230	Calmodulin-Like	>100	>2.0
AB010428	Acyl-CoA Hydrolase	>200	>2.0
AI008638	O-acetyl GD3 ganglioside synthase/Mfge8	>400	>1.5
AI014169	Thioredoxin Interacting Factor (*Vdup1*)	>100	>3.0
AI172064	Lectin, Galactose Binding, Soluble 1 (*Lgals1*)	>400	>1.5
AI178971	Alpha-Globin	>100	>3.0
AI179610	Heme Oxygenase (p32 Protein)	>100	>2.0
AI229637	MYB Binding Protein 1a (*Mybbp1a*)	>50	>1.5
AI230354	Phosphatidate Phosphohydrolase Type 2 (*Ppap2a*)	>100	>2.0
AI232087	(S)-2-Hydroxyacid Oxidase (Glycolate Oxidase)	>1000	>2.0
AI639012	EST	>200	>1.5
AI639058	Nedd4 WW domain-binding protein 4	>1000	>2.0
AI639097	EST	>100	>1.5
AJ010828	Chemokine Orphan Receptor 1 (*RDC-1*)	>100	>1.5
AJ224879	Collagen Alpha 1 Type II	>50	>2.0
D13906	ADH Water Channel (AQP2)	>300	>2.0
D17695	Aquaporin 3 (AQP3)	>300	>2.0
D86297	Erythroid-Specific Delta-Aminolevulinate Synthase	>100	>1.5
J02612	UDP-Glucuronosyltransferase	>100	>1.5
K03243	Phosphoenolpyruvate Carboxykinase (GTP)	>300	>2.0
L19998	Minoxidil Sulfotransferase	>50	>2.0
L23148	Inhibitor of DNA-Binding (*Id1*)	>400	>2.0
M57664	Creatine Kinase-B (*CKB*)	>400	>1.5
M58634	IGFBP-1	>50	>10
M74067	EST	>50	>2.0
M83107	Smooth Muscle 22 Alpha (*SM22*)	>200	>1.5
M83740	Dimerisation Cofactor-HNF-1 Alpha (*DCoH*)	>400	>1.5
S69383	12-Lipoxygenase	>300	>2.0
S72594	Tissue Inhibitor of Metalloproteinase Type 2	>200	>1.5
S75960	Tamm-Horsfall Protein	>900	>1.5
U07971	L-Arginine:Glycine Amidinotransferase	>600	>1.5
U22424	11-Beta-Hydroxysteroid Dehydrogenase Type 2	>900	>1.5
U25805	KAP	>500	>3.0
U44948	Smooth Muscle LIM Protein	>200	>1.5
X17012	IGFII	>200	>1.5
X59864	Adult Skeletal Muscle Gene (*ASM15*)	>200	>1.5
X59961	H2A and H2B Histones	>300	>1.5
X75207	Cyclin D1	>100	>1.5
X94185	MAP Kinase Phosphatase (*MKP-3*)	>50	>2.0
X96437	PACAP-Responsive Gene 1 (*PRG1*)	>50	>2.0
Y09945	Putative Integral Membrane Transport Protein (UST1r)	>100	>2.0
Z75029	Hsp70.2	>100	>1.5

*Values observed in both sets of array data.
- The colour representation is as follows: - Pink, for down-regulated genes
- Blue, for up-regulated genes
- Highlighted genes were subjected to northern blot analyses

The selection of KAP and N4WBP4 for validation was based on the following criteria:

1. Both transcripts are regulated by androgens.
2. KAP is known to be the second-most abundant kidney-specific transcript as determined by SAGE (Virlon et al., 1999).
3. N4WBP4 is a chromosome 3 gene. This selection is based on preliminary fluorescent in situ hybridisation (FISH) analysis, which showed that the mutant integration event is within chromosome 3 (Slade, 2001).

Northern blot analysis showed that KAP expression was decreased in neonatal (P6) male and adult (5-6mo) female mutant kidney compared with their respective wildtypes. In neonatal (P9) mutant female kidney, KAP was not detected at all compared to the low expression in the wildype counterpart. It is of note that KAP was highly expressed in the adult female kidney compared with the moderate expression in neonatal male and low expression in the neonatal female wildtype kidney (Figure 4.13).

Reprobing these northern blots with an N4WBP4 probe revealed that N4WBP4 expression was decreased in neonatal (P6) male and adult (5-6mo) female mutant kidney compared with their respective wildtypes. In neonatal (P9) wildtype and mutant female kidneys, N4WBP4 was not detected at all. In adult females, it is of note that two transcripts of N4WBP4 were detected. The large transcript (*3kb*) was equally expressed in the wildtype and mutant rats, whereas the expression of the small transcript (*1kb*) was down-regulated in the mutant female (Figure 4.14).

4.3.6 Southern Blot Analysis

In the Southern blot analysis used to investigate the N4WBP4 gene, HindIII was found to be the most appropriate enzyme (Figure 4.15A). The N4WBP4 gene signal in neonatal wildtype and mutant males were comparable (Figure 4.15B). However, the bands were not clearly observed in neonatal wildtype and mutant females (Figure 4.15B). This finding is probably explained by partial degradation of the female genomic DNA. Therefore, further studies should be conducted to confirm this result in 78N females.

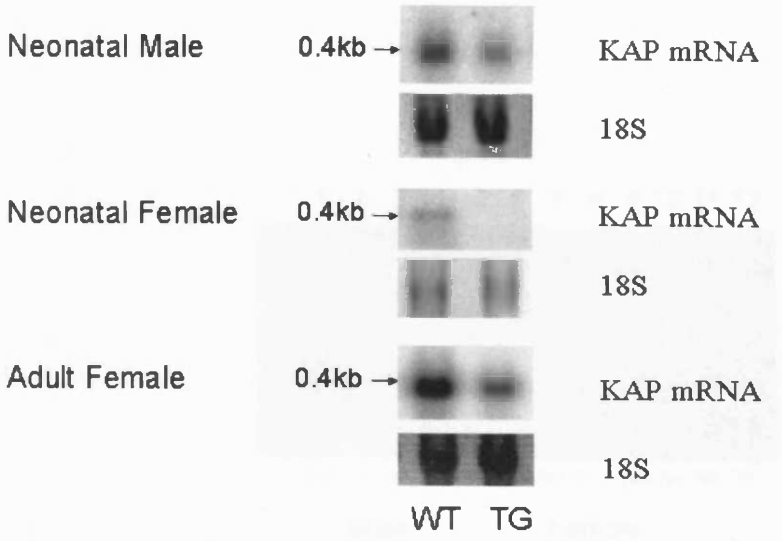


Figure 4.13: Northern blot analysis of neonatal male (P6) and female (P9), and adult female (5-6mo) kidney RNA samples (10 or 15µg). α32P-labelled cDNA's from KAP and 18S RNA genes were used as probes. WT=Wildtype, TG=Transgenic.

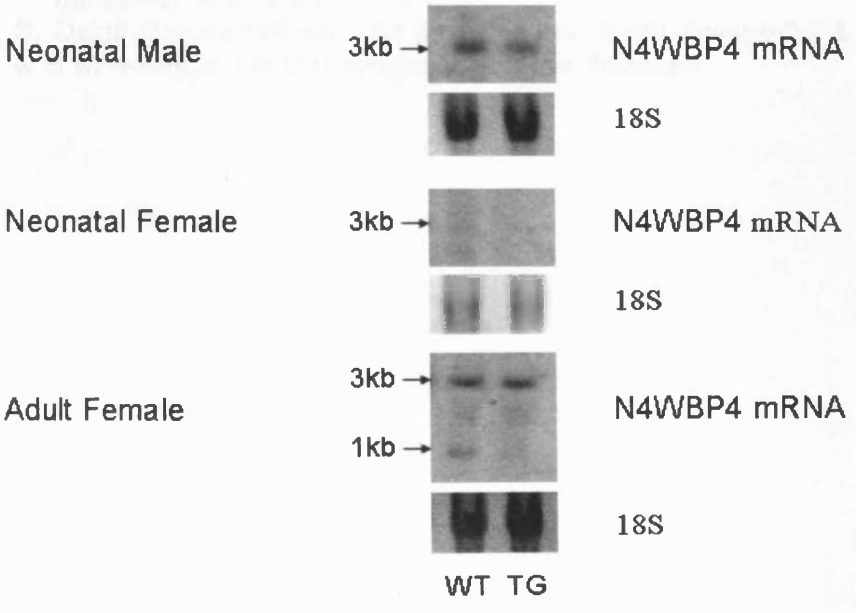


Figure 4.14: Northern blot analysis of neonatal male (P6) and female (P9), and adult female (5-6mo) kidney RNA samples (10 or 15µg). α32P-labelled cDNA's from N4WBP4 and 18S RNA genes were used as probes. WT=Wildtype,TG=Transgenic.

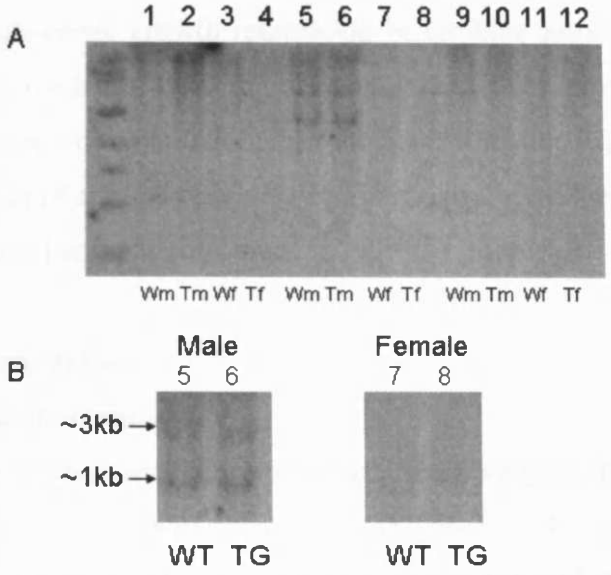


Figure 4.15: Southern blot analysis of neonatal male (P6) and female (P9) genomic DNA samples (20µg). α^{32}P-labelled cDNA's from N4WBP4 were used as probes.

A. Restriction enzyme analysis was carried out using EcoRI (lanes1-4), HindIII (lanes5-8), and XhoI (lanes9-12).

B. Detail of lanes 5&6 and 7&8 depicting the HindIII digest (~3.0 & 1.0 kb).

w or wt=wildtype, t or tg=transgenic, m=male, f=female.

4.4 DISCUSSION

A novel transgenic rat line was generated by Slade et al. (2001) that appears to be an insertional mutant. The main overt phenotypes of this mutant are premature death in males, and juvenile-onset growth retardation in females associated with patchy skin and delayed fur growth. In the first part of the discussion two issues are raised, the aetiology of the growth retardation and the male lethality. In the second part, the possible correlation of altered gene expression (including the sexually dimorphic KAP and N4WBP4 genes) and perinatal male lethality is discussed.

4.4.1 Growth Retardation

4.4.1.1 Idiopathic short stature

The initial study of this line was performed to investigate the cause of the growth retardation (Slade, 2001). It was noticed that the continued retardation in body weight of adult (11 wk) mutant females was associated with reduced skeletal growth reflected in a significant reduction in the nose-anus length and femoral length, while the femoral diameter (anterior-posterior) and strength (failure load) were unaffected. Anterior pituitary weight and pituitary growth hormone (GH) content (assessed by RIA and immunocytochemical analysis) were comparable, and episodic GH secretion was also maintained. Therefore, this postnatal growth-retardation seems not to be related to GH deficiency. It could be classified under the category of idiopathic short stature (ISS) as used in humans. ISS is a term for children with short stature of undefined aetiology where the GH-IGF-I axis is normal (Blair and Savage, 2002).

IGF-I: Free serum IGF-I levels are correlated positively with renal function (Frystyk et al., 1999). In renal failure, free circulating IGF-I is reduced due to an increase in plasma levels of IGF-binding proteins (IGFBPs) (Hirschberg and Adler, 1998; Powell et al., 1999). Elevated levels of IGFBPs, and in particular, BP-1 and -2, have been reported to be correlated inversely with stature in chronic renal failure (Tonshoff et al., 1995). IGFBP levels might contribute to a resistance to GH and IGF-I growth-promoting properties (Roelfsema and Clark, 2001) if the 78N females are suffering from a renal deficiency. Further investigation is needed in this respect, particularly because preliminary serum IGF-I analysis (data not shown) indicated a marked decrease in adult mutant females compared with wildtype.

4.4.1.2 Other considerations

4.4.1.2.1 Correlation between growth retardation and kidney phenotype

The kidney abnormality was noticed in 78N rats during the parallel postmortem analysis. The augmentation in mutant kidney weight reflects kidney enlargement. Kidney size increases in a number of physiological and pathological situations. The growth could be either of a hypertrophic or hyperplastic nature (Liu and Preisig, 2002), and can be associated with structural and functional adaptations to compensate for impairment (Brenner, 1985; Preisig, 1999). Although the decrease in glomeruli number observed in 78N kidneys favours hypertrophy, no conclusion can be drawn unless proper stereological assessement (Nyengaard, 1999) is performed. The diminution in the glomeruli number in mutant kidneys supports the concept that the reduced number of renal elements (i.e. glomeruli and/or nephrons) may contribute to impaired renal function (Lelievre-Pegorier and Merlet-Benichou, 2000; Tulassay and Vasarhelyi, 2002). In the literature, a correlation between low birth weight and renal disturbances has been reported (Tulassay and Vasarhelyi, 2002). Low birth weight neonates have short-term renal-related pathology, manifested by increased risk of acute renal failure and transient imbalance of fluid and electrolyte homeostasis as well. However, low birth weight adults are at risk of developing chronic renal disease. Although birth weight does not appear to be affected in the 78N line, it is reasonable to assume that the renal disturbance may be directly related to the juvenile-onset growth retardation.

4.4.1.2.2 Kidney morphology

Renal damage was assessed by morphological analysis at the light microscope level. In mutants, a decrease in the tissue mass and in glomeruli number was observed. Periglomerular modifications were also noticed. However, when there is a lack of definitive alteration in glomerular structure from that observed in normal tissue, minimal change nephropathy or disease (MCD) is a light microscopy-based definition of idiopathic nephrotic syndrome (Siegel, 2001). Moreover, some cystic changes were detected at the tubular levels. Cysts are common features in kidney disease. They can be encountered in congenital disorders (e.g. autosomal recessive polycystic kidney disease) or inherited polycystic diseases (e.g. autosomal dominant polycystic kidney disease). However, they can also appear as single or a few simple renal cysts unrelated to any disease, and they become more frequent with age (Fick-Brosnahan,

2001) as seen when comparing 78N neonatal male and adult female kidneys. In addition, an increase in renal tubular volume reflecting nephron hypertrophy (an increase in the nephron mass, not number) may indicate a hyperfiltration state (osmotic diuresis) as encountered in diabetes mellitus (Caramori and Mauer, 2001). Furthermore, interstitial cellular infiltrate is a sign of tubulo-interstitial damage and infection of the ascending urinary tract (i.e. Pyelonephritis). In chronic conditions, besides dissolution of normal interstitial architecture, interstitial fibrosis develops accompanied by tubular ectasia, tubular atrophy, and colloid-filled tubules – features of tubulo-interstitial disease (Finn, 2001). To allow proper classification of the proposed renal disease in the 78N model, stereological assessement of the kidney sections concomitant with electron microscopy and clinical tests [i.e. blood pressure, biochemical analyses (glucose, cholesterol, and protein)] should be carried out.

4.4.2 Neonatal Lethality in Males

4.4.2.1 Sexual Dimorphism

Whatever the clinical condition encountered in the 78N mutant, it appears to be acute in males and chronic in females. In general, progression of renal disorders differs between males and females (Seliger et al., 2001). In addition to genetically determined differences between the sexes in renal structure and function, sex hormones may directly influence many of the processes implicated in the pathogenesis of renal disease progression (Neugarten and Silbiger, 1995). In animals, the rate of progression of certain renal diseases (e.g. chronic renal disease, membranous nephropathy, IgA nephropathy, and polycystic kidney disease) is greater in males than in females (Seliger et al., 2001). This gender disparity is underlined by the direct effect of sex hormones (testosterone in male vs oestrogen in female) on kidney cells. By using various animal models of renal disease, investigators have noted the more serious progression of disease due to the presence of testosterone in males compared to the protective role of oestrogen (by exerting potent antioxidant effects) in females (Neugarten and Silbiger, 1995; Silbiger and Neugarten, 2003).

4.4.2.2 Urine and serum creatinine

Routine urine analysis (i.e. chemical tests and microscopic examination of the formed elements of the urine; Swan and Keane, 2001) provides essential and rapid information about a number of primary renal and systemic disorders. Although urine

analysis including urine volume, osmolality, pH, salts (Na^+, K^+, Ca^{++}, and Cl^-), and urea, conducted in adult (20wk) mutant females (Slade, 2001) showed normal values compared to controls (Slade, 2001), the assessment of renal function by urine analysis in neonatal mutant males is difficult. During the postmortem examination, it has been noticed that the bladders of mutant males were devoid of urine, which could be an indication of renal failure. Therefore, in this situation analysis would be limited to blood tests. Urea and creatinine are considered markers of harmful toxins produced by the body. A blood creatinine test is a more reliable guide of kidney function than a urea test since blood urea level is affected by parameters (e.g. amount of dietary protein) other than the kidney (Swan and Keane, 2001; Stein and Wild, 2002). Moreover, serum creatinine levels can reflect both the occurrence of kidney failure and its stage. In rats of both sexes, plasma creatinine values increase during growth and maturation (Tsuchiya et al., 1995). However, in kidney failure, blood creatinine levels start to increase slowly at the beginning, to reach a high level at end stage (Stein and Wild, 2002). Preliminary results (data not shown) showed a decrease in creatinine values in male and female mutants compared with wildtypes. Further analyses are clearly required.

Other factors that may relate to neonatal male lethality are addressed below.

4.4.2.3 Multi-organ failure

One possible cause of death in mutant males is multi-organ failure (MOF). As observed in the mutant rat, the kidney is the earliest organ to deteriorate in human MOF neonates (Avanoglu et al., 1997). The rat model of the human autosomal dominant polycystic kidney disease (ADPKD) (Hannover rat, Han:SPRD cy/+; Gretz et al., 1996), the murine (BALB/c-cpk; Gattone et al., 1996; Ricker et al., 2000) and human autosomal recessive polycystic kidney disease (PKD) also manifest multi-organ pathology (Gattone et al., 2002). Moreover, one transgene-insertion mouse mutant line generated by Hirotsune et al. (2003) shares some phenotypes with 78N mutants. 80% of the heterozygote mice die shortly after birth from multi-organ failure. The survivors exhibit polycystic kidneys and bone deformities accompanied with other pleiotropic phenotypes such as microscopic liver cysts, skin defects in embryos, and early eyelid opening.

4.4.2.4 Hypothyroidism

In a rat model of congenital hypothyroidism, marked postnatal growth retardation is accompanied by perinatal changes in the IGF system (Nanto-Salonen et al., 1991). Moreover, skin and kidney abnormalities were also reported (Hornstein, 1984; Schmitt et al., 2003). In neonatal rat kidney, thyroid hormone differentially regulates cellular development (Slotkin et al., 1992). In hypothyroid [propylthiouracil (PTU)-treated] neonatal rat, primary shortfalls in renal cell number followed by deficits in cell packing density and cell size were observed. Hypothyroidism is also associated with abnormalities in renal water handling. This is reflected by an increase in the expression of aquaporin (AQP) channels (i.e. AQP1-3) (Yeum et al., 2002) and by a reduction of the density of vasopressin receptors per cell (Ali et al., 1988) (see section 4.4.5). More investigation should be carried out on the hypothalamo-pituitary-thyroid axis to determine if any of its elements play a role in the 78N phenotypes and/or death of the males.

4.4.3 Candidate Genes

To study the tissue expression and function of genes, several high-throughput methods have been used (see Cheval et al., 2002). These sophisticated methods make possible the characterisation of tissue-specific gene expression profiles (i.e. Transcriptomes). They are based either on serial, partial sequencing of cDNAs (sequencing of ESTs) and serial analysis of gene expression (SAGE), or on parallel hybridisation of labelled cDNAs to specific probes immobilised on a grid (macro- and micro- arrays).

To identify gene(s) associated with the 78N phenotype, the Affymetrix high-density gene oligonucleotide microarray technology was used to analyse kidney gene expression in the neonatal mutant males. A candidate differentially expressed gene list of 30 'up-regulated', and 17 'down-regulated' genes was produced. The over- and under-expressed genes with an average fold-change value greater than 1.5 were reported. Notable changes have occurred in the renal tissue of the 78N neonatal male. The affected genes appear to be involved in several cellular processes including metabolism, cell structure, protein turnover, signal transduction, transcription, growth, and others (see Table 4.6, for functional categories) (see sections below for more detail).

Table 4.6: Classification of the up-regulated (n=30) and down-regulated (n=17) genes in the *78N* neonatal male rat kidney

Accession no.	*Gene Name*	*Wildtype signal level** *(Average difference value)*	*Fold-Change**
Ribosomal Proteins			
AA891054	4.5 S RNA	>50	>2.0
Protease/Protease Inhibitor			
S72594	Tissue Inhibitor of Metalloproteinase Type 2	>200	>1.5
Heat-shock Proteins			
AA875620	Hsp70-3	>100	>1.5
Z75029	Hsp70.2	>100	>1.5
Metabolic Enzymes			
AA891785	NADP+-Specific Isocitrate Dehydrogenase-Like	>1000	>1.5
AB010428	Acyl-CoA Hydrolase	>200	>2.0
AI008638	O-acetyl GD3 ganglioside synthase/Mfge8	>400	>1.5
AI230354	Phosphatidate Phosphohydrolase Type 2 (*Ppap2a*)	>100	>2.0
AI232087	(S)-2-Hydroxyacid Oxidase (Glycolate Oxidase)	>1000	>2.0
D86297	Erythroid-Specific Delta-Aminolevulinate Synthase	>100	>1.5
J02612	UDP-Glucuronosyltransferase	>100	>1.5
L19998	Minoxidil Sulfotransferase	>50	>2.0
S69383	12-Lipoxygenase	>300	>2.0
U07971	L-Arginine:Glycine Amidinotransferase	>600	>1.5
U22424	11-Beta-Hydroxysteroid Dehydrogenase Type 2	>900	>1.5
Cytoskeletal/Microtubule			
AJ224879	Collagen Alpha 1 Type II	>50	>2.0
M83107	Smooth Muscle 22 Alpha (*SM22*)	>200	>1.5
U44948	Smooth Muscle LIM Protein	>200	>1.5
X59864	Adult Skeletal Muscle Gene (*ASM15*)	>200	>1.5
Immunoglobin Associated			
AI178971	Alpha-Globin	>100	>3.0
AI179610	Heme Oxygenase (p32 Protein)	>100	>2.0
Growth/Growth Factor Related			
AA866419	PDGF-α	>100	>1.5
M58634	IGFBP-1	>50	>10
X17012	IGFII	>200	>1.5
X96437	PACAP-Responsive Gene 1 (*PRG1*)	>50	>2.0

Signal Transduction/Kinase/Phosphatase

AA893230	Calmodulin-Like	>100	>2.0
K03243	Phosphoenolpyruvate Carboxykinase (GTP)	>300	>2.0
M57664	Creatine Kinase-B (*CKB*)	>400	>1.5
X94185	MAP Kinase Phosphatase (*MKP-3*)	>50	>2.0

Transcription

AA800613	TIS11/ Zfp36	>50	>1.5
L23148	Inhibitor of DNA-Binding (*Id1*)	>400	>2.0
M83740	Dimerisation Cofactor-HNF-1 Alpha (*DCoH*)	>400	>1.5
X59961	H2A and H2B Histones	>300	>1.5
X75207	Cyclin D1	>100	>1.5

Transporter/Membrane Proteins/Receptor

AJ010828	Chemokine Orphan Receptor 1 (*RDC-1*)	>100	>1.5
D13906	ADH Water Channel (AQP2)	>300	>2.0
D17695	Aquaporin 3 (AQP3)	>300	>2.0
Y09945	Putative Integral Membrane Transport Protein (UST1r)	>100	>2.0

Binding Proteins

AI014169	Thioredoxin Interacting Factor (*Vdup1*)	>100	>3.0
AI172064	Lectin, Galactose Binding, Soluble 1 (*Lgals1*)	>400	>1.5
AI229637	MYB Binding Protein 1a (*Mybbp1a*)	>50	>1.5
AI639058	Nedd4 WW domain-binding protein 4	>1000	>2.0
S75960	Tamm-Horsfall Protein	>900	>1.5

Unclassified

U25808	KAP	>500	>3.0

EST

AI639012	EST	>200	>1.5
AI639097	EST	>100	>1.5
M74067	EST	>50	>2.0

*Values observed in both sets of array data.
- The colour representation is as follows: - Pink, for down-regulated genes
  - Blue, for up-regulated genes
- Highlighted items were subjected to northern blot analyses

To address the correlation between sexually dimorphic gene expression and perinatal male lethality, further analyses were carried out on the androgen-regulated genes and in particular, the down-regulated KAP and N4WBP4 genes.

4.4.4 Androgen-Regulated Genes

4.4.4.1 KAP

4.4.4.1.1 KAP gene expression

In mouse kidney, KAP is the second-most abundant kidney-specific transcript as determined by SAGE (Virlon et al., 1999). The KAP transcript is exceptional with respect to its disproportional high concentration compared to its rate of synthesis reflecting a particular stability (Watson and Paigen, 1988). The KAP gene is transcriptionally induced by sex steroids (androgen, oestrogen), and thyroid hormone (Meseguer and Catterall, 1990a). In mouse kidney, KAP mRNA represents the most abundant (~ 4% of the total poly(A) RNA) androgen-induced mRNA species (Meseguer and Catterall, 1987) and protein (Takenaka et al., 1998; Virlon et al., 1999; El-Meanawy et al., 2000). Among the genes that are regulated by androgens, KAP expression shows the most complex pattern (Meseguer and Catterall, 1992; Ding et al., 1997; Melia et al., 1998). In different segments of the proximal tubule, KAP gene expression is subject to cell-specific regulation mediated by hormones of both gonadal and pituitary origin (Meseguer and Catterall, 1990a). Androgen regulates KAP expression in all segments [cortical convoluted S1/S2 (Meseguer and Catterall, 1990a) and juxtamedullary straight S3 (Meseguer and Catterall, 1987; 1990b)], whereas oestrogen and pituitary hormone(s) control its expression in S3 segment (Meseguer and Catterall, 1992). However, the functional role of KAP is still unknown.

In male mice, KAP expression is undetectable at birth, appears in the first week and gradually increases to 3 weeks of age with a dramatic rise at 4 weeks of age, reaching a maximum between 6 and 8 weeks of age (Ding and Sigmund, 2001). The KAP expression observed here in wildtype neonatal male kidney is consistent with these findings. In male rodents, KAP expression seems to be under strong temporal androgen regulation, which parallels plasma testosterone levels (Barkley and Goldman, 1977; Martin et al., 1977). In mouse kidney, it has been reported that KAP-mRNA expression is not only age-, but also gender- and adenine phosphoribosyl

transferase (APRT) genotype-dependent (Tzortzaki et al., 2002). KAP expression is reported to be 4-fold lower in adult female compared with male mice (Ding et al., 1997). Moreover, at P30, KAP was detected mainly in S3 proximal tubule segments (and to a lesser extent in the S1/S2 segments) in wildtype male mice, whereas its expression in this area was very low in females. In adult males (3- and 6-month-old), intense cytoplasmic staining in S3 proximal tubules was observed, whereas its expression was undetectable in females (Meseguer and Catterall, 1992). However, KAP gene expression is not eliminated in ovariectomised mice and a slight increase occurs instead (Meseguer and Catterall, 1990a). T3, in cooperation with androgens, promotes cortical (S1/S2 segments) KAP gene expression (Sole et al., 1996). In addition, T3 is necessary for the constitutive expression of the gene in the outer stripe of the outer medulla (S3 segments) (Sole et al., 1994; 1996). However, in females, KAP expression appears to be under the control of TSH and oestrogens in the S3 segments (Meseguer and Catterall, 1992). In the hypophysectomised mouse, the total absence of KAP mRNA implies that pituitary hormone(s) participate in the constitutive expression of the KAP transcript in S3 epithelial cells of the proximal tubules (Meseguer and Catterall, 1992). Recently, global transcription profiling of oestrogen activity in mice has been done by Jelinsky and colleagues (2003). They showed that the kidney had the third largest number of regulated genes by oestrogen receptor α, after the uterus and pituitary. This regulation is localised to the juxtamedullary region of the cortex (tubular epithelial cells) not only in mice, but also in rats. In the dwarf (*dw*) mouse model, known for its GH, PRL and TSH deficiency due to a mutation in the pit-1 gene, the kidney has a complete lack of KAP gene expression (Meseguer and Catterall, 1992). In congenital thyroid hormone (TH)-deficient hyt/hyt female mouse (Beamer et al., 1981), KAP mRNA is not expressed suggesting that thyroid hormones (TH) are required for its expression in female kidneys. It is restored to normal levels by thyroid hormone administration. Males and testosterone-treated females express the transcript throughout the entire proximal tubule (Sole et al., 1994; 1996). Males have normal baseline KAP expression, which is diminished to undetectable levels by castration. In this hypothyroid mouse, the androgen-dependent cortical response is partially impaired. The expression is fully restored after T3 or testosterone administration (Sole et al., 1994; 1996). As for females and castrated males and in contrast to hyt/hyt mouse, the androgen receptor

(AR)-deficient mouse (Tfm/y mouse) shows an exclusive KAP expression in the S3 segment of the proximal tubule (Meseguer et al., 1989).

4.4.4.1.2 KAP expression in 78N rats and other genetic models

Northern blot analysis of KAP gene expression confirmed the microarray result, indicating reduced KAP mRNA levels, in neonatal male and female, and adult female 78N rats. Other rodent models are similar to the 78N mutant rat with respect to KAP expression and phenotype. In the APRT knockout mouse model for human hereditary 2,8 dihydroxyadenine (DHA) nephrolithiasis (Engle et al., 1996), KAP mRNA levels were consistently decreased in the males (2-4-fold; Wang et al., 2000), and undetectable in females. In these mice, the decrease in KAP expression as well as hypertrophy at proximal tubule level (Ouar et al., 1998) may be related to the hormonal changes in DHA-induced nephrolithiasis (Tzortzaki et al., 2002). Renal histopathology demonstrated extensive tubular dilation, inflammation, necrosis, and fibrosis that varied in severity between different mouse backgrounds. Nevertheless, glomerular loss is a known feature of APRT deficiency (Evan et al., 2001). This phenotype is age-dependent and is more severe in male mice. APRT knockout male mice develop severe renal damage by three months of age. However, renal damage in female mice is less pronounced than in males. It would be very helpful to assess (by ISH) the distribution of KAP expression in the wildtype and 78N neonatal male and female and adult female rat kidney sections. This would allow us to detect at which segmental level the down-regulation has occurred, and accordingly which hormone(s) may be involved in each case.

4.4.4.2 N4WBP4

N4WBP4 has been identified recently as one of multiple novel N4WBP's (Jolliffe et al., 2000). The functional significance of the interaction between the ubiquitin-protein ligase Nedd4 and this protein (Murillas et al., 2002) remains to be established. However, since N4WBP4 is a putative membrane-associated/spanning protein, it is believed that it is a strong candidate for Nedd4-mediated regulation. Interestingly, there is evidence to suggest that the N4WBP4 transcript is also regulated by androgens (Xu et al., 2000). Here, northern blot analysis confirmed down-regulation of the N4WBP4 transcript in neonatal male and female, and adult female mutants. Nedd4L (human Nedd4) has been reported to play a role in regulating the epithelial

sodium channel (ENaC) and to be the candidate gene for a blood pressure disorder (i.e. autosomal dominant orthostatic hypotensive; Chen et al., 2001). Konstas et al. (2002) reported a novel Nedd4/Nedd4-2-binding protein, N4WBP5A, to be a potential regulator of ENaC. High expression of N4WBP5A has been noticed in tissues expressing ENaC (e.g. native renal collecting duct). They proposed that this binding protein functions by regulating Nedd4/Nedd4-2 availability and trafficking and is a likely candidate to regulate ENaC function *in vivo*. Further analyses should be carried out on N4WBP4 to elucidate its possible involvement in the regulation of ENaC activity since it is reported that several accessory proteins/factors, where Nedd4 is of central importance, regulate ENaC activity. These proteins act in concert and constitute an intrinsic part of a major mechanism for the ENaC channel down-regulation (Henry et al., 2003). A change in ENaC activity might therefore be involved in the kidney abnormalities observed in the 78N rat.

4.4.4.3 RVP.1

A third candidate gene [EST (M74067); average fold-increase (>2.0) in mutant compared with wildtype] is also androgen-regulated. This transcript (RVP.1, rat ventral prostate) was isolated from the RVP of androgen ablated rats and characterized. It is found to be induced by androgen withdrawal and apoptotic cell death in the rat ventral prostate (Briehl and Miesfeld, 1991). RVP.1. is expressed at very low levels in the RVP and epididymis of normal adult rats and is undetectable in kidney and other tissues (e.g. liver, muscle). However, it appeared to be up-regulated in the 78N mutant male kidney. This gene is therefore worthy of further investigation.

4.4.5 Other Genes

During the first three postnatal weeks of the rat, serum IGF and IGFBP profiles are subjected to a developmental change. The elevated foetal expression pattern of IGF-II and IGFBP-2 is substituted in adulthood by high levels of IGF-I and IGFBP-3 with a decline in IGF-II and IGFBP-2 levels (Feld and Hirschberg, 1996). In the 78N candidate gene list, ***IGF-II*** [(X17012); average fold-increase (>1.5) in mutant compared with wildtype] and ***IGFBP-1*** [(M58634); average fold-increase (>10.0) in mutant compared with wildtype] are up-regulated genes. Like the 78N mutants, IGF-I (Liu et al., 1993), IGFBP-1, and IGFBP-2 (Hoeflich et al., 1999) null mutant mice

manifest growth retardation. The up-regulation of these genes in 78N kidney remains to be explained.

AQPs are responsible for osmotic water movements in kidney (see Knepper et al., 1996). Since epithelial nephrogenesis in rats extends into early postnatal life (P1-P14, Clark and Bertram, 1999; Horster, 2000), expression patterns of several epithelial transporters including AQPs also change during this developmental phase. In 78N neonatal male mutant kidney, the ***ADH water channel*** [AQP-2, (D13906); average fold-increase (>2.0) in mutant compared with wildtype] and ***AQP-3*** [(D17695); average fold-increase (>2.0) in mutant compared with wildtype] are up-regulated candidate genes. In normal mice, the expression of AQP-2, AQP-3 and arginine vasopressin V2 receptors (AVP-V2R) increases between P7-P14. In murine autosomal recessive-infantile (AR) polycystic kidney disease (PKD), the expression of AQP-2, AQP-3 and AVP-V2R is enhanced dramatically at P7 compared with normal mice (Gattone et al., 1999). However, AQP-2 plays a critical role in neonatal renal function in mice unlike AQP-1, -3, and -4. Mutations in AQP-2 cause the hereditary non-X-linked nephrogenic diabetes insipidus (NDI) (Yang et al., 2001). A mouse knock-in model of NDI appears normal 2-3 days after birth, fails to thrive and generally dies by P6 if not supplemented with extra fluid. The mutant kidneys showed collecting duct dilatation, papillary atrophy, and some plasma membrane AQP-2 staining. In 78N mutant kidney, the over-expression of AQP-2 and -3 may reflect a compensatory mechanism and appears interesting for future investigation of their involvement in the early death of 78N males.

Two transcripts identified in the microarray study encode proteins crucial for collecting duct functions, i.e., regulation of water reabsorption by vasopressin and of Na^+ transport by aldosterone (Doucet, 1992). AQP-2 is the most abundant transcript. However, ***11β-hydroxysteroid dehydrogenase type2*** controls aldosterone-dependent regulation of Na^+ transport by transforming glucocorticoids into inactive compounds (Virlon et al., 1999). In 78N, the up-regulation of AQP-2 in mutant compared with wildtype (see above) accompanied by the down-regulation of 11β-hydroxysteroid dehydrogenase type2 [(U22424); average fold-decrease (>1.5) in mutant compared with wildtype] may reflect a disturbance in the regulation of water/Na transport.

In the medullary thick ascending limb of loop of Henle (MTAL) cells, both salt reabsorption rate and Na,K-ATPase activity are high. Another of the microarray-identified genes ***Creatine kinase B***, together with the chloride channel (ClCNKA) are essential proteins for achieving a high rate of salt reabsorption, either directly (ClCNKA) or indirectly (creatine kinase B) by producing the ATP required for active transport processes (Virlon et al., 1999; Elalouf et al., 2002). In 78N male neonates, the over-expression of creatine kinase B [(M57664); average fold-increase (>1.5) in mutant compared with wildtype] may reflect a disruption of this mechanism.

Inhibitors of DNA binding (Id) proteins are helix-loop-helix proteins that form heterodimers with ubiquitous and/or tissue-specific basic helix-loop-helix proteins, and thereby inhibit their DNA-binding activity. The interaction between ***Id-1*** and basic helix-loop-helix proteins, many of which are essential for cellular differentiation, has been proposed as a key regulatory event leading to negative regulation of cell differentiation and as positive regulators of G1 cell cycle control (Norton and Atherton, 1998; Alani et al., 1999). Id-1 is over-expressed in 78N kidney [(L23148); average fold-increase (>2.0) in mutant compared with wildtype] as in Hx kidney (Flores-Morales et al., 2001) reflecting a possible disturbance in the cell cycle control.

Some of the features in the 78N mutant kidney have been encountered in HIV-transgenic rodent models – mouse (Dickie et al., 1991) and rat (Reid et al., 2001) – that were designed to study childhood HIV-1-associated nephropathy (HIVAN). In humans, HIVAN is characterised by the presence of heavy proteinuria, focal or global glomerulosclerosis, and microcystic tubular dilatation leading to renal enlargement, and rapid progression to end-stage renal disease (ESRD). In mutant mice, the renal disease is associated with proteinuria and a high mortality rate. The early histopathological kidney lesion was focal glomerulosclerosis. Moribund animals manifested glomerulopathy (i.e. diffuse glomerulosclerosis) with prominent microcystic tubular dilatation, tubular epithelial degeneration, and interstitial nephritis. Proteinuria was detectable at P24, followed by severe nephrotic syndrome and rapid progression to ESRD. Renal histology showed focal segmental glomerulosclerosis and microcystic tubular dilatation. The kidneys expressed increased steady-state levels of collagen alpha (IV) mRNA when glomerulosclerosis

is present. In young rats, glomerular enlargement with mesangial hyperplasia, mild tubular dilatation, and infiltration of mononuclear cells were common lesions seen during the early stages of the renal disease. In males, uremia was detected (3 males vs 1 female). Moreover, the mutant males developed ESRD within the first month of life (4 males vs 1 female) (Ray et al., 2003). In 78N rats, further studies of the up-regulated ***Collagen Alpha 1 Type II*** transcript [(AJ224879); average fold-increase (>2.0) in mutant compared with wildtype] and its involvement in any structural modification of the renal tissue (i.e. glomerulus) should be undertaken.

4.4.6 Chromosomal Localization of the Transgene Insertion

The perinatal death of 78N mutant males suggests that this sex-linked phenotype may be due to X-linkage. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is lethal in infancy (Bennett et al., 2000). This human fatal recessive disorder is characterised by the neonatal onset of insulin-dependent diabetes mellitus (IDDM), infections, enteropathy, thrombocytopenia and anemia, other endocrinopathy, eczema, and cachexia. Moreover, mononuclear cell and plasmacyte infiltration is accompanied by disappearance of pancreatic islet cells, loss of small bowel mucosa, and disruption of other tissues. This syndrome maps to chromosome Xp11.23-Xq13.3. An insertional mouse mutant – scurfy – caused by Foxp3 mutations, shares many phenotypic features with the human IPEX. The affected males have scaly skin, apparent infection, diarrhea, progressive Coombs-positive anemia, thrombocytopenia, gastrointestinal bleeding, hypogonadism, leukocytocis, lymphadenopathy, and cachexia. They die within four weeks of birth (Wildin et al., 2001). Clearly, these are interesting features of this model that relate to the 78N phenotype.

However, preliminary FISH results have suggested that the 78N integration is on chromosome 3 (Slade, 2001). Since rat chromosome 3 is syntenic with mouse chromosome 2, but with a diversity of human chromosomes: 2, 9, 10, 11, 15, and 20, a typical human disease with its analogous mouse model has not been identified. N4WBP4, a chromosome 3 gene, was subjected to further analysis (i.e. Southern blot) to check if N4WBP4 is the gene that caused the 78N kidney phenotype. Unfortunately, the N4WBP4 gene signal in wildtype and mutant males were similar. More investigation should be carried out on other genes on the candidate list.

4.4.7 Methodological Considerations

The application of microarrays to the whole rat kidney and the analysis of expression profiles in normal and 78N kidneys have clear advantages as well as pitfalls.

The disadvantages/deficiencies of the array analysis are:

(1) The U34A array is not representative of the entire rat genome, therefore genes relevant to this model may be missed.

(2) An effect on gene expression is potentially masked by the use of the whole kidney samples (see concluding remarks).

(3) The array analysis could provide (mutant) gene identification but may not explain the mechanisms that underlie the developmental/physiological defects.

However, the array data is valuable for three main reasons:

(1) The data could potentially identify the gene that is disrupted in this (presumed) insertional mutant (e.g. a gene with a down-regulated expression).

(2) The data will identify genes that exhibit altered expression in the mutant model – either directly as a result of the mutation, or secondarily as a response/compensation to developmental/physiological deficiencies.

(3) The microarray analysis is also important because it represents the first microarray analysis of the postnatal rat kidney – It could potentially identify genes that are not recognized as being important for kidney development/ physiology.

4.5 CONCLUDING REMARKS

More investigation is ongoing to delineate the cause of 78N male death. The presumed insertional mutation in the 78N model may have interfered with kidney development. To endorse the morphological findings and to investigate the genetic basis of this disorder, molecular analysis of the kidney transcriptome was performed. In this study, an oligonucleotide microarray was used to analyse the 78N neonatal kidney transcriptome and a list of differentially expressed candidate genes was generated. Northern blot analyses on two androgen-regulated genes (KAP and N4WBP4) were performed and down-regulation of both genes was validated. Since preliminary FISH results have suggested the transgene integration is on chromosome 3 (Slade, 2001), further analysis (i.e. Southern blot) was carried out on N4WBP4, a chromosome 3 gene. Unfortunately, N4WBP4 does not appear to be the gene that caused the 78N phenotype. More work will be carried out on other genes on the candidate list to reveal the gene responsible for this phenotype. However, this novel transgenic rat may prove to be a useful model of human kidney disease. Moreover, it may provide a model for elucidating KAP and N4WBP4 function.

Since thyroid hormones are required for IGF regulation, AQP expression and maintenance of basal KAP expression, further investigations will be carried out on the hypothalamo-pituitary-thyroid axis. Further analyses will also be carried out to reveal which disrupted genes might have interfered in proper kidney development. One of these approaches would be microarray analysis of 78N ***adult female*** rat kidney. The candidate gene list generated from the mutant adult female kidney will be compared to that from the mutant neonatal male kidney. Hopefully, this will allow us to monitor temporally the progression of the renal abnormality, to identify the disrupted gene(s) causing the kidney phenotype, and to deduce which gene(s), are associated with the early death of the male. Furthermore, for better understanding of the molecular basis of the renal pathophysiology and the most affected structures, transcriptome characterisation could be performed at segmental or single-cell level if possible. Homogeneous or nearly homogeneous cell populations can be isolated from microdissected nephron [proximal tubule sub-segments (convoluted or straight) or cortical and medullary thick ascending limbs] (Takenaka et al., 1998; Virlon et al.,

1999, Elalouf et al., 2002). This analysis would hopefully reveal changes in gene expression that are obscured in the present study.

With regard to the 78N transgene integration site, a PCR-based amplification technique (MacGregor and Overbeek, 1991) optimized according to the observations of Henegariu et al. (1997), was used in an attempt to identify the disrupted endogenous gene in the 78N line, but this approach was not successful (Slade, 2001). Recently, a new methodological approach for rapid genomic identification of transgenic integration sites has been reported (Boyer et al., 2003). It has been used for mouse insertional mutants and is advantageous because previous knowledge of potentially complex transgene rearrangement is not needed. This latest method is promising and will be used for our 78N rat insertional mutant to discover the disrupted gene(s) responsible for the kidney, and also the growth retardation phenotype.

The significance of the 78N rat model with respect to growth retardation will be discussed in the final chapter.

CHAPTER 5.
GENERAL DISCUSSION

The mechanisms of impaired linear growth in conditions of growth retardation are numerous. It is most frequently due to deficiencies in the hypothalamo-pituitary-GH system. Different types of hypothalamic disruptions results in ***dwarfism of hypothalamic GRF origin***. Nevertheless, most of the disorders belong to a genetically heterogeneous group of ***dwarfism of pituitary origin*** in which GH-dependent short stature may be caused by defects in either GH secretion or GH responsiveness (Boguszewski et al., 1997). In humans, *GRF-R* defect results in 'dwarfism of Sindh' which is characterized by pituitary/somatotroph hypoplasia (Baumann and Maheshwari, 1997). A number of known mutations of *hGH gene* results in the production of an inactive GH protein and short stature (Millar et al., 2003). Congenital abnormality of the *GH-R* is a condition known as syndrome of primary GH resistance or insensitivity – the Laron syndrome (reviewed in Laron, 2004). In 'Laron dwarfism', patients are unable to generate IGF-I despite high levels of circulating GH. In 'Pygmy dwarfism', defective IGF-I expression occurs despite normal plasma GH levels and fully functional GH receptors (Merimee et al., 1981). However, in 25-40% of patients with short stature (Carlsson et al., 1994; Root et al., 1998), growth failure is independent of the hypothalamo-pituitary-GH axis and is known as ***idiopathic short stature*** (ISS) (Blair and Savage, 2002). In this thesis, we have investigated the development of phenotypic characteristics associated with dwarfism in two animal models of growth retardation, the *dw/dw* rat and the 78N rat.

In rodent models of GH deficiency (reviewed in Cheng et al., 1983), mutation of genes coding for transcription factors engaged in somatotroph and lactotroph differentiation leads to a reduction in the population of both secretory cell types (Sornson et al., 1996). However, the dwarf (*dw/dw*) rat is unusual, in that profound somatotroph hypoplasia (Downs and Frohman, 1991) is accompanied by not only a relative lactotroph hyperplasia but also a significant increase in PRL content (Carmignac et al., 1998; Thomas et al., 1999; Tierney and Robinson, 2002). This lactotroph hyperplasia is accompanied by the presence of a novel lactotroph subtype, the 'intermediate' lactotroph (Thompson et al., 2002). Since we have shown that a

similar increase in the population of lactotrophs occurs in *lit/lit* mice, which harbour an inactivating mutation in the GRF-R gene (Lin et al., 1993), we investigated the effect of neonatal ablation of GRF neurones on the 'intermediate' lactotroph. MSG treatment of *dw/dw* rats reveals that, whilst the somatotroph population in this model of dwarfism is independent of neonatal GRF, the 'intermediate' lactotrophs are regulated partially in parallel with the traditional lactotroph subpopulations, by this neuropeptide. However, the absence of 'intermediate' lactotroph in the *lit/lit* pituitary reveals the uniqueness of this cell to the *dw/dw* strain. The structure of the GRF-R appears to be normal in the *dw/dw* rat (Zeitler et al., 1998; Frohman et al., 2000), but the reduced cAMP response of *dw/dw* somatotroph to GRF exposure (Downs and Frohman, 1991) indicates a failure of the G-protein-coupled signalling system. In addition to the 'intermediate' lactotroph, the *dw/dw* model also displays several other unusual phenotypic features. Unlike other GH deficiency models, the *dw/dw* rat is peculiar in that it is lean; peripheral fat pads weights and plasma leptin levels are reduced (Unpublished data). However, when the *dw/dw* rats were treated with MSG, this was reversed. Therefore, the identification of the mutation in *dw/dw* rats could shed the light on the mechanisms that originally determine and maintain the relative proportions of GH and PRL cells, and on the mechanisms that regulate peripheral adiposity.

In the growth retarded 78N rat, a kidney abnormality was noticed during parallel postmortem analysis. This was affirmed by light microscopy analysis that revealed an alteration of the normal cortico-medullary architecture, and by microarray analysis that demonstrated an alteration in the expression of kidney genes. An abnormality in renal function could therefore be the cause of the growth retardation. One possibility is that Ca^{2+} homeostasis is impaired. Urine analysis conducted in adult mutant females however revealed normal urinary Ca^{2+} compared to controls (Slade, 2001). Also, examination of the reduction in skeletal growth in adult mutant females showed unaltered femoral diameter (anterior-posterior) and femoral strength (failure load) (Slade, 2001). These results suggest that the regulation of calcium homeostasis and bone mineralisation/remodelling in the 78N model is normal. A second possibility is that GH and/or IGF-I clearance is affected. Episodic GH secretion has been shown to be maintained in adult 78N females (Slade et al., 2001). In addition, the absence of difference in liver weight between 78N and wildtype rats indicates normal GH-GH

receptor function since liver development depends highly on normal GH secretion (Dubreuil et al., 1987). However, quantitative analysis of plasma GH profiles and comparison of GH half-life of 78N rats and their wildtype counterparts is needed to verify if GH clearance is normal. One approach would be to inject rats with iodinated GH and measure GH half-life after blood sampling for a period of time. Alternatively, the growth plate is considered an important target organ for longitudinal growth. Linear growth results from the proliferation and subsequent differentiation of chondrocytes in the epiphyseal growth plates of the long bones. The width of the epiphyseal plate (EPW) is proportionate to the rate of growth. Measuring the tibial EPW of 78N rats would indicate whether GH clearance and the effect of GH on bone are affected. It is of note that EPW is affected by a number of hormones but most markedly by GH, IGF-I, and thyroid hormones (van der Eerden et al., 2003). In the growth plate, GH acts on the germinal zone to cause maturation into proliferative cells, and induces the expression of IGF-I. Locally produced and circulating IGF-I stimulate proliferation of chondrocytes in the proliferative zone (Le Roith et al., 2001). Thyroid hormones stimulate the differentiation of chondrocytes in the hypertrophic zone (Robson et al., 2002). As every zone of the growth plate depends on one main hormonal regulator, measurement of the width of each zone in 78N rats would indicate the relative involvement of each of these hormones. Since the preliminary serum IGF-I analysis conducted here is inconclusive, this method and/or the injection of radiolabelled IGF-I (as mentioned previously for GH) could be adopted to check if IGF-I clearance is normal.

Thus, these two models appear to represent different types of growth retardation, the *dw/dw* rat being a pituitary GH-dependent model whereas the 78N rat remains an idiopathic, possibly GH-independent model. The precise etiology of dwarfism in each model is, as yet, undefined.

APPENDIX A: COMPOSITION OF SOLUTIONS

A1. RIA Solutions

Phosphate Buffered Saline (PBS-RIA) Solution (Assay PBS)
15.5g 50mM NaH_2PO_4
11.6g 100mM NaCl
Dissolve in 1800ml (90%volume) of H_2O
0.484g 0.6mM ***Thimerosal***
Add H_2O to 2L
Adjust to pH 7.2-7.4 with NaOH

0.3% BSA/PBS (Assay Buffer)
3mg Bovine Serum Albumin (BSA)
1ml PBS
Prepare fresh
Store at 4^0C

0.02% Iodogen
Dissolve 200µg iodogen in 1ml Dichloromethane
Aliquot in 40 small glass tubes (5µg/25µl per tube)
Dry aliquots under vaccum pump
Freeze

Tris Buffer
24.288g 100mM Tris
Dissolve in 1800ml (90%volume) of H_2O
0.484g 0.6mM Thimerosal
Add H_2O to 2L
Adjust to pH 8.4 with concentrated HCl

27% Polyethylene Glycol (PEG) Stock Solution
540g PEG (6000)
Dissolve in 50-90% of the volume of Tris buffer
Add Tris to 2L when dissolved (after few hours)

18% PEG Solution for Assays
*To make 600mls of 18% PEG
1.2ml 10%Triton
0.9g Gamma Globulin
400ml 27% PEG
200ml Tris buffer
Dilute Triton in small volume of Tris.
Dissolve Gamma Globulin by layering then add the remaining Tris.
Add the PEG and stir.

A2. Histology

[All constituents were purchased from *Agar Scientific Ltd.*, UK]

0.01M Phosphate Buffered Saline (PBS) Solution

2.883g $Na_2HPO_4.2H_2O$
0.598g $NaH_2PO_4.2H_2O$
18.000g NaCl
0.400g KCl
Add H_2O to 2L
Adjust to pH 7.2-7.4

10% Buffered Formalin

4.0g $NaH_2PO_4.H_2O$
6.5g Na_2HPO_4
100ml Formaldehyde [Formalin (≡ 40% formaldehyde)]
Add H_2O to 1L (store at RT)

Buffered formal saline

4.0g $NaH_2PO_4.H_2O$
6.5g Na_2HPO_4
9.0g NaCl
100ml Formaldehyde
Add H_2O to 1L (store at RT)

EDTA/NaOH

10g Diaminoethanetetra-acetic didodium salt (EDTA)
1.25g NaOH
100ml d.d.H_2O

2.5% Glutaraldehyde

1ml 25%Glutaraldehyde (EM grade)
9ml PBS (0.01M, pH 7.3)

0.2% Tannic Acid

0.2g Tannic Acid
100ml PBS (0.01M, pH 7.3)
Dissolve tannic acid in pre-warmed PBS (37^0C)
N.B. Tannic acid precipitates if the PBS temperature is not 37^0C

Spurrs Resin

3.5g DER 739 (Diglycidyl Ether of Polypropylene Glycol)
13g NSA (Nonenyl Succinic Anhydride)
5g ERL 4206
0.24ml S1 (Diamethylaminoethanol)
Mix well using a syringe

A2.1 Stains *[Raymond A Lamb, England]*

Alum Hematoxylin *(Mayer's)*
1g Hematoxylin Powder
50g Aluminium Ammonium (or Potassium) sulphate
1g Citric Acid
50g Chloral Hydrate
0.2g Sodium Iodate
1L Water
Dissolve the Hematoxylin, Alum, and Sodium Iodate in water using gentle heat
Add the Chloral Hydrate and Citric Acid
Boil the mixture for 5min
Cool and filter

Eosin *(1%)*
10g Eosin Y (Eosin Yellowish Powder)
Dissolve in 1L H_2O

Masson's Trichrome
**Celestine Blue B Stain*
5g Celestine Blue B
50g Ammonium Ferric Sulphate
140ml Glycerol
1L Water
Dissolve the ammonium ferric sulphate in water while stirring
Add the celestine blue B
Boil the mixture for few minutes
Cool and filter
Add glycerol

**Mayer's Hematoxylin*
See recipe above

**Ponceau/Fuschin Stain*
3g Acid Fuschin Powder
7g Ponceau De Xylidine
10ml Acetic Acid
1L Water
Add the acetic acid to the water
Dissolve the two stain powders in the previous solution

*1% *Phosphomolybdic Acid*
5g Phosphomolybdic Acid
Dissolve in 500ml Water

*1% *Light Green Stain*
10g Light Green Powder
20ml Acetic Acid
1L Water
Add the acetic acid to the water
Dissolve the light green powder in the previous solution

*1% *Acetic Acid Solution*
10ml Acetic Acid
Dissolve in 1L Water

A3. Molecular Biology Solutions and Media

Chemicals and media were mainly purchased from *Sigma* or *BDH* unless otherwise stated. Kits were purchased from numerous companies.

A3.1 Solutions

DEPC Treated Water or Solutions
Add 0.1ml DEPC per 100ml of H_2O or solution (0.1%)
Shake vigorously, leave to stand overnight, and autoclave

Sephadex G-50 Solution
30g Sephadex G-50
250ml TE buffer (pH 8.0)
Mix in 500ml bottle and autoclave for 15min
Allow to stand, then decant off supernatant
Replace with equal volume of TE buffer (pH 8.0)
Store at 4^0C

1M Sodium Phosphate (NaPi), pH 7.0
577ml 1M Na_2HPO_4 (85.2g in 600ml H_2O)
423ml 1M NaH_2PO_4 (60g in 500ml H_2O)
(per 1L)

10X Southern Loading Buffer
20%(*w/v*) Ficoll 400
0.1M EDTA (pH 8.0)
1%(*w/v*) SDS
0.25%(*w/v*) Bromophenol Blue
0.25%(*w/v*) Xylene Cyanol

20X SSC Buffer
175.3g 3M NaCl
88.2g 0.3M Trisodium Citrate.$2H_2O$
Add H_2O to 1L
Adjust pH to 7.0 with 1M HCl

STE Buffer
50mM Tris-HCl (pH 8.0)
100mM EDTA (pH 8.0)
0.5%(*w/v*) SDS

Proteinase K/STE (500 µg/ml)
20µl (25µg/µl) Proteinase K
980µl STE

50X TAE Buffer
242g Tris Base
57.1ml Glacial Acetic Acid
37.2g EDTA.$2H_2O$
[or 100ml 0.5M EDTA (pH 8.0)]
Add H_2O to 1L

TE Buffer
10mM Tris-HCl (pH 8.0)
1mM EDTA (pH 8.0)
Autoclave

1M Tris-HCl, pH 8.5
121.1g Tris Base
Add ~750ml H_2O
Adjust pH to 8.5 with concentrated HCl (~20ml)
Add H_2O to 1L

1M Tris-HCl, pH 8.0
88.8g Tris-HCl
53g Trizma Base
Add H_2O to 1L

20% SDS
100g SDS
500ml H_2O
Dissolve at 55^0C overnight

0.5M EDTA, pH 8.0
186.1g EDTA.$2H_2O$
Add H_2O to 1L
Adjust pH to 8.0 with NaOH pellets

Depurination Buffer
(0.25M HCl)
974.2ml H_2O
25.8ml Concentrated HCl (11.6M)

Denaturation Buffer
(1.5M NaCl/0.5M NaOH)
87.7g NaCl
20.0g NaOH
Add H_2O to 1L

Transfer Buffer
(1.5M NaCl/0.25M NaOH)
87.7g NaCl
10.0g NaOH
Add H_2O to 1L

Wash Buffer
(50mM NaPi/0.1%SDS)
50ml 1M NaPi
5ml 20%SDS
945ml H_2O

Hybridisation Buffer
500ml 1M NaPi
350ml 20%SDS
150ml Formamide
2ml 0.5M EDTA (pH 8.0)
Filter and store at RT

20X SSPE
210.4g NaCl
200ml NaPi
40ml 0.5M EDTA
Add H_2O to 1L

20X MAE
41.86g 400mM MOPS (pH 7.0)
4.10g 100mM NaAc
3.72g 20mM EDTA
Add H_2O to 500ml
Adjust pH to 7.0 with NaOH
Cover from light and store at 4^0C

OLB 5X Buffer
**Solution O*
1.25M Tris HCl (pH7.0)
125mM $MgCl_2$

**Solution A*
18µl β-mercapethanol (14.3M)
5µl dTTP (100mM)
5µl dATP (100mM)
5µl dGTP (100mM)
1ml Solution O

**Solution B*
HEPES (pH 6.6)

**Solution C*
Random hexamer oligonucleotides [Pharmacia Pd $(N)_6$]
At 90 OD units/ml in TE

Mix solutions A, B & C in a ratio of 2:5:3 (*v/v/v*)
Store in 100µl aliquots at -20^{0}C

OLB-C STOP Solution
0.05ml 5M NaCl
0.25ml 1M Tris-HCl (pH 7.5)
0.05ml 0.5M EDTA
156.5µl 20%SDS
125µl 100mM dCTP
Add sterile H_2O to a final volume of 12.5ml
Store in 500µl aliquots at 4^{0}C

1% Agarose Gel
1g Agarose
100ml 1xTAE Buffer
Microwave to complete dissolution
Cool down to ~50-60^{0}C
Add 1µl Ethidium Bromide (10mg/ml)
Pour into gel tray
Use after 20-30min

GTC-A Solution
189.1g/400ml 4M Guanidinium Isothiocyanate (GTC)
5ml/L 0.5% Sarkosyl
25ml (of 1M/L) 25mM Sodium Citrate (pH 7.0)
Filter through 0.2µm filter
Add 72µl β-mercaptoethanol per 10ml (final conc. 0.1M) immediately prior to use

A3.2 Media

Luria-Bertani (LB) Agar
Agar Tablet (1.68g)
50ml H_2O
Autoclave
Cool to 55^0C
Add the required antibiotic at the required concentration
Mix and pour into petri dishes (or other container)
Allow to set

LB Broth Base
Broth Base Tablet (1.1g)
50ml H_2O
Autoclave
Cool to 55^0C
Add the required antibiotic at the required concentration
Store at 4^0C

APPENDIX B: CANNULA CONSTRUCTION

Preparation of intravenous single bore cannula: 28cm piece of single bore polyethylene tube [0.5mm ID x 1.0mm OD; SIMS Portex Ltd., UK], was covered with 2.5cm piece of esco rubber [0.5mm Bore x 0.5mm Wall, Bibby Sterilin Ltd., Staffordshire, UK] on one end, and 2.5cm of silicon tube [Thick silastic, 1.0mm OD; SR Medical, Mass., USA] on the other end. Sliding was made easier by expanding the esco and silicon tubing in petroleum ether (Fischer Scientific UK Ltd., UK). The tip of the silicon tube was tapered and rounded to facilitate its insertion into the jugular vein.

REFERENCES

Acampora D, Mazan S, Tuorto F, Avantaggiato V, Tremblay JJ, Lazzaro D, di Carlo A, Mariano A, Macchia PE, Corte G, Macchia V, Drouin J, Brulet P, Simeone A. (1998) Transient dwarfism and hypogonadism in mice lacking Otx1 reveal prepubescent stage-specific control of pituitary levels of GH, FSH and LH. *Development* **125**:1229-39.

Alani RM, Hasskarl J, Grace M, Hernandez MC, Israel MA, Munger K. (1999) Immortalization of primary human keratinocytes by the helix-loop-helix protein, Id-1. *Proc. Natl. Acad. Sci. U S A.* **96**:9637-41.

Alba M, Salvatori R.A (2004) Mouse with targeted ablation of the GHRH gene: a new model of isolated GH deficiency. *Endocrinology* **145**:4134-43.

Aleppo G, Moskal SF 2nd, De Grandis PA, Kineman RD, Frohman LA. (1997) Homologous down-regulation of growth hormone-releasing hormone receptor messenger ribonucleic acid levels. *Endocrinology.* **138**:1058-65.

Ali M, Guillon G, Clos J. (1988) Comparative study of collecting tubules and vasopressin binding capacity in the renal medulla of developing hypothyroid rat. *J. Dev. Physiol.* **10**:297-308.

Alsat E, Guibourdenche J, Couturier A, Evain-Brion D. (1998) Physiological role of human placental growth hormone. *Mol. Cell. Endocrinol.* **140**:121-7.

Anderson, RR. (1968) Lactogenic hormone requirement for pseudopregnancy in normal and hysterectomized rats. *Proc. Soc. Exp. Biol. Med.* **127**:723-5.

Asa SL, Kovacs K, Horvath E, Losinski NE, Laszlo FA, Domokos I, Halliday WC. (1988) Human fetal adenohypophysis. Electron microscopic and ultrastructural immunocytochemical analysis. *Neuroendocrinology* **48**:423-31.

Asa SL, Kovacs K, Stefaneanu L, Horvath E, Billestrup N, Gonzalez-Manchon C, Vale W. (1992) Pituitary adenomas in mice transgenic for growth hormone-releasing hormone. *Endocrinology* **131**:2083-9.

Asa SL, Kelly MA, Grandy DK, Low MJ. (1999) Pituitary lactotroph adenomas develop after prolonged lactotroph hyperplasia in dopamine D2 receptor-deficient mice. *Endocrinology* **140**:5348-5355.

Asami K. (1986) Preservation of ultrastructure in phosphatidylcholine vesicles by tannic acid and OsO4. *J. Ultrastruct. Mol. Struct. Res.* **95**:38-46.

Asonova SN, Migalkin NS. (1996) Use of Masson's trichrome method for staining decalcified bone tissue. *Arkh. Patol.* **58**:66-7.

Avanoglu A, Ergun O, Bakirtas F, Erdener A. (1997) Characteristics of multisystem organ failure in neonates. *Eur. J. Pediatr. Surg.* 7:263-6.

Baird A, Wehrenberg WB, Bohlen P, Ling N. (1985) Immunoreactive and biologically active growth hormone-releasing factor in the rat placenta. *Endocrinology* **117**:1598-601.

Baker BL, Midgley AR Jr, Gersten BE, Yu YY. (1969) Differentiation of growth hormone- and prolactin-containing acidophils with peroxidase-labeled antibody. *Anat. Rec.* **164**:163-71.

Barinaga M, Yamonoto G, Rivier C, Vale W, Evans R, Rosenfeld MG. (1983) Transcriptional regulation of growth hormone gene expression by growth hormone-releasing factor. *Nature* **306**:84-5.

Barkley MS, Goldman BD. (1977) A quantitative study of serum testosterone, sex accessory organ growth, and the development of intermale aggression in the mouse. *Horm. Behav.* **8**:208-18.

Barnard R, Thordarson G, Lopez MF, Yamaguchi M, Edens A, Cramer SD, Ogren L, Talamantes F. (1994) Expression of growth hormone-binding protein with a hydrophilic carboxyl terminus by the mouse placenta: studies in vivo and in vitro. *J. Endocrinol.* **140**:125-35.

Barnea ER, Perlman R, Bick T, Hochberg Z. (1989) Effects of human growth hormone upon term placental hormone secretion in vitro. *Gynecol. Obstet. Invest.* **27**:133-6.

Barta A, Richards RI, Baxter JD, Shine J. (1981) Primary structure and evolution of rat growth hormone gene. *Proc. Natl. Acad. Sci. U S A.* **78**:4867-71.

Bartke A. (1964) Histology of the anterior hypophysis, thyroid and gonads of two types of dwarf mice. *Anat. Rec.* **149**:225-35.

Bartke A. (1973) Differential requirement for prolactin during pregnancy in the mouse. *Biol. Reprod.* **9**:379-83.

Bartke A, Goldman BD, Bex F, Dalterio S. (1977) Effects of prolactin (PRL) on pituitary and testicular function in mice with hereditary PRL deficiency. *Endocrinology* **101**:1760-6.

Baumann G, Maheshwari H. (1997) The Dwarfs of Sindh: severe growth hormone (GH) deficiency caused by a mutation in the GH-releasing hormone receptor gene. *Acta Paediatr.* **423**:33-8.

Beamer WJ, Eicher EM, Maltais LJ, Southard JL. (1981) Inherited primary hypothyroidism in mice. *Science* **212**:61-3.

Bedendi I, Alloatti G, Marcantoni A, Malan D, Catapano F, Ghe C, Deghenghi R, Ghigo E, Muccioli G. (2003) Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. *Eur. J. Pharmacol.* **476**:87-95.

Ben-Jonathan N, Mershon JL, Allen DL, Steinmetz RW. (1996) Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. *Endocr. Rev.* **17**:639-69.

Bennett CL, Yoshioka R, Kiyosawa H, Barker DF, Fain PR, Shigeoka AO, Chance PF. (2000) X-Linked syndrome of polyendocrinopathy, immune dysfunction, and diarrhea maps to Xp11.23-Xq13.3. *Am. J. Hum. Genet.* **66**:461-8.

Bennett PA, Levy A, Sophokleous S, Robinson IC, Lightman SL. (1995) Hypothalamic GH receptor gene expression in the rat: effects of altered GH status. *J. Endocrinol.* **147**:225-34.

Bennett PA, Thomas GB, Howard AD, Feighner SD, van der Ploeg LH, Smith RG, Robinson IC. (1997) Hypothalamic growth hormone secretagogue-receptor (GHS-R) expression is regulated by growth hormone in the rat. *Endocrinology* **138**:4552-7.

Berelowitz M, Szabo M, Frohman LA, Firestone S, Chu L, Hintz RL. (1981) Somatomedin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. *Science* **212**:1279-81.

Berry SA, Pescovitz OH. (1988) Identification of a rat GHRH-like substance and its messenger RNA in rat testis.*Endocrinology* **123**:661-3.

Blair J, Savage M. (2002) The GH-IGF-I axis in children with idiopathic short stature. *Trends Endocrinol. Metab.* **13**:325-330.

Blake AD, Smith RG. (1991) Desensitization studies using perifused rat pituitary cells show that growth hormone-releasing hormone and His-D-Trp-Ala-Trp-D-Phe-Lys-NH2 stimulate growth hormone release through distinct receptor sites. *J. Endocrinol.* **129**:11-9.

Blake CA, Campbell GT, Wagoner J, Rodriguez-Sierra JF, Hendricks SE, Elias KA. (1983) Dissociation between increased growth hormone and prolactin secretion during the morning hours of early pregnancy in the rat. *Life Sci.* **33**:1475-8.

Bloch B, Brazeau P, Ling N, Bohlen P, Esch F, Wehrenberg WB, Benoit R, Bloom F, Guillemin R. (1983) Immunohistochemical detection of growth hormone-releasing factor in brain. *Nature* **301**:607-8.

Bloch B, Ling N, Benoit R, Wehrenberg WB, Guillemin R. (1984) Specific depletion of immunoreactive growth hormone-releasing factor by monosodium glutamate in rat median eminence. *Nature* **307**:272-3.

Boguszewski CL, Carlsson B, Carlsson LM. (1997) Mechanisms of growth failure in non-growth-hormone deficient children of short stature. *Horm. Res.* **48** (Suppl 4):19-22.

Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. (1998) Prolactin (PRL) and its receptor: Actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr. Rev.* **19**:225-268.

Boockfor FR, Hoeffler JP, Frawley LS. (1986) Analysis by plaque assays of GH and prolactin release from individual cells in cultures of male pituitaries. Evidence for functional heterogeneity within rat mammotrope and somatotrope populations. *Neuroendocrinology* **42**:64-70.

Boockfor FR, Frawley LS. (1987) Functional variations among prolactin cells from different pituitary regions. *Endocrinology* **120**:874-9.

Bosse R, Fumagalli F, Jaber M, Giros B, Gainetdinov RR, Wetsel WC, Missale C, Caron MG. (1997) Anterior pituitary hypoplasia and dwarfism in mice lacking the dopamine transporter. *Neuron* **19**:127-38.

Botero-Ruiz W, Biggers WJ, Sanyal MK. (1997) Augmentation of DNA synthesis in placental and fetal tissues in utero by maternal growth hormone treatment. *Early Pregnancy* **3**:272-80.

Bowers CY, Momany FA, Reynolds GA, Hong A. (1984) On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology* **114**:1537-1545.

Boyer A, Paquet M, Raiwet D, Daneau I, Harrison W, Silversides DW. (2003) Rapid genomic identification of transgenic integration sites for mouse insertional mutants. *Biotechniques* **34**:32-4, 36.

Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J, Guillemin R. (1973) Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* **179**:77-9.

Brenner BM. (1985) Nephron adaptation to renal injury or ablation. *Am. J. Physiol.* **249**:F324-37.

Briehl MM, Miesfeld RL. (1991) Isolation and characterization of transcripts induced by androgen withdrawal and apoptotic cell death in the rat ventral prostate. *Mol. Endocrinol.* **5**:1381-8.

Budge H, Mostyn A, Wilson V, Khong A, Walker AM, Symonds ME, Stephenson T. (2002) The effect of maternal prolactin infusion during pregnancy on fetal adipose tissue development. *J. Endocrinol.***174**:427-33.

Butte NF. (2000) Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. *Am. J. Clin. Nutr.***7**:1256S-61S.

Cameron DP, Cutbush L, Opat F. (1978) Effects of monosodium glutamate-induced obesity in mice on carbohydrate metabolism in insulin secretion. *Clin. Exp. Pharmacol. Physiol.* **5**:41-51.

Camper SA, Saunders TL, Katz RW, Reeves RH. (1990) The Pit-1 transcription factor gene is a candidate for the murine Snell dwarf mutation. *Genomics* **8**:586-90.

Caramori ML, Mauer M (2001) Diabetic nephropathy. In: Primer on kidney diseases. 3rd edition. Greenberg A (Ed.). National Kidney Foundation. Academic Press, London, U.K.

Carlsson L, Eriksson E, Seeman H, Jansson JO. (1987) Oestradiol increases baseline growth hormone levels in the male rat: possible direct action on the pituitary. *Acta Physiol. Scand.* **129**:393-9.

Carlsson L, Eden S, Jansson JO. (1990) The plasma pattern of growth hormone in conscious rats during late pregnancy. *J. Endocrinol.* **124**:191-198.

Carlsson LM, Attie KM, Compton PG, Vitangcol RV, Merimee TJ. (1994) Reduced concentration of serum growth hormone-binding protein in children with idiopathic short stature. National Cooperative Growth Study. *J. Clin. Endocrinol. Metab.***78**:1325-30.

Carmignac DF, Bennett PA, Robinson ICAF. (1998) Effects of growth hormone secretagogues on prolactin release in anesthetized dwarf (*dw/dw*) rats. *Endocrinology* **139**:3590-3596.

Caufriez A, Frankenne F, Hennen G, Copinschi G. (1993) Regulation of maternal IGF-I by placental GH in normal and abnormal human pregnancies. *Am. J. Physiol.* **265**:E572-7.

Chandrashekar V, Bartke A, Coschigano KT, Kopchick JJ. (1999) Pituitary and testicular function in growth hormone receptor gene knockout mice. *Endocrinology* **140**:1082-8.

Chapman L, Nishimura A, Buckingham JC, Morris JF, Christian HC (2002) Externalization of annexin I from a folliculo-stellate-like cell line. *Endocrinology* **143**:4330-8.

Charlton HM, Clark RG, Robinson, ICAF, Porter Goff AE, Cox BS, Bugnon C, Bloch BA. (1988) Growth hormone-deficient dwarfism in the rat - A new mutation. *J. Endocrinol.* **119**:51-58.

Chatelain A, Dupouy JP, Dubois MP. (1979) Ontogenesis of cells producing polypeptide hormones (ACTH, MSH, LPH, GH, prolactin) in the fetal hypophysis of the rat: influence of the hypothalamus. *Cell. Tissue Res.* **196**:409-27.

Chen H, Ross CA, Wang N, Huo Y, MacKinnon DF, Potash JB, Simpson SG, McMahon FJ, DePaulo Jr JR, McInnis MG. (2001) NEDD4L on human chromosome 18q21 has multiple forms of transcripts and is a homologue of the mouse Nedd4-2 gene. *Eur. J. Hum. Genet.* **9**:922-30.

Cheng TC, Beamer WG, Phillips JA 3rd, Bartke A, Mallonee RL, Dowling C. (1983) Etiology of growth hormone deficiency in little, Ames, and Snell dwarf mice. *Endocrinology* **113**:1669-78.

Cheval L, Virlon B, Billon E, Aude JC, Elalouf JM, Doucet A. (2002) Large-scale analysis of gene expression: Methods and application to the kidney. *J. Nephrol.* **15**:S170-183.

Chien YH, Thompson EB. (1980) Genomic organization of rat prolactin and growth hormone genes. *Proc. Natl. Acad. Sci. U S A.* 77:4583-7.

Childs GV, Unabia G. (1997) Cytochemical studies of the effects of activin on gonadotropin-releasing hormone (GnRH) binding by pituitary gonadotropes and growth hormone cells. *J. Histochem. Cytochem.* **45**:1603-10.

Chomczynski P, Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-9.

Chomczynski P, Brar A, Frohman LA. (1988) Growth hormone synthesis and secretion by a somatomammotroph cell line derived from normal adult pituitary of the rat. *Endocrinology* **123**:2276-83.

Christian HC, Rolls NJ, Morris JF. (2000) Nongenomic actions of testosterone on a subset of lactotrophs in the male rat pituitary. *Endocrinology* **141**:3111-9.

Clark AT, Bertram JF. (1999) Molecular regulation of nephron endowment. *Am. J. Physiol.* **276**:F485-97.

Clark RG, Jansson JO, Isaksson O, Robinson IC. (1985) Intravenous growth hormone: growth responses to patterned infusions in hypophysectomized rats. *J. Endocrinol.* **104**:53-61.

Clark RG, Robinson IC. (1985) Growth induced by pulsatile infusion of an amidated fragment of human growth hormone releasing factor in normal and GHRF-deficient rats. *Nature* **314**:281-3.

Clark RG, Chambers G, Lewin J, Robinson IC. (1986) Automated repetitive microsampling of blood: growth hormone profiles in conscious male rats. *J. Endocrinol.* **111**:27-35.

Clark RG, Carlsson LM, Robinson IC. (1987) Growth hormone secretory profiles in conscious female rats. *J. Endocrinol.* **114**:399-407.

Clark RG, Carlsson LM, Rafferty B, Robinson IC. (1988) The rebound release of growth hormone (GH) following somatostatin infusion in rats involves hypothalamic GH-releasing factor release. *J Endocrinol.* **119**:397-404.

Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL. (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron.* **37**:649-61.

Cramer SD, Wong L, Kensinger RS, Ogren L, Talamantes F. (1992) Regulation of the hepatic growth hormone receptor and serum growth hormone-binding protein during pregnancy in the mouse: effects of litter size. *Endocrinology.* **131**:2914-20.

Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. (2001) A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* **50**:1714-9.

Dasen JS, Rosenfeld MG. (2001) Signaling and transcriptional mechanisms in pituitary development. *Annu. Rev. Neurosci.* **24**:327-355.

Daughaday WH, Rotwein P. (1989) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr. Rev.* **10**:68-91.

Davenport ML, Clemmons DR, Miles MV, Camacho-Hubner C, D'Ercole AJ, Underwood LE. (1990) Regulation of serum insulin-like growth factor-I (IGF-I) and IGF binding proteins during rat pregnancy. *Endocrinology* **127**:1278-86.

Davenport ML, Pucilowska J, Clemmons DR, Lundblad R, Spencer JA, Underwood LE. (1992) Tissue-specific expression of insulin-like growth factor binding protein-3 protease activity during rat pregnancy. *Endocrinology* **130**:2505-12.

Dawson R Jr, Valdes JJ, Annau Z. (1985) Tuberohypophyseal and tuberoinfundibular dopamine systems exhibit differential sensitivity to neonatal monosodium glutamate treatment. *Pharmacology.* **31**:17-23.

Day R, Dong W, Panetta R, Kraicer J, Greenwood MT, Patel YC. (1995) Expression of mRNA for somatostatin receptor (sstr) types 2 and 5 in individual rat pituitary cells. A double labeling in situ hybridization analysis. *Endocrinology* **136**:5232-5.

De Paul A, Pons P, Aoki A, Torres A. (1997) Different behavior of lactotroph cell subpopulations in response to angiotensin II and thyrotrophin-releasing hormone. *Cell. Mol. Neurobiol.* **17**:245-58.

Di Simone N, Caruso A, Lanzone A, Piccirillo G, Castellani R, Ronsisvalle E, Giannice R, Mancuso S. (1995) In vitro human growth hormone increases human chorionic gonadotropin and progesterone secretion by human placenta at term: evidence of a modulatory role by opioids. *Gynecol. Endocrinol.* **9**:157-64.

Diaz-Torga G, Feierstein C, Libertun C, Gelman D, Kelly MA, Low MJ, Rubinstein, M, Becu-Villalobos D. (2002) Disruption of the D2 dopamine receptor alters GH

and IGF-I secretion and causes dwarfism in male mice. *Endocrinology* **143**:1270-1279.

Dickie P, Felser J, Eckhaus M, Bryant J, Silver J, Marinos N, Notkins AL. (1991) HIV-associated nephropathy in transgenic mice expressing HIV-1 genes. *Virology* **185**:109-19.

Ding Y, Davisson RL, Hardy DO, Zhu LJ, Merrill DC, Catterall JF, Sigmund CD. (1997) The kidney androgen-regulated protein promoter confers renal proximal tubule cell-specific and highly androgen-responsive expression on the human angiotensinogen gene in transgenic mice. *J. Biol. Chem.* **272**:28142-8.

Ding Y, Sigmund CD. (2001) Androgen-dependent regulation of human angiotensinogen expression in KAP-hAGT transgenic mice. *Am. J. Physiol. Renal Physiol.* **280**:F54-60.

Donovan SM, Giudice LC, Murphy LJ, Hintz RL, Rosenfeld RG. (1991) Maternal insulin-like growth factor-binding protein messenger ribonucleic acid during rat pregnancy. *Endocrinology* **129**:3359-66.

Doucet A. (1992) Na-K-ATPase in the kidney tubule in relation to natriuresis. *Kidney Int.* Suppl. **37**:S118-24.

Downs TR, Frohman LA. (1991) Evidence for a defect in growth hormone-releasing factor signal transduction in the dwarf (*dw/dw*) rat pituitary. *Endocrinology* **129**:58-67.

Dubreuil P, Brazeau P, Morisset J. (1987) Effect of somatocrinin and a somatostatin antiserum on liver growth in the rat. *J. Pediatr. Gastroenterol. Nutr.* **6**:128-34.

Eden S. (1979) Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinology* **105**:555-60.

Elalouf JM, Aude JC, Billon E, Cheval L, Doucet A, Virlon R. (2002) Renal transcriptomes: Segmental analysis of differential expression. *Exp. Nephrol.* **10**:75-81.

El-Kasti MM, Christian HC, Huerta I, Hill N, Matthews DR, Wells T. (2004) Pregnancy-induced plasticity in rat pituitary: a morphological and endocrine study of somatotroph and lactotroph function. *The 23rd Joint Meeting of the British Endocrine Societies. Brighton-U.K. Endocrine Abstracts 7,* **P143**.

El-Meanawy MA, Schelling JR, Pozuelo F, Churpek MM, Ficker EK, Iyengar S, Sedor JR. (2000) Use of serial analysis of gene expression to generate kidney expression libraries. *Am. J. Physiol. Renal Physiol.***279**:F383-92.

Engle SJ, Stockelman MG, Chen J, Boivin G, Yum MN, Davies PM, Ying MY, Sahota A, Simmonds HA, Stambrook PJ, Tischfield JA. (1996) Adenine phosphoribosyltransferase-deficient mice develop 2,8-dihydroxyadenine nephrolithiasis. *Proc. Natl. Acad. Sci. USA* .**93**:5307-12.

Enjalbert, A., Bertrand, P., Le Dafniet, M., Epelbaum, J., Hugues, J.N., Kordon, C., Moyse, E., Peillon, F. and Shu, C. (1986) Somatostatin and regulation of prolactin secretion. *Psychoneuroendocrinology* **11**:155-165.

Eriksson L, Eden S, Frohlander N, Bengtsson BA, von Schoultz B. (1988a) Continuous 24-hour secretion of growth hormone during late pregnancy. A regulator of maternal metabolic adjustment? *Acta Obstet. Gynecol. Scand.* **67**:543-7.

Eriksson L, Frankenne F, Eden S, Hennen G, von Schoultz B. (1988b) Growth hormone secretion during termination of pregnancy. Further evidence of a placental variant. *Acta Obstet .Gynecol. Scand.* **67**:549-52.

Eriksson L. (1989) Growth hormone in human pregnancy. Maternal 24-hour serum profiles and experimental effects of continuous GH secretion. *Acta Obstet. Gynecol. Scand. Suppl.* **147**:1-38.

Eriksson L, Frankenne F, Eden S, Hennen G, Vonschoultz B. (1989) Growth-hormone 24-h serum profiles during pregnancy - Lack of pulsatility for the secretion of the placental variant. *Br. J. Obstet. Gynaecol.* **96**:949-953.

Escalada J, Sanchez-Franco F, Velasco B, Cacicedo L. (1997) Regulation of growth hormone (GH) gene expression and secretion during pregnancy and lactation in the rat: role of insulin-like growth factor-I, somatostatin, and GH-releasing hormone. *Endocrinology* **138**:3435-3443.

Evan AP, Bledsoe SB, Connors BA, Deng L, Liang L, Shao C, Fineberg NS, Grynpas MD, Stambrook PJ, Youzhi S, Sahota A, Tischfield JA. (2001) Sequential analysis of kidney stone formation in the Aprt knockout mouse. *Kidney Int.* **60**:910-23.

Farquhar MG, Rinehart, JF (1954) Electron microscopic studies of the anterior pituitary gland of castrate rats. *Endocrinology* **54**:516-541.

Fauquier T, Lacampagne A, Travo P, Bauer K, Mollard P. (2002) Hidden face of the anterior pituitary. *Trends Endocrinol. Metab.* **13**:304-9.

Feld S, Hirschberg R. (1996) Growth hormone, the insulin-like growth factor system, and the kidney. *Endocr. Rev.* **17**:423-80.

Fick-Brosnahan GM, Tran ZV, Johnson AM, Strain JD, Gabow PA. (2001) Progression of autosomal-dominant polycystic kidney disease in children. *Kidney Int.* **59**:1654-62.

Finn WF (2001) Tubulointerstitial disease. In: Primer on kidney diseases. 3rd edition. Greenberg A (Ed.). National Kidney Foundation. Academic Press, London, U.K.

Flavell DM, Wells T, Wells SE, Carmignac DF, Thomas GB, Robinson IC. (1996) Dominant dwarfism in transgenic rats by targeting human growth hormone (GH) expression to hypothalamic GH-releasing factor neurons. *EMBO J.* **15**:3871-9.

Flint DJ, Binart N, Kopchick J, Kelly P. (2003) Effects of different dietary fat types on postprandial appetite and energy expenditure. *Obes. Res.* **11**:1449-55.

Flores-Morales A, Stahlberg N, Tollet-Egnell P, Lundeberg J, Malek RL, Quackenbush J, Lee NH, Norstedt G. (2001) Microarray analysis of the in vivo effects of hypophysectomy and growth hormone treatment on gene expression in the rat. *Endocrinology* **142**:3163-76.

Forsyth IA, Wallis M. (2002) Growth hormone and prolactin--molecular and functional evolution. *J. Mammary Gland. Biol. Neoplasia* 7:291-312.

Frankenne F, Closset J, Gomez F, Scippo ML, Smal J, Hennen G. (1988) The physiology of growth hormones (GHs) in pregnant women and partial characterization of the placental GH variant. *J. Clin. Endocrinol. Metab.* **66**:1171-80.

Frasor J, Gaspar CA, Donnelly KM, Gibori G, Fazleabas AT. (1999) Expression of prolactin and its receptor in the baboon uterus during the menstrual cycle and pregnancy. *J. Clin. Endocrinol. Metab.* **84**:3344-50.

Frawley LS, Boockfor FR, Hoeffler JP. (1985) Identification by plaque assays of a pituitary cell type that secretes both growth hormone and prolactin. *Endocrinology* **116**:734-7.

Frawley LS, Boockfor FR. (1991) Mammosomatotropes: presence and functions in normal and neoplastic pituitary tissue. *Endocr. Rev.* **12**:337-55.

Freeman ME, Kanyicska B, Lerant A, Nagy G. (2000) Prolactin: structure, function, and regulation of secretion. *Physiol. Rev.* **80**:1523-631.

Frohman LA, Downs TR, Heimer EP, Felix AM. (1989) Dipeptidylpeptidase IV and trypsin-like enzymatic degradation of human growth hormone-releasing hormone in plasma. *J. Clin. Invest.* **83**:1533-40.

Frohman LA, Kineman RD, Kamegai J, Park S, Teixeira LT, Coschigano KT, Kopchick JJ. (2000) Secretagogues and the somatotrope: Signaling and proliferation. *Recent Prog. Horm. Res.* **55**:269-291.

Frystyk J, Ivarsen P, Skjaerbaek C, Flyvbjerg A, Pedersen EB, Orskov H. (1999) Serum-free insulin-like growth factor I correlates with clearance in patients with chronic renal failure. *Kidney Int.* **56**:2076-84.

Fuh G, Mulkerrin MG, Bass S, McFarland N, Brochier M, Bourell JH, Light DR, Wells JA. (1990) The human growth hormone receptor. Secretion from Escherichia coli and disulfide bonding pattern of the extracellular binding domain. *J. Biol. Chem.* **265**:3111-5.

Fukamachi S, Sugimoto M, Mitani H, Shima A. (2004) Somatolactin selectively regulates proliferation and morphogenesis of neural-crest derived pigment cells in medaka. *Proc. Natl. Acad. Sci. USA* **101**:10661-6.

Gage PJ, Brinkmeier ML, Scarlett LM, Knapp LT, Camper SA, Mahon KA. (1996) The Ames dwarf gene, df, is required early in pituitary ontogeny for the extinction of Rpx transcription and initiation of lineage-specific cell proliferation. *Mol. Endocrinol.* **10**:1570-81.

Garcia A, Alvarez CV, Smith RG, Dieguez C. (2001) Regulation of Pit-1 expression by ghrelin and GHRP-6 through the GH secretagogue receptor. *Mol. Endocrinol.* **15**:1484-95.

Gargosky SE, Walton PE, Owens PC, Wallace JC, Ballard FJ. (1990) Insulin-like growth factor-I (IGF-I) and IGF-binding proteins both decline in the rat during late pregnancy. *J. Endocrinol.* **127**:383-90.

Gargosky SE, Nanto-Salonen K, Tapanainen P, Rosenfeld RG. (1993) Pregnancy in growth hormone-deficient rats: assessment of insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs) and IGFBP protease activity. *J. Endocrinol.* **136**:479-89.

Gattone VH 2nd, MacNaughton KA, Kraybill AL. (1996) Murine autosomal recessive polycystic kidney disease with multiorgan involvement induced by the cpk gene. *Anat. Rec.* **245**:488-99.

Gattone VH 2nd, Maser RL, Tian C, Rosenberg JM, Branden MG. (1999) Developmental expression of urine concentration-associated genes and their altered expression in murine infantile-type polycystic kidney disease. *Dev. Genet.* **24**:309-18.

Gattone VH, Ricker JL, Trambaugh CM, Klein RM. (2002) Multiorgan mRNA misexpression in murine autosomal recessive polycystic kidney disease. *Kidney Int.* **62**:1560-9.

Gaylinn BD. (1999) Molecular and cell biology of the growth hormone-releasing hormone receptor.*Growth Horm. IGF Res.* **9** Suppl A:37-44.

Geschwind II, Li CH. (1955) The tibia test for growth hormone. In: Smith RW Jr, Gaebler OH, Long NH, eds. *Hypophyseal Growth Hormone, Nature and Actions.* New York: McGraw-Hill Book Co., pp. 28-53.

Gevers E, Pincus SM, Robinson IC, Veldhuis JD. (1998) Differential orderliness of the GH release process in castrate male and female rats. *Am. J. Physiol.* **274**:R437-44.

Gibney J, Smith TP, McKenna TJ. (2004) Routine screening for macroprolactin. *The 23rd Joint Meeting of the British Endocrine Societies. Endocrine Abstracts 7,* **P178**.

Gillies G. (1997) Somatostatin: the neuroendocrine story. *Trends Pharmacol. Sci.* **18**:87-95.

Giros B, Jaber M, Jones SR, Wightman RM, Caron MG. (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379**:606-12.

Giustina A, Veldhuis JD. (1998) Pathophysiology of the neuroregulation of growth hormone secretion in experimental animals and the human. *Endocr. Rev.* **19**:717-797.

Godfrey P, Rahal JO, Beamer WG, Copeland NG, Jenkins NA, Mayo KE. (1993) GHRH receptor of little mice contains a missense mutation in the extracellular domain that disrupts receptor function. *Nat. Genet.* **4**:227-32.

Goffin V, Binart N, Touraine P, Kelly PA. (2002) Prolactin: the new biology of an old hormone. *Annu. Rev. Physiol.* **64**:47-67.

Gourdji D, Kerdelhue B, Tixier-Vidal A. (1972) [Ultrastructure of a clone of hypophyseal cells secreting prolactin (clone GH3). Modifications induced by the hypothalamic hormone releasing thyreotropic hormone (TRF)] *C. R.. Acad. Sci. Hebd. Seances Acad. Sci. D.* **274**:437-40.

Green H, Morikawa M, Nixon T. (1985) A dual effector theory of growth-hormone action. *Differentiation* **29**:195-8.

Gretz N, Kranzlin B, Pey R, Schieren G, Bach J, Obermuller N, Ceccherini I, Kloting I, Rohmeiss P, Bachmann S, Hafner M. (1996) Rat models of autosomal dominant polycystic kidney disease. *Nephrol. Dial. Transplant.* **11** Suppl 6:46-51.

Grow DR. (2002) Metabolism of endogenous and exogenous reproductive hormones. *Obstet. Gynecol. Clin. North. Am.* **29**:425-36.

Gualillo O, Lago F, Garcia M, Menendez C, Senaris R, Casanueva FF, Dieguez C. (1999) Prolactin stimulates leptin secretion by rat white adipose tissue. *Endocrinology* **140**:5149-53.

Gualillo O, Caminos JE, Blanco M, Garcìa-Caballero T, Kojima M, Kangawa K, Dieguez C, Casanueva FF. (2001) Ghrelin, A Novel Placental-Derived Hormone. *Endocrinology* **142**:788-794.

Guerra D, Frizziero L, Losi M, Bacchelli B, Mezzadri G, Pasquali-Ronchetti I. (1996) Ultrastructural identification of a membrane-like structure on the surface of normal articular cartilage. *J. Submicrosc. Cytol. Pathol.* **28**:385-93.

Guillemin R, Brazeau P, Bohlen P, Esch F, Ling N, Wehrenberg WB. (1982) Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. *Science* **218**:585-7.

Hattori N, Saito T, Yagyu T, Jiang BH, Kitagawa K, Inagaki C. (2001) GH, GH receptor, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils. *J. Clin. Endocrinol. Metab.* **86**:4284-91.

Hedinger CE, Farquhar MG. (1957) [Electron microscopic examination of two types of acidophilic anterior pituitary cells in the rat.] *Schweiz Z. Pathol. Bakteriol.* **20**:766-8.

Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. (1997) Multiplex PCR: critical parameters and step-by-step protocol. *Biotechniques* **23**:504-11.

Henry PC, Kanelis V, O'Brien MC, Kim B, Gautschi I, Forman-Kay J, Schild L, Rotin D. (2003) Affinity and specificity of interactions between Nedd4 isoforms and the epithelial Na+ channel. *J. Biol. Chem.* **278**:20019-28.

Hirotsune S, Yoshida N, Chen A, Garrett L, Sugiyama F, Takahashi S, Yagami K, Wynshaw-Boris A, Yoshiki A. (2003) An expressed pseudogene regulates the messenger-RNA stability of its homologous coding gene. *Nature* **423**:91-96.

Hirschberg R, Adler S. (1998) Insulin-like growth factor system and the kidney: physiology, pathophysiology, and therapeutic implications. *Am. J. Kidney Dis.* **31**:901-19.

Hoeffler JP, Boockfor FR, Frawley LS. (1985) Ontogeny of prolactin cells in neonatal rats: initial prolactin secretors also release growth hormone. *Endocrinology* **117**:187-95.

Hoeflich A, Wu M, Mohan S, Foll J, Wanke R, Froehlich T, Arnold GJ, Lahm H, Kolb HJ, Wolf E. (1999) Overexpression of insulin-like growth factor-binding protein-2 in transgenic mice reduces postnatal body weight gain. *Endocrinology* **140**:5488-96.

Hornstein OP. (1984) [The thyroid gland, the parathyroid gland and the skin] *Z. Hautkr.* **59**:1125-6, 1129-32, 1137-43.

Horster M. (2000) Embryonic epithelial membrane transporters. *Am. J. Physiol. Renal Physiol.* **279**:F982-F996.

Horvath E, Lloyd RV, Kovacs K. (1990) Propylthiouracyl-induced hypothyroidism results in reversible transdifferentiation of somatotrophs into thyroidectomy cells. A morphologic study of the rat pituitary including immunoelectron microscopy. *Lab. Invest.* **63**:511-20.

Hosoda H, Kojima M, Matsuo H, Kangawa K. (2000) Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. *J. Biol. Chem.* **275**:21995-2000.

Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Van der Ploeg LH, et al. (1996) A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273**:974-7.

Hreniuk DL, Palyha OC, Howard AD, Woods JM, Smith RG, Feighner SD. (1998) Immunocytochemical localisation of a growth hormone secretagogue receptor in rat pituitary cells. *78th Annual Meeting of the Endocrine Society, New Orleans.* **P2-224**.

Hu Z, Friberg RD, Barkan AL. (1993) Ontogeny of GH mRNA and GH secretion in male and female rats: regulation by GH-releasing hormone. *Am. J. Physiol.* **265**:E236-42.

Huang CY, Kuo WW, Tsai TP, Wu DJ, Hsieh YS, Wang PS, Cheng CK, Liu JY. (2002) Prolactin secretion and intracellular Ca(2+) change in rat lactotroph subpopulations stimulated by thyrotropin-releasing hormone. *J. Cell. Biochem.* **87**:126-32.

Huerta-Ocampo I, Wells T, El-Kasti MM, Christian HC. (2004) The role of growth hormone-releasing hormone (GHRH) on the somatotroph and lactotroph lineage in male and female dwarf (*dw/dw*) rats. *The 2004 Annual Meeting of the British Society for Neuroendocrinology. Glasgow-U.K.* **P13**.

Hull KL, Harvey S. (2001) Growth hormone: roles in female reproduction. *J. Endocrinol.* **168**:1-23.

Ishibashi T, Shiino M. (1989) Co-localization pattern of growth hormone (GH) and prolactin (PRL) within the anterior pituitary cells in the female rat and female musk shrew. *Anat. Rec.* **223**:185-93.

Jacks T, Hickey G, Judith F, Taylor J, Chen H, Krupa D, Feeney W, Schoen W, Ok D, Fisher M, et al. (1994) Effects of acute and repeated intravenous administration of L-692,585, a novel non-peptidyl growth hormone secretagogue, on plasma growth hormone, IGF-1, ACTH, cortisol, prolactin, insulin, and thyroxine levels in beagles. *J. Endocrinol.* **143**:399-406.

Jahn GA, Rastrilla AM, Deis RP. (1993) Correlation of growth hormone secretion during pregnancy with circulating prolactin in rats. *J. Reprod. Fertil.* **98**:327-33.

Jansson JO, Ekberg S, Isaksson OG, Eden S. (1984) Influence of gonadal steroids on age- and sex-related secretory patterns of growth hormone in the rat. *Endocrinology* **114**:1287-94.

Jelinsky SA, Harris, HA, Brown EL, Flanagan K, Zhang XC, Tunkey C, Lai KD, Lane MV, Simcoe DK, Evans, MJ. (2003) Global transcription profiling of estrogen activity: Estrogen receptor a regulates gene expression in the kidney. *Endocrinology* **144**:701-710.

Jenkinson CM, Min SH, Mackenzie DD, McCutcheon SN, Breier BH, Gluckman PD. (1999) Placental development and fetal growth in growth hormone-treated ewes. *Growth Horm. IGF Res.* **9**:11-7.

Jolliffe CN, Harvey KF, Haines BP, Parasivam G, Kumar S. (2000) Identification of multiple proteins expressed in murine embryos as binding partners for the WW domains of the ubiquitin-protein ligase Nedd4. *Biochem. J.* **351**: 557-565.

Josimovich JB, Maclaren JA. (1962) Presence in the human placenta and term serum of a highly lactogenic substance immunologically related to pituitary growth hormone. *Endocrinology* **71**:209-20.

Juarez-Aguilar E, Castro-Munozledo F, Guerra-Rodriguez NE, Resendez-Perez D, Martinez-Rodriguez HG, Barrera-Saldana HA, Kuri-Harcuch W. (1999) Functional domains of human growth hormone necessary for the adipogenic activity of hGH/hPL chimeric molecules. *J. Cell. Sci.* **112**:3127-3135.

Kacsoh, B. (2000) Endocrine physiology, McGraw and Hill, Medical Publishing Division, New York.

Kalina M, Pease DC. (1977) The probable role of phosphatidyl cholines in the tannic acid enhancement of cytomembrane electron contrast. *J. Cell. Biol.* **74**:742-6.

Kamegai J, Minami S, Sugihara H, Hasegawa O, Higuchi H, Wakabayashi I. (1996) Growth hormone receptor gene is expressed in neuropeptide Y neurons in hypothalamic arcuate nucleus of rats. *Endocrinology* **137**:2109-12.

Kaneko T. (1996) Cell biology of somatolactin. *Int. Rev. Cytol.* **169**:1-24.

Kelly PA, Djiane J, Postel-Vinay MC, Edery M. (1991) The prolactin/growth hormone receptor family. *Endocr. Rev.* **12**:235-51.

Kelly MA, Rubinstein M, Asa SL, Zhang G, Saez C, Bunzow JR, Allen RG, Hnasko R, BenJonathan N, Grandy DK, Low MJ. (1997) Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. *Neuron* **19**:103-113.

Kineman RD, Chen TT, Frawley LS. (1989) A cellular basis for growth hormone deficiency in the dwarf rat: analysis of growth hormone and prolactin release by reverse hemolytic plaque assay. *Endocrinology* **125**:2035-40.

Kineman RD, Henricks DM, Faught WJ, Frawley LS. (1991) Fluctuations in the proportions of growth hormone- and prolactin-secreting cells during the bovine estrous cycle. *Endocrinology* **129**:1221-5.

Kineman RD, Faught WJ, Frawley LS. (1992a) The ontogenic and functional relationships between growth hormone- and prolactin-releasing cells during the development of the bovine pituitary. *J. Endocrinol.* **134**:91-6.

Kineman RD, Faught WJ, Frawley LS. (1992b) Steroids can modulate transdifferentiation of prolactin and growth hormone cells in bovine pituitary cultures. *Endocrinology* **130**:3289-94.

Kioussi C, Carriere C, Rosenfeld MG. (1999) A model for the development of the hypothalamic-pituitary axis: Transcribing the hypophysis. *Mech. Develop.* **81**: 23-35.

Kishi K, Hirashiba M, Hasegawa Y. (1991) Gestational profiles of rat placental lactogen-II (rPL-II) and growth hormone (GH) in maternal and fetal serum, amniotic fluid, and placental tissue. *Endocrinol. Jpn.* **38**:589-95.

Knepper MA, Wade JB, Terris J, Ecelbarger CA, Marples D, Mandon B, Chou CL, Kishore BK, Nielsen S. (1996) Renal aquaporins. *Kidney Int.* **49**:1712-7.

Koch BD, Schonbrunn A. (1984) The somatostatin receptor is directly coupled to adenylate cyclase in GH4C1 pituitary cell membranes. *Endocrinology* **114**:1784-90.

Koga M, Nakao H, Arao M, Sato B, Noma K, Morimoto Y, Kishimoto S, Mori S, Uozumi T. (1987) Demonstration of specific dopamine receptors on human pituitary adenomas. *Acta Endocrinol.* **114**:595-602.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**: 656-660.

Konstas AA, Shearwin-Whyatt LM, Fotia AB, Degger B, Riccardi D, Cook DI, Korbmacher C, Kumar S. (2002) Regulation of the epithelial sodium channel by N4WBP5A, a novel Nedd4/Nedd4-2-interacting protein. *J. Biol. Chem.* **277**:29406-16.

Kovacs M, Fancsik A, Hrabovsky E, Mezo I, Teplan I, Flerko B. (1995) Effects of continuous and repetitive administration of a potent analog of GH-RH(1-30)-NH2 on the GH release in rats treated with monosodium glutamate. *J. Neuroendocrinol.* 7:703-12.

Kovacs M, Kineman RD, Schally AV, Flerko B, Frohman LA. (2000) Increase in mRNA concentrations of pituitary receptors for growth hormone-releasing hormone and growth hormone secretagogues after neonatal monosodium glutamate treatment. *J. Neuroendocrinol.* **12**:335-41.

Kukstas LA, Verrier D, Zhang J, Chen C, Israel JM, Vincent JD. (1990) Evidence for a relationship between lactotroph heterogeneity and physiological context. *Neurosci. Lett.* **120**:84-6.

Kumar U, Laird D, Srikant CB, Escher E, Patel YC. (1997) Expression of the five somatostatin receptor (SSTR1-5) subtypes in rat pituitary somatotrophes: quantitative analysis by double-layer immunofluorescence confocal microscopy. *Endocrinology* **138**:4473-6.

Kunkele J, Kenagy GJ. (1997) Inefficiency of lactation in primiparous rats: the costs of first reproduction. *Physiol. Zool.* **70**:571-7.

Laron Z. (2004) Laron syndrome (primary growth hormone resistance or insensitivity): the personal experience 1958-2003. *J. Clin. Endocrinol. Metab.* **89**:1031-44.

Lawrence CB, Snape AC, Baudoin FM, Luckman SM. (2002) Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. *Endocrinology* **143**:155-62.

Legraverend C, Mode A, Wells T, Robinson I, Gustafsson JA. (1992) Hepatic steroid hydroxylating enzymes are controlled by the sexually dimorphic pattern of growth hormone secretion in normal and dwarf rats. *FASEB J.* **6**:711-8.

Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. (2001) The somatomedin hypothesis: 2001. *Endocr. Rev.* **22**:53-74.

Le Tissier P, Magoulas B, Mathers K, McGuinness L, Sesay AK, Carmignac DF, Robinson IC (2002) Ablating GRF neurones in transgenic mice using a novel viral ion channel transgene. *84th Annual Meeting of the Endocrine Society*, San Francisco, USA. Abstract **OR31-1**.

Lee SL, Sadovsky Y, Swirnoff AH, Polish JA, Goda P, Gavrilina G, Milbrandt J. (1996) Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). *Science* **273**:1219-21.

Lelievre-Pegorier M, Merlet-Benichou C. (2000) The number of nephrons in the mammalian kidney: environmental influences play a determining role. *Exp. Nephrol.* **8**:63-5.

Leturque A, Burnol AF, Ferre P, Girard J. (1984) Pregnancy-induced insulin resistance in the rat: assessment by glucose clamp technique. *Am. J. Physiol.* **246**:E25-31.

Leung DW, Spencer SA, Cachianes G, Hammonds RG, Collins C, Henzel WJ, Barnard R, Waters MJ, Wood WI. (1987) Growth hormone receptor and serum binding protein: purification, cloning and expression. *Nature* **330**:537-43.

Li H, Witte DP, Branford WW, Aronow BJ, Weinstein M, Kaur S, Wert S, Singh G, Schreiner CM, Whitsett JA, et al. (1994) Gsh-4 encodes a LIM-type homeodomain, is expressed in the developing central nervous system and is required for early postnatal survival. *EMBO J.* **13**:2876-85.

Li H, Zeitler PS, Valerius MT, Small K, Potter SS. (1996) Gsh-1, an orphan Hox gene, is required for normal pituitary development. *EMBO J.* **15**:714-24.

Li S, Crenshaw EB 3rd, Rawson EJ, Simmons DM, Swanson LW, Rosenfeld MG. (1990) Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature* **347**:528-33.

Lieberman ME, Maurer RA, Claude P, Gorski J. (1982) Prolactin synthesis in primary cultures of pituitary cells: regulation by estradiol. *Mol. Cell. Endocrinol.* **25**:277-94.

Lin SC, Lin CR, Gukovsky I, Lusis AJ, Sawchenko PE, Rosenfeld MG. (1993) Molecular basis of the little mouse phenotype and implications for cell type-specific growth. *Nature* **364**:208-13.

Ling C, Billig H. (2001) PRL receptor-mediated effects in female mouse adipocytes: PRL induces suppressors of cytokine signaling expression and suppresses insulin-induced leptin production in adipocytes in vitro. *Endocrinology* **142**:4880-90.

Liu B, Preisig PA. (2002) Compensatory renal hypertrophy is mediated by a cell cycle-dependent mechanism. *Kidney Int.* **62**:1650-8.

Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. (1993) Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r).*Cell* **75**:59-72.

Lockhart DJ, Winzeler EA. (2000) Genomics, gene expression and DNA arrays. *Nature* **405**:827-36.

Lockhart DJ, Barlow C. (2001) DNA arrays and gene expression analysis in the brain. In: Methods in genomic neuroscience. Chin, H.R. and Moldin, S.O. (Eds.) Boca Raton, Fla. CRC Press.

Lu S, Guan JL, Wang QP, Uehara K, Yamada S, Goto N, Date Y, Nakazato M, Kojima M, Kangawa K, Shioda S. (2002) Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. *Neurosci. Lett.* **321**:157-60.

Lucocq JM, Habermann A, Watt S, Backer JM, Mayhew TM, Griffiths G. (2004) A rapid method for assessing the distribution of gold labeling on thin sections. *J. Histochem. Cytochem.* **52**:991-1000.

Luque EH, Munoz de Toro M, Smith PF, Neill JD. (1986) Subpopulations of lactotropes detected with the reverse hemolytic plaque assay show differential responsiveness to dopamine. *Endocrinology* **118**:2120-4.

MacGregor GR, Overbeek PA. (1991) Use of a simplified single-site PCR to facilitate cloning of genomic DNA sequences flanking a transgene integration site. *PCR Methods Appl.* **1**:129-35.

MacLeod JN, Worsley I, Ray J, Friesen HG, Liebhaber SA, Cooke NE.Human (1991) growth hormone-variant is a biologically active somatogen and lactogen. *Endocrinology* **128**:1298-302.

MacLeod RM. (1969) Influence of norepinephrine and catecholamine-depleting agents on the synthesis and release of prolactin and growth hormone. *Endocrinology* **85**:916-23.

Magoulas C, McGuinness L, Balthasar N, Carmignac DF, Sesay AK, Mathers KE, Christian H, Candeil L, Bonnefont X, Mollard P, Robinson IC. (2000) A secreted fluorescent reporter targeted to pituitary growth hormone cells in transgenic mice. *Endocrinology* **141**:4681-9.

Maiter D, Underwood LE, Martin JB, Koenig JI. (1991) Neonatal treatment with monosodium glutamate: effects of prolonged growth hormone (GH)-releasing hormone deficiency on pulsatile GH secretion and growth in female rats. *Endocrinology* **128**:1100-6.

Maldonado C, Aoki A. (1994) Occurrence of atypical lactotrophs associated with levels of prolactin secretory activity. *Biocell* **18**: 83-95.

Martin CE, Cake MH, Hartmann PE, Cook IF. (1977) Relationship between foetal corticosteroids, maternal progesterone and parturition in the rat. *Acta Endocrinol. (Copenh).* **84**:167-76.

Masson P (1929) Some histological methods. Trichrome stainings and their preliminary technique. *Bull. Int. Assoc. Med.* **12**:75.

Matthews DR, Hindmarsh PC, Pringle PJ, Brook CG. (1991) A distribution method for analysing the baseline of pulsatile endocrine signals as exemplified by 24-hour growth hormone profiles. *Clin. Endocrinol.* **35**:245-252.

Mayhew TM, Desoye G. (2004) A simple method for comparing immunogold distributions in two or more experimental groups illustrated using GLUT1 labelling of isolated trophoblast cells. *Placenta* **25**:580-4.

Mayhew TM, Griffiths G, Lucocq JM. (2004) Applications of an efficient method for comparing immunogold labelling patterns in the same sets of compartments in different groups of cells. *Histochem. Cell Biol.***122**:171-7.

Mayo KE, Vale W, Rivier J, Rosenfeld MG, Evans RM. (1983) Expression-cloning and sequence of a cDNA encoding human growth hormone-releasing factor. *Nature* **306**:86-8.

Mayo KE, Godfrey PA, Suhr ST, Kulik DJ, Rahal JO. (1995) Growth hormone-releasing hormone: synthesis and signaling. *Recent Prog. Horm. Res.* **50**:35-73.

Mayo KE. (1996) A little lesson in growth regulation. *Nat. Genet.* **12**:8-9.

Mayo KE, Miller T, DeAlmeida V, Godfrey P, Zheng J, Cunha SR. (2000) Regulation of the pituitary somatotroph cell by GHRH and its receptor. *Recent Prog. Horm. Res.* **55**:237-267.

Melia MJ, Bofill N, Hubank M, Meseguer A. (1998) Identification of androgen-regulated genes in mouse kidney by representational difference analysis and random arbitrarily primed polymerase chain reaction. *Endocrinology* **139**:688-95.

Merchenthaler I, Vigh S, Schally AV, Petrusz P. (1984) Immunocytochemical localization of growth hormone-releasing factor in the rat hypothalamus. *Endocrinology* **114**:1082-5.

Merimee TJ, Zapf J, Froesch ER. (1981) Dwarfism in the pygmy. An isolated deficiency of insulin-like growth factor I. *N. Engl. J. Med.* **305**:965-8.

Merriam GR, Wachter KW. (1982) Algorithms for the study of episodic hormone secretion. *Am. J. Physiol.* **243**:E310-8.

Meseguer A, Catterall JF. (1987) Mouse kidney androgen-regulated protein messenger ribonucleic acid is expressed in the proximal convoluted tubules. *Mol. Endocrinol.* **1**:535-41.

Meseguer A, Catterall JF. (1990a) Cell-specific expression of kidney androgen-regulated protein messenger RNA is under multihormonal control. *Mol. Endocrinol.* **4**:1240-8.

Meseguer A, Catterall JF. (1990b) Detection of spatially regulated gene expression by hybridization histochemistry. *Rev. Esp. Fisiol.* **46**:69-78.

Meseguer A, Catterall JF. (1992) Effects of pituitary hormones on the cell-specific expression of the KAP gene. *Mol. Cell. Endocrinol.* **89**:153-62.

Meseguer A, Watson CS, Catterall JF. (1989) Nucleotide sequence of kidney androgen-regulated protein mRNA and its cell-specific expression in Tfm/Y mice. *Mol. Endocrinol.* **3**:962-7.

Millar DS, Lewis MD, Horan M, Newsway V, Easter TE, Gregory JW, Fryklund L, Norin M, Crowne EC, Davies SJ, Edwards P, Kirk J, Waldron K, Smith PJ, Phillips JA 3rd, Scanlon MF, Krawczak M, Cooper DN, Procter AM. (2003) Novel mutations of the growth hormone 1 (GH1) gene disclosed by modulation of the clinical selection criteria for individuals with short stature. *Hum. Mutat.***21**:424-40.

Millard WJ, Martin JB Jr, Audet J, Sagar SM, Martin JB. (1982) Evidence that reduced growth hormone secretion observed in monosodium glutamate-treated rats is the result of a deficiency in growth hormone-releasing factor. *Endocrinology* **110**:540-50.

Mirnics K, Lewis DA, Levitt P. (2001) DNA microarrays and human brain disorders. In: Methods in genomic neuroscience. Chin, H.R. and Moldin, S.O. (Eds.) Boca Raton, Fla. CRC Press.

Mizobuchi M, Frohman MA, Downs TR, Frohman LA. (1991) Tissue-specific transcription initiation and effects of growth hormone (GH) deficiency on the regulation of mouse and rat GH-releasing hormone gene in hypothalamus and placenta. *Mol. Endocrinol.* **5**:476-84.

Mori K, Yoshimoto A, Takaya K, Hosoda K, Ariyasu H, Yahata K, Mukoyama M, Sugawara A, Hosoda H, Kojima M, Kangawa K, Nakao K. (2000) Kidney produces a novel acylated peptide, ghrelin. *FEBS Lett.* **486**:213-6.

Muccioli G, Tschop M, Papotti M, Deghenghi R, Heiman M, Ghigo E. (2002) Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity. *Eur. J. Pharmacol.* **440**:235-54.

Mulchahey JJ, Jaffe RB. (1988) Detection of a potential progenitor cell in the human fetal pituitary that secretes both growth hormone and prolactin. *J. Clin. Endocrinol. Metab.* **66**:24-32.

Murillas R, Simms KS, Hatakeyama S, Weissman AM, Kuehn MR. (2002) Identification of developmentally expressed proteins that functionally interact with Nedd4 ubiquitin ligase. *J. Biol. Chem.* **277**:2897-907.

Nagy E, Berczi I. (1991) Hypophysectomized rats depend on residual prolactin for survival. *Endocrinology* **128**:2776-84.

Nakago S, Funakoshi T, Ueda Y, Maruo T. (1999) Regulation of circulating levels of IGF-I in pregnant rats: changes in nitrogen balance correspond with changes in serum IGF-I concentrations. *J. Endocrinol.* **163**:373-377.

Nakane PK. (1975) *The Anterior Pituitary Gland.* Acad. Press, NY, p134.

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. (2001) A role for ghrelin in the central regulation of feeding. *Nature* **409**:194-8.

Nanto-Salonen K, Glasscock GF, Rosenfeld RG. (1991) The effects of thyroid hormone on insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) expression in the neonatal rat: prolonged high expression of IGFBP-2 in methimazole-induced congenital hypothyroidism. *Endocrinology* **129**:2563-70.

Neugarten J, Silbiger SR. (1995) Effects of sex hormones on mesangial cells. *Am. J. Kidney Dis.* **26**:147-51.

Nikitovitch-Winer MB, Atkin J, Maley BE. (1987) Colocalization of prolactin and growth hormone within specific adenohypophyseal cells in male, female, and lactating female rats. *Endocrinology* **121**:625-30.

Nilsson A, Ohlsson C, Isaksson OG, Lindahl A, Isgaard J. (1994) Hormonal regulation of longitudinal bone growth. *Eur. J. Clin. Nutr.* **48** Suppl 1:S150-8; discussion S158-60.

Nishihira M, Yagihashi S. (1978) Immunohistochemical demonstration of somatostatin-containing cells in the human placenta. *Tohoku J. Exp. Med.* **126**:397-8.

Nogami H, Takeuchi T, Suzuki K, Okuma S, Ishikawa H. (1989) Studies on prolactin and growth hormone gene expression in the pituitary gland of spontaneous dwarf rats. *Endocrinology* **125**:964-70.

Nogami H, Yoshimura F. (1980) Prolactin immunoreactivity of acidophils of the small granule type. *Cell Tissue Res.* **211**:1-4.

Nogami H. (1984) Fine-structural heterogeneity and morphologic changes in rat pituitary prolactin cells after estrogen and testosterone treatment. *Cell. Tissue Res.* **237**:195-202.

Nogues N, Del Rio JA, Perez-Riba M, Soriano E, Flavell RA, Boronat A. (1997) Placenta-specific expression of the rat growth hormone-releasing hormone gene promoter in transgenic mice. *Endocrinology* **138**:3222-7.

Norton JD, Atherton GT. (1998) Coupling of cell growth control and apoptosis functions of Id proteins. *Mol. Cell. Biol.* **18**:2371-81.

Nyengaard JR. (1999) Stereologic methods and their application in kidney research. *J. Am. Soc. Nephrol.* **10**:1100-23.

Ochiai K, Kato H, Kelly PA, Rothchild I. (1983) The importance of a luteolytic effect of the pituitary in understanding the placental control of the rat corpus luteum. *Endocrinology* **112**:1687-95.

Ohlsson C, Isgaard J, Tornell J, Nilsson A, Isaksson OG, Lindahl A. (1993) Endocrine regulation of longitudinal bone growth. *Acta Paediatr. Suppl.* **391**:33-40.

Olson LE, Rosenfeld MG. (2002) Perspective: genetic and genomic approaches in elucidating mechanisms of pituitary development. *Endocrinology* **143**:2007-11.

Orgnero de Gaisan EM, Maldonado CA, Aoki A. (1993) Fate of degenerating lactotrophs in rat pituitary gland after interruption of lactation: a histochemical and immunocytochemical study. *Histochem. J.* **25**:150-65.

Ouar Z, Sole E, Bens M, Rafestin-Oblin ME, Meseguer A, Vandewalle A. (1998) Pleiotropic effects of dihydrotestosterone in immortalized mouse proximal tubule cells. Technical note. *Kidney Int.* **53**:59-66.

Owens JA. (1991) Endocrine and substrate control of fetal growth: placental and maternal influences and insulin-like growth factors. *Reprod. Fertil. Dev.* **3**:501-517.

Papka RE, Yu SM, Nikitovitch-Winer MB. (1986) Use of immunoperoxidase and immunogold methods in studying prolactin secretion and application of immunogold labelling for pituitary hormones and neuropeptides. *Am. J. Anat.* **175**:289-306.

Parks JS, Brown MR, Hurley DL, Phelps CJ, Wajnrajch, MP. (1999) Commentary - Heritable disorders of pituitary development. *J. Clin. Endocr. Metab.* **84**:4362-4370.

Pasolli HA, Torres AI, Aoki A. (1994) The mammosomatotroph: a transitional cell between growth hormone and prolactin producing cells? An immunocytochemical study. *Histochemistry* **102**:287-96.

Patel YC, Srikant CB. (1986) Somatostatin mediation of adenohypophysial secretion. *Annu. Rev. Physiol.* **48**:551-67.

Perello M, Gaillard RC, Chisari A, Spinedi E. (2003) Adrenal enucleation in MSG-damaged hyperleptinemic male rats transiently restores adrenal sensitivity to leptin. *Neuroendocrinology* **78**:176-84.

Phelps CJ, Carlson SW, Vaccarella MY, Felten SY. (1993) Developmental assessment of hypothalamic tuberoinfundibular dopamine in prolactin-deficient dwarf mice. *Endocrinology* **132**:2715-22.

Pincus SM, Gevers EF, Robinson IC, van den Berg G, Roelfsema F, Hartman ML, Veldhuis JD. (1996) Females secrete growth hormone with more process irregularity than males in both humans and rats. *Am. J. Physiol.*.**270**:E107-15.

Pioro EP, Scheithauer BW, Laws ER Jr, Randall RV, Kovacs KT, Horvath E. (1988) Combined thyrotroph and lactotroph cell hyperplasia simulating prolactin-secreting pituitary adenoma in long-standing primary hypothyroidism. *Surg. Neurol.* **29**:218-26.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**:143-7.

Pong SS, Chaung LY, Dean DC, Nargund RP, Patchett AA, Smith RG. (1996) Identification of a new G-protein-linked receptor for growth hormone secretagogues. *Mol. Endocrinol.* **10**:57-61.

Porter TE, Dean KJ. (2001) Regulation of chicken embryonic growth hormone secretion by corticosterone and triiodothyronine: evidence for a negative synergistic response. *Endocrine* **14**:363-8.

Porter TE, Hill JB, Wiles CD, Frawley LS. (1990) Is the mammosomatotrope a transitional cell for the functional interconversion of growth hormone- and prolactin-secreting cells? Suggestive evidence from virgin, gestating, and lactating rats. *Endocrinology* **127**:2789-94.

Porter TE, Wiles CD, Frawley LS. (1991) Evidence for bidirectional interconversion of mammotropes and somatotropes: rapid reversion of acidophilic cell types to pregestational proportions after weaning. *Endocrinology* **129**:1215-20.

Powell DR, Durham SK, Brewer ED, Frane JW, Watkins SL, Hogg RJ, Mohan S. (1999) Effects of chronic renal failure and growth hormone on serum levels of insulin-like growth factor-binding protein-4 (IGFBP-4) and IGFBP-5 in children: a report of the Southwest Pediatric Nephrology Study Group. *J. Clin.Endocrinol. Metab.* **84**:596-601.

Preisig PA. (1999) What makes cells grow larger and how do they do it? Renal hypertrophy revisited. *Exp. Nephrol.* 7:273-83.

Ramanathan L, Das NP, Li QT. (1994) Studies on lipid oxidation in fish phospholipid liposomes. *Biol. Trace Elem. Res.* **40**:59-70.

Ray J, Okamura H, Kelly PA, Cooke NE, Liebhaber SA. (1990) Human growth hormone-variant demonstrates a receptor binding profile distinct from that of normal pituitary growth hormone. *J. Biol. Chem.* **265**:7939-44.

Ray PE, Liu XH, Robinson LR, Reid W, Xu L, Owens JW, Jones OD, Denaro F, Davis HG, Bryant JL. (2003) A novel HIV-1 transgenic rat model of childhood HIV-1-associated nephropathy. *Kidney Int.* **63**:2242-2253.

Reger JF, Frase S, Tso P. (1989) Fine structure observations on rat jejunal epithelial cells during fat processing and resorption following L-81 exposure and reversal. *J. Submicrosc. Cytol. Pathol.* **21**:399-408.

Reid W, Sadowska M, Denaro F, Rao S, Foulke J Jr, Hayes N, Jones O, Doodnauth D, Davis H, Sill A, O'Driscoll P, Huso D, Fouts T, Lewis G, Hill M, Kamin-Lewis R, Wei C, Ray P, Gallo RC, Reitz M, Bryant J. (2001) An HIV-1 transgenic rat that develops HIV-related pathology and immunologic dysfunction. *Proc. Natl. Acad. Sci. U S A.* **98**:9271-6.

Reis FM, Florio P, Cobellis L, Luisi S, Severi FM, Bocchi C, Picciolini E, Centini G, Petraglia F. (2001) Human placenta as a source of neuroendocrine factors. *Biol. Neonate* **79**:150-6.

Reis FM, Petraglia F. (2001) The placenta as a neuroendocrine organ. *Front. Horm. Res.* **27**:216-28.

Reitman ML, Bi S, Marcus-Samuels B, Gavrilova O. (2001) Leptin and its role in pregnancy and fetal development--an overview. *Biochem. Soc. Trans.* **29**:68-72.

Rhodes SJ, DiMattia GE, Rosenfeld MG. (1994) Transcriptional mechanisms in anterior pituitary cell differentiation. *Curr. Opin. Genet. Dev.* **4**:709-17.

Ricker JL, Gattone VH 2nd, Calvet JP, Rankin CA. (2000) Development of autosomal recessive polycystic kidney disease in BALB/c-cpk/cpk mice. *J. Am. Soc. Nephrol.* **11**:1837-47.

Rivier J, Spiess J, Thorner M, Vale W. (1982) Characterization of a growth hormone-releasing factor from a human pancreatic islet tumour. *Nature* **300**:276-8.

Robson H, Siebler T, Shalet SM, Williams GR. (2002) Interactions between GH, IGF-I, glucocorticoids, and thyroid hormones during skeletal growth. *Pediatr. Res.* **52**:137-47.

Roelfsema V, Clark RG. (2001) The growth hormone and insulin-like growth factor axis: its manipulation for the benefit of growth disorders in renal failure. *J. Am. Soc. Nephrol.* **12**:1297-306.

Root AW, Kemp SF, Rundle AC, Dana K, Attie KM. (1998) Effect of long-term recombinant growth hormone therapy in children--the National Cooperative Growth Study, USA, 1985-1994. *J. Pediatr. Endocrinol. Metab.* **11**:403-12.

Rosenblatt JS, Mayer AD, Giordano AL. (1988) Hormonal basis during pregnancy for the onset of maternal behavior in the rat. *Psychoneuroendocrinology* **13**:29-46.

Roti E, Christianson D, Harris AR, Braverman LE, Vagenakis AG. (1978) Short" loop feedback regulation of hypothalamic and brain thyrotropin-releasing hormone content in the rat and dwarf mouse. *Endocrinology* **103**:1662-7.

Roux M, Bartke A, Dumont F, Dubois MP. (1982) Immunohistological study of the anterior pituitary gland - pars distalis and pars intermedia - in dwarf mice. *Cell. Tissue Res.* **223**:415-20.

Saiardi A, Bozzi Y, Baik JH, Borrelli E. (1997) Antiproliferative role of dopamine: loss of D2 receptors causes hormonal dysfunction and pituitary hyperplasia. *Neuron* **19**:115-26.

Salacinski PR, McLean C, Sykes JE, Clement-Jones VV, Lowry PJ. (1981) Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenyl glycoluril (Iodogen). *Anal. Biochem.* **117**:136-46.

Samson WK, Taylor MM, Baker JR. (2003) Prolactin-releasing peptides. *Regul. Pept.* **114**:1-5.

Sasaki F, Iwama Y. (1988) Sex difference in prolactin and growth hormone cells in mouse adenohypophysis: stereological, morphometric, and immunohistochemical studies by light and electron microscopy. *Endocrinology* **123**:905-12.

Sato M, Frohman LA. (1993) Differential effects of central and peripheral administration of growth hormone (GH) and insulin-like growth factor on hypothalamic GH-releasing hormone and somatostatin gene expression in GH-deficient dwarf rats. *Endocrinology* **133**:793-9.

Scaglia HE, Colombani M, Zylbersztein C, Margulies M, Alvarez S, Voto LS. (1981) Prolactin, estriol and progesterone levels in frequent blood samples throughout normal pregnancy (lack of prolactin pulsatile secretion). *Ric Clin Lab.* **11**:65-74.

Schmitt R, Klussmann E, Kahl T, Ellison DH, Bachmann S. (2003) Renal expression of sodium transporters and aquaporin-2 in hypothyroid rats. *Am. J. Physiol. Renal Physiol.* **284**:F1097-104.

Scully KM, Gleiberman AS, Lindzey J, Lubahn DB, Korach KS, Rosenfeld MG. (1997) Role of estrogen receptor-alpha in the anterior pituitary gland. *Mol. Endocrinol.* **11**:674-81.

Scully KM, Rosenfeld MG. (2002) Development - Pituitary development: Regulatory codes in mammalian organogenesis. *Science* **295**:2231-2235.

Seifert H, Perrin M, Rivier J, Vale W. (1985) Binding sites for growth hormone releasing factor on rat anterior pituitary cells. *Nature* **313**:487-9.

Seliger SL, Davis C, Stehman-Breen C. (2001) Gender and the progression of renal disease. *Curr. Opin. Nephrol. Hypertens.* **10**:219-25.

Severinghaus AE. (1973) Cellular changes in the anterior hypophysis with special references to its secretory activities. *Physiol. Rev.* **17**:556-588.

Sheng HZ, Westphal H. (1999) Early steps in pituitary organogenesis. *Trends Genet.* **15**:236-240.

Sheppard MS, Bala RM. (1987) Growth hormone secretion during pregnancy: altered effects of growth hormone releasing factor and insulin-like growth factor-I in vitro. *Horm. Res.* **27**:205-10.

Shibata K, Hosoda H, Kojima M, Kangawa K, Makino Y, Makino I, Kawarabayashi T, Futagami K, Gomita Y. (2004) Regulation of ghrelin secretion during pregnancy and lactation in the rat: possible involvement of hypothalamus. *Peptides* **25**:279-87.

Shimon I, Yan X, Melmed S. (1998) Human fetal pituitary expresses functional growth hormone-releasing peptide receptors. *J. Clin. Endocrinol. Metab.* **83**:174-8.

Siegel NJ (2001) Minimal change disease. In: Primer on kidney diseases. 3rd edition. Greenberg A (Ed.). National Kidney Foundation. Academic Press, London, U.K.

Silbiger SR, Neugarten J. (2003) The role of gender in the progression of renal disease. *Adv. Ren. Replace. Ther.* **10**:3-14.

Simon SA, Disalvo EA, Gawrisch K, Borovyagin V, Toone E, Schiffman SS, Needham D, McIntosh TJ. (1994) Increased adhesion between neutral lipid bilayers: interbilayer bridges formed by tannic acid. *Biophys. J.* **66**:1943-58.

Skriver E, Maunsbach AB, Jorgensen PL. (1980) Ultrastructure of Na,K-transport vesicles reconstituted with purified renal Na,K-ATPase. *J. Cell. Biol.* **86**:746-54.

Slade JP. (2001) Characterization of a novel rat mutant: Juvenile-onset growth retardation (Jogr) (Chapter 5). In: Transgenic and physiological investigations of Egr-1. *PhD Thesis*. Cardiff University, Cardiff, UK.

Slade JP, Thompson NM, Davies JS, Wells T, Carter DA. (2001) Juvenile-onset growth retardation: A novel transgenic rat model. *83rd Annual Meeting of the Endocrine Society*, Denver, USA, Abstract **P2-131**.

Slotkin TA, Seidler FJ, Kavlock RJ, Bartolome JV. (1992) Thyroid hormone differentially regulates cellular development in neonatal rat heart and kidney. *Teratology* **45**:303-12.

Smith RG, Van der Ploeg LH, Howard AD, Feighner SD, Cheng K, Hickey GJ, Wyvratt MJ Jr, Fisher MH, Nargund RP, Patchett AA. (1997) Peptidomimetic regulation of growth hormone secretion. *Endocr. Rev.* **18**:621-45.

Smith TP, Gibney J, Kavanagh L, Fahie-Wilson MN, McKenna TJ. (2004) Specificity and clinical utility of methods for the detection of macroprolactin. *The 23rd Joint Meeting of the British Endocrine Societies. Endocrine Abstracts 7,* **P173**.

Snel YE, Brummer RJ, Doerga ME, Zelissen PM, Bakker CJ, Hendriks MJ, Koppeschaar HP. (1995) Adipose tissue assessed by magnetic resonance imaging in growth hormone-deficient adults: the effect of growth hormone replacement and a comparison with control subjects. *Am. J. Clin. Nutr.* **61**:1290-4.

Sole E, Calvo R, Obregon MJ, Meseguer A. (1994) Thyroid hormone controls the cell-specific expression of the kidney androgen-regulated protein gene in S3 mouse kidney cells. *Endocrinology* **135**:2120-9.

Sole E, Calvo R, Obregon MJ, Meseguer A. (1996) Effects of thyroid hormone on the androgenic expression of KAP gene in mouse kidney. *Mol. Cell. Endocrinol.* **119**:147-59.

Sone M, Osamura RY. (2001) Leptin and the pituitary. *Pituitary* **4**:15-23.

Sornson MW, Wu W, Dasen JS, Flynn SE, Norman DJ, O'Connell SM, Gukovsky I, Carriere C, Ryan AK, Miller AP, Zuo L, Gleiberman AS, Andersen B, Beamer WG, Rosenfeld MG. (1996) Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. *Nature* **384**:327-33.

Spady TJ, McComb RD, Shull JD. (1999) Estrogen action in the regulation of cell proliferation, cell survival, and tumorigenesis in the rat anterior pituitary gland. *Endocrine* **11**:217-233.

Spence JW, Sheppard MS, Kraicer J. (1980) Release of growth hormone from purified somatotrophs: interrelation between Ca++ and adenosine 3',5'-monophosphate. *Endocrinology* **106**:764-9.

St John PA, Dufy-Barbe L, Barker JL. (1986) Anti-prolactin cell-surface immunoreactivity identifies a subpopulation of lactotrophs from the rat anterior pituitary. *Endocrinology* **119**:2783-95.

Stefaneanu L, Kovacs K, Horvath E, Asa SL, Losinski NE, Billestrup N, Price J, Vale W. (1989) Adenohypophysial changes in mice transgenic for human growth hormone-releasing factor: a histological, immunocytochemical, and electron microscopic investigation. *Endocrinology* **125**:2710-8.

Stein A, Wild J (2002) Kidney failure explained. Midgley and Milton-Thomson (Eds.). 2nd edition. Class Publishing, London, U.K.

Stoeckel ME, Tappaz M, Hindelang C, Seweryn C, Porte A. (1985) Opposite effects of monosodium glutamate on the dopaminergic and GABAergic innervations of the median eminence and the intermediate lobe in the mouse. *Neurosci. Lett.* **56**:249-55.

Swan SK, Keane WF (2001) Clinical evaluation of renal function. In: Primer on kidney diseases. 3rd edition. Greenberg A (Ed.). National Kidney Foundation. Academic Press, London, U.K.

Takahashi S, Miyatake M (1991) Immunoelectron microscopic study of prolactin cells in the rat - postnatal-development and effects of estrogen and bromocryptine *Zool. Sci.* **8**:549-559.

Takahashi S, Okazaki K, Kawashima S. (1984) Mitotic activity of prolactin cells in the pituitary glands of male and female rats of different ages. *Cell. Tissue Res.* **235**:497-502.

Takenaka M, Imai E, Kaneko T, Ito T, Moriyama T, Yamauchi A, Hori M, Kawamoto S, Okubo K. (1998) Isolation of genes identified in mouse renal proximal tubule by comparing different gene expression profiles. *Kidney Int.* **53**:562-72.

Taniguchi Y, Yasutaka S, Kominami R, Shinohara H. (2001a) Proliferation and differentiation of pituitary somatotrophs and mammotrophs during late fetal and postnatal periods. *Anat. Embryol. (Berl)* **204**:469-75.

Taniguchi Y, Yasutaka S, Kominami R, Shinohara H. (2001b) Proliferation and differentiation of thyrotrophs in the pars distalis of the rat pituitary gland during the fetal and postnatal period. *Anat. Embryol. (Berl)* **203**:249-53.

Tannenbaum GS, Lapointe M, Beaudet A, Howard AD. (1998) Expression of growth hormone secretagogue-receptors by growth hormone-releasing hormone neurons in the mediobasal hypothalamus. *Endocrinology* **139**:4420-3.

Tannenbaum GS, Ling N. (1984) The interrelationship of growth hormone (GH)-releasing factor and somatostatin in generation of the ultradian rhythm of GH secretion. *Endocrinology* **115**:1952-7.

Tannenbaum GS, Martin JB. (1976) Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology* **98**:562-70.

Tannenbaum GS, Painson JC, Lengyel AM, Brazeau P. (1989) Paradoxical enhancement of pituitary growth hormone (GH) responsiveness to GH-releasing factor in the face of high somatostatin tone. *Endocrinology* **124**:1380-8.

Tannenbaum GS, Gurd W, Lapointe M. (1998) Leptin is a potent stimulator of spontaneous pulsatile growth hormone (GH) secretion and the GH response to GH-releasing hormone. *Endocrinology* **139**:3871-5.

Tashjian AH Jr, Barowsky NJ, Jensen DK. (1971) Thyrotropin releasing hormone: direct evidence for stimulation of prolactin production by pituitary cells in culture. *Biochem. Biophys. Res. Commun.* **43**:516-23.

Taylor AD, Christian HC, Morris JF, Flower RJ, Buckingham JC. (1997) An antisense oligodeoxynucleotide to lipocortin 1 reverses the inhibitory actions of dexamethasone on the release of adrenocorticotropin from rat pituitary tissue in vitro. *Endocrinology* **138**:2909-18.

Terkel J. (1986) Neuroendocrinology of coitally and noncoitally induced pseudopregnancy. *Ann. NY. Acad. Sci.* **474**:76-94.

Terry LC, Saunders A, Audet J, Willoughby JO, Brazeau P, Martin JB. (1977) Physiologic secretion of growth hormone and prolactin in male and female rats. *Clin. Endocrinol. (Oxf)* **6** Suppl:19S-28S.

Thomas GB, Phelps CJ, Robinson ICAF. (1999) Differential regulation of hypothalamic tuberoinfundibular dopamine neurones in two dwarf rat models with contrasting changes in pituitary prolactin. *J. Neuroendocrinol.* **11**:229-236.

Thompson MC, Norton NS, Rodriguez-Sierra JF, Lippiello L. (1994) Is glutamate-induced reduction in growth hormone-releasing hormone a neuroendocrine model of aging in the rat? *Proc. Soc. Exp. Biol. Med.* **206**:69-75.

Thompson MC, Norton NS, Rodriguez-Sierra JF, Lippiello L. (1996) Growth hormone-releasing hormone depletion in the female rat: similarities to aging. *J. Gerontol. A. Biol. Sci. Med. Sci.* **51**:B83-90.

Thompson NM, Davies JS, Mode A, Houston PA, Wells T. (2003) Pattern-dependent suppression of growth hormone (GH) pulsatility by ghrelin and GH-releasing peptide-6 in moderately GH-deficient rats. *Endocrinology* **144**:4859-67.

Thompson NM, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC, Wells T. (2004) Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. *Endocrinology* **145**:234-42.

Thompson NM. (2002) Novel and paradoxical effects of ghrelin and the growth hormone secretagogues in growth hormone deficient rats. *PhD Thesis*. Cardiff University, Cardiff, UK.

Thompson NM, Christian, HC, Carmignac, DF, Robinson, ICAF, Wells T. (2002) Female dwarf (*dw/dw*) rats secrete prolactin in response to ghrelin from a pituitary cell type with a novel morphology – The "intermediate lactotroph". *84th Annual Meeting of the Endocrine Society*, San Francisco, USA. Abstract **P2-106**.

Thorpe JR, Wallis M. (1991) Immunocytochemical and morphometric studies of mammotrophs, somatotrophs and somatomammotrophs in sheep pituitary cell cultures. *J. Endocrinol.* **129**:417-22.

Tierney T, Robinson IC. (2002) Increased lactotrophs despite decreased somatotrophs in the dwarf (dw/dw) rat: a defect in the regulation of lactotroph/somatotroph cell fate? *J. Endocrinol.* **175**:435-446.

Tiong TS, Herington AC. (1991) Tissue distribution, characterization, and regulation of messenger ribonucleic acid for growth hormone receptor and serum binding protein in the rat. *Endocrinology* **129**:1628-34.

Tonshoff B, Blum WF, Mehls O. (1995) Insulin-like growth factors (IGF) and IGF binding proteins in children with chronic renal failure. *Prog. Growth Factor Res.* **6**:481-91.

Topilko P, Levi G, Merlo G, Mantero S, Desmarquet C, Mancardi G, Charnay P. (1997) Differential regulation of the zinc finger genes Krox-20 and Krox-24 (Egr-1) suggests antagonistic roles in Schwann cells. *J. Neurosci. Res.* **50**:702-12.

Toshinai K, Mondal MS, Nakazato M, Date Y, Murakami N, Kojima M, Kangawa K, Matsukura S. (2001) Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. *Biochem. Biophys. Res. Commun.* **281**:1220-5.

Travers MT, Madon RJ, Flint DJ. (1993) Regulation of serum insulin-like growth factor-I (IGF-I), hepatic growth hormone binding and IGF-I gene expression in the rat during pregnancy and lactation. *J. Endocrinol.* **139**:89-95.

Tschöp M, Smiley DL, Heiman ML. (2000) Ghrelin induces adiposity in rodents. *Nature* **407**:908-913.

Tsuchiya N, Harada Y, Taki M, Minematsu S, Maemura S, Amagaya S. (1995) [Age-related changes and sex differences on the serum chemistry values in Sprague-Dawley rats--I. 6-30 weeks of age] *Exp. Anim.* **43**:671-8.

Tulassay T, Vasarhelyi B. (2002) Birth weight and renal function. *Curr. Opin. Nephrol. Hypertens.* **11**:347-52.

Tzortzaki EG, Glass D, Yang M, Evan AP, Bledsoe SB, Stambrook PJ, Sahota A, Tischfield JA. (2002) Gender- and age-dependent changes in kidney androgen

protein mRNA expression in a knockout mouse model for nephrolithiasis. *J. Histochem. Cytochem.* **50**:1663-1669.

Vaillancourt C, Petit A, Belisle S. (1998) Expression of human placental D2-dopamine receptor during normal and abnormal pregnancies. *Placenta* **19**:73-80.

Vale W, Rivier C, Brazeau P, Guillemin R. (1974) Effects of somatostatin on the secretion of thyrotropin and prolactin. *Endocrinology* **95**:968-77.

van der Eerden BC, Karperien M, Wit JM. (2003) Systemic and local regulation of the growth plate. *Endocr. Rev.* **24**:782-801.

Velkeniers B, Hooghe-Peters EL, Hooghe R, Belayew A, Smets G, Claeys A, Robberecht P, Vanhaelst L. (1988) Prolactin cell subpopulations separated on discontinuous Percoll gradient: an immunocytochemical, biochemical, and physiological characterization. *Endocrinology* **123**:1619-30.

Vidal S, Horvath E, Kovacs K, Cohen SM, Lloyd RV, Scheithauer BW. (2000) Transdifferentiation of somatotrophs to thyrotrophs in the pituitary of patients with protracted primary hypothyroidism. *Virchows Arch.***436**:43-51.

Vidal S, Lombardero M, Moya L. (1997) Ultrastructural and immunocytochemical studies of prolactin-secreting cells in adenohypophysis of the mink (Mustela vison). *Gen. Comp. Endocrinol.* **107**:311-21.

Vidal S, Roman A, Oliveira MC, De La Cruz LF, Moya L. (1998) Simultaneous localization of Pit-1 protein and gonadotropins on the same cell type in the anterior pituitary glands of the rat. *Histochem. Cell. Biol.* **110**:183-8.

Vidal S, Horvath E, Kovacs K, Lloyd RV, Smyth HS. (2001) Reversible transdifferentiation: Interconversion of somatotrophs and lactotrophs in pituitary hyperplasia. *Mod. Pathol.* **14**:20-28.

Villaplana M, Garcia Ayala A, Garcia Hernandez MP, Agulleiro B. (2001) Immunocytochemical and ultrastructural characterization of somatolactin cells from the gilthead sea bream (Sparus aurata L., Teleostei): an ontogenic study (from newly hatched to adults). *Anat. Embryol.* **203**:449-60.

Virlon B, Cheval L, Buhler JM, Billon E, Doucet A, Elalouf JM. (1999) Serial microanalysis of renal transcriptomes. *P. Natl. Acad. Sci. USA* **96**:15286-15291.

Wakabayashi I, Hatano H, Minami S, Tonegawa Y, Akira S, Sugihara H, Ling NC. (1986) Effects of neonatal administration of monosodium glutamate on plasma growth hormone (GH) response to GH-releasing factor in adult male and female rats. *Brain Res.* **372**:361-5.

Wang G, Lee HM, Englander E, Greeley GH Jr. (2002) Ghrelin--not just another stomach hormone. *Regul. Pept.* **105**:75-81.

Wang L, Raikwar N, Deng L, Yang M, Liang L, Shao C, Evan AP, Stambrook PJ, Sahota A, Tischfield JA. (2000) Altered gene expression in kidneys of mice with 2,8-dihydroxyadenine nephrolithiasis. *Kidney Int.* **58**:528-36.

Watanabe YG, Haraguchi H. (1994) Immunohistochemical study of the cytogenesis of prolactin and growth hormone cells in the anterior pituitary gland of the fetal rat. *Arch. Histol. Cytol.* **57**:161-6.

Watkins-Chow DE, Camper SA. (1998) How many homeobox genes does it take to make a pituitary gland? *Trends Genet.* **14**:284-90.

Watson G, Paigen K. (1988) mRNA synthesis rates in vivo for androgen-inducible sequences in mouse kidney. *Mol. Cell. Biol.* **8**:2117-24.

Webb PD. (1981) The pars distalis (anterior pituitary) in the fetal sheep: an ultrastructural study. *J. Dev. Physiol.* **3**:319-32.

Wehrenberg WB. (1986) The role of growth hormone-releasing factor and somatostatin on somatic growth in rats. *Endocrinology* **118**:489-94.

Wells T, Houston PA. (2001) Skeletal growth acceleration with growth hormone secretagogues in transgenic growth retarded (Tgr) rats: Pattern-dependent effects and mechanisms of desensitization. *J. Neuroendocrinol.* **13**: 496-504.

Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow ME. (2001) X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* **27**:18-20.

Wood DF, Johnston JM, Johnston DG. (1991) Dopamine, the dopamine D2 receptor and pituitary tumours. *Clin. Endocrinol.* **35**:455-66.

Woodall SM, Bassett NS, Gluckman PD, Breier BH. (1998) Consequences of maternal undernutrition for fetal and postnatal hepatic insulin-like growth factor-I, growth hormone receptor and growth hormone binding protein gene regulation in the rat. *J. Mol. Endocrinol.* **20**:313-26.

Wu JT, Kral JG. (2004) Ghrelin: integrative neuroendocrine peptide in health and disease. *Ann. Surg.* **239**:464-74.

Wu WX, Brooks J, Glasier AF, McNeilly AS. (1995) The relationship between decidualization and prolactin mRNA and production at different stages of human pregnancy. *J. Mol. Endocrinol.* **14**:255-61.

Xu LL, Shanmugam N, Segawa T, Sesterhenn IA, McLeod DG, Moul JW, Srivastava S. (2000) A novel androgen-regulated gene, PMEPA1, located on chromosome 20q13 exhibits high level expression in prostate. *Genomics* **66**:257-63.

Yamamuro Y, Aoki T, Sensui N. (2001) Effect of estradiol and FBS on PRL cells, GH cells, and PRL/GH cells in primary cultures of pituitary cells from prenatal rats. *Exp. Biol. Med. (Maywood)* **226**:140-3.

Yang B, Gillespie A, Carlson EJ, Epstein CJ, Verkman AS. (2001) Neonatal mortality in an aquaporin-2 knock-in mouse model of recessive nephrogenic diabetes insipidus. *J. Biol. Chem.* **276**:2775-9.

Yeum CH, Kim SW, Kim NH, Choi KC, Lee J. (2002) Increased expression of aquaporin water channels in hypothyroid rat kidney. *Pharmacol. Res.* **46**:85-8.

Yoshimura F, Harumiya K, Ishikawa H, Otsuka Y. (1969) Differentiation of isolated chromophobes into acidophils or basophils when transplanted into the hypophysiotrophic area of hypothalamus. *Endocrinol. Jpn.* **16**:531-40.

Zapala MA, Lockhart DJ, Pankratz DG, Garcia AJ, Barlow C, Lockhart DJ. (2002) Software and methods for oligonucleotide and cDNA array data analysis. *Genome Biol.* **3**:SOFTWARE0001.

Zeitler P, Stevens P, Siriwardana G. (1998) Functional GHRH receptor carboxyl terminal isoforms in normal and dwarf (*dw*) rats. *J. Mol. Endocrinol.* **21**:363-71.

Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, Lozykowski M, Okada S, Cataldo L, Coschigamo K, Wagner TE, Baumann G, Kopchick JJ. (1997) A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proc. Natl. Acad. Sci. U S A.* **94**:13215-20.