**1**

## DISCOVERY OF GHRELIN, A NOVEL ACYLATED PEPTIDE, AND ITS PHYSIOLOGICAL SIGNIFICANCE

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Small synthetic molecules termed growth hormone secretagogues (GHSs) act through an orphan G-protein coupled receptor, GHS-R, to stimulate growth hormone (GH) release from pituitary. To search for the natural ligand for GHS-R, we constructed a stable CHO cell line expressing rat GHS-R for monitoring intracellular calcium concentration ($[Ca^{2+}]_i$) changes induced by rat tissue extracts. Using this cell line, we purified a peptide from stomach that potently activated GHS-R. The purified peptide was consisted of 28 amino acids, in which the Ser-3 residue was n-octanoylated. Surprisingly, this n-octanoyl modification at Ser-3 was essential to the activity, since des-acyl-peptide could not induce $[Ca^{2+}]_i$ changes. We named the GH-releasing peptide "ghrelin" ("ghre" is the Proto-Indo-European root of the word "grow").

We cloned mammalian ghrelin cDNAs (human, rat, mouse, canine, porcine, ovine and bovine) and determined their structures. Precursor structures of mammalian ghrelin are highly homologous. In particular, N-terminal 10 amino-acids are identical, which supports the importance of octanoyl modification for ghrelin's activity.

Ghrelin induced GH release in a dose-dependent manner both in vitro and in vivo. ICV injection of ghrelin also stimulated GH release. Ghrelin immunoreactive cells in the stomach were found in X/A-like cells, an endocrine cell in stomach. Ghrelin is found in the secretary granules of X/A-like cells whose hormonal product and physiological functions have not been previously clarified. In rat brain, ghrelin immunoreactive neurons were found to be localized in the hypothalamic arcuate nucleus, a region where regulates food intake. In fact, ICV injection of ghrelin strongly stimulated feeding in rats and increased body weight gain. On the other hand, anti-ghrelin IgG robustly suppressed feeding. These facts suggest that ghrelin is a new physiological regulator of nutritional homeostasis.

Recently we purified ghrelin peptides from chicken, frog (bullfrog) and fish (eel and trout). Amino-acid sequences of ghrelin peptides in all vertebrates are well conserved. Moreover, the ghrelin peptides are all modified by octanoic acid. These results suggest that ghrelin plays a fundamentally important role in vertebrates.

(Rreference)

Kojima M, Hosoda H, Matsuo H, Kangawa K

Ghrelin, discovery of the natural endogenous ligand for the growth hormone secretagogue receptor

Trends Endocrinol Metab. (2001) 12:118-122.

**2**

## EMBRYO DEVELOPMENT AND BIOTECHNOLOGY

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Mammalian preimplantation embryos are the only stage of development that may be grown in vitro independent of the maternal environment. The grown oocyte formed in the maturing follicle can be extracted from the ovary and either matured in vitro, or recovered after maturation in vivo, to produce viable embryos with a high developmental competence. This has enabled the in vitro production of mammalian embryos that may be fertilized and grown to the blastocyst stage in culture. While control of the germ cell, primary oocyte and growing oocyte in the preantral follicle is less well understood, it is possible to manipulate ovarian follicular dynamics by ovarian transplantation and may be controlled in the future by interference in the normal selection mechanisms of primordial follicle recruitment. Data is being generated from expression libraries in the human and other species to identify the key regulatory molecules for follicular and oocyte recruitment. This may be used to alter the reproductive life-span for the female.

The production of oocytes and embryos provides the basis for reproductive biotechnologies in domestic animal species and the human. Cattle reproductive technologies are well developed for production and human IVF is the foundation for the treatment of human infertility. The cattle industry accepts embryo production, embryo cryopreservation and embryo transfer as an integral part of the breeding strategies used. More recently, the cloning of animals, particularly cattle, has been explored with variable outcomes. The reprogramming of somatic cells by ooplasm doesn't completely reset the epigenetic regulators of gene expression, so that failure to erase the somatic epigenetic signature can result in abnormalities of development, including both placental and fetal compartments. This results in high rates of implantation failure, fetal growth and both anatomical and functional abnormalities which are often lethal. However, around50% of offspring are viable and healthy despite the altered epigenetics. In the second generation the epigenetic irregularities are corrected during gametogenesis. The ability to clone animals enables the production of transgenic animals in one generation. Other methods of transgenesis in animals include sperm-mediated transfection which involves intracytoplasmic sperm injection (ICSI). Other new and interesting methods are being designed that involve the manipulation of gametogenesis.

Human IVF has evolved into a successful treatment for female and male infertility. ICSI may used for testicular sperm and elongated spermatids. However, success with round spermatids or their precursors has not been clinically useful. The new and rapidly growing area of genetic diagnosis of point mutations and trinucleotide repeat disease in preimplantation embryos is an important new biotechnology in human medicine. These new molecular techniques are also being applied for aneuploid screening of IVF embryos, reducing the need for multiple embryo transfer for maintaining high pregnancy success rates. It is now possible to fingerprint sibling embryos to test new culture systems etc. With these capabilities, it is possible to identify genes that correlate with breast cancer and HLA type embryos for compatible transplants. Expression screening and apoptosis markers may also be used for determining embryo viability.

Preimplantation embryos are also a source of pluipotential embryonic stem (ES) cells. These may be derived from fertilized oocytes, parthenogenetic embryos and nuclear transfer embryos. It is of interest that embryos that have little or no developmental competence can produce ES cell lines with apparently complete ability to produce all types of terminal tissue types and to integrate in vivo into all tissues of interest. This may be similar to the ability of some adult cell types to trans differentiate under experimental conditions in vitro and in vivo. These extraordinary cells may enable a new medical biotechnology of cell therapy for tissue repair and regeneration. These cells mayalso allow for gene therapy to be applied safely for the treatment of a wide range of genetic diseases.

These have been exciting times for biological sciences and promise a wonderful future research opportunity for young scientists. I thank all the scientists who have worked with me over the decades for the joy it has brought me for the contributions they have all made. I also acknowledge the funding of our creativity by numerous agencies and commercial bodies over the decades.

**3**

## ENDOCRINE -IMMUNE-INTERACTIONS

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Corticotropin releasing hormone (CRH) is a key regulator of the hypothalamic-pituitary-adrenal (HPA) axis and coordinator of the stress response. As in stress, intracerebroventricular administration of CRH suppresses the immune system and causes a Thelper 1 to Thelper 2 predominance indirectly, via glucocorticoid and sympathetic system-mediated mechanisms. Also, during inflammatory stress, the cytokines TNF, IL-1 and IL-6 stimulate hypothalamic CRH and/or vasopressin secretion, as well as adrenal cortisol secretion, as a way of preventing the inflammatory reaction from overreacting. Recently, CRH receptors were described in peripheral sites of the immune system and CRH was found to promote several immune functions. We examined the effect of systemic CRH immunoneutralization in an experimental model of carrageenin-induced aseptic inflammation in Spague-Dawley rats. Intraperitoneal administration of rabbit anti CRH sera caused suppression of both inflammatory exudate volume and cell concentration by approximately 50-60%. Large amounts of immunoreactive (IR) CRH, designated immune CRH, were detected by specific immunohistochemistry and/ or radiommunoassay (300-500 pg/g wet tissue) in the inflammatory area, but not in concurrent plasma samples from the systemic circulation (<10 pg/ml). Glucocorticoids and somatostatin inhibited production of IR CRH in the inflammatory site. We

extended these observations to other forms of experimental inflammation, including streptococcal cell wall polysacharide- and adjuvant-induced arthritides and peptide R16 (epitope of the interphotoreceptor retinoid-binding protein)-induced uveitis in Lewis rats. We also studied human disease states, including rheumatoid arthritis, Hashimoto thyroiditis and ulcerative colitis. Inflamed tissues contained large amounts of IR CRH. The concentrations of the peptide in the synovial fluid of the rat and human arthritic joints were elevated to 200-500 pg/ml, levels similar to those observed in the hypophysial portal system. We also demonstrated the presence of CRH mRNA and CRH receptors in inflammatory cells and identified the skin and meningeal vassel mast cell as an immune target for peripheral CRH. In addition to production by immune cells, the peripheral nervous system, including the postganglionic sympathetic neurons and the sensory fibers type C, appears to contribute to IR CRH production in inflammatory sites. The production of CRH from the postganglionic sympathetic neurons may be responsible for the stress-induced activation of allergic/autoimmune phenomena, such as asthma and eczema, or vasokinetic phenomena, such as migraine headaches, via mast cell degranulation. The proinflammatory actions of CRH were antagonized by antalarmin, a nonpeptidic CRH receptor type 1 receptor antagonist. We recently demonstrated presence of active estrogen responsive elements in the regulatory region of the hCRH gene. This might explain the sexual dimorphism of the stress response and the inflammatory/immune reaction. We conclude that in the periphery, CRH acts directly on cells of the immune system to stimulate the inflammatory reaction, in contrast to its central, indirect immunosuppressive/anti-inflammatory actions on innate and cellular immunity and stimulatory actions on certain aspects of humoral immunity. Changes in the activity of the HPA axis and in the secretion of "immune" CRH may predispose individuals to certain infectious, neoplastic, and autoimmune/inflammatory diseases.

**4**

## PRE-RECEPTOR METABOLISM OF CORTISOL BY 11β-HYDROXYSTEROID DEHYDROGENASES: IMPLICATIONS FOR HUMAN DISEASE.

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Clinical observations on patients with Cushing's syndrome highlight the importance of cortisol in regulating blood pressure and body fat mass and distribution. However, patients with the prevalent diseases - hypertension and obesity - invariably have normal circulating cortisol concentrations. We have focussed on the concept of "pre-receptor" metabolism as a mechanism of modulating the action of cortisol in a tissue-specific fashion. Two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) catalyse the interconversion of hormonally active cortisol (F) to inactive cortisone (E). 11β-HSD1 is an oxoreductase expressed in human liver, pituitary, gonad and adipose tissue, responsible for E to F conversion. Specifically within human adipose tissue, expression is higher in omental compared to subcutaneous fat. Here the generation of F via 11β-HSD1 stimulates adipocyte differentiation and this may explain the predilection of glucocorticoids for visceral obesity. 11β-HSD1 within adipose tissue is inhibited by IGF-1 and enhanced expression in hypopituitary, GH-deficient, subjects may explain the visceral obesity of this condition. "Cushing's disease of the omentum" may be a novel mechanism underpinning central obesity and raises the exciting possibility that inhibition of the 11β-HSD1 enzyme could offer a novel therapeutic therapy for this condition.

By contrast, 11β-HSD2 is a high affinity dehydrogenase expressed in adult mineralocorticoid target tissues (kidney, colon) and fetal tissues including the placenta. By inactivating F to E in the kidney it protects the mineralocorticoid receptor from cortisol excess. Mutations in the human 11β-HSD2 gene explain an aggressive form of inherited hypertension – the syndrome of "Apparent Mineralocorticoid Excess" in which cortisol acts as a potent mineralocorticoid. Liquorice ingestion inhibits 11β-HSD2 and results in an identical form of cortisol-induced hypertension. "Milder" mutations in the human 11β-HSD2 gene have been reported in patients with "essential" hypertension, but at present the true prevalence of defects in cortisol metabolism in hypertensive patients is unknown.

11β-HSD's by activating or inactivating cortisol in peripheral tissues, can amplify or inhibit corticosteroid hormone action at an autocrine level. The manipulation of 11β-HSD expression in peripheral tissues may offer a novel therapeutic option for diseases such as obesity and hypertension without the deleterious systemic effects of cortisol excess or deficiency.

**5**

## COMPARATIVE 3D STRUCTURES OF THE IGF-1 AND EGF RECEPTORS

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The extracellular regions of both the insulin and EGF tyrosine kinase receptor families have a similar arrangement of two homologous domains (L1 and L2) separated by a cys-rich region. The C-terminal half of the IR ectodomain dimer is comprised of three fibronectin type 3 (Fn3) repeats, and an insert domain which contains the α-β cleavage site. The C-terminal portion of the EGFR ectodomain consists solely of a second cys-rich region.

A fragment comprising the L1-cysteine-rich-L2 region of the IGF-1R ectodomain has been crystallised and its structure determined (1). The L domains consist of a single stranded right-handed beta-helix. The cys-rich region is composed of eight disulphide-bonded modules, seven of which form a rod-shaped domain with modules associated in a novel manner. At the centre of this extended structure is a space of sufficient size to accommodate a ligand molecule.

Recently we have solved the structure of a high affinity, truncated form of human EGFR ectodomain bound to TGFalpha (2). sEGFR501 is consists of the L1, CR1 and L2 domains plus the first module from the second cys-rich region CR2. The overall size of the 2:2 TGFalpha/sEGFR501 complex is approximately 33 x 78 x 103 Å. The L domains and cys rich region resemble the corresponding domains of the IGF-1R as expected. Each TGFalpha molecule is clamped between the L1 and L2 domains from the same sEGFR501 molecule making many main chain contacts with L1 and interacting with L2 via key conserved residues. Analysis of these interactions indicates how EGFR family members can bind a family of highly variable ligands. The ligands are located on opposite sides of the complex and each makes contact with only one receptor molecule in the dimer. The dimer interface predominantly involves back-to-back receptor-receptor interactions involving residues from the first cys-rich region. The TGFα:sEGFR501 crystal structure offers new insights into receptor signalling.

1. Garrett TPJ, McKern NM, Lou M, Frenkel MJ, Bentley JD, Lovrecz GO, Elleman TC, Cosgrove LJ. & Ward CW (1998*) Nature* 394: 395-399

2. Garrett TPJ, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Zhu HJ, Walker F, Hoyne PA, Jorissen RN, Nice EC, Burgess AW & Ward CW (2002) *Cell* In press.

**6**

## 60

### FETAL FAT, LEPTIN AND THE PROGRAMMING OF ADULT OBESITY

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People who were small at birth tend to have a more abdominal distribution of obesity and a high body fat content in adult life despite a lower BMI (1). Lambs with low birth weights are also fatter at any given body weight and this may be attributable to an increased voluntary food intake or hyperphagia during the early postnatal period, coupled with relatively lower energy requirements (2). Whilst growth restriction before birth is associated with increased adult adiposity, increased fetal nutrient supply is also associated with the development of obesity in later life. In this context, there has been considerable recent interest in the potential role of the hormone leptin in the early onset of adult obesity. Leptin, a polypeptide hormone, is synthesised and secreted principally by adipocytes and acts in the adult as a circulating signal of fat mass. Leptin binds to specific receptors to decrease food intake and increase energy utilisation thereby maintaining energy balance homeostasis. Serum leptin concentrations are elevated early in the development of childhood-onset obesity and obese children have a high serum leptin even when normalised to fat mass (4). There have been relatively few experimental studies, however, on the specific impact of alterations in fetal nutrient supply on the synthesis, secretion and actions of leptin before and after birth. We have recently demonstrated that circulating leptin concentrations are positively related to leptin mRNA expression in fetal adipose tissue in fetal sheep during late gestation and that there is a significant relationship between fetal adiposity, adipocyte cell size and circulating leptin concentrations in fetal sheep of ewes fed at or above maintenance energy requirements. We have also demonstrated that leptin infusion into fetal sheep in late gestation results in changes in the structural and functional characteristics of fetal fat. Thus leptin can act as a signal of adiposity in fetal life and alterations in the synthesis, secretion or actions of leptin may therefore play an important role in the early programming of adult obesity.

1. Law C et al (1992) J Epid Com Health 46:184
2. Greenwood P et al (1998) J Anim Sci 76: 2354
3. Dorner G et al (1994) Horm Metab Res 26: 213
4. Lahlou N et al (1997) Diabetes 46: 989.

## 61

### PERINATAL PROGRAMMING OF SYNDROME X IN THE ADULT

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Insulin resistance and compensatory hyperinsulinaemia are common and are frequently associated with a cluster of abnormalities, which predispose the individual to cardiovascular disease (1). These include glucose intolerance and diabetes, obesity, dyslipidaemia and hypertension and are collectively termed Syndrome X or the metabolic syndrome (1). Genetic and lifestyle factors are implicated in the development of the syndrome and that of what appears to be its primary and initial defect, insulin resistance (1). Recently a novel risk factor for Syndrome X and its individual components has been identified, that of small size at birth for gestational age, which reflects poor growth and an adverse environment before birth (1). Numerous studies have shown that small size at birth, in terms of reduced weight or length or thinness, predicts insulin resistance, hyperinsulinaemia, impaired glucose tolerance or diabetes and hypertension in the adult, and not unexpectedly, an increased risk of developing or dying from cardiovascular disease (1). This has led to the hypothesis that an adverse prenatal environment alters the functional development and postnatal function of key regulatory systems and/ or their target tissues, leading to impaired metabolic and cardiovascular homeostasis in the adult (1). This cluster of abnormalities associated with small size at birth can also occur with or without dyslipidaemia and central obesity, which in part appears to reflect the extent and timing of accelerated or catch-up growth postnatally. This suggests that altered postnatal growth after poor prenatal growth may also program later function. Support for perinatal programming of Syndrome X has been provided by studies in the guinea pig, where experimental and spontaneous restricted fetal

growth result in adult onset whole body and peripheral insulin resistance, compensatory hyperinsulinaemia, glucose intolerance and diabetes, hypertension, impaired cholesterol homeostasis and atherosclerosis (2, 3). In addition, the extent of some of these, such as insulin resistance and central obesity, is exacerbated by catch-up growth postnatally. Identification of the mechanisms by which restricted prenatal growth and accelerated postnatal growth impair adult function may reveal new approaches to reducing the burden of Syndrome X and associated diseases in adult life.

1 Barker DJP et al (2002) Diabetologia 45: 342-348

2 Kind KL et al (1999) Am J Physiol 277: R1675-1682

3 Kind KL et al (2002) Exp Physiol 87: 469-477

**62**

## NEUROENDOCRINE PROGRAMMING OF CANCER: THE ROLE OF PRE NATAL AND POSTNATAL PHYSIOLOGICAL STRESSORS.

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Infection poses a serious threat to the neonate in the short term. Accumulating evidence is now indicating that neonatal infection may also have serious long-term implications. Development of the foetus is determined by interactions between, amongst others, the neurochemical, immune and endocrine systems. Cell-mediated and humoral immunity continues to develop throughout the early post-natal period and is dependent on appropriate immune-endocrine signalling. Normal development of the HPA and hypothalamic-gonadal axes is influenced by early immune activation. Research from our laboratory has demonstrated that neonatal exposure to bacterial endotoxin permanently alters the development of the HPA axis and retards growth, alters social development, and potentiates ACTH and corticosterone responses to stress in adulthood. Altering the responsivity of the HPA axis has potentially important implications given that glucocorticoids have potent immunosuppressant effects which may impair disease resistance in later. In support of this proposal research from our laboratory has recently provided evidence that exposure to bacterial endotoxin, or a psychological stressor, maternal separation, during the pre- and postnatal period, impairs tumour immunity in the adult rat, depressing the activity of natural killer (NK) cells, cells critically involved in the surveillance and eradication of tumour cells, and markedly potentiates tumour metastasis in adulthood. We have further demonstrated that these animals have an impaired ability to respond to stress, as reflected in behavioural changes, and this is associated with an impaired immunological response to immune challenge in adulthood. The findings of these studies are significant in that they indicate that pre- or postnatal exposure to immunological or non-immunological stressors, both common occurrences for human neonates, may have long term implications for health, with specific reference to cancer susceptibility.

**63**

## THE PLACENTAL GLUCOCORTICOID BARRIER AND FETAL PROGRAMMING: A LEPTIN CONNECTION?

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Programming effects of the fetal environment are now recognised as key determinants of the adult phenotype. Among the range of endocrine signals potentially involved in fetal programming, leptin and glucocorticoids appear to be of particular importance. For example, leptin exposure in early life has recently been proposed as a determinant of adult obesity (1), and glucocorticoids are well known for their potent differentiation effects in several tissues. Accordingly, exposure of the fetus to excess glucocorticoids has a major impact on the postnatal phenotype, most notably in regard to hypertension and insulin sensitivity (2). Fetal exposure to endogenous glucocorticoids is normally limited by the 'placental glucocorticoid barrier' that results from placental expression of the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes. We have demonstrated that increased fetal glucocorticoid exposure induced by inhibition of placental 11β-HSD (with carbenoxolone) or maternal treatment with dexamethasone reduces birthweight and delays the onset of puberty in postnatal life (3). Although this

puberty delay was not linked to postnatal changes in either plasma leptin (a key determinant of normal puberty onset) or hypothalamic leptin receptor (Ob-R) expression, marked reductions in fetal plasma leptin and placental Ob-R were observed (4). These effects occurred despite unchanged maternal leptin (CBX-treated mothers) or a five-fold increase in maternal leptin (DEX-treated mothers), suggesting that transplacental leptin passage was compromised by glucocorticoids. Therefore, we measured the transfer of $^{125}$I-leptin from mother to fetus *in vivo*, and showed this to be reduced by increased placental-fetal glucocorticoid exposure (by DEX or CBX). In contrast, inhibition of maternal corticosterone synthesis by metyrapone (MET) enhanced birthweight, placental Ob-R expression and transplacental passage of $^{125}$I-leptin, and also advanced the subsequent timing of puberty onset. Collectively, these data indicate that glucocorticoid effects on placental leptin transport and the programming of puberty onset in postnatal life operate across the full physiological range of corticosterone concentrations. It now needs to be established whether the disturbances in fetal leptin exposure *per se* mediate the programming effects of glucocorticoids in relation to puberty onset and adult conditions including hypertension, insulin resistance and obesity.

(1) Schmidt I *et al* (2000). *Physiol Genomics* 3:113; (2) Seckl JR (1997). *Steroids* 62:89; (3) Smith JT, Waddell BJ (1998). *Endocrinology* 141:2422; (4) Smith JT, Waddell BJ (2002) *Biol Reprod* (In Press).

**64**

## OOCYTE-DERIVED GROWTH FACTORS AND OVULATION RATE IN MAMMALS

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Ovulation rate in mammals is determined by a complex exchange of hormonal signals between the pituitary gland and the ovary, and by a localised exchange of hormones within ovarian follicles including the oocyte and its adjacent somatic cells. Studies from mice lacking certain growth factor genes and sheep with naturally-occurring genetic mutations show that the oocyte plays an essential role in regulating ovarian follicular growth and ovulation rate. If the oocyte has such a major effect on ovulation rate, then it can be hypothesised that the relative importance and indeed actions of certain oocyte-derived growth factors may vary between mammals with a high ovulation rate phenotype (e.g. mice, rats, dogs and pigs) and those with a low ovulation rate phenotype (e.g. humans, cattle and sheep). The evidence from studies of two oocyte-derived growth factors, namely bone morphogenetic protein 15 (BMP15; otherwise known as growth differentiating factor 9B or GDF9B) and GDF9 in mice and sheep suggest that this hypothesis may have some validity. For example, mice lacking the GDF9 gene are infertile with ovarian follicular growth blocked at the primary stage of development. However, mice lacking a functional BMP15 gene are fertile with normal numbers of preovulatory follicles. Mice heterozygous for either gene have apparently normal ovulation rates. By contrast, sheep that are heterozygous for inactivating mutations in BMP15 have higher ovulation rates than normal. Moreover, sheep with modest antibody responses to BMP15 or GDF9 can have higher than normal ovulation rates (i.e. 2 to 12 versus 1 or 2). Sheep that are homozygous for inactivating mutations in BMP15 or with high antibody responses to BMP15 or GDF9 are infertile with completely impaired follicular development beyond the primary stage of development. Also passive immunisation studies in sheep with specific antiplasma to either BMP15 or GDF9 show that both are essential for normal follicular development before ovulation. The molecular forms of BMP15 and GDF9 in biological fluids (e.g. monomers, homodimers, heterodimers etc) as well as the specific receptors to these growth factor remain be determined. With these caveats in mind, the current evidence is consistent with the hypothesis that both follicular growth and ovulation rate are profoundly influenced by the dose of BMP15 or GDF9 delivered to the somatic cells of the follicle in mammals with a low ovulation rate phenotype, whereas in those with a high ovulation rate phenotype, the follicular somatic cells are relatively insensitive to changing the doses of BMP15 but have an absolute requirement for GDF9.

**65**

## INTRAOVARIAN REGULATION OF FOLLICULOGENESIS: SIGNAL MOLECULES AND THEIR CELLULAR SOURCES

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Folliculogenesis in mammals is a continuous and dynamic process that begins during embryonic development in utero and continues until the end of adult reproductive life. The overall process is regulated by a complex array of interacting chemical signals originating both within and external to the ovaries. The identities and relative importance of specific signals change as follicle growth progresses through a series of morphologically distinct stages, beginning with initiation of growth of the dormant primordial follicles, and ending in either atresia or ovulation. The initiation of follicle growth occurs when a primordial follicle is activated by as yet unknown mechanisms and enters a pool of growing follicles. Recent evidence has established the essential role of the oocyte as the driving force in promoting follicle growth throughout pre-antral and early antral stages, mediated via recently identified members of the TGFβ superfamily of growth factors, GDF9 and GDF9B, which have essential roles in both proliferation and differentiation of granulosa cells. The follicular somatic cells, including both granulosa and thecal cells, also contribute to regulation of follicle growth during these stages, through secretions of several other peptide growth factors, in particular bFGF, IGF-I, TGFβ, activin, and inhibin. Upon reaching antral stage of development, follicles acquire responsiveness to follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and these gonadotrophins then assume primary importance in regulation of growth to the pre-ovulatory stage, with growth factors continuing to play supportive and modulating roles. The actions of FSH and LH are mediated in part via androgens and oestrogens secreted by the thecal and granulosa cells, respectively. With the approach of ovulation, migratory cells derived from the circulation, including macrophages and to a lesser extent other leukocyte subsets, infiltrate the ovarian stroma and thecal layer of the pre-ovulatory follicle, where they play essential roles in the tissue remodelling that leads to rupture of the follicle and its transformation to a corpus luteum at ovulation. Their actions are mediated via pro-inflammatory molecules including cytokines, chemokines and eicosanoids secreted by the macrophages and by the follicle cells themselves. Recent evidence implicates signals initiated in the endometrium in response to semen exposure, in enhancing this leukocytic infiltration of the ovary, and influencing their pattern of cytokine secretion, with important consequences in enhancing ovulation and luteinization.

**66**

## OESTROGEN TARGET SITES IN HUMAN TESTIS

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It has been proposed that oestrogens play a role in regulating germ cell function in adulthood and during fetal life. Oestrogen action is mediated via high affinity intracellular receptors expressed in target tissues [1]. Two subtypes of oestrogen receptor known as ERß (NR3A1) and ERß (NR3A2), have been cloned and hERß variant isoforms identified. In target cells these receptors can exist as homo- or heterodimers [1]. We have used immunohistochemistry to examine the patterns of expression of ERs in human fetal and adult testis to determine the cellular targets for oestrogen action. In parallel studies we have prepared ER constructs and used these to examine steroid binding to ER homo- and heterodimers in transfected cells.

In human testicular tissues (fetal or adult) we have never detected ERß mRNA or protein. With a polyclonal antibody we detected ERß protein in multiple testicular cell nuclei including those of Sertoli cells, Leydig cells and peritubular myoid cells as well as some germ cells [2]. Additional studies using monoclonal antibodies capable of discriminating between human wild type ERß and the human ERßcx/ß2 [3] splice variant revealed discrete patterns of expression of the subtypes. In adult testis immunoexpression of wtERß was most intense in pachytene spermatocytes and round spermatids, whilst low levels of expression were detected in Sertoli cells,

spermatogonia, preleptotene, leptotene, zygotene and diplotene spermatocytes. Expression of ERßcx/ß2 protein was highest in Sertoli cells and spermatogonia with low/variable expression in preleptotene, pachytene and diplotene spermatocytes [4]. In the fetal testis wtERß was undetectable in gonocytes whereas these cells expressed the highest levels of ERßcx/ß2 compared with other testicular cell types. Both ERß1 and ERßcx/ß2 were detected in some but not all Sertoli cells, peritubular cells and Leydig cells. Transfection studies with ERß and ERß constructs confirmed that wtERß and ERß were activated following exposure to oestogenic ligands but that the ERßcx/ß2 variant did not bind oestrogens and was not capable of activating an ERE-containing reporter construct.

The testicular cells most likely to be targets for oestrogens in adulthood are round spermatids in which levels of expression of wtERß1 are high. In contrast, expression of ERcx/ß2, an isoform that may act as a dominant negative inhibitor of ER action, in adult Sertoli cells and spermatogonia, and in fetal gonocytes could prevent these cells responding to oestrogens.

1. Nilsson, S., et al., Phys Rev, 2001. **81**: 1535. 2.Saunders, P.T.K., et al., Mol Hum Reprod, 2001. **7**: 227. 3.Ogawa, S., et al., NAR, 1998. **26**: 3505. 4.Saunders, P.T.K., et al., JCEM, 2002. **87**: 2706.

**67**

## MALE CONTRACEPTION – APPROACH TO HORMONAL METHODS

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Worldwide men participate actively in contraception through natural methods, condom usage and vasectomy but no new acceptable, safe and reversible methods have become available. Male hormonal contraception (MHC) is the most promising avenue of research. Testosterone (T) treatment suppresses pituitary LH and FSH secretion, the primary endocrine signals required for spermatogenesis. Large multicentre WHO-sponsored trials have shown that high doses of T reduce sperm counts to zero (azoospermia) in ~70% Caucasian men while over 90% have sperm counts < 1 million/m (severe oligospermia), a level at which contraceptive efficacy appears adequate. But the need for frequent injections and androgenic side effects (acne, mood changes, reduced HDL-cholesterol) demand alternative methods of T administration. Testosterone implants provide the prototype for more physiological T treatment but pellet insertion/extrusion limit their wide application. New long acting intramuscular preparations, such as T undecanoate in oil, are most promising and may allow 8-12 weekly injection intervals.

Co-administration of other gonadotropin-suppressing agents, such as GnRH antagonists or progestins, enhance spermatogenic suppression and permit the use of lower T doses. In MHC, progestin action is probably via gonadotropin suppression but direct actions within the testis have not been excluded. A range of progestins have been used including levonorgestrel, desogestrel and depot medroxy-progesterone acetate, and studies have shown that ~75% and ~95% of men become azoospermic or severely oligospermic, respectively. The only T plus progestin efficacy study to date has reported excellent contraceptive efficacy over a 1 year exposure (Turner et al, ESA 2002, abstract). The stage is set for pharmaceutical company involvement, sadly lacking in the past, in order to bring the first MHC regimen to market. About 5% of men fail to suppress adequately and understanding the reason(s) for their failure to respond is important in gaining the widest acceptance of MHC.

We have explored the MHC effects on spermatogenesis in men using stereological techniques for germ cell quantification and showed that type Apale→B spermatogonial development and sperm release (spermiation) are the key sites of action. The spermatogonial defect probably results from FSH withdrawal but the subtypes affected & its mechanism of action are unknown. Spermiation failure is a feature of both acute (accounting for dramatic falls in sperm output within 3 weeks) and chronic MHC treatment. Both FSH and T regulate rodent spermiation but nothing is known in man. Cell-cell adhesion/communication and regulation of gene expression in later germ cell types can now be explored using laser capture microdissection and in vitro culture. From an endocrine regulatory viewpoint, MHC suppresses serum LH to <0.3% of control and intra-testicular T levels to 2% but dihydrotestosterone (DHT) levels are maintained – a possible role may exist for DHT and the up-regulation of testicular 5α-reductase type 1 in supporting some degree of spermatogenesis. FSH levels are

suppressed to 1-3% but remain detectable in most MHC-treated men and again may support some degree of continued spermatogonial development.

**68**

## DIABETES AND THE IGF SYSTEM – FOR BETTER OR FOR WORSE

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The insulin-like growth factor system (IGF) is ubiquitous and essential to life, involved in development, maintenance and repair of every tissue, as well as playing a role in many disease processes. It is exquisitely regulated via its ligands (IGF-I and IGF-II), its specific cell surface receptors, and its family of six circulating and tissue-specific binding proteins. The actions of the IGF system are growth-promoting, enhancing cellular proliferation and cell survival, as well as insulin-like. The IGF system thus plays multiple roles in the disordered metabolism and tissue damage of diabetes, variously having beneficial and adverse effects. Circulating IGFs, bound to IGF binding protein-3 are depressed in poorly controlled diabetes, reflecting their hepatic regulation by nutrition, mediated by insulin deficiency. IGFBP-1 is acutely regulated by insulin, playing a role as a glucose counter-regulatory hormone by binding "free" IGFs. These changes may all contribute to the growth failure of diabetes in children. Secondary insulin resistance also occurs in Type I diabetes due to elevated growth hormone levels resulting from lack of IGF-I feedback consequent upon hepatic insulin deficiency. Thus in both Type 1 and Type 2 diabetes, IGF-I therapy has improved insulin sensitivity and diabetic control. In tissues affected by microvascular damage in diabetes (eyes and kidneys), disturbances in expression of components of the IGF system have pointed to potential roles in the pathogenesis of damaging proliferative changes. These involve up-regulation of IGF-I and binding proteins potentially involved in targeting IGF to its receptors. Conversely, in peripheral nerves adversely affected by diabetes, the inevitable neuronal death by apoptosis is preventable by IGF-I treatment. Hypoglycemia is a major adverse effect of insulin therapy in diabetes, leading to potential brain damage in affected young children. We have recently utilised neuroblastoma cell lines as developing neuron models to examine effects of glucose deprivation, and potential roles of the IGF system. In low glucose conditions, neuronal survival is impaired, and IGF-I is able to enhance survival. Mechanisms involved include up-regulation of glucose transporters (GLUT1), similar to those occurring at the blood-brain barrier, as well as enhancement of anti-apoptotic mechanisms. These findings point to potential novel approaches to the management of hypoglycemic brain insults. In conclusion, the IGF system plays multiple and complex roles in diabetes, in both early and later life. Manipulation of the IGF system may provide novel therapies for prevention of some of the adverse effects of diabetes, both metabolic and tissue-damaging, so that the IGF system can operate in diabetes for "better" rather than for "worse".

**69**

## SUPPRESSORS OF CYTOKINE SIGNALLING

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Suppressor of cytokine signalling-1 (SOCS-1) is an important negative regulator of IFNγ signal transduction. While SOCS1 deficient (SOCS1-/-) mice die before weaning of a severe inflammatory disease, mice lacking both SOCS1 and IFNγ survive normally to adulthood, but succumb to a range of diseases in their second year of life. In addition to an SH2 domain which regulates interaction with tyrosine phosphorylated signalling proteins such as JAKs, SOCS1 contains a 40 amino acid motif, termed a SOCS box. Biochemical studies have demonstrated that the SOCS box interacts with elongin B and C, suggesting that SOCS proteins act as part of a ubiquitin ligase complex and that the termination of signal transduction by SOCS1 may occur in part by targeting signalling

proteins for proteasomal degradation. To test this, we have generated mice (SOCS1Δ/Δ) in which the SOCS-box of SOCS1 has been specifically deleted. SOCS1Δ/Δ mice display a phenotype that is intermediate between SOCS1-/- and wild type mice. SOCS1Δ/Δ mice survive weaning but succumb to inflammatory disease at 2 to 6 months of age. In vivo and in vitro, cells from these mice respond to IFNγ for longer than cells from wild type mice, but not for as long as cells from SOCS1-/- mice. This suggests that while important, regulation of protein degradation by the SOCS box is not the only mechanism by which SOCS1 attenuates signalling.

**70**

**71**

**72**

## MANAGEMENT STRATEGIES FOR MEDULLARY THYROID CARCINOMA (MTC)

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Medullary thyroid carcinoma (MTC), a malignancy arising from C-cells of the thyroid, is primarily treated by surgery. Many patients develop metastatic disease that is poorly responsive to chemotherapy and radiotherapy. Targeted gene therapy approaches may provide new strategies for treatment.

C-cells express the polypeptide hormone calcitonin (CT) almost exclusively, although an alternative spliced calcitonin gene related peptide (CGRP) is produced in neural tissue. The splicing choice in thyroid C cells is to include exon 4 to generate CT mRNA (exons 1-4) and in neuronal cells exon 4 is excluded and CGRP mRNA (exons 1-3,5-6) is generated.

In developing an adenoviral mediated gene therapy that selectively targets and induces killing of malignant C-cells, we have firstly enhanced CT expression level and improved the specificity by modification of the CT/CGRP promoter using a dual tandem tissue specific element (TSE) placed upstream of the basal promoter (TSE2.CP1). To further increase specific expression in C cells we have constructed an expression mini gene cassette containing only CT specific exons and introns, driven by our enhanced CT promoter (TSE2.CP1) or the constitutive RSV.LTR promoter. The pro-drug activating enzyme E.coli purine nucleoside phosphorylase (PNP) gene has been inserted within exon 4 of the CT mini gene. PNP can act as a cytotoxic gene by activating the pro-drugs 6 methylpurine-2-deoxyriboside and fludarabine.

RT-PCR analysis of transiently transfected cell lines has shown the CT mini gene containing PNP within exon 4, driven by the constitutive RSV-LTR promoter was spliced to include the PNP gene in the MTC cell line, TT, whereas in the neuronal cell line model T98G the chimeric mRNA excluded the PNP gene. Furthermore PNP enzyme levels were increased 1.4-fold and tissue specificity maintained when the CT/PNP construct is driven by the TSE2.CP1 promoter compared to RSV.LTR promoter. In the presence of pro-drugs 6-MPDR and fludarabine, specific cytotoxicity was demonstrated in TT cells expressing the CT/PNP chimera under the TSE2.CP1 promoter and no cytotoxicity was seen in the neuronal model, T98G. We are currently introducing the TSE2.CP1-CT/PNP hybrid mini gene into recombinant replication defective adenovirus to examine the ability of these viruses to exclusively express the cassette specifically in MTC cells and xenograft tumours.

## 73

### THE ROLE OF THE TSH RECEPTOR IN THYROID ASSOCIATED OPHTHALMOPATHY.

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Graves' disease (GD) is a common endocrine autoimmune condition in which simulating autoantibodies to the thyroid stimulating hormone receptor (TSHR) induce hyperthyroidism. Graves' ophthalmopathy (GO), also referred to as thyroid associated ophthalmopathy (TAO), is clinically characterised by proptosis, chemosis, periorbital oedema, conjunctivitis and extraocular muscle (EOM) dysfunction leading to diplopia. In rare cases, EOM enlargement at the apex of the orbit may impinge upon the optic nerve and cause visual disturbance and even visual loss. The precise pathogenic target of TAO is unknown although a logical target might be the TSHR residing in retroorbital tissues, since this is the target antigen of GD itself. Thus, stimulating autoantibodies to the thyroidal TSHR might also stimulate retroorbital tissue, inducing some of the phenotype of TAO. Our group has looked for the presence of the TSHR in retroorbital tissues. Using highly sensitive RT-PCR, Southern blot analysis, in vitro autoradiography and immunohistochemistry we have been successful in localising the TSHR to normal human EOM, in addition to connective tissues surrounding EOM and, consistent with the literature, connective tissues in other sites. However, the TSHR is not expressed in non-ocular skeletal muscles i.e., there appears to be differential expression of TSHR gene and protein in EOM but not in non-ocular skeletal muscle. RT-PCR amplicons have been excised and the gene sequence suggests that the EOM TSHR is identical to that found in the thyroid. We have also confirmed that TSHR immunoreactivity is present in normal human extraocular myoblasts in culture; biological responses to TSH and Graves' serum are currently being examined. In conclusion the TSHR, the target autoantigen of GD, is expressed generally in connective tissue and is expressed in EOM but not non-ocular muscle, suggesting a specific functional and immunologic role of the TSHR in EOM. We are currently examining the biological and immunologic relationship of the TSHR in normal EOM in culture. This information should lead to important new insights into the role of the TSHR in EOM and TAO.

## 74

### SIGNIFICANCE OF EYE MUSCLE AUTOIMMUNITY IN THYROID-ASSOCIATED OPHTHALMOPATHY

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Thyroid associated ophthalmopathy (TAO) is a progressive autoimmune disorder of the extraocular muscle and orbital connective tissue. The best characterized antigens are flavoprotein (Fp), G2s, a thyroid and eye muscle shared antigen now identified as the transcription factor Foxp1, and the TSH receptor (TSHr) which is expressed in the orbital preadipocyte and extraocular muscle cell. Antibodies against Fp (antiFp) are good markers of eye muscle damage in patients with Graves' hyperthyroidism whilst those targeting G2s (antiG2s) seem to be produced in the context of an autoimmune reaction at the eye muscle fiber surface. AntiFp and antiG2s are detected mainly in patients with ocular myopathy, manifest as double vision, eye muscle volume increase and reduced eye movement. Antibodies against TSHr and collagen Type XIII, the only member of the protein family to have a transmembrane domain, are markers of the more common congestive ophthalmopathy. We have developed an experimental model for TAO in BALB/c and CD-1 mice by genetic immunization with TSHr and G2s. In CD-1 mice, antiFp were detected at 12, 14 and 16 wk, in all experimental groups, including those immunized with G2s only, and greatest in the group immunized with TSHr alone. AntiTSHr levels were greatest in mice immunized with G2s + TSHr in the presence of IL4, but not IL12. Histological changes in the orbit included eye muscle fiber dissociation by edema, and mast cell infiltration, especially in mice immunized with G2s + TSHr. Thus Foxp1 and TSHr may both be primary antigens in TAO. The finding of negative antiG2s but positive antiFp in some mice suggests that eye muscle damage and Fp release were mediated by T lymphocytes targeting G2s or some other cell membrane antigen. In both BALB/c and CD-1 mice there was spleen T cell sensitization to G2s, but only minimal eye signs and no lymphocyte infiltration in the thyroid. Attempts to enhance the model to more closely resemble human TAO are in progress. The possibility that radioactive iodine

treatment of patients with Graves' hyperthyroidism leads to worsening of eye disease is being addressed. So far we have shown increased production of eye muscle antibodies following radioiodine, but not anti-thyroid drugs, in the first cohort of patients tested.

**75**

**SODIUM-IODIDE TRANSPORTER: ITS ROLE IN THYROIDAL AND EXTRATHYROIDAL DISEASE.**

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The thyroid gland is unique amongst the endocrine glands since its function is directly dependent on a range of environmental micronutrients in particular, the ambient level of iodine intake. If ambient levels of micronutrients are perturbed for prolonged periods this leads to the development of nodular thyroid disease or goitre. There is also some evidence that such perturbations may be contributing factors to thyroid malignancy as well as to autoimmune thyroid disease. The thyroid gland has evolved mechanisms, termed autoregulation, to protect itself against rapid fluctuations in thyroid hormone secretion secondary to changes in the ambient level of iodine. Although these physiological mechanisms were known for many years and that a putative iodide transporter (NIS) was required for these effects, the nature and structure of this transporter was only described in 1996. NIS is located in the basolateral membrane of thyroid follicular cells, and accumulated I- is organified to molecular iodine through the action of the enzyme thyroid peroxidase (TPO) in the presence of H2O2, which takes place mainly at the apical membrane of the follicular cell. Organified iodine iodinates the thyroid protein thyroglobulin, which when stored in the lumen of thyroid follicles represents a storage site for the hormones thyroxine (T4) and triiodothyronine (T3). Possession of NIS enables the thyroid to concentrate I- 20–40-fold. Although the thyroid is the most efficient organ concentrating iodide, it is not the only one to have this ability as NIS expression and the ability to concentrate I- has been demonstrated in the salivary and lacrimal glands, breast, gastric mucosa, placenta and kidney. Initial interest was centred on the role of NIS in thyroid cancer, where variable results were identified. Despite divergent reports a high positive predictive value was reported for therapeutic outcome between NIS expression by immunohistochemistry and tumour 131I uptake in both primary and recurrent thyroid carcinoma. Further, because of the critical role sodium iodide symporter plays in thyroid function, its potential role as a novel thyroid autoantigen in the pathogenesis of autoimmune thyroid disease has become the focus of many studies. Recent reports involving large series of patient samples and specific and quantitative binding assays were unable to confirm early, promising data that suggested a pathogenic role of sodium iodide symporter in autoimmune thyroid disease. On the other hand, although further studies are needed to determine the clinical importance of sodium iodide symporter as a thyroidal autoantigen, sodium iodide symporter expression detected in mammary glands may have important diagnostic and therapeutic implications in breast cancer.

**76**

**THE NEUROENDOCRINOLOGY OF STRESS**

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The stress system coordinates the adaptive responses of the organism to stressors of any kind. The main components of the stress system are the CRH and Locus Ceruleus-Norepinephrine (LC/NE)-Autonomic systems and their peripheral effectors, the pituitary-adrenal axis, and the limbs of the autonomic system. Activation of the stress system leads to behavioral and peripheral changes that improve the ability of the organism to adjust homeostasis and increase its chances for survival. The CRH and LC/NE systems stimulate arousal and attention, as well as the mesocorticolimbic dopaminergic system, which is involved in anticipatory and reward phenomena, and the hypothalamic β--endorphin system, which suppresses pain sensation and, hence, increases analgesia. CRH inhibits appetite and activates thermogenesis via the catecholaminergic system. Also, reciprocal interactions exist between the amygdala and the hippocampus and the stress system, which stimulates these elements and is regulated by them. CRH plays an important role in inhibiting GnRH secretion during stress, while, via somatostatin, it also inhibits GH, TRH and TSH secretion, suppressing, thus, the reproductive, growth, and thyroid functions. Interestingly, all three of these functions receive and depend on positive catecholaminergic input. The end-hormones of the hypothalamic-pituitary-adrenal (HPA) axis, glucocorticoids, on the other hand, have multiple roles. They simultaneously inhibit the CRH, LC/NE and □-endorphin systems

and stimulate the mesocorticolimbic dopaminergic system and the CRH peptidergic central nucleus of the amygdala. In addition, they directly inhibit pituitary gonadotropin, GH and TSH secretion, render the target tissues of sex steroids and growth factors resistant to these substances and suppress the 5' deiodinase, which converts the relatively inactive tetraiodothyronine (T4) to triiodothyronine (T3), contributing further to the suppression of reproductive, growth, and thyroid functions. They also have direct as well as insulin-mediated effects on adipose tissue, ultimately promoting visceral adiposity, insulin resistance, dyslipidemia and hypertension (Metabolic syndrome X) and direct effects on the bone, causing "low turnover" osteoporosis. Central CRH, via glucocorticoids and catecholamines, inhibits the inflammatory reaction, while directly secreted by peripheral nerves stimulates local inflammation. Antalarmin, a novel CRH receptor type 1 antagonist, decreases the activity of the HPA axis, suppresses neurogenic inflammation and blocks CRH-induced skin mast cell degranulation, in addition to blocking the development and expression of conditioned fear and stress-induced colonic hyperfunction. Chronic administration of antalarmin is not associated with glucocorticoid deficiency. These data suggest that such antagonists may be useful in human pathologic states, such as melancholic depression and chronic anxiety, associated with chronic hyperactivity of the stress system, along with predictable behavioral, neuroendocrine, metabolic and immune changes, based on the interrelations outlined above. Conversely, we will need potentiators of CRH secretion/action to treat atypical depression, postpartum depression and the fibromyalgia/chronic fatigue syndromes, all characterized by low hypothalamic-pituitary-adrenal axis and LC/NE activity, fatigue, depressive symptomatology, hyperalgesia and increased immune/inflammatory responses to stimuli.

**77**

**STRESS AND STRESSORS: A CONCEPTUAL FRAMEWORK**

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Excessive stress, whether due to excessive exposure or sensitivity to stressors, can both cause illness and exacerbate existing illnesses. The rational development of drugs that prevent development of stress-related illness involves accurate identification of brain pathways that control stress responses. One of the greatest impediments to progress in this area is conceptual rather than technical. In particular, the way in which researchers define stress and categorise stressors inevitably shapes the design of their experiments and thus the maps that they construct of CNS stress control pathways. This in turn shapes their views on how and where to probe the CNS for new drug targets.

Biomedical researchers still commonly define stress as the body's response to an actual or threatened disturbance of homeostasis. However, this is inadequate on many levels, particularly when one considers "psychological" stress. Firstly, it fails to offer useful insights as to the identity of stimuli or situations that can act as stressors and, secondly, it fails to explain why a stimulus that constitutes a powerful stressor for one individual may have no effect upon another individual. One way forward may be to consider stress from an evolutionary perspective.

Natural selection favours the emergence of species with characteristics that improve the odds of personal survival and reproductive success, and the survival and reproductive success of genetic relatives (Hamilton's theory of "inclusive fitness"). Many convergent lines of evidence suggest that these characteristics are underpinned by neural networks organised to facilitate the achievement of certain fundamental goals, e.g. (i) maintenance of immediate homeostatic state; (ii) anticipation and thus avoidance of future disturbances of homeostatic state; (iii) attainment and maintenance of social relationships; (iv) attainment and maintenance of hierarchical status; and (v) mating opportunities. In this presentation I will discuss how this leads to the proposal that: (a) stress is elicited by threats to the achievement of these genetically programmed goals; (b) the neural pathways that generate a stress response vary with the goal being threatened, and whether or not recognition of the stressor is innate or learned; (c) a stress response is a co-ordinated physiological, behavioural, cognitive and affective state that is conceptually indistinguishable from a negative emotional state. The implications of this proposal for biomedical scientists investigating stress will be illustrated by examples drawn form our own work on neuroendocrine stress responses.

78

## STRESS AND THE REPRODUCTIVE AXIS: SEX DIFFERENCES AND CONSEQUENCES FOR FERTILITY

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The mechanisms by which stress influences the activity of the reproductive axes in males and females are not well understood. Many physiological systems are activated by stress and any of these could potentially impinge on reproduction at any level of the hypothalamo-pituitary gonadal axis. A predominant impact of stress is on the secretion of gonadotrophin releasing hormone (GnRH) from the brain and the gonadotrophins, luteinising hormone and follicle stimulating hormone, from the anterior pituitary gland. Prolonged stress is likely to suppress the secretion of the gonadotrophins but the effects of acute stress or repeated acute stress are not clear. Investigations of the mechanisms by which stress impacts on reproduction should consider sex differences, because there are differences between males and females in physiological responses to stress (1) and these differences are influenced by the type of stressor (2). Furthermore, the mechanisms by which stress affects reproduction vary with sex and the predominance of particular sex steroids in the circulation (1). Specifically, these factors influence the extent to which stress suppresses the secretion of GnRH and reduces the responsiveness of the pituitary to the actions of GnRH. Stress may also modify the feedback actions of gonadal hormones. These mechanisms are likely to involve complex interactions between a number of central and peripheral pathways. An understanding of these mechanisms requires determination of the stress pathways that are activated by different stressors and identification of how they impact on the secretion and actions of GnRH.

(1) Tilbrook, AJ, Turner, AI & Clarke, IJ 2002 Stress and reproduction: central mechanisms and sex differences in non-rodent species. *Stress* **5** 83-100.

(2) Turner, AI, Canny, BJ, Hobbs, RJ, Bond, JD, Clarke, IJ & Tilbrook, AJ. 2002 Influence of sex and gonadal status of sheep on cortisol secretion in response to ACTH and on cortisol and LH secretion in response to stress: importance of different stressors. *Journal of Endocrinology* **173** 113-121.

79

## STRESS INDUCED DISTURBANCES OF THE HPA AXIS: A PATHWAY TO TYPE 2 DIABETES

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Type 2 diabetes is the most common form of hyperglycaemic state. The disease exists in all populations, but in developed societies, the prevalence has risen as the population ages and above all becomes more obese. In the prediabetic state, type 2 diabetes involves two defects, peripheral insulin resistance and hyperinsulinemia, which is followed by failure of insulin secretion to compensate for the insulin resistance. There is convincing evidence linking visceral obesity to the development of insulin resistance, and virtually all obese subjects are insulin resistant (1), but the association is complex.

Throughout newly developed methods we could single out subgroups of the functional status of the HPA axis within a general population of noncushingoid middle-aged men. The first group was characterized by a high morning cortisol peak, a normal circadian rhythm (variability) and feedback regulation (dexamethasone) along with a brisk cortisol response to lunch. Such men are in general lean, measured as body mass index and waist-hip ratio, with higher values of insulin-like growth factor I (IGF-I) than average, and low total and low-density lipoprotein (LDL) cholesterol as well as blood pressure (Table 1) (2-4).

The other group identified was characterized by the absence of a morning cortisol peak and circadian rhythm, cortisol escape from dexamethasone suppression and a poor lunch-induced cortisol response. Such men suffer from obesity with a predominance of centrally located body fat, low testosterone and IGF-I concentrations, high glucose, insulin, triglycerides, total and LDL cholesterol, blood pressures and heart rate, while high-density lipoprotein cholesterol is low (Table 1) (2-4).

Over the years, we have come to recognize that a number of psychosocial and socio-economic handicaps that are known to activate the HPA axis are also associated with visceral obesity. The most prominent factors are low

education, unemployment, poor economy, being divorced and solitude (5). Moreover, low socio-economic status is associated with both visceral obesity and perturbed cortisol secretion (6). In addition, we have been able to identify a subgroup of visceral obesity, where a blunted dexamethasone response is found, associated with such symptoms (7).

In conclusion, distressing events or situations evokes prominent HPA system activation, and after long-term exposure, the HPA axis will eventually become dishabituated, resulting in a disruption of central regulatory systems. Psychosocial and socio-economic impairments are most likely important triggers for the perturbations of the HPA axis described herein, which in genetically susceptible individuals are followed by visceral obesity, insulin resistance, dyslipidemia and type 2 diabetes.

Table 1

The status of the HPA axis in relation to visceral obesity, glucose, insulin, and lipids

| | Normal HPA axis regulation | Disturbed HPA axis regulation |
|---|---|---|
| Visceral obesity | ↓ | ↑ |
| Fasting glucose | 0 | ↑ |
| Fasting insulin | 0 | ↑ |
| Triglycerides | 0 | ↑ |
| Total cholesterol | ↓ | ↑ |
| Low-density lipoprotein cholesterol | ↓ | ↑ |
| High-density lipoprotein cholesterol | 0 | ↓ |


1. Hodge AM, de Courten MP, Zimmet P. Obesity and type 2 diabetes mellitus. In: Björntorp P, editor. The International Textbook of Obesity. Chichester: John Wiley & Sons; 2001. p. 351-364.

2. Rosmond R, Dallman MF, Björntorp P. Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. J Clin Endocrinol Metab 1998;83:1853-1859.

3. Rosmond R, Björntorp P. Blood pressure in relation to obesity, insulin and the hypothalamic- pituitary-adrenal axis in Swedish men. J Hypertens 1998;16:1721-1726.

4. Rosmond R, Holm G, Björntorp P. Food-induced cortisol secretion in relation to anthropometric, metabolic and haemodynamic variables in men. Int J Obes Relat Metab Disord 2000;24:416-422.

5. Rosmond R. Visceral obesity and the metabolic syndrome. In: Björntorp P, editor. The International Textbook of Obesity. Chichester: John Wiley & Sons; 2001. p. 339-350.

6. Rosmond R, Björntorp P. Occupational status, cortisol secretory pattern, and visceral obesity in middle-aged men. Obes Res 2000;8:445-450.

7. Rosmond R, Björntorp P. Endocrine and metabolic aberrations in men with abdominal obesity in relation to anxio-depressive infirmity. Metabolism 1998;47:1187-1193.

**100**

## SIGNALLING THROUGH THE SMAD PATHWAY BY INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 IN BREAST CANCER CELLS

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*J. Biol. Chem. 277: 7255-7261 (2002)*

We previously demonstrated in T47D human breast cancer cells transfected to express the type II transforming growth factor-β receptor (TGF-βRII) that insulin-like growth factor binding proein-3 (IGFBP-3) could stimulate Smad2 and Smad3 phosphorylation, potentiate TGF-β1-stimulated Smad phosphorylation, and cooperate with exogenous TGF-β1 in cell growth inhibition (Fanayan, S., Firth, S.M., Butt, A.J. and Baxter, R.C. (2000) *J. Biol. Chem.* **275**: 39146-39151). This study further explores IGFBP-3 signalling through the pathway. Like TGF-β1, natural and recombinant IGFBP-3 stimulated the time- and dose-dependent phosphorylation of TGF-βRI as well as Smad2 and Smad3. This effect required the presence of TGF-βRII. IGFBP-3 mutated in carboxyl-terminal nuclear localisation signal residues retained activity in TGF-βRI and Smad phosphorylation, whereas IGFBP-5 was inactive. Immunoneutralisation of endogenous TGF-β1 suggested that TGF-β1 was not essential for IGFBP-3 stimulation of this pathway, but it increased the effect of IGFBP-3. IGFBP-3, like TGF-β1, elicited a rapid decline in immunodetectable Smad4 and Smad4-Smad2 complexes. IGFBP-3 and nuclear localisation signal mutant IGFBP-3 stimulated the activity of the plasminogen activator inhibitor-1 promoter but was not additive with TGF-β1, suggesting that this end point is not a direct marker of the IGFBP-3 effect on cell proliferation. This study defines a signalling pathway for IGFBP-3 from a cell surface receptor to nuclear transcriptional activity, requiring TGF-βRII but not dependent on the nuclear translocation of IGFBP-3. The precise mechanism by which IGFBP-3 interacts with the TGF-β1 receptor system remains to be established.

**101**

## PROSTATE-SPECIFIC ANTIGEN (PSA) OVER-EXPRESSION INCREASES THE MIGRATORY POTENTIAL OF THE PROSTATE CANCER CELLS, PC-3.

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Prostate-specific antigen (PSA) is a seminal fluid liquefaction enzyme and the clinical marker of choice for prostate cancer. Whilst serum assays of PSA levels are widely used for the diagnosis of patients with prostate cancer, it remains to be determined whether it has a functional role in the progression of this disease. Recent evidence suggests that it may be involved in prostate cancer progression as a tumour promoter by releasing bioactive IGF-1. Conversely, it may act as a tumour repressor through apoptosis and anti-angiogenic activity. E-cadherin is a 120kDa transmembrane glycoprotein that regulates cell-to-cell adhesion. In prostate cancer, decreases in E-cadherin expression have been associated with an aggressive phenotype. Additionally, down-regulation of E-cadherin in cancer can result in a decrease in adhesive properties, and correlate to higher mobility and invasiveness of tumour cells. Thus, inactivation of E-cadherin may allow cells to become more motile and promote progression and metastasis. In order to clarify the role of PSA in prostate cancer, we have created stably transfected PC-3 cell lines which over-express PSA. Several functional assays were performed to determine the effect of PSA over-expression when compared to the parent cell line and vector only clones. Expression of PSA appeared to have no effect on proliferation; however, clones producing PSA were associated with increased invasion and migration when compared with cell lines stably transfected with empty vector. Consistent with this, PSA expressing cells showed compromised adhesion to culture dishes. This evidence suggested that a cell adhesion molecule might be involved, such as E-cadherin. In vitro degradation assays confirmed that seminal purified PSA was able to degrade E-cadherin, and that these events are inhibited by the serine protease inhibitor, PMSF. Analysis of E-cadherin by immunofluorescence demonstrated that PC-3 cells expressing PSA had reduced staining for E-cadherin, in contrast to the native and vector controls, which exhibited classical membrane staining. In summary, transfection of the prostate cancer cell line, PC-3, with PSA, appears to increase the invasive and migratory potential of cells expressing the gene, potentially via degradation of E-cadherin, an important cell adhesion molecule.

## 102

### A MOUSE MODEL OF SPINAL BULBAR MUSCULAR ATROPHY (SBMA)

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SBMA is a late onset, progressive neuromuscular disorder, characterised by wasting of the proximal muscles and signs of androgen insensitivity, including testicular atrophy and infertility. The underlying genetic mutation is the expansion of a trinucleotide repeat (CAG repeat) in exon 1 of the androgen receptor (AR) gene. This disorder is one of a group of neurodegenerative diseases associated with expanded CAG repeats, which includes Huntington disease (HD) and several spinocerebellar ataxias. The CAG repeat in the AR is polymorphic, ranging from 8-31 repeats in normal individuals, with the disease phenotype associated with more than 38 CAG repeats. An experimental animal model of SBMA has been generated carrying a full-length human AR cDNA with 20, 65, or 120 CAG repeats. These cDNAs are driven by the CMV promoter, resulting in expression in most tissues, with the notable exception of germ cells. Mice with either 65 or 120 CAG repeats displayed progressive behavioural and motor dysfunction, reminiscent of abnormalities exhibited by HD mice. These phenotypic changes were more dramatic in the mice with the longer repeats, indicating that disease severity is directly related to trinucleotide repeat length. By 4 months of age male mice displayed signs of progressive muscle weakness, with the onset being delayed in females, possibly a hormonal component to disease progression. Histological signs of denervation atrophy were evident, from 3 months of age and included, mild fibre-type grouping, small angular fibres, and regions of degeneration, with compensatory hypertrophy observed in many of the remaining fibres. These changes were most likely caused by a progressive loss of motor neurons (approximately 65% by 6 months of age) in the anterior horn, as shown by stereological analysis. Further, these mice were shown to have a 50% reduction in daily sperm production, with no obvious changes in testis weight, T, LH, or FSH levels. This data supports SBMA being a dominant disorder, affecting motor neurons, muscle, and late stage spermatogenesis. In humans, females appear to be protected by X-inactivation, and possibly lower androgen levels.

## 103

### GENE-ENVIRONMENT INTERACTION INFLUENCES THE RELATIONSHIP BETWEEN ALCOHOL CONSUMPTION AND ABDOMINAL OBESITY IN HEALTHY FEMALE TWINS

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Moderate alcohol consumption is associated with reduced coronary morbidity and mortality and is purported to contribute to the low rates of coronary disease in some populations with high saturated fat intakes ("French paradox") (1). This has primarily been attributed to increased HDL-cholesterol (1,2), although improved insulin sensitivity (2), haemostatic activity (1) and post-prandial lipaemia (3) have also been proposed. The association between these factors and abdominal obesity in the 'Metabolic Syndrome' led us to investigate the relationship between alcohol consumption and body fat distribution in 334 non-diabetic British female twins (aged 57.7±6.7 yrs) from the St Thomas' UK Adult Twin Registry, London, UK, after excluding energy under-reporters. Alcohol intake was measured by food-frequency questionnaire and total and central abdominal fat (CAF) by dual-energy X-ray absorptiometry. The Generalised Estimating Equation corrected for intra-pair correlations. Subjects were grouped according to daily alcohol consumption (g): abstainers (21%), 0.1–5.9 (48%), 6–11.9 (20%), 12–17.9 (6%) ('moderate alcohol consumers') and ≥18g/d (5%). Compared to abstainers, moderate alcohol consumers had less total (34.3±6.5 *vs* 39.0±7.4%, $P<0.05$) and abdominal (31.9±10.6 *vs* 37.6±10.5%, $P<0.05$) fat. These differences persisted after controlling for smoking and physical activity. In multiple regression models, only

physical activity and alcohol consumption predicted lower total and abdominal adiposity. With genetic, age, gender and environmental confounders excluded in monozygotic twin pair analyses, moderate alcohol consumption accounted for ~300g less abdominal fat than abstinence and intermediate-level drinking. In gene-environment interaction analyses (*n*=150 twins concordant for smoking and hormone replacement therapy), in which genetic risk was based on co-twin phenotype, moderate alcohol consumption was associated with lower abdominal adiposity in twins at high genetic risk of abdominal obesity (%CAF: 37.0±8.9 *vs* 45.5±6.8%, P<0.05), with no difference in twins at low genetic risk (31.5±10.9 *vs* 27.8±6.2% respectively, NS) (P<0.05 for interaction). In conclusion, our results suggest that the association between moderate alcohol consumption and reduced abdominal adiposity occurs predominantly in subjects genetically predisposed to abdominal obesity. Lower abdominal fat may be an intermediate between moderate alcohol consumption and cardiovascular protection.

1 Renaud S *et al*. *Lancet* 1992;339:1523-6.

2 Bell RA *et al*. *Diabetes Care* 2000;23:1630-6.

3 Sharrett AR et al. Arterioscler Thromb Vasc Biol 2001;21:275-81.

**104**

## SIGNAL TRANSDUCTION VIA 1-O-PHOSPHATIDYLINOSITOL-3 KINASE (PI3K) IS NECESSARY FOR THE SURVIVAL OF THE PREIMPLANTATION EMBRYO.

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The preimplantation embryo requires the action of survival factors for their development by the 2-cell stage (1). Survival signalling is often transduced by PI3K. The role of PI3K in the survival of the early embryo has not been investigated. The aim of this study was to determine whether PI3K is expressed in the preimplantation mouse embryo, its actions are required for normal embryo survival and if it acts by transducing the signalling induced by the embryonic survival factor PAF (1). mRNA (RT-PCR) for regulatory subunits p85$\alpha$ and p85$\beta$, and catalytic subunits p110$\alpha$, p110$\beta$ and p110$\gamma$ of PI3K were expressed in the 2-cell embryo. Blocking PI3K activity with LY 294002 or Wortmannin caused a dose-dependent reduction in embryonic survival. Zygotes cultured in LY 294002 (3$\mu$M) or Wortmannin (10nM) for 96h had a 32% (P <0.0001) and 47% (P <0.0001) reduction in the number of embryos reaching the blastocyst stage, respectively. Of those that reached the blastocyst stage their was a significant reduction (P<0.0001) in the number of total cells/blastocyst, due to a reduced number of normal cells/blastocyst, and a corresponding increase in the number of apoptotic cells/blastocyst. When zygotes were placed in LY 294002 (a reversible PI3K inhibitor) for only 24h, and then allowed to develop in inhibitor-free media for a further 72h, a significant reduction (P < 0.0001) in embryo development still occurred. This is consistent with earlier observations that survival factor signalling was required by the 2-cell stage of development. PAF induces receptor-dependent intracellular calcium transients in zygote and 2-cell stage embryos (2). Both LY 294002 and Wortmannin inhibited PAF-induced intracellular calcium transients in 2-cell embryos with the same potency as they had on embryo survival. LY 303511 (an inactive analogue of LY 294002) had no effect of PAF-induced signalling. This study shows: (a) that several different classes of PI3K were expressed in the early embryo; (b) PI3K activity was required for normal survival of preimplantation embryo; and (c) PI3K acts to transduce the signal of at least one well defined embryonic survival factor by inducing transient rises in intracellular calcium.

Reference:

(1) O'Neill C. Biol Reprod. 1998; 58(5): 1303-9.

(2) Emerson M *et al*., J Biol Chem. 2000; 275(29): 21905-13.

## 105

### INHIBITION OF LIVER RECEPTOR HOMOLOGUE-1 (LRH-1) BY SMALL HETERODIMER PARTNER (SHP) IN PREADIPOCYTES.

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Liver Receptor Homologue-1 (LRH-1) and Steroidogenic Factor-1 (SF-1) are the two human homologues of the drosophila nuclear receptor Ftz-F1. The role of SF-1 in regulating transcription of genes of the reproductive axis is well established. Considerably less is known regarding functions of LRH-1. We have previously shown that LRH 1, but not SF-1, is expressed in the preadipocyte fraction of human breast adipose tissue where it stimulates transcription from the proximal promoter (promoter II) of the CYP19 gene that encodes aromatase. The overall aim of this work is to identify mechanisms that regulate LRH-1 activity or expression, which in turn could modify CYP19 expression and, in turn, oestrogen synthesis in breast adipose. The present study focuses on the small heterodimer partner (SHP), an atypical nuclear receptor that lacks a DNA binding domain and inhibits transcriptional activity of a range of nuclear receptors. To assess the effects of SHP on LRH-1 transcriptional activity, a reporter of LRH-1 activity was constructed by replacing the DNA binding domain (DBD) of LRH-1 with the DBD of the yeast transcription factor GAL4 (GAL4LRH). This construct, or a construct encoding the GAL4 DBD only, was transfected into 3T3-L1 preadipocytes in the presence or absence of an SHP expression vector and a GAL4-responsive luciferase reporter gene. Luciferase activity in the presence of the GAL4-LRH fusion gene was approximately 15-fold higher than in the presence of the GAL4 DBD alone, indicating that LRH-1 exhibits constitutive transcriptional activity. Transfection of SHP completely inhibited activity of GAL4LRH-1. To assess the functional effects of LRH-1 and SHP on CYP19 promoter II, 3T3-L1 cells were transfected with a CYP19 promoter II – luciferase reporter gene in the presence or absence of LRH-1 and increasing concentrations of SHP. SHP dose-dependently and completely inhibited LRH-1 – induced CYP19 promoter II activity. Cells transfected with LRH-1 and treated with forskolin and phorbol ester, which maximally stimulates CYP19 promoter II activity (~60-fold), also showed a dose-dependent and complete inhibition of promoter II activity in the presence of SHP. We conclude that CYP19 expression and thus oestrogen synthesis in breast preadipocytes is determined, at least in part, by the ratio of LRH-1: SHP. We are currently investigating the hormonal regulation of these nuclear receptors.

## 106

### ROLE OF β1-INTEGRIN IN SPERMIATION AND SPERMIATION FAILURE

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Spermatogenesis (sperm production) is completed when elongated spermatids undergo their final maturation and disengage from the supporting Sertoli cells prior to their transport to the epididymis. This process of disengagement, termed spermiation, involves a complex series of events including removal of adhesion junctions between Sertoli cells and spermatids, and remodelling of the spermatid. Spermiation is hormone sensitive; testosterone (T) and FSH withdrawal causes spermatids to be retained and phagocytosed by Sertoli cells instead of being released (1). Spermiation failure occurs in response to gonadotrophin suppression in rodents (1) monkeys (2) and men (3), and thus is of clinical relevance when considering the development of male contraceptives. The molecular mechanisms involved in spermiation and spermiation failure are largely unknown.

This study aimed to characterise a) the ultrastructural events involved in spermiation and spermiation failure and b) the role of the cell adhesion molecule $\beta_1$-integrin in this process. Spermiation failure in rats was induced by FSH&T suppression and light and electron microscopic analysis undertaken. The localisation of $\beta_1$-integrin, its associated kinase ILK, and vinculin and espin, which are localised within the specialised "ectoplasmic specialisation" (ES) adhesion junction between spermatids and Sertoli cells, were investigated using immunohistochemical methods.

Following FSH&T suppression, the majority of ultrastructural events associated with normal spermiation occurred however the final disengagement of spermatids from Sertoli cells was impaired. During normal spermiation, ES junctions were removed ~20hrs prior to spermatid disengagement and ILK, which is associated

with $\beta_1$-integrin in ES junctions, was removed along with the ES. However, $\beta_1$-integrin persisted at the junction between spermatids and Sertoli cells after ES removal until the point of disengagement, suggesting that integrin-mediated, ILK-independent processes are part of the disengagement process. During spermiation failure, ES junctions were removed normally, and thus a persistence of ES cannot explain spermiation failure. However, the association of $\beta_1$-integrin with retained spermatids, suggests that the normal integrin-mediated "loss of adhesion" that occurs during spermiation may be impaired during spermiation failure.

We conclude that spermiation failure during gonadotrophin suppression may be caused by functional changes in integrin-containing cell adhesion complexes between mature spermatids and Sertoli cells.

1. Saito *et al* 2000 Endocrinology 141:2779

2. O'Donnell *et al* 2001 JCEM 86:1814

3. McLachlan *et al* 2002 JCEM 87: 546

**107**

## CORTICOSTEROID BINDING GLOBULIN POLYMORPHISMS IN CHRONIC FATIGUE SYNDROME

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Background: Chronic fatigue syndrome (CFS) is an idiopathic disorder characterised by disabling fatigue of greater than 6 months duration and complex associated symptoms. The NIH neuroendocrine data show that patients tend to exhibit low plasma and urine cortisol and reduced adrenocorticotropin (ACTH) and cortisol responses to stimuli, suggesting a defect of CRH release.[1] 28% of CFS patients respond completely to low-dose hydrocortisone.[2] Twin and family studies indicate a substantial genetic contribution to the aetiology of CFS. Recent studies have shown that severe corticosteroid-binding globulin (CBG) gene mutations are associated with CFS in isolated kindreds.[3] Hence, we hypothesised that CBG gene polymorphisms may act as a genetic risk factor for CFS.

Methods: 248 patients with CFS defined by CDC criteria and 198 controls were recruited. Full sequencing of CBG gene coding exons revealed a common exon 3 polymorphism (c825G→T, Ala→Ser$^{224}$). This was confirmed in CFS patients and controls using restriction enzyme analysis of genomic DNA. Plasma CBG, total and free cortisol (calculated and measured) were ascertained in single samples taken between 8-10AM.

Results: There was a 35% excess of serine$^{224}$ homozygotes among the CFS patients compared to controls (P=0.004). Plasma IR-CBG levels were higher in serine heterozygous subjects and patients, and higher again in serine homozygotes, although this reached significance only in the male controls; Ser/Ser: 46.1 ± 1.8 (n=31, P=0.03) *vs* Ser/Ala: 42.4 ± 1.0 (n=56, P= 0.05) *vs* Ala/Ala 40.8 ± 1.7 mcg/mL (n=21). Despite higher CBG levels, there was a non-significant trend towards lower total and free plasma cortisol in serine allele positive patients, total cortisol: Ser/Ser: 364 ± 36 (n=34) *vs* 390 ± 20 (n=66) *vs* 417 ± 26 nmol/L (n=23), leading to a reduced cortisol:CBG ratio (Ser/Ser *vs* Ala/Ala, P= 0.05).

Conclusion: Homozygosity for the CBG serine allele may predispose to CFS, perhaps due to an effect on HPA axis function related to elevated IR-CBG levels. Further studies of the neuroendocrine effect of CBG phenotype are required.

[1]Demitrack MA, et al. J Clin Endocrinol Metab 1991; 73:1224-1234.

[2]Cleare AJ et al. Lancet 1999; 353:455-458.

[3]Torpy DJ et al. J Clin Endocrinol Metab 2001; 86:3692-3700.

## 108

### GLUCOCORTICOID RECEPTOR POLYMORPHISMS AND POST TRAUMATIC STRESS DISORDER

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Background: Post traumatic stress disorder (PTSD) affects 10-15% of combat exposed Vietnam War Veterans. Development of the syndrome is not predictable on the basis of stressor alone and PTSD patients have a characteristic stress system abnormality, with activation of the sympathetic nervous system but unexplained relative hypocortisolism. Glucocorticoid feedback sensitivity where cortisol levels are hypersuppressible to exogenous glucocorticoid (dexamethasone) has been documented by several investigators. A familial tendency towards PTSD and high concordance in monozygotic twins, if stress exposed, suggests a genetic predisposition to PTSD. Relative hypocortisolism and glucocorticoid hypersensitivity has also been noted in unaffected relatives of patients with PTSD. Recent studies have shown that polymorphisms in exon 2 of the glucocorticoid receptor (GR) which alter the N-terminal transactivation domain may be associated with altered glucocorticoid sensitivity. In particular the N363S polymorphism has been associated with glucocorticoid hypersensitivity and a tendency towards abdominal obesity and low bone mineral density.1

Methods: We studied 58 PTSD patients diagnosed by psychological assessment (Clinician administered PTSD scale for DSM-IV-current diagnostic version), self-report measures and psychiatric interview. Low-dose 0.25-mg (n=34) and 0.5-mg (n=32) dexamethasone suppression testing (DST) was compared as a marker for glucocorticoid sensitivity at the hypothalamic-pituitary unit. The GR exon 2 (1404bp) was sequenced using an ABI 377 DNA sequencer.

Results: The heterozygous form of the N363S polymorphism was detected in three of 58 PTSD patients (5.2%). Thirty-four PTSD patients received 0.25-mg DEX and 32 received 0.5-mg DEX. Cortisol levels were reduced by > 40% in 59% of patients after 0.25-mg DEX compared to 97% of patients after 0.5-mg DEX ($P<0.01$).

Conclusions: The frequency of the N363S polymorphism in these PTSD patients was not higher than that noted in community surveys. 0.25-mg DEX testing resulted in a wider range of suppressibility than 0.5-mg and will allow comparison of subtle variations in glucocorticoid sensitivity. Further studies are aimed at comparing all known and novel polymorphisms of the GR N-terminal transactivation domain, and relating these, and cortisol responses to low-dose DEX, to psychological variables in combat exposed Vietnam veterans with and without PTSD.

1 Huizenga NATM, et al. J Clin Endocrinol Metab 1998; 83:144-151.

## 109

### PLASMA FREE, SALIVARY AND TOTAL CORTISOL RESPONSES TO VESTIBULAR CALORIC STIMULATION IN NORMAL HUMANS

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Stressors stimulate dopaminergic projections to the medial prefrontal cortex (mPFC) in animals, leading to hypothalamic-pituitary-adrenal (HPA) axis stimulation. This effect is asymmetric, with certain psychological stressors eliciting predominantly right-lateralized dopamine release, which correlates with the degree of HPA axis activation. In humans, selective activation of the right mPFC by left visual field stimulation, results in greater cortisol responses than left mPFC activation. We hypothesized that vestibular caloric stimulation (VCS) could provide an easily controlled means of mPFC stimulation in humans and that the ensuing HPA axis response would be greater with left ear (i.e. right mPFC) stimulation.

Six normal volunteers (2 M, 4 F) underwent separate vestibular caloric stimulation tests in the left and right ears (i.e. L-VCS, R-VCS). Tests occurred randomly, a week apart, in mid-afternoon. We infused ice-cold water into the external auditory canal by syringe, ceasing at the beginning of nystagmus. Blood and saliva for hormone

assay (plasma RIA) was collected from 5 min before to 60 min after infusion. Results are expressed as mean ± SEM.

Side-effects (dizziness, pain) and the times of onset and duration of nystagmus were not different in L- and R-VCS. No nausea was reported. Both L- and R-VCS stimulated a prompt plasma cortisol response [mean time of peak 20.0 ± 3.2 vs. 19.2 ± 5.2 min] which was left lateralized in 5/6 subjects, with the mean AUC response to L-VCS twice that to R-VCS [P<0.05, Mann-Whitney]. Plasma free cortisol (left lateralized in 5/6) rose in parallel with total cortisol, peaking at 20.0 ± 3.2 and 20.0 ± 3.4 min in L-and R-VCS, respectively. Peak salivary cortisols reached only 50% of peak plasma free cortisol levels and were more variable, with some basal levels at or near the detection limit of the assay (1.4 nmol/L). As well, salivary cortisol levels peaked at a later time [27.5 ± 7.2 vs. 35.0 ± 6.3 min in L-and R-VCS, respectively].

In conclusion, we showed that L-VCS (right mPFC stimulation) elicited greater total cortisol responses than R-VCS in 5/6 subjects. Equilibration of cortisol in saliva appeared delayed compared to that of plasma total and free cortisol following this rapid stimulus.

## 110

## DIETARY COMPOSITION IN RESTORING REPRODUCTIVE AND METABOLIC PHYSIOLOGY IN OVERWEIGHT WOMEN WITH POLYCYSTIC OVARY SYNDROME

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Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic condition in women of reproductive age. This randomised control trial examined the metabolic effects of a high protein diet (HP) (n = 14) (40 % carbohydrate, 30 % protein) (n = 14) compared to a low protein diet (LP) (n = 14) (55 % carbohydrate, 15 % protein) (n = 14) in overweight women with PCOS. The intervention consisted of 12 weeks energy restriction (~ 6000 kJ/day) followed by 4 weeks weight maintenance. Improvements in menstrual cyclicity, insulin homeostasis and lipid profile and decreases in weight (7.5 %) and abdominal fat (12.5 %) occurred independent of diet composition. 3 pregnancies additionally resulted (2 HP, 1 LP). Improvements in menstrual cyclicity were associated with greater decreases in insulin resistance, as measured by the homeostasis model, and fasting insulin (p = 0.011). On the LP diet, high-density lipoprotein cholesterol (HDL-C) decreased 10 % in energy restriction (p = 0.008) and free androgen index (FAI) increased 44 % in weight maintenance (p = 0.027). There were no changes in HDL-C in energy restriction or FAI in weight maintenance for the HP diet. Test meal AUC glucose was higher for the LP compared to the HP diet (week 0 3.5 times higher p = 0.02, week 16 4 times higher p = 0.009). Weight loss from dietary and exercise intervention leads to improvements in cardiovascular and endocrine reproductive parameters which appear to be mediated by improvements in surrogate measures of insulin sensitivity. A high protein/low carbohydrate diet during weight loss may result in minor differential endocrine and metabolic improvements.

## 111

## THE EFFECT OF INCREASED DIETARY PROTEIN ON WEIGHT LOSS AND INSULIN RESISTANCE IN OBESE MEN AND WOMEN.

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The benefits of high protein diets for weight loss and insulin resistance remain unclear. The aim of this study was to determine the effect of an increased dietary protein-to-carbohydrate ratio on weight loss and insulin resistance.

A parallel, clinical intervention study of 12 weeks energy restriction (6.4 MJ/day) and 4 weeks energy balance compared 2 groups of obese, hyperinsulinemic subjects (14 male and 43 females, aged 22-65 yrs, BMI 27-42.6 kg/m$^2$, fasting insulin 12-47.8 mU/L) who were randomly assigned to either a high protein (HP) (27% energy as protein, 44% carbohydrate) or a standard protein (SP) (16% energy as protein, 57% carbohydrate) diet. Body weight, fat mass [DEXA], and fasting plasma glucose, serum insulin and serum triglycerides were measured at

weeks 0, 4, 8, 12 and 16. Resting energy expenditure (REE) and the thermic effect of feeding (TEF) [Deltatrac], and postprandial glucose and insulin responses to a HP or SP meal were determined at weeks 0 and 16.

After 16 weeks, weight loss (-7.9 ± 0.5 kg) and total fat loss (-6.9 ± 0.4 kg) was similar in the HP and SP groups. REE fell similarly with each diet (-719 ± 106 kJ/day). The TEF was 15.7% greater after the HP than the SP meal ($p = 0.008$) at both weeks 0 and 16. Fasting glucose increased by 3% on the HP diet and decreased by 2% on the SP diet ($p = 0.017$). Fasting insulin concentrations decreased by 32% and 35% in response to the HP and SP diets respectively ($p < 0.0001$). The glycaemic response to the HP meal was less than to the SP meal at weeks 0 and 16 ($p = 0.027$), and after weight loss the decrease in glycaemic response was greater in the HP group ($p = 0.08$). The insulin response to the test meals was reduced similarly in both diet groups ($p < 0.001$). Serum triglyceride concentrations decreased more on the HP diet (23%) than on the SP diet (10%) ($p < 0.05$).

We conclude that in obese subjects with hyperinsulinemia, (i) energy restriction is the major determinant of weight loss (ii) an increase in the protein-to-carbohydrate ratio is associated with lower postprandial glucose and decreased plasma triglyceride concentrations.

**112**

## THE EXTRAOCULAR MUCLE THYROID STIMULATING HORMONE RECEPTOR (TSHR) AND ITS RELEVANCE TO GRAVES' OPTHALMOPATHY

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Graves' ophthalmopathy (GO) is one of the main manifestations of Graves' disease (GD). The pathology of GO is primarily due to enlargement of the extraocular muscles (EOM). Presently we understand little of the cause of the disease at the molecular level and research into the mechanisms of GO may provide a target for drug development. We have previously shown that the thyroid target of GD, the TSH receptor (TSHR), is expressed at the mRNA level in EOM but not in other skeletal muscles (1). Due to GO's close association with GD we therefore hypothesise that TSHR autoantibodies, which stimulate the TSHR in the thyroid and cause the hyperthyroidism of GD, also act upon ocular tissues to induce the clinical features of GO. The aim of the present studies is therefore to verify the presence of the TSHR protein in EOM and to determine its function at this site. We have used three different TSHR antibodies for immunohistochemistry of normal human EOM. MCID has been used to quantify differences to control skeletal muscle and these studies were verified using in vitro autoradiography with 125I-TSH as the ligand, also quantified by MCID. Normal human eye muscle cultures have been established to verify the presence of the TSHR and to investigate its function in EOM. Results indicate that TSHR protein is localised to the normal thyroid (as expected) and also to EOM (as detected by immunohistochemistry and in vitro autoradiography), but not in control skeletal muscle, supporting our previous data (1,2). Cell culture studies are underway; preliminary results from immunohistochemistry on cultures also confirm the expression of the TSHR protein within myoblasts. The next step in our investigations is to use our ocular myoblast cultures to test functional responses of myoblasts to TSH and Graves' serum.

1. Major BJ, Cures A. and Frauman AG.(1997)Biochemical & Biophysical Research Communications 230: 493-6.

2. Busuttil, B., Frauman, AG. (2001) Journal of Clinical Endocrinology & Metabolism 86: 2315-9

## 113

## MANAGEMENT OF MULTINODULAR GOITRE IN AUSTRALIA: DIFFERENCES BETWEEN ENDOCRINOLOGISTS AND ENDOCRINE SURGEONS

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Recent European and American surveys showed a lack of consensus on the management of multinodular goitre. There are no comparable data from Australia. In addition, it is not known if endocrinologists and endocrine surgeons differ in their management of multinodular goitre. A questionnaire, adapted from the previous surveys, was sent to Australian endocrinologists and endocrine surgeons. An index case was presented of a 42 year old female with an irregular, non-tender bilaterally enlarged thyroid of 50-80g, with no clinical suspicion of malignancy or thyroid dysfunction and moderate neck discomfort. Eleven variations on the case were then introduced to evaluate how these affected management. The overall response rate was 55%, including 128 endocrinologists and 45 endocrine surgeons who regularly managed multinodular goitre. For the index case, conservative management was recommended by most endocrinologists and endocrine surgeons (65% and 67% respectively), surgery by 10% of endocrinologists and 31% of surgeons, and thyroxine suppression by 22% of endocrinologists and 2% of surgeons ($P<0.001$ for comparison). Treatment recommendations also differed significantly between endocrinologists and endocrine surgeons for 6 of the 11 variations. In particular, for a patient with suppressed TSH, most endocrinologists (60%) recommended radioiodine treatment, whereas there was no consensus among endocrine surgeons (surgery 40%, no treatment 36%, radioiodine 21%) ($P<0.001$). For a patient with a partly intrathoracic goitre, 83% of endocrine surgeons recommended surgery, whereas there was no consensus among endocrinologists (surgery 45%, no treatment 34%, thyroxine suppression 13%, radioiodine 9%) ($P<0.001$). The data show a lack of consensus between endocrinologists and endocrine surgeons in the management of multinodular goitre. The treatment patients receive may depend to some extent on the type of specialist they consult.

## 114

## ENDOCRINE RESPONSE TO TRAUMATIC HEAD INJURY: A PROSPECTIVE STUDY.

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Anterior pituitary deficiency, with or without diabetes insipidus, may be an immediate or delayed consequence of traumatic head injury (1). Diagnosis is difficult because early clinical features and laboratory findings lack specificity. In order to establish the prevalence and clinical importance of post-traumatic pituitary deficiency, we initiated a prospective study with endocrine assessment 7 days, 14 days, 2-3 months and 1 year after severe head injury in subjects who required intensive care admission and survived for at least 7 days. No routine hormone treatment or high dose dexamethasone was given, but subjects were treated with phenytoin for 7 days. We report results of the first 25 subjects (18 males, 7 females, initial Glasgow coma score <9, intensive care stay 2-36 days). One male had clear hypopituitarism with refractory hypotension responsive to glucocorticoid and florid diabetes insipidus, but there was no other clinically obvious example of pituitary failure. The remaining 24 subjects had basal plasma cortisol 350-950 nmol/l at 7 and 14 days; at 14 days, plasma cortisol 60 min after 0.25 mg Synacthen was >900 nmol/l. In 15 of 18 males, plasma testosterone at days 7-14 was <7 nmol/l, with LH <3U/l and FSH <3U/l. At days 7-14 gonadotrophin excess was temporarily absent in two post-menopausal women. In 9 of 11 subjects so far followed to 3 months, gonadal status has returned to normal. Assessment of thyroid function at 7 days showed abnormal results in 24 of 25 subjects. The free T4 estimate (AxSYM, Abbott) was subnormal in 22, an effect attributable in part to phenytoin (2). Unexpectedly, serum TSH was slightly increased (4-7 mu/l) in 7/25 subjects at 7 days, consistent with a phase of recovery from hypothyroxinemia. Conclusions: Standard tests of endocrine function show a high prevalence of apparent abnormality in the pituitary- gonadal and pituitary-thyroid axes in the first weeks after severe traumatic head injury. The impact of transient androgen deficiency on tissue recovery remains unknown. No early laboratory finding can so far predict endocrine deficiency.

1. Benvenga S et al , J Clin Endocrinol Metab 2000; 85: 1353

2. Surks Mi, Defesi CR, JAMA 1996; 275: 1495

## 115

### SEX DIFFERENCES IN THE NEURONAL INPUTS FROM THE HYPOTHALAMUS AND BRAINSTEM TO THE REGION OF THE GONADOTROPHIN-RELEASING HORMONE (GNRH) NEURONES IN THE MEDIAL PREOPTIC AREA

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In sheep, the majority of GnRH neurones are located in the rostral preoptic area and are regulated by a complex network of neurones. The location of these neurones in the ram remains unknown. Retrograde tracing studies in the ewe have mapped the location of cells that project to the rostral preoptic area (1), but similar data are not available for the ram. The regulation of GnRH secretion in the sheep is sexually dimorphic and GnRH neurones in ram lambs receive about half the number of synaptic inputs as do ewe lambs (2). Thus, it cannot be assumed that the inputs to the GnRH neurones are the same in males and females. The retrograde tracer, FluoroGold, was used to trace the neuronal inputs from the hypothalamus and brainstem to the rostral preoptic area of the ram and to compare this with the ewe. Retrogradely labelled cells were observed in the same hypothalamic and brainstem regions as previously reported for the ewe (1) but the ram had a significantly ($P<0.05$) greater number of retrogradely labelled cells/section in the dorsomedial nucleus than the ewe and fewer ($P<0.05$) retrogradely labelled cells in the ventromedial nucleus. These nuclei have been implicated in the regulation of GnRH secretion (3) and our results may partially explain the sex differences in how GnRH secretion is regulated. Fluorescence immunohistochemistry was used to determine the neurochemical identity of some of these cells in the ram. Very few tyrosine hydroxylase-containing neurones in the A14 group (<1%), adrenocorticotrophic hormone (<1%) and neuropeptide Y-containing neurones (1-5%) in the arcuate nucleus contained FluoroGold. Within the brainstem, virtually all FluoroGold-containing cells in the A1 region and about half in the A2 region co-stained for dopamine β-hydroxylase. No other retrogradely labelled cells in the brainstem were noradrenergic. Although dopamine, β-endorphin, and neuropeptide Y have been implicated in the regulation of GnRH secretion in males, it is unlikely that these neurotransmitters regulate GnRH secretion via direct inputs to GnRH neurones.

(1) Tillet et al. 1993. J Comp Neurol 330:195-220. (2) Kim et al. 1999. Biol Reprod 61:599-605.

(3) Scott et al. 2000. Anim Reprod Sci 60/61:313-326.

## 116

### IN VITRO REGULATION OF ACTIVIN / INHIBIN SUBUNITS, FOLLISTATIN AND BAMBI IN THE 3 DAY OLD RAT TESTIS BY ACTIVIN, FOLLISTATIN AND FSH

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Members of the transforming growth factor (TGF)β superfamily and their antagonists are produced by several cell testicular types during early postnatal development. We have observed a discrete switch from activin to follistatin expression within gonocytes as they transform into spermatogonia, and we have illustrated their functional effect on somatic and germ cells (Meehan et al 2000. Dev Biol 220:225). This lead to the hypothesis that these proteins may regulate their own expression and thereby modulate this stage of testicular differentiation. Using 24 hour cultures of 3 day old rat testis fragments and real-time PCR analysis, expression levels of activin and inhibin subunits (α,βA and βB), follistatin and BAMBI mRNAs were measured in response to activin A, follistatin and FSH relative to media alone. BAMBI is a pseudoreceptor that antagonises signalling by activin, TGF□□and some bone morphogenetic proteins, while both follistatin and inhibin can inhibit activin signalling. The inhibin β subunit mRNA was increased 3-fold by FSH (to 280% of control, $p<0.001$), while FSH had no significant effect on the other target mRNAs. Addition of follistatin to these cultures reduced follistatin mRNA to 38% ($P<0.001$) of the control group value. Activin A and the combination of FSH and follistatin also partially reduced levels of follistatin mRNA. In contrast, activin A and follistatin treatments each increased activin βB mRNA (180% $p<0.001$ and 160% $p<0.05$, respectively). BAMBI mRNA was significantly reduced by the addition of activin A (67% $p<0.05$), but neither follistatin nor FSH alone or in combination affected BAMBI expression. This study provides evidence that activin and follistatin may regulate their own actions during postnatal testis development. The data indicate the presence of a negative auto-feedback loop involving follistatin. In contrast, the effect of activin A to inhibit expression of BAMBI and to stimulate activin βB subunit

mRNA levels would reinforce the maintenance of activin bioactivity. This finding correlates with the upregulation of BAMBI mRNA observed in germ cells using in situ hybridisation as gonocytes transform into spermatogonia and activin A protein is lost. This approach has identified the potential for interactions between activin and its regulatory molecules at the onset of spermatogenesis.

**117**

## REGULATION OF SERTOLI CELL ACTIVIN AND INHIBIN SECRETION BY HORMONES, CYTOKINES AND THE SEMINIFEROUS EPITHELIAL CYCLE

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Activin A and the inflammatory cytokines, interleukin-1α (IL-1α) and IL-6, are produced by the Sertoli cell and are implicated in control of spermatogonial and spermatocyte development. Activin A also inhibits IL-1 and IL-6 production and action in several other systems, and therefore is a potential negative regulator of testicular inflammation. However, there have been few studies describing the production and regulation of activin A in the testis. Sertoli cells from immature (20 day-old) or adult rats were cultured for 48h in the presence of IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-6, the inflammatory mediator lipopolysaccharide (LPS from E. Coli), testosterone and/or ovine FSH or dibutyryl cAMP (cAMP). Adult rat seminiferous tubules were stage-dissected and incubated with test substances for 72h. Dimeric activin A and inhibin B in the culture media were measured by specific ELISAs. In immature and adult Sertoli cells, activin A secretion was stimulated by IL-1, and inhibited by FSH/cAMP. Conversely, inhibin B was stimulated by FSH/cAMP and inhibited by IL-1. LPS induced a large increase in activin A, which was partially inhibited by IL-1ra, and a decrease in inhibin B secretion by the Sertoli cells. In cultured seminiferous tubules, activin A secretion occurred across all stages, with a distinct peak of secretion at stage VIII. This secretion was almost completely blocked by IL-1ra. In contrast to isolated Sertoli cells, activin A secretion by cultured seminiferous tubules at stages VII-VIII was stimulated by cAMP. Inhibin B secretion was stimulated in all stages by cAMP, but not by IL-1. These data confirm that Sertoli cell activin A production is positively regulated by endogenous and exogenous IL-1 via a cAMP-independent pathway, although other locally-produced cytokines also may be involved. Activin A is negatively regulated by FSH/cAMP, but this arm of the regulation is modulated by specific germ cell associations. Inhibin B, on the other hand, is positively regulated by FSH/cAMP, and negatively regulated by IL-1. There is reciprocal control of activin A and inhibin B throughout the cycle of the seminiferous epithelium by IL-1 and FSH, which has important implications for control of spermatogonial development and the effects of inflammation on the pituitary-testicular axis.

**118**

## CHARACTERISATION OF PLASMA INHIBIN FORMS IN FERTILE AND INFERTILE MEN

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Inhibin B is the major feedback regulator of FSH in the male. In vitro data suggest that precursor high mol wt forms of inhibin have reduced bioactivity. The aim of this study was to determine the levels of high mol wt forms of inhibin B and inhibin α-subunit in plasma in fertile men and men with various forms of infertility. Plasma from control fertile men (n=11), and men classified by testicular histology as hypospermatogenesis (HypoSG, n=4), Sertoli Cell Only (n=4), germ cell arrest (n=2) and Klinefelter's Syndrome (n=3), and following chemotherapy (n=4) were fractionated using a combined immunoaffinity chromatography, preparative SDS-PAGE and electroelution procedure. Inhibin forms were determined by ELISAs for inhibin B, total inhibin (all forms of inhibin containing the αC fragment) and pro-αC. In fertile men and men with HypoSG, inhibin B was identified as mature (26-30k) and precursor (60k) forms with similar proportions (29.1% vs 25.2%, respectively) of the 60k form in both groups. Inhibin B levels were too low to be assessed in the other infertile groups.

Precursor forms of pro-αC (46k, pro-αN-αC) also showed no differences between control (7.4%) and all infertile groups (7.4-11%). To establish if the Pro-αC ELISA was detecting the precursor forms of inhibin B containing the pro- fragment, normal male plasma was repeatedly immunoabsorbed with antiserum (INPRO) to the pro-region and the remaining immunoabsorbed plasma then immunoabsorbed with antiserum to the αC subunit. No inhibin B was detected in the INPRO-absorbed sample and the profile of immunoactivity as determined by Pro-αC and total inhibin ELISAs were similar. Similarly, the profiles of inhibin B and total inhibin in the α subunit-absorbed sample were identical. These data indicate that the pro-αC ELISA is detecting all forms of the monomeric αC subunit but not the precursor inhibin B forms and that the inhibin B ELISA is detecting all dimeric inhibin B. The two ELISAs are thus separately detecting all the free α subunit and inhibin B in male plasma. It is concluded that a) the Pro-αC and inhibin B ELISAs provide an unambiguous assessment of the levels of these inhibin forms in the circulation and b) the proportion of precursor inhibin B forms in plasma (25-30%) is unchanged in men with sub-fertility.

**119**

## CONTRACEPTIVE EFFICACY OF A DEPOT ANDROGEN AND PROGESTIN COMBINATION IN MEN

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Hormonal male contraception was theoretically feasible for decades with formal proof of concept demonstrated in landmark WHO male contraceptive efficacy studies in early 1990s. Combined testosterone plus progestin treatment is the most promising strategy, but no contraceptive efficacy studies are reported. The superior efficacy plus lesser demands on compliance suggest depot regimens are ideal for hormonal male contraception. We completed the first study estimating the contraceptive efficacy of a depot hormonal regimen, an androgen/progestin combination, in men. Fifty-five men in stable relationships seeking a change in contraceptive method were administered testosterone (four 200 mg implants, 4 or 6 monthly) and a progestin (300 mg DMPA, 3 months). Once sperm output was suppressed (<1M/mL, two consecutive months), men entered a 12 month efficacy period when all other contraception was ceased. Sperm output was monitored monthly. There were no pregnancies in 426 person-months (35.5 person-years) of efficacy exposure (upper 95% CL for contraceptive failure rate, 8% per annum). Mean sperm density fell rapidly (by 88% @1 month and 98% @2 months) allowing nearly all men to enter efficacy within 3 months (50% @ 1 month, 83% @ 2 months, 94% @ 3 months). Only 2/55 (3.6%) men were unable to enter efficacy due to insufficient suppression of sperm output. A few men treated with T implants at 6 month intervals demonstrated androgen deficiency symptoms &/or escape of spermatogenic suppression (predicted by suboptimal gonadotropin suppression) between months 5-6; men receiving T implants at 4 months intervals had no androgen deficiency nor loss of gonadotropin and sperm output suppression. Recovery was slower than anticipated (median time 6-9 months to sperm density 20M/mL) presumably due to DMPA kinetics. Discontinuations were for protocol (12), personal (10) and medical (5) reasons but there were no serious adverse effects related to drug exposure. We conclude that the first prototype depot androgen/progestin combination regimen provides high contraceptive efficacy with satisfactory short-term safety but slow recovery of spermatogenesis. Larger studies with purpose-developed depot combinations are required to clarify the overall safety and efficacy of the most promising approach to hormonal male contraception. This study was supported by CONRAD

**120**

## PRESENCE OF IMMUNE MODULATING MOLECULES IN BOAR SEMINAL PLASMA

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Factors in seminal plasma are recognised as potential modulators of reproductive success in the pig. Experiments in rodents and more recently in pigs indicate roles for specific proteins in seminal plasma in inducing cellular and molecular changes in the female reproductive tract. An active constituent of murine seminal plasma has been

identified as the potent immune modulating cytokine transforming growth factor beta (TGFβ)1. In vitro experiments suggest the response to TGFβ is further influenced by interferon (IFN)γ and lipoplysaccharide (LPS). To investigate whether immune modulating molecules are present in pig semen, the content of TGFβ1, TGFβ2, IFNγ and LPS in boar seminal plasma were measured. Semen was collected from 42 Large White boars of similar age and of known fertility, and seminal plasma was prepared by centrifugation. Commercial ELISA assays were used to measure TGFβ1, TGFβ2 (both Promega) and IFNγ (Endogen) and LPS was measured by Limulus Amebocyte Lysate assay (Bio Whittaker). TGFβ1 and TGFβ2 were detected in all samples [median (range) = 185 (91-423) and 50.1 (26-101) ng/ml respectively], IFNγ was detectable in only 2 samples (39 & 40 pg/ml) and endotoxin was present in 30 samples [12.5 (11–193) EU/ml]. Variation over time and effect of frequency of collection on TGFβ content was also evaluated. Content of both isoforms varied <18 % within individual boars over 4 months (n = 42 boars, 4-8 collections). When semen was collected 3 times within a week (n = 3 boars), TGFβ content was diminished by up to 60% by the third collection. This data demonstrates that cytokines, in particular TGFβ are present in large quantities in boar seminal plasma and that the level of this cytokine varies between boars. Cytokine content remains relatively constant within an animal over time but decreases in response to frequent collection of semen. These findings show that immune modulating moieties, with the potential to cause beneficial type 2 skewing of the maternal immune response in early pregnancy, might be the active fertility modulating constituents of seminal plasma. This information might provide the basis for development of prognostic assays for the evaluation of fertility in pigs. Supported in part by the Australian Pork Limited.

1. Tremellen KP, Seamark RF and Robertson SA: Biol. of Reprod. 58 (5) 1217-1225 (1998).

**121**

## INFLUENCE OF OXYTOCIN ON OXYTOCIN RECEPTOR AND 5 α-REDUCTASE TRANSCRIPTION IN THE RAT PROSTATE

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Dihydrotestosterone (DHT) is essential for normal function and growth of the prostate. DHT is produced by the reduction of testosterone, catalysed by 5 α-reductase. Two isoforms of the 5 α-reductase enzyme exist. In the rat prostate type I is expressed in the epithelium and type II in stromal cells. Oxytocin increases the activity of this enzyme but it is unclear how. Neither expression nor distribution of oxytocin receptor have previously been described in the rat prostate. This study aimed to determine the distribution of oxytocin receptor, and to investigate if oxytocin influences expression of its receptor and the isoforms of 5 α-reductase.

Adult male Wistar rats were treated daily with either 5 μg/Kg oxytocin, 2 μg/Kg of a specific oxytocin antagonist (OTA), both oxytocin and OTA, or saline (control) respectively for 3 days. On day 4 rats were euthanased by $CO_2$ inhalation and the ventral prostate removed. Western blot and immunohistochemistry, employing a polyclonal antibody to the 3$^{rd}$ intracellular loop of the oxytocin receptor, identified a specific peptide of ~60 kDa and localised oxytocin receptor to both the stromal tissue and epithelium. Total RNA was used in a semi-quantitative RT-PCR approach to estimate relative levels of expression of 5 α- reductase type I and II, and oxytocin receptor with levels normalised against a β-actin amplicon as internal control. Oxytocin treatment significantly decreased ($P<0.05$) oxytocin receptor mRNA to 50% that of control. In contrast, oxytocin treament significantly increased ($P<0.001$) 5 α-reductase II expression by 44%. Both changes were inhibited by OTA, indicating a specific action. No significant difference was found in levels of 5 α-reductase I expression between treatment groups.

In conclusion, the localisation of the oxytocin receptor would potentially allow oxytocin to affect both isoforms of 5 α-reductase. The increase in only type 2 expression suggests that activity of both enzymes are regulated differently by oxytocin. Furthermore, downregulation of oxytocin receptor by its ligand provides another site of control.

## 122

### THE IDENTIFICATION AND EXPRESSION PATTERN OF A GHRELIN VARIANT THAT ENCODES A NOVEL PEPTIDE IN THE HUMAN AND MOUSE

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Ghrelin is a novel growth hormone (GH)-releasing peptide consisting of 28 amino acids with an n-octanoyl modification at the third residue serine, which is essential for its biological activity. Ghrelin's actions are mediated via growth hormone secretagogue receptors (GHS-R) which are expressed in peripheral, hormone-responsive tissues including breast and prostate. Ghrelin is encoded by the ghrelin gene which consists of 4 exons in humans, and 5 exons in the mouse. We report for the first time the identification of a new ghrelin mRNA variant in human cell lines and tissues and mouse tissues that has a complete deletion of exon 3. The deletion of exon 3 in the human and mouse ghrelin cDNA results in a frameshift and the generation of unique peptide sequences. Expression of this exon 3-deleted ghrelin variant was analysed at the mRNA level by RT-PCR and at the protein level by immunohistochemistry. The prostate cancer cell lines ALVA41, DU-145, LNCaP and PC3 expressed mRNA for both wild-type ghrelin and the exon 3-deleted variant, as did normal prostate tissue. Breast cancer cell lines MCF-7, MD-AMB-231 and T47D express ghrelin mRNA transcripts and the exon 3-deleted variant, however, only the variant mRNA was detected in normal breast tissue. RT-PCR using primers specific for mouse ghrelin demonstrated the expression of the wild type and exon 3-deleted ghrelin mRNA variants of ghrelin in mouse tissues and embryos at different stages of embryogenesis. Immunohistochemistry, employing an antibody generated against the putative unique human peptide sequence encoded by the exon 3-deleted cDNA, demonstrated the expression of the variant in normal and malignant prostate glands. Staining in the cancer glands was greater than in the normal prostate tissue. Expression was also demonstrated in the glands and stroma of breast cancer tissues, with the most intense staining shown in high-grade carcinoma specimens. Normal breast sections displayed weak glandular staining and no stromal staining. In conclusion, we report the identification of a novel human and mouse ghrelin variant which is expressed in normal and malignant tissues. This ghrelin variant may be involved in an autocrine/paracrine pathway capable of stimulating growth in these tissues, and may represent a potential marker for prostate and breast cancer.

## 123

### OESTROGEN AND SELECTIVE OESTROGEN RECEPTOR MODULATORS EXERT DIFFERENTIAL EFFECTS ON GROWTH HORMONE SIGNALLING

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Growth hormone (GH) acts by binding to specific receptors (GHRs) and activating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. We have previously demonstrated that oestrogen inhibits the GH/JAK2/STAT signalling cascade by suppressing JAK2 phosphorylation. Whether GH signalling is affected by other oestrogenic compounds such as selective oestrogen receptor modulators (SERMs) has not been studied. SERMs, exemplified by tamoxifen and raloxifene, are a group of synthetic ligands for oestrogen receptors (ERs), which differ from classical oestrogens by exhibiting tissue-specific oestrogen-agonistic and antagonistic actions. We compared the effects of 17β-oestradiol ($E_2$), 4-hydroxytamoxifen (4HT; an active form of tamoxifen) and raloxifene (Ral) on GH activation of the JAK2/STAT5 pathway in human embryonic kidney 293 cells stably expressing human GHR (293GHR) and in ER-negative human breast cancer cells (MDA-MB-231). The cells were transiently transfected with an expression plasmid for human ERα and a luciferase reporter containing the STAT5 binding element. MDA-MB-231 cells were co-transfected with a GHR expression plasmid. The cells were then treated with 500ng/ml GH and with $E_2$ or SERMs (1, 10, 100nM) for 6h before measuring luciferase activity.

GH alone increased the reporter activity by 6-8 fold. Co-treatment with $E_2$ resulted in a dose-dependent reduction of the activity in both 293GHR and MDA-MB-231 cells, to a maximum of 56±1% ($P$=0.0005) and 61±6% of control ($P$<0.0001) at 100nM, respectively. In contrast, 4HT and Ral increased the transcriptional activity of GH in 293GHR cells maximally by 88±11% ($P$<0.0005) and 70±18% ($P$<0.01), respectively, but did not affect the GH activity in MDA-MB-231 cells. The enhancing effects of 4HT and Ral were completely attenuated by co-

treatment with the anti-oestrogen ICI182780, revealing the specificity of ER in mediating the SERM action. We next studied the effects of $E_2$ and SERMs on GH-induced JAK2 phosphorylation in 293GHR cells by Western blotting. The level of phosphorylation was reduced to 60% of control by $E_2$ but increased to 200% by 4HT and Ral. In summary, we provided the first evidence that SERMs enhanced the transcriptional activity of GH by promoting JAK2 phosphorylation, an effect opposite to that of oestrogen. This effect of SERMs may be cell-type dependent. (Supported by the NHMRC of Australia and Eli Lilly Australia)

**124**

## INSULIN-LIKE GROWTH FACTOR-I PREVENTS APOPTOSIS IN GLUCOSE DEPLETED NEURONAL CELLS.

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Glucose is the major source of energy for neurons that undergo cell death when exposed to glucopenic insults. Decreased glucose availability is particularly damaging to the developing brain. IGF-I is able to protect neuronal cells from apoptosis, however whether IGF-I can protect neurons from glucopenic stress is not known.

Two related human neuroblastoma cell lines were used: (i) the *SK-N*-SH-SY5Y cells, (ii) the *SK-N*-SHEP cells with five fold fewer IGF-IR than the SY5Y, and no detectable IGFs, (iii) SHEP cells transfected with *bcl-2* or *IGF-IR* genes. Both express glucose transporter GLUT1. Cells were cultured for up to 72h in serum free media containing glucose at 0-25mM, +/- IGF-I at 25ng/ml. In SY5Y and SHEP cells low glucose (0-2.5mM) reduced cell number, mitochondrial activity and survival. In low glucose IGF-I increased cell number, mitochondrial activity and inhibited apoptosis in SH-SY5Y cells, while IGF-I had minimal effect in the SHEP cells. Responses in SHEP-IGF-IR cells were comparable to those seen in the SY5Y cells. Over-expression of bcl-2 (SHEP-bcl-2 cells) was sufficient to maintain cell number, mitochondrial activity and inhibit apoptosis. Following 6 hours of glucopenic stress GLUT1mRNA was increased in both SY5Y and SHEP and further increased by IGF-I. At 24h (IGF-I 25ng/ml) GLUT1 expression was up-regulated dose responsively by glucose in the SY5Y cells and inversely by glucose in the SHEP cells. GLUT1 mRNA was increased by low glucose in both the SHEP/bcl-2 and SHEP-IGF-IR cells with no further potentiation by IGF-I. The PI-3K inhibitors LY294002 and Wortmannin blunted the IGF-I induced increase of GLUT1 mRNA. Deoxy-D-14C-glucose uptake was increased by 15mins in low glucose and further potentiated by IGF-I suggesting possible IGF-I regulation of GLUT1 translocation.

Gene-array showed that: i) antiapoptotic bcl-2, bcl-x mRNAs levels were increased (~50-70%) in low glucose and further increased by IGF-I. ii) proapoptotic caspase-10, -1, -3, Fas/Apo1 and Trail mRNAs levels were increased (20-500%) in low glucose but were inhibited by IGF-I. Our findings indicate a potential role for IGF-I in protecting neuronal cells exoposed to low glucose. Likely mechanisms involve both up-regulation of intracellular glucose transportation and induction of antiapoptotic genes. Thus *in vitro,* IGF-I efficiently protects neurons from glucose deprivation suggesting that IGF-I might have *in vivo* applications in the prevention of hypoglycaemic brain damage.

**125**

## UROKINASE TYPE PLASMINOGEN ACTIVATOR RECEPTOR (UPAR) IS INVOLVED IN INSULIN-LIKE GROWTH FACTOR I AND II-INDUCED MIGRATION OF RHABDOMYOSARCOMA (RMS) CELLS IN VITRO

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Urokinase-type plasminogen activator (uPA), a serine proteinase, becomes activated when bound to its receptor, uPAR. uPA, in turn, activates plasmin, which cleaves extracellular matrix proteins, facilitating cell migration. Plasminogen activator inhibitor type 1 (PAI-1) inhibits uPA proteolytic activity and competes with uPA/uPAR for binding to vitronectin. Thus uPAR enhances cell migration via proteolytic and non-proteolytic mechanisms. Rhabdomyosarcomas (RMS) are tumours thought to arise from skeletal muscle precursors in young people. RMS cell lines such as RD secrete high levels of insulin-like growth factor II (IGF-II), suggesting autocrine IGFs play

a major role in the proliferation and metastasis of RMS. The aim of these studies was to study RMS migration in response to IGF-I and IGF-II with regard to the uPA/uPAR/plasmin system. In a wound migration assay, IGF-II (200ng/mL) and IGF-I (200ng/mL) increased migration of RD cells to 124±9% ($P<0.01$) and 131±8% ($P<0.05$) of control, respectively. IGF-II-induced migration was inhibited by insulin-like growth factor binding protein-6 (IGFBP-6) and PAI-1 to 88±6 % ($P<0.01$) and 98±5% of control ($P<0.05$), respectively. Aprotinin, a plasmin inhibitor, and mannosamine, an inhibitor of uPAR, decreased IGF-II- but not IGF-I-induced migration to 93±3% ($P<0.05$ vs IGF-II) and 113±5% ($P< 0.05$ vs IGF-II) of control, respectively. An anti-uPAR antibody blocked IGF-II-induced migration to 105±2% of control ($P<0.05$). [$Arg^{54}$, $Arg^{55}$]IGF-II (200ng/mL) and [$Leu^{27}$]IGF-II (200ng/mL), which preferentially bind the type I and II IGF receptors respectively, upregulated RD cell migration to 146±8% ($P<0.01$) and 120±7% ($P<0.05$) of control, respectively. This implicated both type I and II IGF receptors in IGF-II-mediated RD migration. IGF-II (50ng/mL) and IGF-1 (10ng/mL) also maximally increased specific $^{125}$I-uPA binding to RD cells in a dose-dependent manner to 166±20% ($P<0.01$) and 174±33% of control ($P<0.01$), respectively, but did not alter uPAR mRNA levels. Increased $^{125}$I-uPA binding and inhibition of IGF-II-induced cell migration by inhibitors of uPA, uPAR and plasmin suggest uPAR is acting in a proteolytic manner. In contrast, the plasmin-independent effect of IGF-I suggests uPAR is acting in a non-proteolytic manner. These results implicate the uPA/uPAR/plasmin system during IGF-stimulated RMS migration which may contribute to metastasis.

**126**

## FGF-2 PROMOTES DIFFERENTIATION OF NEUROBLASTOMA CELLS BY INHIBITION OF IGF MITOGENIC ACTIVITY AND MODULATION OF THE APOPTOTIC SIGNALING PATHWAYS.

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A better understanding of the mechanisms involved in neuronal differentiation would potentially be of great clinical benefit to patients affected by brain injury,neuro-degenerative diseases and neoplasms including neuroblastomas. A number of growth factors, including FGF-2 and IGF-I, are known to influence the differentiation of neuronal cells.

The aim of this study was to investigate some of the mechanisms by which the interactions of two potent neurogenic systems, FGF-2 and IGF, promote neuronal differentiation.

The model system was the neuroblastoma cell line SK-N-MC, which possesses a complete IGF system and responds to FGF-2. Cells were cultured for up to 72 h in SFM +/- IGF-I (25-100 ng/ml) or FGF-2 (25-50 ng/ml) alone or in combinations. The IGF-I analogues des(1-3)IGF-I and QYAL IGF-I (25 ng /ml) (reduced IGFBPs affinity) were also used. End-points determined were: IGF system profile, cell proliferation (MTT assays), cell differentiation (neuronal markers, TUJ-1, NEU-N), apoptosis (ELISA, gene-array).

SK-N-MC cells treated with FGF-2 over 72h underwent a process of neuronal differentiation (TUJ-1 & NEU-N +ve at 48-72h) and showed decreased proliferation, even in the presence of IGF-I. In addition to IGFBP-2 proteolysis and increased IGFBP-3 level, IGFBP-6 CM levels were also increased. Whether alteration of the IGFBP profile might affect IGF-I responses remains unclear. The IGF-I analogues alone potently stimulated cell proliferation (0.5-2 fold above IGF-I), however their activity was ablated in the presence of FGF-2. Following FGF-2 alone or FGF/IGF treatments these cells underwent apoptosis due to an increased expression (0.5-4 fold) of genes in pro-apoptotic pathways (p53, caspases, Fas/Trail/TNF families). IGF-I alone was strongly anti-apoptotic (bcl-2 up 2-3 fold), however, IGF-I failed to protect SK-N-MC cells from apoptosis when combined with FGF-2.

Neuroblastoma is a paediatric tumour which is largely insensitive to conventional therapy and its growth is supported by an IGF autocrine loop. Our findings thus demonstrate that FGF-2 overcomes the proliferative/anti-apoptotic effects of IGF-I in neuroblastoma tumour derived cells and potentially induces differentiation/apoptosis of these cells in a clinical setting.

## 127

### TNFα AND IGF SYSTEM CROSS-TALK IN A HUMAN KERATINOCYTE CELL LINE: IMPLICATIONS FOR EPIDERMAL HOMEOSTASIS.

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Tumour necrosis factor-α (TNFα) and the insulin-like growth factor (IGF) system regulate skin homeostasis, particularly the epidermis. Absence of IGF-I receptors (IGF-IR) results in a thin, disorganised epidermis while IGF-IR activation stimulates keratinocyte cell proliferation, migration and cell survival. A reduction of IGF-IRs, via antisense oligonucleotides, normalises the hyperplastic epidermis and thus confirms its role in the aetiology of psoriatic lesions. In normal skin, IGF binding protein-3 (IGFBP-3) is expressed by specific basal keratinocytes but its distribution is severely altered in psoriatic skin. IGFBP-3 is reduced upon keratinocyte differentiation and modulates IGF-stimulated proliferation. Hence IGFBP-3 may also modulate epidermal morphology. TNFα is over-expressed in skin inflammation, particularly psoriasis, but its role in the hyperplastic epidermis is unknown. To examine the potential epidermal interaction between TNFα and the IGF system, we utilised the human keratinocyte cell line, HaCaT, to assess the effects of TNFα, +/- IGF-I, on cell proliferation and modulation of IGFBP-3 and IGF-IR.

Keratinocytes were exposed to basal medium (KBM) plus IGF-I (0-100ng/ml), TNFα (0-100ng/ml) or IGF-I (0-100ng/ml) +/- TNFα (0.1, 1, 5, or 50ng/ml). Proliferation was determined by XTT assay. IGFBP-3 abundance in conditioned medium (CM) was assessed by Western ligand blot (WLB) or dot blot (DB). IGFBP-3 and IGF-IR mRNA abundance was assessed by real time PCR.

When compared with KBM, IGF-I (5, 10, 50, 100ng/ml) stimulated and TNFα (50, 100ng/ml) inhibited cell growth. TNFα at 50 ng/ml inhibited the IGF-I proliferative response at all IGF-I concentrations. In contrast, at 5 ng/ml TNFα only 5 or 10 ng/ml of IGF-I-induced proliferation was inhibited. WLB and DB revealed that neither IGF-I nor TNFα significantly altered IGFBP-3 abundance when compared with KBM. In comparison, TNFα (0.1, 1, 5 or 50ng/ml) plus IGF-I (0.1, 1, 5, 10, 50, 100ng/ml) reduced IGFBP-3 abundance. TNFα (50ng/ml) alone altered IGF-IR mRNA abundance (increased 50%) when compared with KBM.

In conclusion, these data confirm cross-talk and opposing actions of TNFα and IGF-I in keratinocyte proliferation. Furthermore, reduced IGFBP-3 and elevated IGF-IRs induced by TNFα, +/- IGF-I, may compensate for the overall inhibitory actions of TNFα. Finally, the modulation of IGFBP-3 and IGF-IR abundance by TNFα +/- IGF-I may relate to their altered distribution in psoriatic skin.

## 128

### SERUM INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-5 IS RE-DISTRIBUTED IN HUMAN DIABETES MELLITUS.

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Human serum IGFBP-5 exists in binary complexes when it is bound to IGF-I or IGF-II, and in high molecular weight ternary complexes, when it is also bound to a third protein, termed ALS. Whilst binary complexes are tissue bioavailable, ternary complexes are thought to be restricted to the circulation. In healthy human serum, IGFBP-5 exists mainly in ternary complexes, possibly acting as a circulating reservoir of IGFs. IGFBP-5 levels in diabetic serum have previously been reported to be reduced compared with levels in healthy controls. We hypothesised that a decrease in the amount of IGFBP-5 in diabetic serum exists due to a relative shift of IGFBP-5 from ternary complexes to tissue bioavailable complexes of lower molecular weight in diabetic patients, secondary to increased protease activity towards IGFBP-5 in diabetic serum.

The amount of IGFBP-5 in each serum sample was determined by an in-house IGFBP-5 RIA. The cross-sectional samples analysed included 30 healthy controls, 40 samples from subjects with type 2 diabetes, and 40 subjects with type 1 diabetes. Serum IGFBP-5 levels were decreased (median; [25th-75th centile]) in both type 1 (88; [72-

138] ng/mL) and type 2 diabetes (92; [64-141] ng/mL), compared with non-diabetic controls (205; [179-236] ng/mL); each P<0.01 vs controls. Size fractionation of pooled serum samples was then performed by FPLC using a Superose-12-column, and column fractions were analysed by IGFBP-5 RIA. A size-shift was detected in the molecular distribution of IGFBP-5 in type 1 and type 2 diabetes, away from ternary complexes, and in contrast to healthy control serum, the predominant peak of IGFBP-5 immunoreactivity in diabetes was present in low molecular weight complexes (P<0.001 for both fractionation curves vs control serum). To then determine serum protease activity towards IGFBP-5, 1µg rhIGFBP-5 was co-incubated with control and diabetic serum (each 125 µL, diluted 1:2) for various time intervals (0-24h), and sub-aliquots of each mixture were then analysed by IGFBP-5 Western Immunoblot. The rate of protease activity towards rhIGFBP-5 was increased in diabetic serum in subjects with type 1 diabetes, and to a lesser extent, in type 2 diabetes, in comparison with healthy control serum.

These results show that serum IGFBP-5 is size-shifted to lower molecular weight in diabetes, which may be secondary to increased protease activity towards IGFBP-5 in diabetic serum. This may cause IGFs bound to IGFBP-5 to be more tissue bioavailable in diabetes than in non-diabetic subjects.

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**129**

## THE MORBIDITY AND MORTALITY IN GH-DEFICIENT ADULTS: EFFECTS OF LONG-TERM GH REPLACEMENT THERAPY

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Several studies suggest that overall and cardiovascular mortality are increased in adult patients with hypopituitarism (1-5). As GH replacement therapy improves several of the cardiovascular risk factors identified in this patients group untreated GH deficiency has been suggested to be the link to the above observations. The present study consisted of three studies. First, a retrospective comparison was performed between 1411 hypopituitary adults and the normal population in terms of cardiovascular disease and malignancy. In the second study, the rates of cardiovascular disease and malignancy were determined prospectively in 289 hypopituitary patients receiving long-term GH replacement therapy (1443 patient years) in one treatment centre and compared with that in the normal population. In the third study, overall mortality in the 289 patients on GH replacement therapy was compared with that in the hypopituitary patients without GH replacement previously reported (1).

In the 1411 hypopituitary patients without GH replacement therapy, the incidence of stroke and malignancy was increased in both sexes, and the incidence of myocardial infarction was increased in women. In the 289 hypopituitary patients on GH replacement therapy, cardiovascular morbidity and mortality as well as the incidence of malignancy were similar as that in the normal population. The relative risk for overall mortality in the 289 patients, based on previously reported data in hypopituitary patients without GH treatment, was 0.51 (95% confidence interval 0.23-1.14; p=NS).

In conclusion, in the retrospective analysis cardiovascular morbidity and mortality was increased in hypopituitary adults without GH replacement therapy. In the prospective analysis, hypopituitary adults on long-term GH replacement therapy, cardiovascular morbidity and mortality was similar as in the normal population.

1. Rosén T, Bengtsson B-Å. Premature mortality due to cardiovascular diseases in hypopituitarism. Lancet. 1990;336:285-288.

2. Bates AS, et al. The effect of hypopituitarism on life expectancy. J Clin Endocrinol Metab. 1996;81:1169-1172.

3. Bülow B, et al. Increased cerebrovascular mortality in patients with hypopituitarism. Clin Endocrinol (Oxf). 1997;46:75-81.

4. Bates AS, et al. Life expectancy following surgery for pituitary tumours. Clin Endocrinol (Oxf). 1999;50:315-319.

5. Tomlinson JW, et al. Association between premature mortality and hypopituitarism. West Midlands Prospective Hypopituitary Study Group. Lancet. 2001;357:425-431.

## 130

### USE OF LONG ACTING SOMATOSTATIN IN ACROMEGALY: AN AUDIT

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OBJECTIVE: To describe our experience with a long acting somatostatin analogue (Sandostatin-LAR®) for the treatment of Acromegaly

DESIGN AND METHODS: A retrospective review of all individuals treated with a long acting somatostatin analogue at a tertiary referral centre.

RESULTS: 41 patients received treatment between January 2000 to March 2002. The mean age at diagnosis was 43 years (22-68 years). 61% were male. At diagnosis, there were 27 cases of macroadenoma and 10 cases of microadenoma. The size of the tumour was unknown in 3 cases and there was one case of an ectopic growth hormone secreting tumour. The mean duration between the commencement of Sandostatin-LAR® and diagnosis was 7 years. The mean age at commencement of treatment was 50 years. 83% had previously undergone surgery. All (34%) individuals that had radiotherapy also had surgery. 7 patients (17 %) had long acting somatostatin analogue as their primary treatment (3 macroadenomas, 2 microadenomas and 2 unknown).

At the commencement of treatment, the mean IGF-1 level was 55 ± SD 30 nmol/L (14-145nmol/L). The mean dose of Sandostatin-LAR® was 22 ± SD 8mg per month (5-50mg). 56% and 25% of individuals were having 20mg/month and 30 mg/month doses respectively. The mean duration of follow up was 18 months (range 1-30 months). 68% of the patients achieved age-adjusted normalisation of IGF-1 (28 ± 6nmol/L in this group). In a subgroup analysis, Sandostatin LAR® treatment achieved normal age-adjusted IGF-1 levels in 11/20 patients with previous surgery alone, 12/14 patients with previous surgery and radiotherapy, and 5/7 patients who had medical therapy as their only treatment.

CONCLUSION: Long acting somatostatin is an effective means of normalising IGF-1 levels in active acromegaly and can be effective as primary therapy.

## 131

### LONG-TERM EFFECTS OF ANDROGEN REPLACEMENT THERAPY ON BONE MINERAL DENSITY (BMD) IN MEN

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Androgen deficiency decreases BMD in men; however, the long-term effects of androgen replacement therapy on BMD are not well described. In this study we have evaluated prospectively collected BMD among 154 men, aged 17-79 years, with established androgen deficiency requiring regular androgen replacement therapy, mostly using testosterone implants. At first BMD observation, most (85%) were untreated or inadequately treated and they underwent further BMD observations at 2-3 year intervals with 50 having two, and 16 having three BMD measurements, up to a maximum follow-up of 10 years. BMD (g/cm2 and t and z scores) was measured for lumbar (L1-L4) and total hip using dual energy x-ray absorptiometry (DEXA). BMD at lumbar vertebra increased significantly (1.76% per year at L2) with most of the benefit being seen at the first follow-up visit (mean 2.8 years after 1st BMD). There was minimal or no significant improvement in hip BMD. Further follow up is required to determine whether long-term testosterone replacement therapy increases BMD at the hip and whether these site-specific changes in BMD due to androgen deficiency and replacement are reflected in fracture rates.

## 132

### ACTIVATING AND INACTIVATING MUTATIONS IN THE CALCIUM SENSING RECEPTOR ASSOCIATED WITH DISORDERS OF CALCIUM HOMEOSTASIS

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The calcium-sensing receptor (CaR), a G-protein coupled receptor, plays an integral role in regulating extracellular calcium concentrations. The activation of the human CaR induces a signalling pathway that results in a decrease in parathyroid hormone secretion in parathyroid cells and inhibits the reabsorption of Ca2+ in kidneys. There are three distinct inherited disorders associated with mutations in the CaR. Autosomal dominant hypocalcaemia (ADH) and familial hypocalciuric hypercalcaemia (FHH) are benign disorders caused by activating and inactivating mutations of the receptor, respectively. The third disorder, neonatal severe hyperparathyroidism (NSHPT), occurs when there is an inactivating mutation in each CaR allele. NSHPT can be life threatening without parathyroidectomy.

A key aim of our research program is to identify and functionally characterize CaR mutations associated with these disorders. DNA sequence analysis of the CaR of probands biochemically diagnosed with CaR associated disorders led to the detection of several novel single point mutations in the CaR. Mutations detected in patients diagnosed with FHH include those we have previously reported, L174R, E250K and R648Stop, as well as recently identified mutations, R25Stop, K323Stop and G778D. The mutations R25Stop, L174R, E250K and K323Stop are all located in the extracellular domain of the receptor. The R648Stop mutation is located in the first intracellular loop, while the G778D mutation is located in the fifth transmembrane domain. In a case of NSHPT, we reported compound heterozygous truncation mutations, R648Stop and G94Stop. A point mutation, V836I, located just prior to the seventh transmembrane domain was found in a patient diagnosed with ADH. Mutations cosegregate with disease status in all ADH and FHH families and were shown to not be common polymorphisms. In order to confirm biochemical phenotype, these mutations were introduced by site directed mutagenesis into the pcDNA1 recombinant vector for wild-type human CaR tagged with the FLAG epitope. The CaR mutant constructs were transfected into HEK293 cells and examined for expression by Western analysis. An assay measuring phosphoinositide hydrolysis, a signalling event downstream of CaR activation, is currently being used to determine the functional effects of each mutation on the CaR.

## 133

### HYPOVITAMINOSIS D - A FORGOTTEN EPIDEMIC IN THE ELDERLY WITH OSTEOPOROTIC FRAGILITY FRACTURES

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Hypovitaminosis D is a common problem in the elderly. Advancing age is associated with decreased sun exposure, intake and skin activation of vitamin D, and decreased vitamin D absorption. All these factors may cause low vitamin D levels. Hypovitaminosis D worsens osteoporosis and is associated with a proximal myopathy affecting antigravity muscles, which increases fall risk and makes post fracture rehabilitation more difficult. In a metropolitan general hospital setting we have retrospectively examined medical records for the period of year 2001 for all patients >64 years of age admitted with fractures. Out of 941 such patients after excluding traumatic fractures, fractures due to bony metastasis and Pagets Disease, patients with renal failure and patients who died during admission, 424 were selected as they have had minimal trauma or spontaneous fractures due to osteoporosis. Incidence and severity of Hypovitaminosis D and frequency of initiation of Vitamin D supplementation during hospital stay were examined for these patients. Mean age of patients was 80.1 years. Of them on admission 62% were community dwelling, 17%were from hostels or independent living units and 22% were nursing home residents. Out of these patients 8.5% were on some form of Vitamin D supplementation. Of

patients who were not on Vitamin D supplementation 16% were screened for Vitamin D deficiency during the admission. Vitamin D insufficiency (<50nmol/L) was found in 80%, with 58% of patients having Vitamin D levels <30nmol/L. Among those who were screened for Hypovitaminosis D, 36% were also screened for evidence of secondary hyperparathyroidism with PTH levels. Of these, 35% had Secondary Hyperparathyroidism. Vitamin D supplementation was initiated during the hospital stay in 12%. We conclude that Vitamin D insufficiency is common in elderly patients with osteoporotic fractures, and it is grossly under recognized and under treated.

## 134

### SEVERE VITAMIN D DEFICIENCY IN YOUNGER ADULTS - EFFECTS ON THE SPINE.

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We report 3 non-smoking adults aged 46-49 yr in whom profound vitamin D deficiency occurred due to lack of sunlight exposure. Two cases had undergone lumbosacral fusion with pedicle screws and segmental fixation plates but fusion was unsuccessful and the internal fixation devices were removed. In case 1, recurrence of severe pain 1 month after surgery necessitated exploration with findings that the screws were loose and the bone soft. He had deeply pigmented skin (Indian ethnicity), followed a vegetarian diet and had worked night-shift for 10 yr. His 25OHVitD was <12nmol/L (N 35-155), ALP 179U/L (N<120) and GGT 195U/L (N<65) whilst taking carbamazepine. Case 2, a white woman with persisting pain 2 yr after instrumented lumbosacral fusion underwent removal of screws and plates. Flexion/extension lumbosacral spine xrays indicated significant movement. Bone biopsy showed large areas of woven bone. Her 25OHVitD was <12nmol/L and ALP 79 U/L. She confirmed active avoidance of sunlight throughout adult life. Swabs in both cases 1 and 2 were sterile. Vitamin D repletion resulted in symptomatic improvement in both cases and imaging demonstrated sound fusion and new bone formation 6-12 months later. Case 3, a white woman had undergone lumbar laminectomy for low back pain 10 yr earlier. Due to persistence of pain, she became depressed and increasingly housebound. She presented with acute severe sacral pain on rising from bed. MRI revealed a sacral insufficiency fracture. Bone densitometry T-scores were –1.85 (femoral neck), -2.02 (Ward's triangle), -0.96 (trochanter) and –2.17 (L1-3). 25OHVitD was 11 nmol/L, Ca 2.06 mmol/L, albumin 42g/L and urine Ca 1.4 mmol/24hr. ALP was 142U/L and fell to 65 after treatment with ergocalciferol and active sunlight exposure for 3 months, with resolution of sacral pain. No other factor predisposing to osteopenia or osteomalacia was identified in any of these patients. These cases highlight the occurrence of severe vitamin D deficiency in Australian adults <50 years of age. It may have significant consequences including failure of spinal fusion surgery, osteoporosis and fracture and should be considered in all such cases.

## 135

### SEX-SPECIFIC CHANGES IN PLACENTAL FUNCTION AND FETAL GROWTH AND DEVELOPMENT IN PREGNANCIES COMPLICATED BY ASTHMA

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Asthma is associated with fetal growth restriction but the mechanisms for this effect are unknown. To determine if asthma or its treatment with glucocorticoids is responsible for the described growth effects the following studies were undertaken:

Pregnant women were prospectively recruited and classified as control (non-asthmatic, n=31), no glucocorticoid (asthmatic, no inhaled steroid use, n=49) and glucocorticoid (asthmatic, inhaled steroid use, n=93). Glucocorticoid intake during pregnancy was recorded and neonatal weight and sex determined at birth. Placental 11β-HSD2 enzyme activity and umbilical vein plasma cortisol and estriol concentrations were measured. Gene expression of placental cytokines was determined using quantitative RT-PCR.

Birth weight of female neonates in the no glucocorticoid group was significantly reduced compared to neonates in the control or glucocorticoid groups (ANOVA, $P$=0.035). Male neonates from all asthmatic groups were of

similar size and not significantly different from the control group. Asthmatic women using moderate or high doses of steroids significantly increased their steroid use as gestation progressed ($P<0.03$), while those pregnant with a male fetus did not, suggesting that maternal asthma may worsen in the presence of a female fetus. 11β-HSD2 activity was significantly reduced in placentae from female neonates of the no glucocorticoid group compared to placentae from control and glucocorticoid female neonates (ANOVA, $P=0.002$). Placentae from all male neonates had lower 11β-HSD2 activity, which was not different between groups. Fetal cortisol was higher, but not significantly increased, in females from the no glucocorticoid group compared to the control group. Fetal estriol concentrations, a marker of fetal adrenal activity, were significantly reduced in females from the no glucocorticoid group (ANOVA, $P=0.02$). Changes in maternal inflammation may contribute to an altered cytokine environment in the placenta which may affect 11β-HSD2 activity. Placentae from women pregnant with females in the no glucocorticoid group had reduced levels of TNF-α, IL-1β, IL-4 and IL-8, but a significantly increased ratio of Th2 type cytokines to Th1 type cytokines (IL-5:TNFα, $P<0.005$).

These data suggest that increases in maternal inflammation may be involved in altering fetal growth in asthmatic women pregnant with female fetuses who are not treated with inhaled steroids, through changes in placental function and the metabolism of cortisol

**136**

## PREVALENCE AND PREDICTORS OF ANDROGEN ABUSE IN AUSTRALIAN HIGH SCHOOLS

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Androgen abuse for cosmetic, recreational, sporting and occupational purposes is increasingly recognized but difficult to detect or monitor due to its illicit nature. Regular high school drug and alcohol surveys provide a convenient opportunity to survey secondary students as a representation of the general adolescent population. Large-scale surveys using stratified random sampling of secondary schools were conducted initially in NSW and Victoria in 1992 and nationally in 1996 and 1999 using standardised methodology including questions on androgen ("anabolic steroid") abuse. This study aimed to compare the findings from the 1996 and 1999 surveys with published findings from the 1992 survey1 to identify trends in prevalence or predictors of androgen abuse. In 1996 and 1999 a random sampling of high schools from all Australian states, stratified by education sectors (government, Catholic, independent) were studied aiming to select students in years 7-12 (80 per school) to complete an anonymous questionnaire using validated questions on drug and alcohol use and related variables. The surveys involved 29,700 (1996) and 25,486 (1999) students representing 93% of target participation both years. The lifetime ("ever") prevalence of androgen abuse was 2.8% in 1996 and 3.3% in 1999 compared with 3.2% in 1992 among boys and for recent (last month) use the prevalence was 1.6% in 1996 and 1.8% in 1999 compared with 1.7% in 1992. The predictors of lifetime abuse remained stable with males more likely than females (OR = 3.3 (1996) and 2.1 (1999) compared with 2.8 in 1992) and the strongest predictors of lifetime androgen abuse remained 'truancy or unsupervised recreation' with 9.9% in 1992 (OR = 8.4), 5.4% in 1996 (OR = 5.7) and 9.6% in 1999 (OR = 7.3). Another predictor in the 1996 and 1999 surveys was 'perceived school ability by the student' in which both high and low self-perceived performance was associated with greater 'recent' use (low ability: 12.2% OR = 12.7; CI 8.2-19.3 in 1999; 11.2% OR = 16.5 CI 10.5-25.2 in 1996; high ability: 3.1% OR = 3.0 CI 2.0-4.1 in 1999; 2.9% OR = 3.9 CI 2.7-5.5 in 1996). We conclude that the prevalence and predictors of androgen abuse use amongst Australian adolescents have been relatively stable over the last decade.

1 Handelsman & Gupta, International Journal of Andrology, 20:159-164 (1997)

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## 137

### OVARIAN FOLLICULAR DEVELOPMENT IS COMPROMISED IN TRANSGENIC (MREN-2)27 RATS AND ANGIOTENSIN II-INFUSED SPRAGUE DAWLEY RATS

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Angiotensin II (ANG II) is implicated in ovulation, vascularisation and atresia, and may influence these processes by a local ovarian renin-angiotensin system (RAS), which we have previously localised in rat ovarian stroma and its blood vessels. The aim of this study was to determine the role of ANG II in follicular development. Three groups of 12 week old normally cycling, female rats were used: 1) untreated Sprague Dawley (SD), 2) SD infused with ANG II (145 ng/kg/min for 12-14 days), and 3) hypertensive homozygous (HMZ) (mRen-2)27 transgenic rats, which display amplified extra-renal tissue renin and ANG II. Ovaries were collected at proestrus for histological analysis of follicular type and number, immunolocalisation of renin and ANG II, *in situ* hybridisation for AT1a receptor (AT1a-R) mRNA and radioimmunoassay of ovarian renin content.

Systolic blood pressure (SBP; tail cuff) was highest in HMZ Ren-2 followed by SD+ANG II and SD. HMZ Ren-2 and SD+ANG II had more antral follicles than SD, but fewer large antral and pre-ovulatory follicles. Litter size was reduced in HMZ Ren-2 compared to SD. Consistent with the high RAS of the HMZ Ren-2, ovarian renin content and renin and ANG II immunolabelling were increased and AT1a-R mRNA reduced. SD+ANG II exhibited a reduction in ovarian renin content and AT1a-R mRNA gene expression, indicating a negative feedback on the RAS.

| N = 4-6 rats per group. | SD | SD+ANG II | HMZ (mRen-2)27 |
|---|---|---|---|
| SBP (mmHg) | 125±1 | 199±12* | 233±5*† |
| Antral Follicles:150-390µm (% Total) | 37.5±4.5 | 53.5±6.3* | 52.8±2.2* |
| Large Antral Follicles: 390-500µm (%Total) | 7.7±1.8 | 2.4±1.1* | 2.4±0.9* |
| Pre-ovulatory Follicles: >500µm (% Total) | 17.1±3.6 | 3.8±1.5* | 4.5±1.9* |
| Litter Size | 13.7±0.7 | NA | 10.3±0.6* |
| Ovarian Renin Content (mGU/g) | 4.8±0.5 | 2.7±0.3* | 18.8±2.1*† |
| AT1a-R mRNA (Relative Optical Density) | 75.3±1.8 | 64.0±2.4* | 60.8±2.3* |

Values are mean ± sem. *$p<0.05$ compared to SD, †$p<0.05$ compared to SD+ANG II. NA: not analysed.

These findings indicate that ANG II compromises follicular development, by the accumulation of antral follicles and a reduction in large antral and pre-ovulatory follicles. The presence of RAS on stromal blood vessels suggests that follicular development and litter size are influenced by an ovarian RAS, possibly through effects on ovarian vascularisation and local blood flow which are essential for follicular growth, antral formation, enlargement and subsequent ovulation.

## 138

### EFFECT OF SUB-BURSAL FAS ANTIBODY ADMINISTRATION ON FOLLICULAR CASPASE-3 ACTIVATION AND OVARIAN VOLUME

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Fas is a transmembrane receptor complex belonging to the TNF-α superfamily which, when ligated by its naturally produced ligand (FasL), activates downstream caspases resulting in the execution of apoptosis. Studies have recently demonstrated that caspase-3, an apoptotic effector enzyme, is present and active in granulosa cells and oocytes of atretic follicles (1). To investigate the possible involvement of FasL in the initiation of atresia in vivo, an anti-Fas monoclonal antibody was injected beneath the ovarian bursa in peripubertal mice (n=9). Animals were killed and the ovaries removed after 1, 3, and 5, days following surgery. Ovaries were compared with saline treated counterparts (n=8) and examined immunohistochemically for evidence of caspase-3 activity and the volume determined for each ovary using the Cavalieri method. Histological observation revealed that corpora lutea had disappeared in 7/9 anti-Fas treated animals and that cystic structures had developed in 3 ovaries. No significant differences in ovarian volume were found within or between treatment groups. An

increase in the number of atretic antral follilces was apparent, as evidenced by active caspase-3 immunoreactivity in granulosa cells. Also, a larger number of degenerating oocytes from preantral follicles were observed in anti-Fas treated ovaries. Results from this study suggest that the Fas signal is a potent inducer of caspase-3 activity and follicular atresia in vivo, and that the cell type governing the mechanism of atresia could be dependent on the stage of follicle development. Furthermore, overstimulation of the Fas/FasL pathway may lead to aberrant cyst formation in the ovary.

1. Fenwick, M.A. and Hurst, P.R. (2002) Reproduction (submitted)

**139**

## GRANULOSA CELLS MATURE BEFORE DEATH IN BASAL ATRESIA

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Most ovarian follicles undergo atresia, yet this process is still poorly characterised. Recently we described a previously unrecognised type of atresia (1). Within the membrana granulosa cell death first occurs near the basal lamina and progresses antrally (basal atresia), the opposite of that found in classic atresia (antral atresia). Basal atresia is rarely seen in follicles >5 mm in diameter and occurs in approximately 20% of follicles <5 mm. Follicular fluids from atretic small bovine follicles reportedly have elevated progesterone concentrations (2, 3). We investigated the hypothesis that only basal atretic follicles have elevated follicular fluid progesterone.

Antral follicles were harvested from the ovaries (one per cow) of young non-pregnant Bos taurus cows. Follicles (2-5 mm, n = 24, 2 per ovary) were dissected and snap-frozen in OCT compound. Frozen follicles were bisected and one half immersed in 2.5% glutaraldehyde for histological classification. Cholesterol side-chain cleavage cytochrome P450 (SCC) (0/11 follicles examined) and 3β-hydroxysteroid dehydrogenase (3β-HSD) (0/15) were not expressed in the granulosa cells of healthy follicles or antral atretic follicles. On atresia where the basally-located granulosa cells died first, surviving antrally-situated granulosa cells expressed SCC (3/8) and 3β-HSD (8/8) suggesting that these enzymes had been switched on during the process of atresia.

A further 32 ovaries were collected as above. Follicles (3-5 mm) were dissected (n = 111, 1-4 per ovary) and snap frozen. A portion of the follicle wall was immersed in 2.5% glutaraldehyde and the remaining follicle stored at $-70^{o}$C for subsequent collection of follicular fluid. Healthy follicles had either rounded or columnar basal cells (4). Follicular fluids from healthy (rounded basal cells, n = 15; columnar basal cells, n = 13) and atretic (antral, n = 9; basal, n = 15) follicles were measured by RIA. Follicular fluid progesterone was significantly greater (468 ± SEM 49 nmol/ml, $P<0.001$, ANOVA and SNK) in basal atretic follicles compared to antral atretic follicles (96 ± 21) or healthy follicles with either rounded (112 ± 23) or columnar basal cells (137 ± 26).

Thus in basal atretic follicles granulosa cells undergo maturational changes before dying.

1. Reproduction 2001: 122, 761-775

2. Biol Reprod 2001: 65, 726-732

3. J Reprod Fert 1984: 72, 39-53

4. J Reprod Fert 2000: 118, 221-228

**140**

## ESTROGEN PROMOTES ANGIOGENESIS IN ERα-EXPRESSING HUMAN MYOMETRIAL MICROVASCULAR ENDOTHELIAL CELLS BY UPREGULATING VEGF RECEPTOR-2

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Angiogenesis is the growth of new blood vessels from pre-existing vessels and involves proliferation of microvascular endothelial cells (MEC). VEGF is a major promoter of angiogenesis and mediates angiogenic effects primarily through interaction with VEGF receptor-2 (VEGF-R2/KDR) present on the surface of MEC. Estrogen promotes bFGF-induced angiogenesis, but its effect on VEGF-mediated angiogenesis is unknown. We have demonstrated that MEC derived from human myometrium constitutively express estrogen receptor-β (ERβ), while ERα varies between subjects and is only expressed in approximately 60% of MEC isolates (1). The aims of

the present study were to determine whether (a) estrogen increases VEGF-R2 expression in ERα positive and ERα negative myometrial MEC, (b) ER mediates this effect and (c) estrogen promotes VEGF-induced MEC proliferation. Myometrial MEC were isolated from hysterectomy tissue obtained from ovulating women, purified with UEA-1-coated Dynabeads, cultured and used between passages 1-3 (purity >98% CD31+ cells) (1, 2). ERα and ERβ mRNA were determined by RT-PCR (3) and protein by flow cytometry or Western blotting. VEGF-R2 expression was measured by flow cytometry using either VEGF-R2 antibody or biotin-rhVEGF$_{165}$ binding. MEC proliferation was determined by MTS bioassay (2). Estrogen (1 and 10 nM) significantly increased rhVEGF$_{165}$ binding and VEGF-R2 receptor expression in ERα positive ($P<0.05$, n=4) but not in ERα negative MEC samples (n=6). There was a significant association between ERα mRNA and protein expression in myometrial MEC and the ability of estrogen to upregulate VEGF-R2 ($P=0.03$ and $P=0.025$ respectively). ER mediated the effect of estrogen on VEGF-R2 expression, since the ER antagonist ICI 182,780 blocked this effect ($P<0.05$, n=3). Similarly, estrogen augmented VEGF-induced proliferation of ERα-expressing MEC ($P<0.05$, n=5), which was blocked by ICI 182,780 ($P<0.01$, n=5). These observations suggest that estrogen promotes proliferation of human myometrial MEC by increasing VEGF-R2 expression, an effect that varies between subjects and appears to be mediated primarily by ERα.

Gargett CE, Bucak K, Zaitseva M, Chu S, Taylor N, Fuller PJ, Rogers PAW (2002) Molec Hum Reprod 8 (in press)

Gargett CE, Bucak K, Rogers PAW (2000) Hum Reprod 15:293-301

Chu S, Mamers P, Burger HG, Fuller PJ (2000) J Clin Enocrinol Metab 85:1200-1205

**141**

## DIETARY ESTROGENS HAVE SELECTIVE EFFECTS ON THE REPRODUCTIVE AXIS IN ESTROGEN DIFFICIENT ARKO MICE

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Phytoestrogens, or isoflavones such as genistein, are a significant component of many diets including commercial rodent chow that contains soya meal. Estrogen deplete Aromatase knockout (ArKO) mice were used to test the capacity of these sources of estrogen activity to influence components of the female reproductive axis.

Wildtype (Wt), Heterozygote (Het) and ArKO mice (n =7-14/group) were maintained from birth (and in utero) until 16 weeks of age on either (i) standard commercially available pelleted rat chow containing 14% Soya meal (0.146mg/g isoflavones: genistein and diadzein) (Soy plus), (ii) a soya free (Soy minus) rodent chow with undetectable levels of isoflavones, or (iii) a soy minus diet to which we added 250mg/kg of Genistein (Gen). Diets were corrected for amino acids, vitamins and minerals per kg of feed. ArKO mice displayed increased body weight compared to Wt and Het littermate controls when maintained on either S- or Gen diets ($P<0.001$), and no effect was observed in S+ mice. ArKO-Gen mice had increased body weight compared to ArKO S- ($P=0.005$). ArKO mice had reduced ovarian and uterine weights compared to Wt and Het littermates when maintained on any of the diets ($p<0.001$) and no effect of diet within genotype (WT, Het or ArKO) was noted. ArKO mice had significantly elevated serum LH and FSH compared to Wt and Het controls, when maintained on Gen or Soy minus diets ($P<0.001$ and $P= 0.003$ respectively). ArKO-Gen mice had decreased LH ($p=0.046$) and FSH ($p<0.001$) compared to ArKO Soy minus mice, an effect not observed in Wt and Het littermates. In summary, ArKO mice were heavier than their littermate controls and possessed hypoestrogenic uteri and ovaries even when maintained on estrogenic diets. The addition of Genistein to the diet reduced serum LH and FSH in ArKO mice. In conclusion, in states of hypoestrogenicity isoflavones can selectively alter different components of the female reproductive axis which may have implications for hormone replacement therapy for menopausal symptoms, and in experimental animals consuming a diet containing phytoestrogens.

## 142

### A LONG TERM FOLLOW-UP OF A FEMALE WITH AROMATASE DEFICIENCY.

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In humans, natural mutations in the CYP19 gene are a rare occurrence. In the last decade, ten patients with different mutations were described. All mutations translated into inactive forms of aromatase and consequently a lack of estrogens in these patients. In both sexes, epiphyseal closure was delayed, osteopenia and reduced bone mineral density and bone age were observed. Females were presented with pseudohermaphroditism at birth and progressive virilization at puberty, whereas males were presented with normal pubertal development.

We describe here an aromatase deficient female, who was followed for 18 years since birth. She has never showed elevated levels of androgens or progressive virilization during puberty. The patient had ambiguous genitalia at birth. Her parents are first cousins. Basal estradiol was consistently undetectable, while LH followed the typical pattern with an elevation during infancy and a drop in the following years. FSH was consistently elevated. The explorative laparotomy at age 5 years revealed a normal appearance of the uterus, fallopian tubes and ovaries. Histology of both ovaries showed fibrotic stroma with a remarkably decreased number of cells surrounding primordial, primary, secondary and tertiary follicles. Often there was a discrepancy between the large size/morphology of the germ cells and the flat granulosa cells with reduced cytoplasm. At age 5, the bone age was delayed by one year. Despite high levels of gonadotropins at age 13.5 years, breast development and other signs of puberty were absent. The biopsy of the left ovary showed fibrotic ovarian stroma with many antral follicles with loss of adequate germ cells and an increased number of atretic follicles. Bone maturation was delayed, corresponding to a 10 year old. DNA analysis revealed a homozygous point mutation in exon X, resulting in R457X and deletion of 47 amino acids at the carboxyl terminus. The same mutation was described in an unrelated female infant by Portrat-Doyen (1). In vitro activity of this truncated aromatase was zero. With oestrogen therapy, bone maturation has improved and regular menstruations have been established confirming the aromatase deficiency.

(1) Portrat-Doyen 1996, Horm. Res. 46(2): 4

## 143

### LIVER RECEPTOR HOMOLOGUE-1 REGULATES AROMATASE EXPRESSION IN PREADIPOCYTES.

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Estrogen biosynthesis from C19 steroids is catalysed by the enzyme aromatase cytochrome P450. Aromatase is expressed in breast adipose tissue through the use of a distal, cytokine-responsive promoter (pI.4). In the presence of breast tumours, however, aromatase is over-expressed in response to tumor-derived factors that induce the proximal, cAMP-responsive promoter II (pII). In other tissues, transcription from promoter II requires the presence of the Ftz-F1 homologue Steroidogenic Factor-1 (SF-1, NR5A1). Adipose tissue, however, expresses little or no SF-1. We have explored the hypothesis that in adipose tissue, an alternative Ftz-F1 family member Liver Receptor Homologue-1 (LRH-1, NR5A2) can substitute for SF-1 in driving transcription from pII. 3T3-L1 preadipocytes were cotransfected with a fusion gene comprising -516/-17 bp of human CYP19 pII 5'-flanking DNA linked to the luciferase reporter, and a mouse LRH-1 expression construct. Cells were then incubated in the presence or absence of forskolin (FSK) and phorbol ester (PMA) for 16 hours. In the absence of exogenous LRH-1, FSK+PMA increased luciferase activity 3-fold. In the presence of LRH-1, basal activity increased 2.5-fold, and FSK+PMA increased activity to over 30-fold. This stimulatory effect of LRH-1 required the presence of a nuclear receptor half-site within promoter II, to which LRH-1 was shown to bind in gel shift analysis. To explore the relevance of LRH-1 induced pII activity in preadipocytes we quantified LRH-1 mRNA in various tissues by real-time PCR. LRH-1 mRNA levels in whole adipose tissue were low - approximately 10% that of liver. However, levels in isolated preadipocytes were approximately 2.5 times that of liver, suggesting that LRH-1, like aromatase, is expressed specifically in preadipocytes but not in mature adipocytes. To test this hypothesis, cultured human preadipocytes were differentiated into lipid-laden adipocytes by incubation in an adipogenic

medium for 12 days. Differentiating cells displayed a time-dependent induction of PPAR with concomitant loss of LRH-1 and aromatase expression. We conclude that LRH-1 is expressed at high level in human preadipocytes, and is a likely regulator of aromatase transcription from pII. Alterations in LRH-1 expression and / or activity in adipose tissue could therefore have considerable effects on local estrogen production and breast cancer development.

## 144

### MECHANISMS UNDERLYING THE REDUCED SENSITIVITY TO PROLACTIN NEGATIVE FEEDBACK DURING LACTATION

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Prolactin negative feedback maintains normal, low prolactin concentrations in non-lactating individuals. Prolactin stimulates the tuberoinfundibular dopaminergic (TIDA) neurons of the hypothalamus to release dopamine which suppresses prolactin secretion from lactotrophs. During lactation, however, the sucking stimulus reduces the sensitivity of TIDA neurons to prolactin feedback and consequently high prolactin levels are observed. The mechanisms involved in this loss of negative feedback are unknown, but may involve changes in prolactin signal transduction pathways. Activation of prolactin-receptors normally results in phosphorylation and nuclear translocation of Stat proteins, particularly Stat5, so we investigated Stat5 signalling in TIDA neurons of lactating rats.

Lactating rats with or without pups (removed for 16h) were given a sc injection of either oPRL (250µg) or saline vehicle (n=4/treatment), and after 1h were killed and perfused. Hypothalamic sections were immunostained for tyrosine hydroxylase (Chemicon, MAB318) and Stat5 (Santa Cruz, Sc835) and confocal images of the dorsomedial arcuate nucleus were captured for quantitation. Stat5 intracellular distribution in TIDA neurons was determined by the ratio of Stat5 fluorescence intensity in the nucleus expressed as a proportion of that in the cytoplasm (N/C ratio). In lactating rats with pups removed for 16h, oPRL injection significantly ($P<0.05$) increased Stat5 N/C ratio (2.37±0.52) compared to vehicle treated mothers (1.14±0.04). In contrast, oPRL injection did not increase Stat5 N/C ratio in lactating mothers with pups (oPRL 1.41±0.04 vs vehicle 1.37±0.10). These results demonstrate that the TIDA neurons exhibit reduced Stat5 signalling in response to exogenous prolactin when the mother is being suckled.

To investigate possible mechanisms involved in this reduced prolactin signalling, we examined the expression of SOCS (suppressors of cytokine signalling) proteins that negatively regulate prolactin signalling through the Jak2/Stat5 pathway. Northern analysis on whole hypothalamus showed that CIS (cytokine-inducible SH2 protein), but not SOCS-1, -2 or -3, mRNA expression was upregulated in suckled lactating rats. Moreover, using dual label immunofluorescence, we found that CIS is localised to TIDA neurons of lactating rats. Together these results support the hypothesis that diminished sensitivity to prolactin negative feedback during lactation is a result of increased expression of CIS in TIDA neurons.

## 145

### ANALYSIS OF TRANSCRIPTION FACTORS REGULATING PLACENTAL CRH THROUGH THE CRE.

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Corticotropin releasing hormone (CRH) is a neuropeptide involved in the HPA axis and stress response that is also expressed in peripheral sites including the placenta. There is a progressive increase in placental CRH production throughout pregnancy with levels peaking at labour. Studies suggest that there is a relationship between CRH concentrations and the length of gestation. This is particularly significant in predicting pre term birth. Therefore, investigating the molecular mechanisms regulating CRH expression is important. Using

methods such as site-directed mutagenesis and transfection of primary cultures of placental syncytiotrophoblast cells, key regulatory DNA sequences controlling placental CRH gene expression have been identified.

The CRE is one of the most important regulatory elements determining up regulation of CRH expression in the placenta. Data has shown that the CRE forms DNA-protein complexes with placental nuclear proteins in vitro. Studies have shown that CREB and c-Jun are part of the placental nuclear protein complex that binds to the CRE, but there are also other unidentified proteins in this complex. To identify the placental nuclear proteins that bind the CRE we used a yeast-one hybrid system with the CRE as the target. Screening of a placental cDNA library yielded a cDNA clone with a predicted protein sequence that includes a leucine-zipper motif similar to known transcription factors with DNA binding properties, but the DNA sequence is not homologous with any known transcription factor. The capacity for this new putative transcription factor to specifically bind to the CRE was confirmed by EMSA using the DNA binding domain expressed in yeast cells. This protein has been named CREAP for cAMP regulatory element associated protein.

A human placental cDNA library was screened and several CREAP clones were isolated. From these a full-length CREAP cDNA clone was prepared. A CREAP expression vector and vectors containing CREB or AP1 have been used in cotransfection studies with the CRH promoter, in primary placental syncytiotrophoblast cell cultures, to determine the regulatory effect that these proteins have on CRH gene expression in the placenta.

This work is characterising a novel transcription factor and advancing our knowledge of the factors determining the rate of rise of maternal plasma CRH and the consequent timing of delivery.

**146**

## CORTICOSTERONE BINDING GLOBULIN EXPLOITS SERPIN MECHANISMS TO DELIVER CORTISOL TO TARGET CELLS.

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Corticosteroid binding globulin (CBG), is a glycoprotein belonging to the SERPIN family and is found in the blood of all vertebrate species studied to date. CBG has both passive and active roles in steroid hormone action. In its passive roles it serves to increase the biological half-life as well as provide a buffering capacity for corticosteroids in the blood. An active role for CBG is mediated by specific target cell membrane receptors that are involved in a signalling system for cortisol at the cell membrane. This paper aims to determine the structure and function of the receptor binding site on CBG. We present results showing no involvement of the 6 N-linked oligosaccharides in receptor binding. Further we have identified a C-terminus peptide region as the binding site. CBG was digested with trypsin, and the resulting peptides separated and analysed by HPLC and ESI-MS connected in line. Each peptide was assayed for its ability to compete for 125I-CBG binding to rat liver membranes. A 1494.7 Da peptide was found to inhibit CBG binding. Interestingly this peptide, "FNKPFILLLFDK" shows strong sequence homology to 16 members of the SERPIN family as well as CBG's from other species and is placed a few amino acids from the $\alpha$1-PI reactive centre. It is possible to speculate that the CBG has retained the relaxed and stressed conformations of the serpins in its steroid hormone delivery mechanism. Because the specific CBG receptor on target cell membranes binds at the identified peptide, we postulate that the receptor may cleave CBG in a manner similar to neutraphil elastase which cleaves CBG at the homologous reactive centre. This would cause CBG to release its steroid which can diffuse into its target cell. Thus, the CBG receptor would serve a dual function - signal transduction at the cell membrane and release of bound cortisol for entry into cells.

## 147

### REGULATION OF P450C17 MRNA BY TGF-β SUPERFAMILY MEMBERS IN A MOUSE ADRENAL CELL LINE

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Cytochrome P450c17 catalyses steroid 17α-hydroxylase and 17,20-lyase activities, and therefore plays an important role in steroidogenesis by controlling the determining step between glucocorticoid and sex hormones biosynthesis. Expression of the P450c17 gene (*Cyp17*) is developmentally regulated and shows tissue-specificity in different animal species. In porcine Leydig cells, expression of *Cyp17* mRNA was stimulated both by activin A and inhibin A [1]. Inhibin, a member of the TGF-β superfamily, is classically thought to provide endocrine feedback to suppress pituitary FSH synthesis and secretion by antagonising activin's actions. Parallel actions of activin and inhibin on Leydig cell *Cyp17* expression raises the possibility that inhibin also has local and activin-independent actions. Our model for studying the local specific actions of inhibin is the mouse adrenal AC cell line, which expresses inhibin α and $\beta_B$ subunits along with novel inhibin-specific binding proteins. We found, using real-time pcr quantification, that this cell line also expresses *Cyp17* mRNA. Inhibin A treatment did not significantly alter *Cyp17* mRNA expression in the presence of insulin suggesting that inhibin alone has no direct agonist action. We then examined whether other TGF-β superfamily members are able to modify adrenal steroidogenesis. BMP-2 and BMP-7 suppressed *Cyp17* mRNA expression, and this effect was dose-dependent (ED50 of ~1-2 nM) with BMP-2 being more effective than BMP-7 (~1.6 fold). Activin B (1 nM) also strongly suppressed *Cyp17* mRNA levels after 18 h. TGF-$\beta_1$, however, did not alter *Cyp17* mRNA expression in these mouse adrenal cells, in contrast to the strong suppression seen in bovine [2], ovine [3] and human adrenal cells [4]. Thus, multiple factors are involved in the cell-type and species-specific expression of *Cyp17* mRNA. In this context, endocrine inhibin A and autocrine/paracrine inhibin B may modify the regulation of *Cyp17* expression by other TGF-β superfamily members. We are currently investigating whether inhibin can rescue the inhibitory effects of activin or BMP on *Cyp17* mRNA expression.

[1] Lejeune, H. et. al. Endocrinology 138:4783, 1997

[2] Feige, J.J et al. Biochem. Biophys. Res. Commun. 139:693, 1986

[3] Rainey, W.E et al. Endocrinology 127:1910, 1990

[4] Lebrethon, M-C. et al. J. Clin. Endocrinol. Metab. 79:1033, 1994

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## 148

### METABOLISM OF SYNTHETIC GLUCOCORTICOIDS USED FOR THE TREATMENT OF ASTHMA BY PLACENTAL 11β-HYDROXYSTEROID DEHYDROGENASE TYPE 2

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Asthma complicates 12% of Australian pregnancies, and is associated with an increased incidence of low birth weight neonates. The mechanism by which fetal growth is reduced in asthmatic pregnancies is unknown, though the use of inhaled glucocorticoids for treatment of asthma may contribute to this outcome. Glucocorticoids are necessary for normal fetal growth and development, however overexposure results in decreased fetal growth and altered hypothalamic-pituitary-adrenal function. Fetal glucocorticoid exposure is regulated by the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which converts bioactive glucocorticoids to their 11-keto metabolites. Previous studies have shown that the synthetic glucocorticoids betamethasone and dexamethasone are not metabolised by placental 11β-HSD2, however, the metabolism of semi-synthetic glucocorticoids used for the treatment of asthma, such as prednisolone, beclomethasone dipropionate, budesonide and fluticasone propionate, by the placenta is unknown. If these steroids are not metabolised by the placenta it is possible that they could cross into the fetal compartment and alter development. Thus, an investigation was

carried out to determine whether the placenta is able to metabolise semi-synthetic glucocorticoids used for the treatment of asthma. A thin-layer chromatography (TLC) procedure was optimised for separation of synthetic steroids from their 11-keto metabolites. Placental homogenates from normal pregnancies were incubated with a saturating concentration of each synthetic steroid (5μM) for 15 minutes at 37°C, and steroids visualised on F-254 silica gel plates under UV light at 254 nm wavelength. The optimal mobile phase chosen for the TLC procedure was chloroform:methanol 93:7 (v/v) , allowing separation of all steroids from their 11-keto metabolites. Preliminary qualitative analyses indicate that beclomethasone dipropionate, fluticasone propionate and prednisolone are metabolised by the placenta into their 11-keto forms, though betamethasone, dexamethasone and budesonide are not metabolised. These studies suggest that some synthetic glucocorticoids used for the treatment of asthma may be more suitable for use during pregnancy than others in relation to fetal outcome.

**149**

## TRANSFORMING GROWTH FACTOR β1 (TGFβ1) – PUTTING THE BRAKES ON ADRENAL STEROIDOGENESIS BEFORE BIRTH

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In most mammalian species, periods of active adrenal steroidogenesis may be interrupted by periods of "quiescence" in which the fetal adrenal gland is unresponsive to ACTH, and adrenal steroidogenesis is suppressed. Whilst there is a body of literature describing the activators of adrenal function, the factor(s) which inhibit adrenal responsiveness remain to be elucidated. In the present study, we have investigated the developmental expression of transforming growth factor beta 1 (TGFβ1; a putative inhibitor of steroidogenesis) and cytochrome P450 17α hydroxylase (CYP17; a key enzyme adrenal steroidogenesis). Fetal sheep adrenal glands were collected from fetuses between 102 and 120d gestation when the adrenal is relatively quiescent (102-103d; n=5 and 117-120d; n=5) and between 140 d gestation and the first day after birth during the peripartum period of adrenal activation (140-141d; n=6 and PN1; n=6), Northern blot analysis was performed using a 32P-labelled cDNA probes to porcine TGFβ1 and bovine CYP17 and a 32P-labelled rat 18S rRNA oligo-probe was used to verify equal RNA loading. TGFβ1 mRNA was detected, with a major transcript size of 2.5kb and 1 minor bands at ~4kb in all fetal and postnatal adrenals. Adrenal expression of TGFβ1 mRNA:18S rRNA was significantly higher at 102-103d (167.9 ± 5.7) than at 117-120d gestation (100.3 ± 7.8). There was a further significant decrease in adrenal TGFβ1:18S expression between 140-141d (99.1 ± 15.8) and PN1 (58.1 ± 13.8). In contrast, adrenal CYP17:18S was low at 102-103d (74.6 ± 17.8), increased almost 10 fold between 117-120d (67.0 ± 18.7) and 140-141d (632.6 ± 87.0) and there was a further 2 fold increase by PN1 (1302.1 ± 181.0). There was an inverse correlation (r=0.76; p=0.000) between adrenal TGFβ1 and CYP17 expression when adrenals from all age groups were combined. In summary, we found that high expression of TGFβ1 occurs at ~102-103d gestation, at a time when fetal sheep adrenal is quiescent. Therefore, it may be that TGFβ1 plays a novel role in suppressing adrenal steroidogenesis in the mid-gestation fetus.

**150**

## NOVEL ASPECTS OF CRH AND AVP SIGNALLING IN PITUITARY CELLS

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Corticotropin releasing hormone (CRH) is a key hormone controlling the body's stress response as well as being important in the onset of human labour. In the pituitary, CRH stimulates ACTH release from corticotrophs by increasing intracellular cAMP and activating cAMP-dependent protein kinase (PKA). Arginine vasopressin (AVP) activates protein kinase C (PKC) and synergises with CRH to increase ACTH release but the point of interaction of these signalling pathways is unclear. AtT20 mouse corticotroph pituitary tumour cells were treated with CRH (10 nM), AVP (50 nM) and CRH/AVP together, forskolin (10 μM), phorbol ester (PMA, 1 μM) and forskolin/PMA together for 15 or 60 minutes. Cells were lysed, run on SDS-PAGE, transferred to nitrocellulose membrane and probed with an antibody to the catalytic subunit (C-subunit) of PKA. CRH/AVP and forskolin/PMA treatment for 15 or 60 minutes decreased C-subunit levels 10 and 2 fold respectively. This

suggests that C-subunit levels are controlled by rapid degradation or down regulation when both cAMP and PKC pathways are simultaneously activated. Increased levels of C-subunit were detected after 15 minutes with CRH or after 60 minutes with forskolin/PMA, suggesting that cAMP signalling can rapidly stimulate translation of PKA C-subunit. A protein kinase assay was used to show whether the changes in protein levels correlate with changes in total activity of PKA. This assay measured PKA able to be stimulated above basal counts. In the conditions where total C-subunit levels were reduced, no difference in total PKA activity could be determined. This suggested a stable activation of PKA had occurred. The small amount of C-subunit left in these samples must have been modified in some way to make it "more active" for example by phosphorylation. This has not been described before for PKA. Total PKC activity increased after CRH treatment for 15 minutes and decreased after 60 minutes with PMA or PMA/forskolin. β-endorphin levels were measured in the media collected from the stimulated cells and showed that the AtT20 cells used were functionally responsive to CRH stimulation. This stimulation was rapid, occurring after 15 minutes. This demonstrated that the unusual changes in C-subunit levels and activity occurred in the context of normal β-endorphin release.

**151**

## IDENTIFICATION OF AMINO ACID RESIDUES WITHIN THE TETRATRICOPEPTIDE REPEAT DOMAIN OF CYCLOPHILIN 40 THAT MEDIATE HSP90 RECOGNITION

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Cyclophilin 40 (CyP40) is a TPR-containing immunophilin and a modulator of steroid receptor function through its binding to heat shock protein 90 (hsp90) (1). Critical to this binding are the C-terminal MEEVD motif of hsp90 and the TPR domain and flanking acidic and basic domains of CyP40 (2). We have previously determined the crystal structure of CyP40 (3) and have now modelled the MEEVD peptide onto the CyP40 protein. Results from these studies were used as the basis for a comprehensive mutational analysis of the hsp90-interacting domain of CyP40. Using a C-terminal CyP40 construct as template, 24 amino acids were individually mutated by site-directed mutagenesis. These mutants were used as bait in a yeast two hybrid assay with a C-terminal hsp90b prey construct and qualitatively assessed for hsp90 binding. For quantitative assessment, mutants were expressed as GST-fusion proteins and assayed for binding to C-terminal hsp90b using conventional pulldown and ELISA microtitre plate assays. We identified five TPR-domain residues (K227, N231, N278, K308, R312) essential for hsp90 binding, four of which contribute to a binding groove for the MEEVD peptide predicted from modelling experiments. Another five TPR-domain residues (F234, S274, L284, K285, D329), two of which also help define the binding groove, are required for efficient hsp90 binding. None of nine acidic and basic domain residues that were mutated, reduced hsp90 binding. This data confirms the critical importance of amino acids in the MEEVD binding groove for hsp90 recognition and reveals that additional residues within the CyP40 TPR-domain are required for hsp90 binding.

References: (1) Pratt, WB and DO Toft, Endocrine Reviews 18, 306-360 (1997)

(2) Carrello, A et al, J Biol Chem 274, 2682-2689 (1999)

(3) Taylor, P et al, Structure 9, 431-438 (2001)

## 152

### SPECIFIC LEUCINE RESIDUES WITHIN AN HSP90 C-TERMINAL MOTIF TARGETED BY THE HSP90 ANTAGONIST NOVOBIOCIN ARE ESSENTIAL FOR HSP90 DIMERISATION AND INTERACTION WITH STEROID RECEPTOR-ASSOCIATED IMMUNOPHILINS

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The structurally related immunophilins cyclophilin 40 (CyP40), FKBP51 and FKBP52, have been implicated as modulators of steroid receptor function through their association with hsp90, a molecular chaperone with a key role in steroid hormone signalling. Within steroid receptor complexes these immunophilins compete via conserved tetratricopeptide repeat (TPR) domains for a common site located in the C-terminal region of hsp90. An α-helical microdomain, incorporating residues 649-670 and located upstream of the critical MEEVD interaction motif, is important for hsp90 dimerization and may constitute a second interaction site for TPR-containing immunophilins. Novobiocin, a coumarin antibiotic, interacts with this site and disrupts hsp90 function by interfering with regulatory interactions between the C-terminal region and the N-terminal domain of hsp90. The aims of this study were to perform a mutational analysis of selected residues within this microdomain to assess their role in hsp90-CyP40 interaction and hsp90 dimerization, and to determine if novobiocin interferes with CyP40 recognition by hsp90.

Using site-directed mutagenesis, we introduced single point alanine substitutions for lysine and large hydrophobic residues within this microdomain of human hsp90β, the C-terminal domain (520-724) of which was cloned into the yeast 2-hybrid prey vector pVP16. Mutants were cotransformed into yeast strain L40 with a bait pBTM116 plasmid containing a LexA DNA-binding domain fused to a wild-type human CyP40 (185-370) or wild-type human hsp90β (520-724) C-terminal sequence. CyP40 binding or hsp90β dimerization was analysed using a β-galactosidase assay. Single substitutions at L657, L658, L664 and L670 markedly inhibited hsp90-CyP40 binding and hsp90 dimerization. Other introduced mutations had little impact on either of these functions. Using an ELISA assay, novobiocin (1mM) was found to prevent the binding of a GST-hsp90β (520-724) fusion protein to full-length hCyP40. These results identify specific leucine residues within this microdomain that play a key role in both CyP40 binding and hsp90 dimerization. They also link hsp90 antagonism by novobiocin to the disruption of hsp90-immunophilin interaction.

## 153

### ZONAL AND DEVELOPMENTAL VARIATION OF PROGESTERONE- AND GLUCOCORTICOID RECEPTOR MRNA EXPRESSION IN THE RAT PLACENTA.

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Fetal growth and development are critically dependent on adequate growth of the placenta, the control of which involves a range of endocrine signals. Glucocorticoids potently inhibit placental growth, whereas progesterone exerts opposite, trophic effects. Recent studies in human placenta and rat corpus luteum indicate that progesterone exerts some effects via interaction with the glucocorticoid receptor (GR). Thus, interaction of glucocorticoids and progesterone in the placenta likely depends on the relative expression of GR and the two known forms of progesterone receptor (PR-A and PR-B). Therefore, in the present study we used real-time RT-PCR to quantitate GR, PR-A and PR-B mRNA in the two morphologically- and functionally-distinct regions of the rat placenta, the basal and labyrinth zones. Placentas were obtained at days 16 and 22 of gestation (term=23 days), thus covering the period of maximal fetal growth. PR-A and PR-B mRNA levels in labyrinth zone on both days were extremely low, close to the limits of detection. The basal zone exhibited markedly higher levels of mRNA expression in comparison to the labyrinth zone for both PR-A (>7 fold at days 16 and 22, $P<0.01$) and PR-B (day 16: >9 fold, $P<0.05$; day 22: >3 fold, $P<0.05$). There was also an increase in PR-A mRNA expression within the basal zone from days 16 to 22 (1.6 fold, $P<0.05$). Moreover, basal zone expression of PR-A exceeded that of PR-B (4-5 fold) on both days of pregnancy. These expression patterns are consistent with previous reports

of readily detectable progesterone binding in the basal but not labyrinth zone. GR mRNA levels were similar in basal and labyrinth zones on day 16, then increased 2.6 fold in labyrinth zone by day 22 ($P<0.01$) but remained unchanged in basal zone. These data show that mRNAs encoding GR, PR-A and PR-B are all expressed in the rat placenta with substantial zone- and stage-specific variation. Importantly, the very low expression of PR-A and PR-B in the labyrinth zone is consistent with progesterone effects in this tissue being mediated via the GR. Further studies are required to assess how progesterone and glucocorticoids interact with their cognate and non-cognate receptors in a physiological setting to control placental function and thus fetal growth.

**154**

## HUMAN MYOMETRIAL GENES ARE DIFFERENTIALLY EXPRESSED IN LABOUR: A SUPPRESSION SUBTRACTIVE HYBRIDISATION (SSH) STUDY

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We aim to identify the genes that are changed by labour in the human myometrium by suppression subtractive hybridisation (SSH). Term myometrial tissues were obtained from labouring and non-labouring women undergoing Caesarean section upon obtaining informed consent. Total RNA was used in SSH (Clontech PCR Select) to produce two subtracted cDNA libraries enriched for genes expressed during or before labour, "labour" and "not-in-labour" libraries respectively. Dot blot screening of 400 positive clones, constituting 20% of the two subtracted libraries, revealed 30 differentially expressed clones. Of 10 known genes that were identified to be up-regulated in labour, 6 had apparent immune regulatory and inflammatory roles. Three are well-known inflammatory mediators and modulators previously linked with parturition, interleukin-8, manganese superoxide dismutase (MnSOD) and metalloproteinase-9. Three others, interferon-inducible 1-8d gene, elongation factor 1$\alpha$ and nucleophosmin (B23) have not been previously linked with labour. Constitutively expressed genes including cyclophilin and $\alpha$-actin were found to be altered by labour. Quantitative real-time RT PCR (QRT-RTPCR) using Taqman$^{TM}$ probes further confirmed the up-regulation of some of these genes. The amounts of the specific genes assayed were standardised to 18S ribosomal RNA and are expressed as mean ± SEM. QRT-RTPCR showed that IL-8 mRNA rose from 0.003 ± 0.002 in non-labouring samples (n=38) to 0.24 ± 0.11 (n=20) in gestational-age-matched spontaneously labouring women ($P$=0.035). Similarly, MnSOD rose from 0.11 ± 0.02 (n=24) to 1.23 ± 0.56 (n=24) in gestational-age-matched women ($P$=0.047). Additionally, cyclophilin, often used as a constitutive or housekeeping gene marker, increased from 0.0008 ± 0.0002 (n=6) to 0.002 ± 0.0004 (n=6, $P$= 0.008) during labour. Induced labour further showed significantly higher levels of IL-8 (0.63 ± 0.21, n=14) than spontaneous labour (0.22 ± 0.11, n=20) ($P$ =0.046), but not MnSOD ($P$=0.1). This work identifies both novel as well as known genes not previously associated with parturition. The data support the view that inflammatory pathways are activated in both spontaneous and induced labour.

**155**

## INTERLEUKIN 11 SECRETION BY HUMAN ENDOMETRIAL STROMAL CELLS IS STIMULATED BY PROSTAGLANDIN E2 IN PART VIA A CYCLIC ADENOSINE MONOPHOSPHATE DEPENDENT PATHWAY.

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Decidualization of human endometrial stromal cells (HESC) is crucial for embryo implantation and placentation. Interleukin 11 (IL-11) signalling is essential for decidualization and implantation in the mouse[1]. Both IL-11 and prostaglandin (PG) $E_2$ enhance stromal cell decidualization in the human[2,3]. This study examined the effect of $PGE_2$ on the regulation of IL-11 secretion by HESC. Primary HESC were cultured for 24 hours with or without either $PGE_2$ or indomethacin (cyclo-oxygenase inhibitor). Following 24 hours of culture with indomethacin, $PGE_2$ was added to the cells and these were further cultured for 24 hours. HESC were also cultured with either a cyclic adenosine monophosphate (cAMP) inhibitor, Rp—adenosine 3,5-cyclic monophosphioate (rp-cAMP) or

$PGE_2$ and rp-cAMP for 24 hours. At each time point culture medium was collected and IL-11 measured by ELISA. Addition of $PGE_2$ to HESC increased IL-11 secretion compared with controls (550±65 vs 250±52 respectively, $P<0.01$ compared with controls, no $PGE_2$), and this returned to control levels following $PGE_2$ washout. Addition of indomethacin to HESC caused a significant suppression of IL-11 secretion by the cells compared to controls (95±32 vs 245±25 respectively, $P<0.01$ compared to controls, no indomethacin). However, the addition of $PGE_2$ to indomethacin treated cells completely reversed this inhibition of IL-11 secretion. Rp-cAMP caused a reduction in IL-11 secretion compared to controls (180±23 vs 290±35 respectively, $P<0.05$ compared with controls, no rp-cAMP). Similarly, IL-11 secretion by HESC following the addition of $PGE_2$ and rp-cAMP was significantly reduced compared to controls (420±58 vs 620±70, $P<0.05$ compared to controls, no $PGE_2$ or rp-cAMP). The results demonstrate that $PGE_2$ stimulates IL-11 secretion by HESC and suggest endogenous $PGE_2$ acts as an autocrine stimulus for IL-11 secretion which is mediated in part via a cAMP-dependent pathway. Supported by NH&MRC and CONRAD/CICCR.

1. Robb L, Li R, Hartley L, Nandurkar HH, Koentgen F, Begley CG (1998) *Nat Med* 3:303-308

2. Dimitriadis E, Robb L, Salamonsen LA (*in press*) *Mol Hum Reprod*

3. Frank GR, Brar AK, Cedars MI, Handwerger S (1994) *Endocrinology* 134:258-263

**156**

## IDENTIFICATION OF THE TRANSCRIPTION FACTOR E2F4 AS A NOVEL BINDING PARTNER FOR THE GONADOTROPIN-RELEASING HORMONE RECEPTOR

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The mammalian gonadtropin-releasing hormone receptor (GnRHR) is a unique G-protein coupled receptor (GPCR) in both its structure and mechanism of regulation. This is the only GPCR cloned to date that lacks an intracellular C-terminal domain and consequently its desensitization and internalization is not regulated by an arrestin-dependent mechanism. The GnRHR is also known to exhibit anti-proliferative properties in both tumor and non-tumor cells. Thus, there are numerous questions regarding the mechanisms, or more specifically the proteins, directly utilized by this receptor. The yeast two hybrid (Y2H) system has been used as a powerful tool to identify GPCR-interacting proteins involved in receptor internalization, signaling and cytoskeletal association. Therefore, the aim of this study was to utilize the Y2H system to identify novel partner proteins capable of selectively binding to the GnRHR. The third intracellular loop (3iL) of the rat GnRHR was utilized as a 'bait' to screen a mouse embryonic library. Five positive clones with high homology to the transcription factor, E2F4, were identified. These only interacted with the 3iL and not the 2iL of the GnRHR, suggesting specificity with this domain of the receptor. An interaction between the GnRHR and E2F4 was confirmed in mammalian cells by using bioluminescence resonance energy transfer (BRET), a sensitive method which measures protein-protein interactions in live cells. Cells expressing tagged GnRHR and E2F4 constructs (GnRHR-EYFP and E2F4-Rluc) produced a BRET signal that was modulated following treatment with GnRH. No interaction of E2F4 with other GPCRs (TRH and □2-adrenergic receptor) was detectable. Functional analysis revealed that over expression of E2F4 did not affect GnRHR binding, signaling, or internalization. However, reporter gene analysis demonstrated that GnRH resulted in an increase in the transcriptional activity of E2F4. As E2F4 is a critical regulator of the cell cycle, the role of E2F4 in GnRH mediated growth effects was investigated by FACS and 3H-thymidine incorporation. We propose that E2F4 is a novel protein partner of the GnRHR and this association may provide a previously unrecognised mechanism for regulating the proliferative actions of this receptor.

## 157

### THE INTRACELLULAR CONSEQUENCES OF THE CAG EXPANSION AND ANDROGEN RECEPTOR TRUNCATION ON THE PATHOGENESIS OF KENNEDY'S DISEASE

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An expansion of CAG repeat within the androgen receptor (AR) gene results in a motoneurone disease known as Kennedy's disease (KD), or spinobulbar muscular atrophy. The major manifestation of KD includes progressive degeneration of the spinal and bulbar motoneurones, resulting in weakness of the proximal musculature. While the genetic basis of KD is known, the pathogenic mechanisms leading to neurodegeneration remain elusive. Studies have suggested that truncated AR fragments with expanded CAG repeats may be more toxic to neurones, possibly by initiating the transcription of specific genes in the absence of hormonal control. The aim of this study is to investigate the effects of AR truncation and CAG repeat expansion on the intracellular trafficking of the AR. We examined the intracellular localization, cytoplasmic-nuclear translocation, and cell viability of AR in green monkey kidney cell line, COS, in vitro. Using enhanced green fluorescence protein (EGFP) (Clontech), we constructed four EGFP-human AR chimeric expression vectors, including the full length proteins with CAG repeat in the normal (n=20) and KD range (n=59); and the C-terminally truncated protein fragments that mimic fragments generated by caspase cleavage at the first cleavage site Asp146, with the normal and mutant CAG repeats. These were transiently transfected into COS cells and localisation of the AR was visualised by the EGFP fluorescence using confocal microscopy 3 days post-transfection. Temperature and CO2 level were maintained at physiological range throughout the observation. The GFP-AR was found primarily in the cytoplasm, irrespective of the size of the CAG repeat or protein length. However, while fluorescence in the cells transfected with the wild type construct were uniformly distributed throughout the cytoplasm; a large proportion of those that contained the mutant constructs exhibited vacuole-like vesicles of various sizes within the cytoplasm. Trypan blue exclusion showed that viable cells decreased in number when transfected with the truncated fragments, compared to those containing the full length proteins. The degree of toxicity induced by the truncated fragments was similar to that caused by the expanded full length AR protein. Our studies showed that both expanded CAG repeat size of the AR gene and AR truncation confer higher toxicity to COS cells. However, it appears that the presence of a truncated AR with expanded CAG repeat is more toxic than a truncated AR with normal CAG repeat. Therefore, the increased vulnerability for neurodegeneration may be determined by the presence of truncated proteins in the spinal and bulbar motoneurones.

## 158

### EXPRESSION AND REGULATION OF CYCLO-OXYGENASE ISOFORMS IN THE ADULT RAT TESTIS

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Data from our laboratory suggest that deficiency of immune responses within the rat testis may be due in part to regulation of the pro-inflammatory and anti-inflammatory functions of the resident testicular macrophages. For example, the pro-inflammatory or "inducible" form of nitric oxide synthase (iNOS) is constitutively expressed by Leydig cells, but is not produced by testicular macrophages under normal or inflammatory conditions. In contrast, both $PGE_2$ and $PGF_{2\alpha}$, which are important autocrine regulators of macrophage inflammatory functions, are produced by the testicular macrophages. Significantly, the key enzymes responsible for prostaglandin (PG) production, cyclo-oxygenase-1 (COX-1) and COX-2, are known to be stimulated by NO in macrophages from other tissues. Consequently, we hypothesised that constitutive production of testicular NO may stimulate local COX expression, particularly the expression of COX-2, which is the inducible form of the enzyme produced by macrophages, thereby regulating the local immune environment. Expression of the COX isoforms, as well as the pro-inflammatory cytokine interleukin-1β (IL-1β) and the anti-inflammatory cytokine IL-10, were examined in the testis and liver (as control tissue) of adult male rats using quantitative real-time PCR. Rats were also treated

with an inflammatory stimulus, lipopolysaccharide (LPS; 5 mg/kg), the NO inhibitor L-nitro-arginine-methyl-ester (L-NAME; 30 mg/kg) or LPS and L-NAME combined. Both COX-1 and COX-2 were found to be expressed in the normal adult rat testis, but whereas COX-2 mRNA expression was similar in the testis and liver, COX-1 mRNA expression in the testis was significantly lower. LPS stimulated an increase in expression of COX-2, IL-1 β and IL-10, but not COX-1 in both testis and liver. LPS-stimulated IL-1β and IL-10 mRNA expression in the testis was considerably lower (>10-fold) when compared to the liver, even though these tissues contain similar numbers of resident macrophages. However, treatment with L-NAME had no significant effect on either basal or LPS-stimulated COX-1, COX-2, IL-1 β and IL-10 expression. The data indicate a significant role for COX-2 in the testis, and provide further proof of the fact that testicular macrophages are poor producers of pro-inflammatory cytokines. The data did not support an essential role for iNOS or local NO in regulating either testicular COX-2 or cytokine expression *in vivo*, suggesting that other factors may be more important.

**159**

## THE NOVEL G PROTEIN-COUPLED RECEPTOR LGR8 IS A POTENTIAL RECEPTOR FOR INSULIN 3

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Insulin 3 (INSL3, relaxin-like factor, RLF) and relaxin are both members of the insulin/IGF superfamily of peptide hormones. Both peptides have distinct patterns of expression and sites of action. INSL3 is primarily a product of the testicular Leydig cells and is essential for testicular descent. Problems with testis descent, leading to cryptorchidism, are very common, with 2-5% of boys requiring corrective surgery and having an increased risk of testicular cancer and infertility. INSL3 is essential for the development of a ligament called the gubernaculum, however the mechanisms by which it exerts its effects are not known. Furthermore the receptor for INSL3 has not been identified.

Recently relaxin was shown to activate two orphan leucine-rich repeat-containing G protein-coupled receptors (LGR), LGR7 and LGR8, resulting in cAMP release [1]. The LGR7 is the relaxin receptor, however evidence suggested that the LGR8 should be the receptor for INSL3 [1]. Using the human LGR7 and LGR8 expressed in 293T cells together with relaxin and INSL3 peptides we have studied whether the LGR7 or LGR8 will bind INSL3. Furthermore the action of INSL3 and relaxin were tested in whole organ cultures of fetal rat gubernaculum and the expression of LGR7 and LGR8 was examined in this structure.

Cells transfected with human LGR7 bound relaxin with high affinity (EC50 = 0.28 nM) but did not bind INSL3. In contrast although cells transfected with human LGR8 bound relaxin with high affinity (EC50 = 0.65nM) they had a similar affinity for INSL3 (EC50 = 0.92 nM). Both relaxin and INSL3 induced the release of cAMP from LGR8, but only relaxin stimulated cAMP release from LGR7 transfected cells. INSL3 stimulated growth of whole organ cultures of fetal rat gubernaculum while relaxin only had a modest effect. LGR8 but not LGR7 transcripts were detected in this structure indicating that relaxin was acting through LGR8 receptors. The LGR8 therefore fullfills all the criteria of being the INSL3 receptor. Further studies on this unique receptor will increase our understanding of the mechanisms of gubernacular development and, hence, testicular descent.

1. S.Y. Hsu et al, Science 295, 671 (2002)

## 160

### STEREOLOGICAL EVALUATION OF SPERMATOGENESIS INDUCED BY TESTOSTERONE (T) OR HUMAN CHORIONIC GONADOTROPHIN (HCG) IN GONADOTROPHIN DEFICIENT (HPG) MICE.

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We established that testosterone (T) alone induces (1) and maintains (2) spermatogenesis in a mutant hypogonadal (*hpg*) mouse, lacking postnatal circulating LH, FSH and testosterone. Although T induced qualitatively complete spermatogenesis, testis weight remained only 30-40% of non-*hpg* controls (1). In addition to T, Leydig cells under LH stimulation produce other steroids and peptides that could influence germ cell and testis development. We therefore investigated whether LH-dependent Leydig cell products other than T influenced the shortfall in testis development and germ cell populations by comparing the effects of treatment with T alone or hCG in *hpg* mice. Immature *hpg* mice (21days) were treated with either T (1cm silastic implant) or hCG (1U, 3 x wk) for 6 weeks. Stereological evaluation of the perfusion-fixed testis was carried out using the optical dissector technique and CAST image analysis. Testis weight increased from untreated (2.0 ± 0.1 mg, n=13) to a similar extent with both T (24.8 ± 0.7 mg, n=15) and hCG (28.5 ± 2.5mg, n=6) treatment. Histology of the seminiferous epithelium of both T treated and hCG mice appeared similar, with all stages of spermatogenesis present within tubules with well developed lumina and mature, basally situated Sertoli cells (SC). Stereological evaluation revealed no significant difference in Sertoli cells or post-meiotic, haploid germ cell population (round (RS) or elongated (ES) spermatids), however hCG treatment produced more spermatogonia (SG) and primary spermatocytes (Sc) but fewer pachytene spermatocytes (PS) than T treatment.

| | SC $x10^6$/testis | SG $x10^6$/testis | Sc $x10^6$/testis | PS $x10^6$/testis | RS $x10^6$/testis | ES $x10^6$/testis |
|---|---|---|---|---|---|---|
| hpg | 1.5 ±0.1 | 0.9 ±0.1 | 0.5 ±0.1 | 0 | 0 | 0 |
| *hpg* + T | 2.0 ±0.1 | 1.5 ±0.1 | 2.4 ±0.2 | 3.9 ±0.2 | 11.1 ±0.5 | 9.1 ±0.6 |
| *hpg* + hCG | 2.1 ±0.1 | 2.7 ±0.3* | 3.3 ±0.2* | 2.8 ±0.3* | 10.2 ±0.9 | 8.8 ±1.2 |

* significant hCG vs T, ± SEM

We conclude that testosterone is the major Leydig cell (LC) product contributing to the output of spermatozoa but that other LH-dependent Leydig cell products may have additional effects on early (pre-meiotic) germ cell populations.

1) Singh et al, Endocrinology 136:5311, 1995

2) Handelsman et al, Endocrinology 140:3938,1999.

## 161

### DOES BETACELLULIN-INDUCED ERBB ACTIVATION INHIBIT CYTOKINE-INDUCED APOPTOSIS OF PANCREATIC β-CELLS?

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Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterized by the infiltration of mononuclear cells into pancreatic islets. These cells produce and release the cytokines TNF-α, IFN-γ and IL-1β that contribute to β-cell destruction through a process known as death-receptor induced apoptosis. The ErbB family of receptor tyrosine kinases is thought to be involved in cell survival, proliferation and differentiation. Betacellulin (BTC) is a novel growth factor that signals through the ErbB receptor family and has been implicated in islet growth and pancreas development. In this study, we characterised ErbB receptor expression in the pancreatic β-cell line, NIT-1, and subsequently investigated whether BTC-induced ErbB activation could inhibit cytokine-induced (death receptor-induced) apoptosis of these cells. ErbB1, ErbB2 and ErbB3 were expressed as both mRNA and protein transcripts in the NIT-1 cell line, as determined through RT-PCR, southern blot and western blot. BTC induced NIT-1 cell proliferation, most likely through a pathway involving ErbB1 phosphorylation and activation. The effects of insulin-like growth factor-I (IGF-I), which promotes β-cell

survival in an animal model of type 1 diabetes, alone and in combination with BTC, were also examined. Whilst BTC and/or IGF-1, did not inhibit cytokine-induced apoptosis, both growth factors either alone or in combination inhibited basal levels of NIT-1 apoptosis. Therefore, while BTC did not protect β-cells from death receptor-induced (caspase-8 dependent) apoptosis, its action with IGF-1 did reduce stress-induced (caspase-9 dependent) apoptosis. It is possible experimental conditions were sub-optimal for BTC to act as a survival factor in response to cytokine treatment, or activation of ErbB1 by BTC may be insufficient to activate downstream anti-apoptotic cascades such as the PI3K pathway. However, BTC and IGF-1 used in combination may be useful as media supplements in the culture of isolated islets, which are currently being trialled for transplantation studies for IDDM treatment, and are known to undergo a high level of apoptosis in culture.

**162**

## PREDIALYSIS CHRONIC RENAL INSUFFICIENCY IS ASSOCIATED WITH ELEVATED GLUCOSE LEVELS AND INSULIN RESISTANCE

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Insulin resistance is an important risk factor for cardiovascular disease in subjects with end-stage renal failure on dialysis. It is unclear whether insulin resistance is present in patients with chronic renal insufficiency pre-dialysis. The aim of this study was to determine whether insulin resistance is present in patients with non-end stage chronic renal insufficiency. Patients with diabetes mellitus were excluded. Firstly, we compared 46 patients (17 females, 29 males) with chronic renal insufficiency (creatinine 0.26 ± 0.02 mmol/L) to 62 normal subjects (45 females, 17 males) without renal impairment (creatinine 0.08 ± 0.01 mmol/L) matched for BMI (27.1 ± 0.6 vs 26.4 ± 1.0 kg/m$^2$). Chronic renal insufficiency was characterised by higher fasting glucose (5.5 ± 0.1 vs 4.9 ± 0.1 mmol/L, p = 0.0001) and insulin levels (14.3 ± 0.8 vs 7.3 ± 1.0 mU/L, p = 0.0001). Insulin resistance, determined by HOMA (1), was also higher in chronic renal insufficiency (3.5 ± 0.2 vs 1.6 ± 0.2, p = 0.0001).

To further characterise insulin action in chronic renal insufficiency, 12 patients (body fat = 29.4 ± 3.0 %, determined by DEXA) and 12 normal subjects (body fat = 36.6 ± 3.2 %) underwent a meal tolerance test (caloric equivalent of 30 % basal energy expenditure). Fasting glucose (6.3 ± 0.3 vs 5.4 ± 0.2 mmol/L, p = 0.02) and mean post-prandial glucose levels (7.6 ± 0.4 vs 6.6 ± 0.3 mmol/L, p = 0.04) were significantly higher in chronic renal insufficiency. Fasting insulin levels (16.2 ± 3.8 vs 5.8 ± 0.6 mU/L, p = 0.02) were higher in chronic renal insufficiency where as mean post-prandial insulin levels were similar (33.3 ± 7.9 vs 24.1 ± 2.7 mU/L, p = 0.28). Diet-induced thermogenesis, an indirect measure of insulin action was similar between groups.

In summary, chronic renal insufficiency is associated with higher fasting and post-prandial glucose levels and higher fasting insulin levels and insulin resistance as determined by HOMA. These findings support the notion that insulin resistance is present in chronic renal insufficiency and therefore may contribute to increased cardiovascular disease.

1. Matthews et al, Diabetologia 1985;28:412-19.

**163**

## CHOLESTEROL FEEDING RESCUES THE FATTY LIVER PHENOTYPE OF THE AROMATASE KNOCKOUT (ARKO) MOUSE.

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Aromatase knockout (ArKO) mice lack a functional aromatase gene, therefore are unable to synthesize endogenous oestrogens. ArKO and WT males were fed either a normal diet (soy free), 0.2% or 2% cholesterol diet for 90 days. ArKO body and liver masses were heavier compared to wildtype (WT) on the control diet. Interestingly there was a decrease in body and liver mass with increasing cholesterol in the diet. ArKO males on the control diet had pronounced hepatic steatosis, but this was no longer present with cholesterol-supplemented diets. There was a significant decrease in serum cholesterol, triglyceride and HDL levels ($p<0.05$) between control and 2% cholesterol diet. ArKO males on the control diet had 2-fold higher levels of liver cholesterol

compared to WT control, and both groups showed increases in liver cholesterol levels when fed cholesterol. Control ArKOs had significantly elevated levels of liver triglycerides compared to WT controls ($p<0.05$); both ArKO and WT on the cholesterol diet showed significant reductions in liver triglyceride levels when fed cholesterol ($p<0.05$). To further understand this phenotype, gene expression of key regulators in the cholesterol pathway was examined using real time PCR. HMG CoA reductase and HMG CoA synthase, enzymes involved in de novo synthesis of cholesterol showed no difference between ArKO and WT on either diet, however there was a reduction HMG CoA reductase transcripts levels in both groups fed 2% cholesterol. LDLR and SREBP2 showed no difference between ArKO and WT, regardless of diet. Cholesterol 7□ hydroxylase, the enzyme responsible for regulating bile acid excretion showed no differences between ArKO and WT on the control diet, but there was a significant increase in transcript levels for the WT males on the 2 % cholesterol diet compared to the control group. ABCG8, a transporter involved in the excretion of cholesterol, was increased in both ArKO and WT when fed a 2% cholesterol diet compared to controls. In conclusion, ArKO males display hepatic steatosis, which is reduced when they are fed high cholesterol diets. Mechanisms which allow excretion of cholesterol are up-regulated in both ArKO and WT mice when there is excess cholesterol in the diet.

**164**

## THE EFFECT OF FETAL SERUM ON THE SYNTHETIC FUNCTION OF THE BRONCHIAL SMOOTH MUSCLE

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Recent studies have indicated that maternal asthma severity is increased during gestation in the presence of a female fetus. This data would suggest that a factor derived from the female fetus alters bronchial smooth muscle (BSM) function. Nevertheless, the specific mechanisms that cause these outcomes in asthmatic pregnancies are unknown. We hypothesise that a factor derived from the female fetus alters the synthetic function of maternal bronchial smooth muscle through the upregulation of factors involved in inflammation such as cell adhesion molecules (CAMs) and cytokines and through inhibition of pathways involved in BSM relaxation such as the β2-adrenergic receptor (β2-AR). Cultured bronchial smooth muscle cells were incubated with 20% male or female fetal serum for 6 and 24 hours. Asthmatic serum and serum free media were used as control treatments in this study. mRNA was extracted and real time RT-PCR was used to measure the message abundance of ICAM-1, VCAM-1 and β2-AR. At 6 hours, ICAM-1 mRNA (n=5) expression was upregulated whereas VCAM-1 mRNA levels (n=5) were inhibited in the presence of both male and female serum. β2-AR mRNA (n=5) was upregulated in the presence of male fetal serum but significantly down regulated in the presence of female fetal serum. Results at 24 hours showed an enhanced increase in ICAM-1 and β2-AR mRNA expression by female serum (n=5). No change was observed with VCAM-1mRNA in the presence of both male and female serum. Our preliminary results indicate that male and female fetal serum differentially affects synthetic function in the bronchial smooth muscle. Maternal asthma may be significantly altered during pregnancy in the presence of a female fetus due to alterations in β-2AR expression.

**165**

## PERIPUBERTAL INCREASES IN PLASMA LEPTIN LEVELS IN FEMALE MERINO SHEEP

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Leptin is produced by adipose tissue and plasma concentrations are correlated with fat stores in many species including the sheep (1). Leptin has been reported to be important in many systems including the reproductive system. In women, leptin levels vary over the menstrual cycle (2), as they do during the oestrous cycle in Merino ewes, with highest concentrations being recorded at the end of the luteal phase. The onset of fertility in maiden

ewes is influenced by their nutritional status (1,3), and the aim of this study was to investigate the changes in circulating leptin levels in young Merino ewes prior to the first sign of behavioural oestrus.

A flock of maiden Merino ewes (n=16) was run on open pasture with vasectomised rams (n=2) fitted with a harness and marking crayon to determine the onset of first behavioural oestrus. Blood samples were collected biweekly during this period via venipuncture. Plasma leptin concentrations were determined using an ovine leptin ELISA (4) and the data synchronised to the first day of behavioural oestrus. The onset of fertility in this group of ewes, ranged from 450 to 505 days of age. Plasma leptin levels were significantly elevated on days 43 (6.43±0.34 ng/ml) and 24 (6.14±0.4 ng/ml) prior to the onset of behavioural oestrus (4.33±0.33 ng/ml) ($P<0.05$). The interval of approximately 20 days between these leptin peaks and behavioural oestrus approximately corresponds to the duration of a normal oestrous cycle in Merino ewes. The timing of these peaks in plasma leptin may correspond to the occurrence of 'silent" oestrous cycles and may serve as a primer for the gonads. It remains to be determined if these peaks are unique to maiden ewes prior to the onset of fertility or if they also occur in mature ewes prior to their first behavioural oestrus each breeding season.

References:

(1) Blache D *et al*. 2000. *Journal of Endocrinology* **165**, 625-637

(2) Cella F et al. 2000. European Journal of Endocrinology **142**, 174-178

(3) Daniel JA *et al* 2002. *Journal of Animal Science* **80**, 1083-1089

(4) Kauter KG *et al.* 2000. *Journal of Endocrinology* **166**, 127-135

**166**

## PLACENTAL RESTRICTION AND ONTOGENY OF INSULIN-REGULATED GLUCOSE HOMEOSTASIS IN SHEEP

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Insulin sensitivity is reduced in children[1], young[2] and middle-aged adults[3] who were light or thin at birth, which may predispose them to their increased risk of diabetes in later life[4]. We have investigated the effects of placental restriction of fetal growth on the ontogeny of glucose tolerance, insulin secretion and insulin sensitivity, in the sheep.

Placental and hence fetal growth was restricted (PR) by surgical removal of most endometrial caruncles before pregnancy[5]. Studies were performed in 23 control (12 male, 11 female) and 24 PR sheep (8 male, 16 female). A hyperinsulinaemic euglycaemic clamp (120 minutes, 2 mU insulin/min/kg bodyweight) was performed at 35, 65, 127 and 373 days (adult) of age. Insulin sensitivity was calculated as the steady-state glucose infusion rate required to maintain euglycaemia, corrected for plasma insulin. An intravenous glucose tolerance test (IVGTT) (2 mg/kg bodyweight) was performed at 38, 67, 130 and 376 days of age. Glucose intolerance was calculated as area under the glucose profile (AUCG) and insulin secretion was calculated as area under the insulin profile divided by AUCG. Effects of age, gender and PR were examined by repeated measures ANOVA.

PR reduced birth weight (3.9 ± 1.1 kg vs. 5.3 ± 0.8 kg; $P<0.001$). Glucose tolerance, insulin secretion and insulin sensitivity decreased with age, and insulin secretion was higher in males than in females. PR exacerbated the impairment of glucose tolerance with age in males ($P=0.03$). In adult males, PR increased the initial rise in glucose during the IVGTT ($P<0.05$). Reduced size at birth and slow neonatal growth each independently predicted poor glucose tolerance in the adult, and predicted reduced insulin sensitivity in control adults alone ($P<0.05$ for each). Impaired glucose tolerance following reduced perinatal growth in the sheep thus appears to reflect defects in insulin sensitivity and/or glucose effectiveness, rather than insulin secretion.

1. Yajnik CS et al. 1995 *Diabetic Med* 12:330-336

2. Flanagan DE et al. 2000 *Am J Physiol* 278: E700-E706.

3. Phillips DIW et al. 1994 *Diabetologia* 37:150-154.

4. Barker DJP 1998 Mothers, babies and health in later life, 2nd ed. Churchill Livingston, Edinburgh.

5. Robinson JS et al. 1979 *J Dev Physiol* 1:379-398.

## 167

## PROGRAMMING OF APPETITE AND BODYWEIGHT BY PERINATAL DIETARY OMEGA-3 FATTY ACIDS AND ANGIOTENSIN.

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Pregnant Sprague-Dawley rats were fed one of two diets that differed only in the presence or absence of omega-3 polyunsaturated fatty acids (PUFA) during gestation and lactation. After weaning, male pups born to these mothers were either continued on the maternal diet (n=12) or switched to the opposite diet (n=12). Rats that were omega-3 PUFA deficient during gestation and lactation had increased food intake and body weight post-weaning compared with rats supplied with omega-3 PUFA during this time. Repletion of dietary omega-3 PUFA in perinatally deficient animals after weaning did not change the body weight profile of the rats in adulthood, indicating a programming effect of perinatal omega-3 fatty acids in the maternal diet. At 14 weeks of age, rats raised on deficient diets weighed 12% more than rats raised on deficient diets (P>0.01) and displayed increased daily food intake. We repeated the experiment using Sprague-Dawley females crossed with male homozygote m(Ren-2) rats, which have an increased expression of renin and angiotensin activity. Half of the male heterozygote pups were switched to the alternative diets after weaning. We discovered that the body weight profiles of the offspring in relation to dietary omega-3 PUFA supplementation were opposite to those found in the first study that used pure Sprague-Dawley rats. At 14 weeks of age, omega-3 PUFA sufficient m(Ren-2) heterozygote rats (n=24) weighed 12% more than omega-3 PUFA deficient (n=24) rats (P>0.001) and displayed increased food intake. Once again, switching of the diet after weaning had no effect on adult body weight. These two studies indicate that the presence of dietary omega-3 PUFA during gestation and lactation has a programming effect on adult food intake and body weight (irrespective of the diet that the rats received once they had been weaned). The opposite effect of dietary omega-3 fatty acid deficiency that was observed in a genetic model of renin-based hypertension (mRen-2) indicates that the programming effect is influenced by factors such as maternal diet and genetic background. The interaction between genetic and environmental factors during foetal and neonatal development can produce radically different effects on appetite and body weight outcomes in adult life.

## 168

## THE EFFECT OF COMMON VARIANTS IN MTHFR ON TWINNING AND EMBRYO LOSS.

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Folic acid is essential for the synthesis of nucleotide precursors for DNA replication and for cellular methylation reactions. During pregnancy, folate requirements increase. Folate deficiency influences embryonic development and increases the risk of neural tube defects (NTD) and recurrent early pregnancy loss. Supplementation with folate around conception reduces NTD, but may increase twinning rates. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism. Common variants of this enzyme result in the synthesis of an enzyme with reduced catalytic activity leading to elevated plasma concentrations of homocysteine, especially when dietary folate intake is low. Research has shown that certain MTHFR haplotypes are present in fetal tissue, but not amongst neonates, suggesting that MTHFR may influence embryo survival. A recent case-control study investigated the C677T polymorphism in mothers of dizygotic twins (MODZT) and found a higher frequency of the 677C allele amongst MODZT in comparison to women who gave birth to singletons. The authors suggest that the 677T allele of MTHFR is protective against multiple pregnancy. We investigated the presence of linkage and association between MTHFR and hereditary DZ twinning by examining the allelic transmission of two common MTHFR polymorphisms (C677T and A1298C) in 454 families with a total of 735 MODZT. Allele frequencies for the 677T and 1298C alleles were 0.33 and 0.31 respectively. Inherited twinning was not associated with segregation of the common variants at MTHFR. We also typed the variants in 462 families of DZ and MZ twins. Allele frequencies for the 677T and 1298C alleles were similar in this population (0.35 and 0.29 respectively).

There was no excess allele sharing among DZ twins that would be expected if MTHFR variants contributed to variation in DZ twinning. There was strong linkage disequilibrium between the two variants. The 677T/1298C haplotype was very rare. There was statistically significant evidence of segregation distortion with reduced transmission of the 1298C allele in both groups and consequently reduced transmission of the 677C/1298C haplotype. The data show no association between MTHFR and DZ twinning, but some variants of MTHFR may affect embryonic survival.

**169**

## THE THRESHOLD FOR ANDROGEN DEFICIENCY SYMPTOMS IN MEN WITH HYPOGONADISM OF DIFFERING ETIOLOGIES

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The schedule of administration of androgen replacement therapy (ART) is usually determined by reference to standard intervals based on average pharmacokinetics and pharmacodynamics of different, mostly short-acting testosterone (T) preparations which produce marked fluctuations in plasma T concentrations. By contrast, testosterone implants feature a depot, steady-state release of T over long periods exhibiting a gradual decline in T concentrations over months which might permit an unique view of the onset of the timing of androgen deficiency symptoms. The present study evaluated the timing and blood T concentrations of men returning for re-implantation of subdermal T implants to determine the symptomatic threshold for androgen deficiency symptoms, as defined by the blood T concentration when men's familiar androgen deficiency symptoms required them to return for re-implantation. We reviewed prospectively obtained data from 136 androgen deficient men undergoing 600 implantation procedures over 5 years using a standard T dose (800 mg) per procedure, who had blood samples taken when returning for re-implantation. Among men presenting for multiple reimplantations, the symptomatic threshold was highly consistent with no significant within-subject difference in total ($p=0.53$) and free ($p=0.57$) blood T concentrations at the time of re-implantations whereas there were striking and consistent between-subject differences. The total and free T concentrations at the time of reimplantation for all diagnoses were $11.3 \pm 0.6$ nmol/L and $165 \pm 11.4$ pmol/L, respectively, which were higher than the nadir values ($5.5 \pm 0.2$nmol/L and $81.1 \pm 4.3$ pmol/L), as expected ($p=<0.001$), reflecting their return for symptomatic treatment before reaching nadir blood T concentrations. The symptomatic threshold was significantly lower among men with secondary (hypogonadotrophic) hypogonadism compared with men with primary ($p=<0.001$) or mixed ($p=<0.05$) hypogonadism although the time between procedures was similar. Free testosterone was higher ($p=<0.05$) and SHBG was lower ($p=<0.05$) in the group with mixed hypogonadism as compared with the other two diagnoses. We conclude that onset of symptoms of androgen deficiency occurred at consistent blood T concentrations for each person, at about the lower limit of the eugonadal reference range for blood T concentrations. These unique empirical findings have implications for other potential androgen deficiency states such as men with systemic diseases and ageing.

**170**

## THE EFFECT OF 12 MONTHS ORAL TESTOSTERONE UNDECANOATE ON BODY COMPOSITION, MUSCLE STRENGTH AND PROSTATE FUNCTION IN ELDERLY MEN.

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Loss of muscle mass leads to frailty in older men. The decline in testosterone over the lifespan may contribute to this muscle loss. We studied the effect of low dose oral testosterone on muscle mass in older men over a twelve-moth period. Testosterone undecanoate (80mg twice daily) or placebo was administered for one year to 76 healthy men 60 years or older with a free testosterone index of 0.3 – 0.5. Body composition, muscle strength, hormones, and safety parameters were obtained at 0, 6, and 12 months. Twelve participants from the placebo and six from the testosterone group withdrew prior to completion.

At month 6, lean body mass increased 2.2% in the testosterone group and decreased 1.5% in the placebo group (P = 0.0001). Fat mass decreased 4.3% in the testosterone group and increased 4.2% in the placebo group (P = 0.0001). After 12 months, there was an overall increase in lean body mass of 1.5% in the testosterone group and a decrease of 1.6% in the placebo group (P = 0.0001) and fat mass had decreased 1.1% in the testosterone group and increased 4.6% in the placebo group (P = 0.02). There were no significant effects of testosterone treatment on muscle strength. At 12 months, sexual quality of life was better in the testosterone as compared to the placebo group (P = 0.069). The change in International Prostate Symptom Scores did not differ between the groups (testosterone -0.3 ± 4.0; placebo 0.9 ± 5.1, P = 0.25). Haematocrit increased 0.02% in the testosterone group (P = 0.03). Plasma triglycerides, total and LDL cholesterol levels were similar in both groups, but there was a 0.1 mmol/L decrease in HDL cholesterol in the testosterone group (P = 0.026). There were no differences in prostate specific antigen, systolic or diastolic blood pressures between the groups.

Low dose oral testosterone administration to older men with low-normal gonadal status results in increased muscle mass and decreased body fat, without an adverse effect on prostate function or urine flow. The clinical significance of the small decline in HDL cholesterol is not known.

**171**

## THE ACUTE EFFECTS OF HIGH DOSE TESTOSTERONE ON SLEEP IN AGEING MEN

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There are no placebo-controlled studies evaluating the effects of testosterone on breathing during sleep, although worsening sleep apnea is cited as a risk of testosterone therapy. Since such therapy is increasingly being evaluated in older men and age is associated with worsening sleep, we evaluated the safety of acute high dose intramuscular testosterone on sleep in a randomised placebo-controlled cross-over study. Seventeen ambulatory men over 60 years old without significant co-morbidities were randomised to receive 3 injections of mixed testosterone esters (500, 250, 250 mg at weekly intervals) or matching oil-based placebo. After a washout period of 8 weeks, subjects crossed-over to the other treatment. Sleep, body composition and physical activity was assessed at entry and at the end of each treatment period. The key polysomnographic variable is the total Respiratory Disturbance Index (RDI) which is the number of apnoeic events per hour of sleep. At baseline, mean (SE) age was 69 (1) years, Body Mass Index (BMI) was 27 (1) kg/m2 and total RDI was 15 (3) events/hour. Data was analysed by standard crossover (Grizzle) techniques: period and carryover effects were not detected. Testosterone therapy worsened breathing during sleep: total RDI (~7 events/hour, P=0.05) and non-REM RDI (~7 events/hour, P=0.02) were significantly increased, but no effect on REM RDI (P=0.80) was seen. Testosterone treatment reduced the time slept in total (57 minutes), non REM (41 min) and REM (16 min) sleep (all P<0.05). Sleep efficiency (time asleep/time in bed) was also significantly reduced (13%, P<0.05). A small but significant increase in BMI of 0.8 kg/m2 occurred in association with an increase in weight and lean body mass (Lukaski, bioimpedance) (all P<0.05). Physical activity assessed objectively (accelerometry) and by self-report (PASE questionnaire) and quality-of-life (SF36) were not changed by treatment. Blood pressure, PSA and lower urinary tract symptoms (IPSS questionnaire) were unchanged. We conclude that testosterone treatment in elderly men is associated with a reduction in total sleep time and sleep efficiency as well as an increase in sleep disordered breathing. This study shows that short-term supraphysiological testosterone supplementation can worsen sleep, particularly non-REM sleep, but does not exclude the possibility that lower dose supplementation in older men may be safe.

## 172

### THE RELATIONSHIP OF SERUM TOTAL TESTOSTERONE TO BODY COMPOSITION IN HEALTHY AGEING MEN

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Male ageing is associated with a decline in total and free testosterone [1] with current guidelines for the diagnosis of hypoandrogenism based on total testosterone levels [2]. To date randomized controlled data showing benefits of testosterone replacement in older men are limited. We are undertaking studies to evaluate the effects of testosterone replacement in healthy older men presenting with symptoms consistent with, but not diagnostic of, androgen deficiency with total testosterone levels <15nM. As obesity is an important determinant of total testosterone (levels falling with increasing body mass index) [3] we are treating only men with confirmed body mass index (BMI)<30kg/$m^2$ and abdominal circumference (AC) ≤102cm, although our screening procedure included subjects with BMI up to 35kg/$m^2$. This allowed us to examine the relationship of body composition and total testosterone levels across a wide range of body mass indices. Telephone interview of non-smoking men aged ≥55 years who responded to community advertisement was used to exclude those with significant medical illness. The arbitrary threshold for further assessment was a self reported BMI<35kg/$m^2$. Eligible men with symptoms in each of the categories of sexual function, strength and mood/cognition attended for assessment of BMI and AC, and 2 morning blood samples for total testosterone, SHBG, LH, FSH and free testosterone [4]. 177 men were identified with mean age 63.0 years (range 54-83) and mean total testosterone 13.9nM. 106 men had a BMI<30 and an AC ≤102cm. The mean total testosterone of this group (Group A) was 14.8nM (range 5.4 -34.2) compared to 12.6nM (range 4.7-26.7) for their peers with higher BMI and/or AC (Group B) (p=0.0008); there was no difference in the age distribution. Regression analysis showed that AC had a stronger relationship to total testosterone than BMI. The mean SHBG levels for the groups were 40.3nM (Group A) and 34.4nM (Group B) (p=0.005). The difference in calculated free testosterone levels just failed to reach significance; Group A 293.7pM, Group B 273.4pM (p=0.06). BMI and AC are significantly related to total testosterone and SHBG but not to free testosterone. This suggests that the use of total testosterone in the assessment of hypogonadism in older men may be confounded by obesity.

References

1. Gray et al. 1991 J Clin Epidemiol 44(7):671.
2. Conway et al. 2000 MJA 172:220.
3. Gray et al. 1991 JCEM 73(5):1016.
4. Sodergard et al. 1982 J Steroid Biochem 16(6):801.

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## 173

### TESTOSTERONE ENHANCES THE ANTINATRIURETIC AND METABOLIC EFFECTS OF GROWTH HORMONE

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Testosterone enhances growth of growth hormone (GH) deficient children during GH treatment. To determine whether testosterone augments the biological effects of GH in adult life, we have investigated the impact of GH alone and of combined GH and testosterone on extracellular water (ECW) and protein metabolism in hypopituitary men. Seven subjects were randomised to receive either GH (1.5 IU/d) or GH and testosterone (250mg IMI every 3 weeks) for one month, and then crossed over to the alternate treatment for the second month. Plasma testosterone and IGF-I, ECW and protein turnover were measured before and at the end of each treatment. ECW was measured using the bromide dilution technique. Protein turnover was studied using a 3 h primed constant infusion of 1-[13C] leucine, from which rates of leucine appearance (leucine Ra, an index of protein

breakdown), leucine oxidation (Lox, an index of irreversible oxidative loss of protein) and non-oxidative leucine disposal (NOLD, an index of protein synthesis) were estimated. Lox and NOLD are expressed as a percentage of Ra. GH significantly increased plasma IGF-I (7±1 to 24±3 nmol/L, $p<0.005$), and ECW (15±1 to 18±1 kg, $p<0.002$). Co-administration of testosterone increased the testosterone level into the normal range (2±1 to 12±2 nmol/L, $p<0.005$), and resulted in a further increase in IGF-I (to 26±3 nmol/L, $p<0.05$) and ECW (to 19±1 kg, $p<0.05$). In the protein turnover studies, GH increased NOLD (68±2 to 74±2%, $p<0.05$), and reduced Lox (32±2 to 26±2%, $p<0.05$). Addition of testosterone resulted in a further increase in NOLD (to 79±1%, $p<0.05$), and a further reduction in Lox (to 21±1%, $p<0.05$). There was no change in leucine Ra following either treatment. In summary, GH replacement in hypopituitary adults increased circulating IGF-I, ECW and protein synthesis, and reduced irreversible oxidative protein loss. Concurrent testosterone replacement further amplified these effects. We conclude that testosterone, in physiologic doses, enhances the anti-natriuretic and protein anabolic effect of GH, mediated in part through increased IGF-I production.

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## 174

### ENDOGENOUS OESTROGENS BENEFICIALLY INFLUENCE ENDOTHELIAL FUNCTION IN HEALTHY YOUNG MEN

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Oestrogens are known to influence endothelial function favourably in women, in spite of the fact that the clinical role of hormonal therapies after menopause remains uncertain. Males produce oestrogens endogenously from androgenic precursors via the enzyme aromatase. Previous studies have suggested a role for oestrogen supplementation in improving cardiovascular function in elderly men. To address question of a possible role for endogenous oestrogens in normal male physiology we examined the effects of aromatase inhibition on endothelial function and lipid levels in healthy young men. The study followed a placebo controlled, double blind, randomised design. Twenty healthy men, aged 18 to 32, were randomised to receive either the aromatase inhibitor anastrozole (1mg) or matching placebo. Hormone and lipid levels were measured and endothelial function was assessed by flow mediated dilation (FMD) of the brachial artery at baseline and after 6 weeks of treatment. No significant adverse effects of anastrozole were experienced. There was one withdrawal in the treatment group for unspecified reasons. Compared with baseline, a significant decrease was observed in FMD in subjects receiving anastrozole (median 6.1 (5.2-13.4) to 3.5 (2.0-5.7); $p=0.034$), but not in the placebo group. No changes were observed in GTN-induced endothelial independent dilation in either group. There was a significant decrease in oestrogen concentrations with aromatase inhibition (mean 85.4±4.2 pmol/L to 64.3±8.1 pmol/L; $p=0.042$). No significant changes occurred in total cholesterol, triglycerides, total testosterone or DHEA levels in either the anastrozole or the placebo group. We conclude that suppression of endogenous oestrogens with an aromatase inhibitor results in impairment of endothelial function, as measured by flow mediated dilation. Our results suggest that endogenous oestrogens may play a significant role in maintaining endothelial function in healthy young men. The clinical significance of these findings remains to be determined.

## 175

### PURIFIED ISOFLAVONES IMPROVE ARTERIAL FUNCTION IN MEN AND WOMEN.

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Previous studies have suggested that estrogen improves arterial reactivity. More recently, many other compounds aside from estrogen have been demonstrated to have estrogenic activity. Isoflavones are phytoestrogens with binding affinity for the estrogen receptors, especially ER-□ in the arterial wall. Included in the isoflavone compounds are biochanin, a precursor of genistein and formononetin a precursor of daidzein, both found in high concentration in red clover. In this trial, we have extended previous studies of arterial biomechanics by investigating the effects of these 2 purified isoflavones, biochanin and formononetin, on arterial functional

markers. These vascular functional markers have been shown to be accurate, reproducible, to correlate with cardiovascular risk factor status and to predict clinical cardiovascular events. They include arterial compliance (SAC), pulse wave velocities (PWV) in the central (aorto-femoral A-F) and peripheral circulations (F-D) as well as endothelial function, reflected by flow-mediated dilation (FMD). In a randomized, double-blind, placebo-controlled trial, 46 men and 34 women took 40mg/d of either biochanin or formononetin in a 2x5wk cross-over with placebo. Isoflavones versus placebo (n=80) significantly improved central arterial distensibility (elasticity) with raised SAC and lowered PWV A-F (P=0.041 and 0.021 respectively; RM ANOVA adjusted by Bonferroni procedure). This was primarily attributable to formononetin, where SAC rose (P=0.016, adjusted =0.063) and PWV A-F fell (P=0.023, adjusted 0.069). The effect appeared to be mediated by a change in total peripheral resistance (P=0.016, adjusted 0.032), with no change observed in 24hr ambulatory blood pressures with treatment. In conduit arteries there was a trend for biochanin to improve FMD; P=0.061 unadjusted) but PWV F-D was unaffected. There was no gender interaction. Moderate consumption of purified isoflavones beneficially affects the central arterial circulation, more so than the muscular conduit arteries.

**176**

## ASSESSING THE PROGNOSTIC POTENTIAL OF CD82, A METASTASIS SUPPRESSOR, IN PRIMARY PROSTATE CANCER

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Prostate cancer (CaP) is a common disease worldwide and its impact on the male community is significant. Acquisition of the metastatic phenotype is the most fatal aspect of CaP progression, thus detection of CaP while it is still localised is of great importance. Unfortunately, current prognostic methods cannot accurately predict all patients who are at highest risk of metastases. In this study, expression of the CD82 metastasis suppressor gene was examined in benign prostatic hyperplasia (BPH) and primary CaP specimens to determine whether its expression correlates with tumour grade and clinical outcome. CD82 protein levels were measured by quantitative immunohistochemical analysis in BPH not associated with cancer (n=6), BPH associated with CaPs (n=39) and well (n=13), moderately (n=15) and poorly (n=11) differentiated CaPs. Altogether, CaPs exhibited significantly higher levels of CD82 expression compared to BPH not associated with cancer (P=0.022, unpaired two sample t-test). Increased CD82 expression in well and moderately differentiated CaP specimens, above levels seen in BPH, were also observed (1). CD82 levels in poorly differentiated cancers, though increased, tended towards levels in BPH. These results mirror our previous findings of biphasic CD82 mRNA expression in primary CaP (2). Interestingly, BPH surrounding CaPs exhibited significantly higher CD82 levels than that observed in BPH not associated with any cancer (P=0.009, paired t-test), even though the BPH appeared histologically similar in both situations. In conclusion, CD82 is overexpressed in BPH associated with CaP and early stage CaP, but is lost in poorly differentiated CaP. Thus, CD82 overexpression may restrain cancer onset, whilst its loss may predispose the patient to more aggressive cancer behaviour and metastatic disease. Altered CD82 expression in primary CaPs and BPH associated with cancer could have important diagnostic and prognostic roles. The future aim is to correlate these data with prediction of clinical outcome.

1. Lijovic et al. Cancer Detect Prev 2002; 11:1-9.

2. Bouras & Frauman. J Pathol 1999;188: 382-388.

## 300

### USE OF A HUMAN COMMERCIAL KIT TO ASSAY OESTROGEN DURING BOVINE CYCLES WITH DIFFERENT FOLLICULAR WAVES

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Oestrogen is a difficult hormone to measure in the bovine plasma because most commercial kits are specifically designed for the range of concentrations that is typical of humans. The aim of this study was to measure plasma concentrations of oestrogen by radioimmunoassay, using human commercial kits (DPC MEDLAB – solid phase), and link the data to follicular dynamics during oestrous cycles of Nelore heifers.

The samples were collected every day and were analyzed at Days 0 (oestrus), 1, 2, 3, and then every 3 days, until the Days -1 and 0 of the following cycle. Sample numbers for each day differed according to variation in the duration of the oestrous cycles. The data were transformed in log to get the results. Follicular growth curves and ovulation were observed daily by ovarian ultra-sound.

Heifers displayed two, three or four follicular waves during their cycle and these corresponded respectively to two, three and four peaks of oestrogen (Table 1). The day of each oestrogen peak during the luteal phase coincided with the day of maximum diameter of the morphologically dominant follicle and the highest oestrogen peak was observed during oestrus. In this case, it was not up to do statistical analyze because of these fluctuations and of wide variation individual, where oestrogen peaks followed follicular growing, whose have growing rate self and are different to each animal. Then, while a heifer had a functional dominant follicle with maximum oestrogen production, another ones had follicles beginning their growing or decreasing at same day of the cycle.

Despite difficulty to dosage oestrogen in bovines with human kit because it displays low results, it was possible to use them. However, it would be important to dosage oestrogen using bovine antibody to compare the data, becoming useful to use those kits in laboratories that don't produce oestrogen bovine antibody.

## 301

### OPTIMIZATION OF SOLID PHASE EXTRACTION PROCEDURE FOR ANALYSIS OF ESTROGENS AND PROGESTOGENS IN BIOLOGICAL SAMPLES

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Solid Phase Extraction (SPE) is a sample preparation technique for the pre-purification, isolation and concentration of components from a complex matrix of either liquid or solid form. SPE procedures are more efficient and less time consuming than classical liquid/liquid extraction, particularly when working with biological samples **[1,2]**. The aim of this work is to demonstrate the capability of SPE for the isolation and pre-purification of wide range of steroid hormones from urine samples.

Studies were conducted using three progestogens (progesterone, 17α-hydroxyprogesterone and 20α-hydroxyprogesterone) and six estrogens (estetrol, estriol, 17α-estradiol, 17β-estradiol, estrone and equilin). Each individual steroid was selected because of its biological importance during pregnancy. The SPE system used was composed of an octadecylsilane stationary phase (C-18, 500 mg, 6ml Supelclean$^{TM}$ cartridges; Supelco) and binary methanol-water mobile phases from 0 to 100%(v/v) with increments of 10%. The extraction was performed using a vacuum manifold (Visiprep$^{TM}$, Supelco). The breakthrough volumes of steroids were determined by recording a frontal chromatogram produced by discretely monitoring the appearance of the individual steroids at the outlet of the SPE cartridges.

Based on the breakthrough curves, the volumes of washing and elution solvents were optimised and a final extraction protocol was established. For this extraction of steroids, the SPE cartridges were equilibrated with 1%

of methanol in water. After sample application the cartridges were washed with a 30% (v/v) methanol solution and sucked dry. The isolated compounds were eluted with 4 x 0.5 mL of 100% methanol. Samples were evaporated and then reconstituted in mobile phase for further separation and quantification using high-performance liquid chromatography (HPLC). The influence of the urine matrix and the effect of sample pre-treatment, on extraction efficiency, were also studied. Under optimal conditions the total recovery of steroids ranged from 82% (estrone) to 100% (17α-hydroxyprogesterone). The results were compared with published previously data **[2,3]**.

The described Reversed-Phase Solid Phase Extraction method is rapid, accurate and reproducible and could be a reliable alternative to other isolation methods for steroids including liquid-liquid extraction or thin-layer chromatography. Due to high efficiency in recovery of steroids from the biological matrix, the sample volume can be significantly reduced. The procedure has been successfully applied to extraction of estrogens and progestogens from human urine.

**[1]** D. S. Seibert, Colin F. Poole; J. High Resol. Chromatogr., 21 (1998) 481-490.

**[2]** Guide to Solid Phase Extraction, Bulletin 910, Supelco 1998.

**[3]** H. Lamparczyk, P. K. Zarzycki, J. Nowakowska, R. J. Ochocka; Chromatographia, 38 (1994) 168-172.

**302**

## ROLE OF LOCAL ESTROGEN METABOLISM IN THE CONTROL OF ESTROGEN ACTION IN HUMAN PARTURITION

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In most mammals, increased estrogenic activity at parturition is required for labour and delivery and is achieved by a rise in circulating estrogens. However, in humans, circulating estrogens [estrone (E1), estradiol (E2) and estriol (E3)] are high for most of pregnancy and target tissues (uterus, cervix and fetal membranes) appear to be refractory to estrogen action except at parturition. Therefore, we hypothesised that in human pregnancy, estrogen actions are controlled by changes in target tissue responsiveness. A possible mechanism for this is by the interconversion of circulating estrogens to more (E1 to E2) or less (E2 to E1) active forms by the family of 17βhydroxysteroid dehygrogenase (17βHSD) enzymes. To test this hypothesis, we determined whether the capacity of the amnion, chorion, decidua and myometrium to interconvert circulating E1 and E2 changes with the onset of labour and if so, whether that change is consistent with increased E2 availability to target cells and expression of specific 17ßHSD isozymes. 17ßHSD oxidative (E2 to E1) and reductive (E1 to E2) activities were measured in specimens of amnion, chorion, decidua and myometrium collected from labouring (n=6) and non-labouring (n=6) term deliveries. In the labouring specimens, the oxidative and reductive activities were highest in the chorion, followed by amnion and then decidua. In non-labouring samples, activity in the amnion and decidua were similar and higher than that of chorion. In both labouring states, 17βHSD activity in the myometrium was barely detectable. In amnion, decidua and myometrium there was no significant difference in the 17ßHSD activities before and during labour. However, in labouring chorion the reductive (E1 to E2) activity was significantly ($P<0.017$) increased. This increase in 17βHSD reductive activity in the chorion was not due to increased expression of the 17ßHSD isozyme genes as assessed by quantitative real-time RT-PCR. This indicates that human parturition is associated with a shift towards increased estradiol availability in the chorion but not in the other estrogen targets examined and that this change is not attributable to altered expression of known 17ßHSD genes. This is consistent with the hypothesis that estrogen action in human parturition is, at least in part, modulated by local metabolism.

## 303

### GLUTAMATERGIC NEURONS IN THE ARCUATE/VENTROMEDIAL COMPLEX EXPRESS ESTROGEN α RECEPTOR AND PROJECT TO PREOPTIC AREA IN EWES

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During the follicular phase of the estrous cycle, plasma estrogen (E) levels rise and act on the brain to cause a pre-ovulatory surge of gonadotropin releasing hormone (GnRH) secretion. Although GnRH neurons, located mostly in the rostral preoptic area (POA), do not possess high levels of estrogen receptor (ER), cells of the arcuate/ventromedial complex (ARC/VMH) express this receptor and implantation of E into this region causes GnRH/gonadotropin surges. Furthermore, E injection induces Fos responses in neurons of the ARC/VMH . Glutamate is the major excitatory neurotransmitter in the brain and is implicated in the regulation of GnRH secretion. The aim of the present study was to determine whether glutamatergic neurons in the ARC/VMH express ERα and project to the POA. Four ewes were killed by pentobarbitone overdose and their heads perfused with 10% formalin. Forty µm sections were cut from frozen blocks of POA and processed for immunohistochemistry. Immunolabeling for ERα was revealed with Ni-enhanced diaminobenzidine (Ni-DAB) and this was followed by labeling with an antibody against vesicular glutamate transporter-2 (VGluT-2) a marker for glutamatergic neurons that was revealed by DAB. Eighty percent of glutamatergic neurons in the ARC/VMH complex co-expressed ERα. Based on these findings, the retrograde tracer, FluoroGold was microinjected (50nl) into the rostral POA of 3 ewes which were killed 3 weeks later. The brains were perfused with Zamboni's fixative and 40µm sections were processed for immunohistochemistry using VGluT-2 antibody that was visualized with a red fluorescent tag. Of the FluoroGold-labeled cells found in the ARC/VMH complex, 20% expressed glutamate, indicative of a projection to the rostral POA. In conclusion, most glutamatergic neurons in the ARC/VMH possess ERα and some glutamatergic neurons in this region project to the rostral POA, where GnRH cells are found; these may be involved in the mediation of the feedback effects of E on GnRH secretion.

## 304

### SEASONAL CHANGES IN EXPRESSION OF PREPROOREXIN (PPORX), MELANIN CONCENTRATING HORMONE (MCH) AND LEPTIN RECEPTOR (OB-RB) MRNA IN OVARIECTOMISED (OVX) CORRIEDALE EWES IN RELATION TO APPETITE AND BREEDING SEASON

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The breeding season of Corriedales extends from July to December, whereas VFI is maximal between December and April and minimal in August/September. Seasonal VFI correlates with neuropeptide Y (NPY) expression in the arcuate nucleus (ARC). (1). The aim of the present study was to examine gene expression of ppORX, MCH and Ob-Rb across the year in relation to the seasonal breeding and VFI cycles. OVX Corriedale ewes (4-5/group) were maintained under natural conditions and killed in February (breeding season) , September, October and December (anestrus). Mean (±SEM) VFI (g/kg/day) was greater ($P<0.05$) in February (21.7±0.8) and December (24.5±1.0) than September (14.4±2.7) and October (14.9±3.5). Sheep were killed (overdose of pentobarbital) and brains collected for in situ hybridization. Sections were selected from the perifornical area (PFA), the dorsomedial hypothalamus (DMH), the lateral hypothalamic area (LHA) and the ARC. In situ hybridization was performed with $^{35}$S-labeled ppORX, MCH or Ob-Rb riboprobes and subjected to image analysis. Expression of ppORX, MCH and Ob-Rb was greater in the breeding season (Feb) than in anestrus (Table 1). Similar results were obtained in the PFA, DMH and LHA. Some differences were also seen between Oct and Dec. Expression of the genes for ppORX, MCH and Ob-Rb did not correlate with VFI. We conclude that these alterations in gene expression relate to seasonal changes in reproductive status rather than being involved in appetite regulation. NPY still appears to be the dominant regulator of appetite in the seasonal ewe (1).

Table 1 Expression of ppORX, MCH and Ob-Rb across the year for OVX Corriedale ewes.

| Time of Year | | February | September | October | December |
|---|---|---|---|---|---|
| ppORX in DMH | Cell # | 89±9.5 [c] | 66±3.1 | 64.8±6.9 | 66±6.5 |
| | Grains/cell | 933.6 ±58.2 [a] | 579.6±20 | 605.2±18 | 418.1±17.2 [d] |
| MCH in LHA | Cell # | 192.6±12.7 [b] | 87.8±18 | 95.8±8.2 | 119.3±15.9 |
| | Grains/cell | 1543.4±125.5 [b] | 778.5±139.3 | 862.1±93.9 | 649.5±57.9 |
| Ob-Rb in ARC | Cell # | 132.8±2.8 [c] | 88±8.4 [f] | 67.8±6.4 | 51.2±3.1 [g] |
| | Grains/cell | 377.9±11.8 [b] | 226.4±15.1 | 172.2±5.5 [e] | 166±7.9 [e] |

a:P<0.001, b:P<0.01, c:P<0.05 compared to all other groups.

d:P<0.001 vs September and October.

e:P<0.01 vs September.

f:P<0.05 vs October and g:P<0.05 vs September and October.

Reference 1: Clarke et al, (2000) J. Neuroendocrinology 12, 1105

**305**

## REPRODUCTIVE HORMONE RESPONSES TO ACUTE EXERCISE IN YOUNG EUMENORRHEIC WOMEN

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The impact of physical activity on female reproductive function has focused primarily on the association of chronic (long term) exercise with reproductive dysfunction in athletes. There have been relatively few studies, however, on the impact of acute (single bout) exercise on female reproductive function. The aim of this study was to determine the independent effects of acute maximal exercise and acute sub-maximal exercise on circulating female hormone concentrations in the follicular and luteal phases of the menstrual cycle young, sedentary women.

Fourteen healthy, sedentary females (21.8 ± 1.0 yrs; mean ± SEM) with normal menstrual function participated. Subjects performed 2 incremental exercise tests to exhaustion and 2 submaximal steady state exercise tests (20min at 25% $VO_{2peak}$, 20min at 75% $VO_{2peak}$) in the follicular (day 5-7) and luteal (day 23-25) phases of the menstrual cycle. Exercise testing days were determined from menses calendars and hormonal determination of ovulation as confirmed by serum hormone concentrations. Blood samples were taken for plasma 17β-estradiol ($E_2$), progesterone (P), follicle stimulating hormone (FSH) and lutenising hormone (LH) derterminations using an automated chemiluminescent assay system (ADVIR Centaur System, Bayer). Plasma $E_2$ concentrations (conc.) increased in both the follicular (pre: 131.7 ± 34.9, post: 169.5 ± 40.4; pmol·$L^{-1}$, p< 0.01) and luteal (pre: 348.0 ± 61.6, post: 448.7 ± 81.4; pmol·$L^{-1}$, p< 0.01) phases following acute maximal exercise. In contrast, there was no change in the plasma $P_4$ conc. in response to acute maximal exercise in either the follicular or luteal phase. During the luteal phase, high intensity exercise increased plasma $E_2$ conc. when compared to low exercise intensity exercise or basal conditions (p< 0.01). There was no change in either plasma FSH or LH conc. following steady state submaximal exercise at either low or high intensity levels.

In summary, maximal exercise to exhaustion and moderate duration high intensity exercise is an effective stimulus for increasing circulating 17β-estradiol conc in the absence of changes in the plasma rogesterone, FSH and LH conc. We conclude that therefore the alterations in circulating estradiol with exercise are intensity dependent, are independent of hypothalamic-pituitary control and may relate to exercise induced changes in the clearance of this hormone from the circulation.

## 306

### ESTROGEN HAS A ROLE IN REGULATING APOPTOSIS IN THE BRAIN

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Recent published literature has demonstrated increasing evidence on the possible protective role of estrogen on the brain. In order to evaluate such reports, we have utilised a unique model, the aromatase knockout (ArKO) mouse. The ArKO mouse is unable to synthesise estrogen, as the gene encoding the enzyme aromatase has been disrupted, rendering it non-functional. Therefore, this model allows us to examine the effect of a total lack of estrogen on the brain, whilst previous studies have been limited by using gonadectomized models, which still have some level of estrogen in the brain. In order to study the effects of estrogen on the brain, four groups of female mice were used: young (16 wks) and old (1yr) ArKO, and young and old wild type littermate controls. Brains were sagittally sectioned at 5μm and TUNEL (TdT-mediated dUTP nick end labelling) staining was performed to label apoptotic cells. ArKO mice, both young and old, showed apoptosis occurring in the frontal cortex of the brain, whilst none was detectable in wild type mice. This evidence of apoptosis occurring in ArKO mice was further confirmed at the molecular level by conducting an RNase protection assay on RNA extracted from the forebrains of ArKO (10-12wk old) and WT mice. RNase protection assays was applied to measure the differential expression of apoptotic genes, using two templates: mAPO-2, containing the mitochondrial pathway or bcl-2 family of proteins, and mAPO-3, containing the death receptor proteins e.g. FAS, FASL. These templates contained inhibitors and promotors of apoptosis, and in addition two control probes, GAPD and L32. Statistically significant down-regulation of bcl-W ($p=0.0317$) and bfl-1($p=0.0079$) transcript levels (both anti-apoptotic proteins) were observed in the female ArKO when compared with WT, while statistically significant up-regulation of TRADD ($p=0.0159$) transcript levels (a pro-apoptotic protein) was seen in ArKO when compared with wild type forebrains. Three week exogenous administration of 17β-estradiol partially recovered the phenotype in young ArKO female mice. These results suggest that indeed estrogen has a major role to inhibit apoptosis in the brain, and hence estrogen has neuroprotective functions. This is the first *in vivo* evidence that estrogen is essential for the integrity of neurons.

## 307

### IMPAIRED MAMMARY PTHRP CONTENT AND FUNCTION AND NEWBORN GROWTH DURING LACTATION IN THE SHR

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Parathyroid hormone-related protein (PTHrP) has proposed important roles during lactation including stimulation of mammary gland and newborn growth and differentiation; increasing calcium transport from blood to milk; regulation of mammary blood flow and myoepithelial cell tone; and regulation of maternal and neonatal calcium homeostasis. The spontaneously hypertensive rat (SHR) is associated with hypertension and intrauterine and postnatal growth restriction. The aim of this study was to determine the relative roles of the newborn suckling and maternal lactational environment on newborn growth and mammary and milk PTHrP and calcium concentrations in the SHR and its control, the Wistar Kyoto (WKY) rat.

On days 1, 3, 6, 14 and 28 after birth, WKY and SHR mothers and their pups were studied. SHR pups were also cross fostered (on the day of birth and studied on day 6) onto WKY mothers (SHR-on-WKY) while WKY pups were cross fostered onto SHR mothers (WKY-on-SHR) and were compared to normally fostered WKY (WKY-on-WKY) and SHR (SHR-on-SHR) pups. Plasma and milk were collected and analysed for PTHrP and calcium concentrations. Mammary tissue was analysed for PTHrP content and morphology. Data were analysed by ANOVA.

SHR newborns were lighter compared to WKY after 6 days ($P<0.05$). SHR mammary tissue PTHrP content was 35-70% lower and morphological development impaired compared with WKY in the first two weeks of postnatal life ($P<0.01$). SHR newborns were exposed to milk with reduced PTHrP and calcium concentrations compared to WKY ($P<0.0001$). Cross fostering a SHR pup onto a WKY mother increased newborn weight at 6 days

(compared to SHR-on-SHR) in association with normal mammary function and higher milk PTHrP and calcium concentrations ($p<0.05$). In contrast, the WKY pups who suckled on a SHR mother with impaired mammary function and morphological development had a reduced body weight at 6 days ($p<0.05$). The cross fostering studies demonstrated that mammary PTHrP content and milk PTHrP concentrations are regulated independently and are influenced by maternal mammary development and function as well as newborn suckling.

Our results suggest that impaired SHR mammary function and milk composition as well as newborn suckling contributes to the reduced postnatal growth of the SHR. We conclude that the postnatal lactational environment is critical to ensure normal postnatal growth and may play a role in the development of hypertension in the SHR.

**308**

## DEVELOPING OF AN ANALYTICAL METHOD BASED ON SUPRAMOLECULAR CHEMISTRY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR SEPARATION AND QUANTIFICATION OF SEX STEROIDS

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The simultaneous quantification of wide range of steroid hormones relevant to parturition in humans is still an unsolved analytical problem. In clinical practice compounds like sex steroids are usually determined using radioimmunoassay (RIA) methods. However, the increasing proliferation of RIA has raised serious problems associated with the handling of radiolabelled material and the waste disposal of radioactivity. Chromatographic techniques may be employed for quantification of steroids in biological fluids as an alternative to RIA methods.

The objective of this work is to study the chromatographic behaviour of selected sex steroids and their inclusion complexes with β-cyclodextrin using stationary phases with different carbon loads to determine if an improvement in resolution power for key steroids can be achieved [1-3].

Separation studies of both free estrogens and progestogens and their compexes with β-cyclodextrin were conducted using C-18 HPLC columns with different carbon loads. The difference in carbon load between investigated materials was about 50%. The mobile phases were composed of a 30% v/v acetonitrile-water mixture without and with addition of cyclodextrin (12mM). The temperature of chromatographic column was controlled using a water jacket connected to circulating thermostat.

The experimental data revealed that retention of the steroids was significantly reduced on the column with the lower carbon load. Moreover, it was found that this column offers better separation power. The retention and separation of the steroids on the investigated chromatographic systems can be controlled in two independent ways. These are the interaction of steroids with the C-18 chains of the stationary phase and the intensity of host-guest interactions in the mobile phase. In terms of the practical application, the total analysis time of the steroid mixture can be reduced two-fold by using a column with a low carbon load and six-fold by addition of β-cyclodextrin to the mobile phase. Under these conditions the battery of sex steroids was quantified in urine extracts of non-pregnant and pregnant subjects.

The proposed analytical procedure can be used as an effective tool for separation and quantification of wide range of steroids in biological samples. The method is specific, reproducible and should be useful for therapeutic drug monitoring as well as for clinical and biomedical investigations.

[1] H. Lamparczyk, P. K. Zarzycki, J. Nowakowska, R. J. Ochocka; Chromatographia, 38 (1994) 168-172.

[2] P. K. Zarzycki, R. Smith, J. Chromatogr. A., 912 (2001) 45-52.

[3] P. K. Zarzycki, K. M. Kulhanek, R. Smith, J. Chromatogr. A., in press

## 309

### THE ACUTE ADMINISTRATION OF THE SELECTIVE SEROTONIN REUPTAKE INHIBITOR (SSRI) ANTIDEPRESSANT, SERTRALINE, ACTIVATES THE HPA AXIS IN FEMALE, BUT NOT MALE, SHEEP

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Evidence is plentiful for a link between depression and dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis and many effective antidepressant medications have been shown to activate the HPA axis in normal individuals. This effect is likely to be mediated through monoamines such as noradrenaline and serotonin, since these transmitters regulate the HPA axis, and brain levels of both monoamines are affected by antidepressants. As the incidence of depressive illness and the effectiveness of antidepressant therapy may differ between males and females, the aim of the current study was to compare the effects of the acute administration of impramine (a classical tricyclic antidepressant) and sertraline (an SSRI) on the HPA axis in male and female sheep. Gonadectomised male (n=5) and female (n=5) sheep were given s.c. injections of imipramine (2 and 5 mg/kg), sertraline (2 and 5 mg/kg) and vehicle (50% DMSO, 0.2 mL/kg). Blood samples were collected every 10 mins for 1 h prior and 2 h after the injection. Plasma ACTH and cortisol concentrations were measured by RIA.

When compared with vehicle administration, sertraline increased ($P<0.05$) the concentrations of both ACTH and cortisol in females, but had no effect in males. In contrast, neither dose of imipramine affected the concentrations of either hormone. Both sertraline and imipramine, however, stimulated an increase in the plasma concentrations of prolactin, an effect consistent with their actions on serotonin turnover.The apparent sensitivity of the ewes to sertraline, as well as the substantially higher incidence of depression in women, may argue for a sex-related difference in the serotoninergic control of the HPA axis. It is unclear, however, if this differential activation of the HPA axis between the sexes is related to either the aetiology or therapy of depressive illness.

## 310

### THE PHYSIOLOGICAL ROLE OF GLUCOCORTICOIDS DURING EMBRYONIC DEVELOPMENT: ANALYSIS IN GLUCOCORTICOID RECEPTOR NULL MICE

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Glucocorticoids regulate many physiological processes including aspects of embryonic development. The action of glucocorticoids in target tissues is predominantly mediated by the glucocorticoid receptor (GR) which after activation by hormone functions as a nuclear transcription factor. We have previously investigated the specific role of GR in adrenal steroid action in vivo using mice with a gene-targeted disruption of exon 2 of the GR gene (1-4). Isogenic GR-null 129sv mice all die at birth from pulmonary atelectasis and have defects in adrenal, liver, and hypothalamic-pituitary-adrenal axis function. In the following studies we have further investigated developmental defects in the embryonic lung of GR null mice and further analyzed the role of GR signaling in thymic T-cell development. In the lungs of GR null mice prior to birth analysis by electron microscopy shows marked reduction in numbers of differentiated type-1 alveoli cells yet relatively normal numbers of surfactant-producing type 2 alveoli cells. There is clear evidence of surfactant production and secretion, with normal expression of the surfactant protein genes. We have shown previously that in fetal GR-null mice GR signaling is not required for normal T cell development and selection in the embryonic thymus (2, 3). We have now generated GR-null thymocyte-reconstituted adult mice and analysis both in the thymus and spleen show no alterations in thymocyte populations clearly indicating that glucocoticoid signaling via GR is also not required for normal T cell development in the adult thymus of the mouse.

1. Cole T.J., Blendy J.A., Monaghan A.P., Krieglstein K., Schmid W., Aguzzi A., Fantuzzi, G., Hummler E., Unsicker K., Schütz G. (1995) Genes Dev. 9, 1608-1621.

2. Purton J.F., Boyd, R., Cole T.J., Godfrey D.I. (2000) Immunity 13, 179-186.

3. Godfrey D.I., Purton J.F., Boyd, R., Cole T.J. (2000) Immunol Today 21, 606-611.

4. Cole T.J., Myles K., Purton J. F. Brereton P. S., Solomon N. M., Godfrey D. I., Funder J. W. (2001) Mol Cell Endocrinol 173, 193-202.

## 311

### CORTICOTROPIN RELEASING HORMONE CAUSES VASODILATION IN HUMAN SKIN VIA MAST CELL DEPENDENT PATHWAYS

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Corticotropin-releasing hormone (CRH) plays a central role in orchestrating the hypothalamic-pituitary-adrenal (HPA) axis and stress response, and is known to be a potent vasodilator in a number of vascular beds. CRH is produced predominantly in the central nervous system, however skin also expresses CRH and CRH receptors, which may suggest a potential role for the peptide in the regulation of microvascular tone. Previously we have shown that CRH acts via a CRH receptor to cause dilation in human skin (1) and that CRH induced dilation was more potent in premenopausal females than males. However the mechanism by which CRH causes dilation is presently unknown. We have examined the mechanism by which CRH may cause dilation in human skin using laser Doppler and iontophoresis. CRH (1nM) was administered subcutaneously by iontophoresis to a small area of skin in the female forearm and laser Doppler flowmetry measured blood flow in the same area simultaneously. Human CRH (1nM) caused a dose dependent vasodilatation in skin circulation (n=6) and was significantly reduced in the presence of the mast cell inhibitor, sodium cromoglycate (ANOVA $P<0.01$, n=6), and the histamine H1 receptor antagonist, promethazine hydrochloride, (ANOVA $P<0.01$, n=6). However, CRH-induced dilation was not inhibited by the H2 receptor antagonist, ranitidine, (n=4). CRH-induced dilation was also significantly reduced in the presence of the nitric oxide synthase inhibitor, N□-Nitro-L-arginine methyl ester, (ANOVA $P<0.01$, n=6). CRH-induced dilation was not inhibited by the cyclooxygenase inhibitor, piroxicam, (n=6). These findings suggest that CRH causes vasodilation in human skin via mast cell degranulation, and is principally mediated by histamine (H1) receptors and nitric oxide. This study contributes to our understanding of the regulatory mechanisms in the peripheral circulation. The gender specific nature of this CRH response may be implicated in the difference in cardiovascular risk seen between males and females.

(1) Clifton et. al. (2002) J Clin Endo Metab. 87:267-70.

## 312

withdrawn

## 313

### EFFECT OF SIZE AT BIRTH ON SALIVARY CORTISOL IN THE ADULT GUINEA PIG.

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Studies in humans have shown that small size at birth is associated with increased hypothalamo-pituitary-adrenal (HPA) axis activity in later life, which could in turn contribute to the associated increased risks of developing diabetes, hypertension and cardiovascular disease. The precise timing and extent of such prenatally induced alterations in HPA axis activity, in terms of cortisol secretion and circulating cortisol, in the adult human, is unclear however. There is also limited and conflicting experimental evidence for this in other species to date. We therefore hypothesised that reduced size at birth would increase salivary cortisol, a marker of circulating free cortisol, in the adult guinea pig. Male and female offspring of known size at birth were studied every 60 days between 120 and 400 days of age, when saliva was sampled at two-hourly intervals from 8 am to 4 pm. Salivary cortisol concentration was determined by specific radioimmunoassay without extraction. The effects of age, gender, size at birth and time of day on individual, mean, integrated, maximum and minimum salivary cortisol

concentrations were examined by analysis of variance, co-variate and correlation analysis. Mean, integrated, minimum and maximum salivary cortisol concentrations varied with age, gender and their interaction ($p<0.05$ for all). Individual salivary cortisol concentrations also varied with time of day (T), birth weight tertile (W) and differently with age (A) and gender (G) ($T \times W \times A \times G$, $p=0.001$). In general, salivary cortisol decreased in males and increased in females with age and was higher in males than in females ($p<0.02$ for all). Decreasing weight, length or increasing thinness at birth predicted increasing mean, afternoon and maximum salivary cortisol concentrations in early adult life in males (120 days of age) and in later adult life in females (240-300 days) ($p<0.05$ for all). Therefore restricted fetal growth increases HPA axis activity, as indicated by increased salivary cortisol concentrations, in the adult guinea pig. The presence of these prenatally induced increases in cortisol abundance varied with age and gender however, suggesting that so may their contributions to impaired metabolic and cardiovascular homeostasis. Whether fetal growth restriction also contributes to the latter by inducing altered sensitivity to cortisol postnatally remains to be determined.

## 314

## CHANGES IN ADRENAL RESPONSIVENESS DURING RAT PREGNANCY: THE ROLES OF ACTH RECEPTOR AND STAR.

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We have previously identified sexual dimorphism and pregnancy-induced changes in the association between plasma ACTH and corticosterone. Specifically, the adrenal appears more responsive to ACTH in females compared with males, and during late pregnancy. The mechanism for such alterations in adrenal responsiveness to trophic stimulation is unknown but may relate to ACTH receptor expression and/or the corticosterone synthetic pathway. To investigate this, we measured levels of the ACTH receptor (MC2-R) and the Steroidogenic Acute Regulatory Protein (StAR) in adrenals at various stages of rat pregnancy and lactation, and in normal males and non-pregnant females. StAR was investigated due to its key role in adrenal steroidogenesis where it serves as a cholesterol transporter. Quantification of MC2-R mRNA indicated a trend of increasing levels in gestation from day 12 ($2.8 \pm 0.6$) to a maximum at day 22 ($5.2 \pm 0.3$), but this did not reach statistical significance (ANOVA: $p = 0.27$). Levels of MC2-R expression in males ($3.7 \pm 0.6$) and non-pregnant females ($3.5 \pm 0.8$) were comparable. StAR protein levels were significantly different among groups (ANOVA: $p < 0.001$) and demonstrated a steady increase from early (day 7: $42 \pm 3$) to late gestation (day 22: $80 \pm 6$) reaching a peak at day 12 post partum ($98 \pm 3$). Amounts of StAR protein in the male were significantly lower than females at every stage examined (LSD test: $p < 0.05$) except day 7 of gestation. StAR mRNA levels were also significantly different among groups (ANOVA: $p < 0.05$) but did not reflect the pattern observed in StAR protein. Levels were lowest at day 22 of gestation ($79 \pm 23$) and males and females at several stages showed comparable amounts of StAR mRNA. These results indicate that levels of StAR protein, but not mRNA, parallel adrenal corticosterone output. The 30 kDa immunoreactive StAR protein, however, is an indicator of steroidogenesis that has already occurred rather than active expression. Our preliminary results on MC2-R transcription indicating higher levels of mRNA at times of maximal adrenal corticosterone synthesis, suggest a more likely mechanism for changes in adrenal responsiveness.

## 315

## PRENATAL ENDOTOXIN EXPOSURE REDUCES STRESS RESPONSE OF PRE-WEANED GUINEA PIGS IN SEX-SPECIFIC MANNER.

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The experience of stress in pregnancy is capable of producing developmental changes in the fetus. This study specifically examined the effect of prenatal exposure to a physiological stressor (bacterial endotoxin) on the offspring's HPA response to stress (novel environment). Pregnant guinea pigs (tri-colour strain) received injections of endotoxin (salmonella enteritidis, 50ug/kg, s.c.), or equivalent volume of saline, on gestational days

46, 48, 50 and 52 (term=68days). At postnatal age 17-20 days, each litter was placed in a novel environment for 60minutes, either alone or with their mother. Cortisol levels were measured in the offspring immediately following removal from the novel environment. Four to six days later, the procedure was repeated in the alternate condition. A significant interaction was observed ($p<0.05$) between prenatal treatment and novel environment condition. Animals treated prenatally with endotoxin exhibited reduced cortisol levels following exposure to the novel environment compared to those treated prenatally with saline. Cortisol levels were further reduced in both treatment groups when the offspring experienced the novel environment with their mother. An interesting sex-effect also existed, with cortisol levels significantly higher in the female offspring ($p<0.05$) compared to those in the males' and the attenuating effect of neonatal endotoxin treatment on cortisol was more pronounced in the male. These results indicate that while the presence of the mother buffers the offspring's response to stress, exposure to bacterial infection during pregnancy results in a reduced stress response in the male offspring. This may imply that male pups having been exposed to bacteria are less able to mount a stress response, and this may have long term consequences of HPA ontogeny.

**316**

## NEONATAL BACTERIAL ENDOTOXIN EXPOSURE ALTERS PRO-INFLAMMATORY AND FEBRILE RESPONSES IN ADULT RODENTS.

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Neonatal exposure to gram negative bacterial endotoxin has been shown to alter the functional dynamics of neuroendocrine-immune interactions. In the current experiment the long term effect of neonatal lipopolysaccharide exposure on hypothalamic pituitary adrenal function, temperature regulation and the production of the pro-inflammatory cytokine Interlukin-1 were assessed. Fischer 344 rats were treated with endotoxin (50 µg/kg *Salmonella enteritidis*, i.p.) or the vehicle on postpartum days 3, and 5. At 8 months of age adult animals were injected with either endotoxin (100ug/kg *Salmonella enteritidis*, i.p.) or the vehicle. The peak fever response was greatest in animals neonatally exposed to *Salmonella enteritidis* ($p < 0.05$). Consistent with this finding, circulating levels of IL-1 diverged significantly from baseline in the neonatally treated endotoxin group ($p < 0.05$). Interestingly, and in contrast to previous findings, circulating levels of corticosterone were not found to be influenced by neonatal treatment. The results suggest that neonatal endotoxin exposure can induce long term changes in the dynamics of the pro-inflammatory and febrile response to an acute bacterial challenge.

**317**

## THE EFFECT OF METYRAPONE INFUSION ON ADRENAL STEROIDOGENESIS IN THE LATE GESTATION FETAL SHEEP

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Cortisol is known to be essential for the development of key organs in the fetal sheep during late gestation and for the initiation of parturition. There is also a maturation of the hypothalamic-pituitary- adrenal axis in the fetal sheep during late gestation. The aims of this study were to determine the effect of suppressing the synthesis of fetal cortisol during late gestation on markers of activation of the HPA axis including fetal plasma ACTH concentrations and adrenal expression of the MC2 receptor, StAR and CYP11A1 mRNA. Surgery was performed at 119-120d on fourteen pregnant ewes to insert vascular catheters into the fetus and ewe for infusion studies and collection of daily blood samples for hormone analysis. Metyrapone (0.9M/h; a competitive inhibitor of 11β hydroxylase; n=8) or vehicle (tartaric acid 0.6M/h; n=6) were infused into the fetal jugular vein from 125d gestation until 140d (term = 147± 3 d). At 140d a post mortem was performed during which fetal adrenals were weighed and collected for Northern blot analysis. Ovine MC2 receptor cDNA, ovine StAR cDNA, and CYP11A1 cDNA probes were radiolabelled with 32P-dCTP. An 18S rRNA oligonucleotide probe, was used to verify equal

RNA loading. Fetal adrenals were significantly larger in metyrapone infused fetuses (0.83 ± 0.05 g) when compared to vehicle infused controls (0.42 ± 0.03 g) ($p<0.001$). There was a significant increase in plasma ACTH concentrations from 135-140d gestation in the metyrapone infused fetuses (184 ± 27 pg/ml) when compared to vehicle infused controls (70 ± 12 pg/ml) ($p<0.01$). There was no significant difference in the expression of MC2R mRNA:18S, between the two groups (vehicle 3217.3 ± 914; metyrapone 5697.9 ± 3122). There was, however a significant decrease in StAR mRNA:18S in metyrapone infused adrenals (275.3 ± 123.1) when compared with controls (553.7 ± 85.8). CYP11A1 mRNA:18S was significantly higher in metyrapone infused adrenals (2495.8± 507.4) when compared with controls (1575.4 ± 320.5). There was also a significant correlation between mean plasma ACTH concentrations from 135-140d gestation and expression of CYP11A1 mRNA in the metyrapone infused fetuses but not in the vehicle infused controls. Our data demonstrate that modulation of endogenous cortisol synthesis increases fetal plasma ACTH concentrations, adrenal growth and differentially modulates the expression of key enzymes in the adrenal steroidogenic pathway.

**318**

## INHIBIN INCREASES MDA-MB-468 BREAST CANCER CELL ADHESION TO FIBRONECTIN.

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Inhibins and activins are members of the TGF-β superfamily that play an important role in the regulation of follicle-stimulating hormone expression in the pituitary. They are expressed in a wide variety of tissues, and are involved in the proliferation and differentiation of numerous cell types, including mammary epithelial cells [1]. Inhibin has been implicated as a tumour-suppressor, and expression of immunoreactive inhibin α- and β-subunits is reduced in breast carcinoma tissues [2]. Their physiological roles in mammary cell physiology are unknown, although activin can inhibit growth of some breast cancer cells [3]. In addition, BMP-2, another TGF-β superfamily member, shares high homology with inhibin α-subunit in the loop 1 domain of the molecule, a region important for receptor binding. BMP-2 has anti-proliferative effects on breast cancer cells, and regulates cancer cell adhesion to extracellular matrix proteins, including fibronectin [4]. We now show that inhibin can regulate cell adhesion of the highly metastatic MDA-MB-468 human breast cancer cells. Tissue culture wells coated with fibronectin in the presence or absence of the appropriate hormones were seeded with $5 \times 10^4$ cells and incubated at 37C for 1 hr. Following incubation, unattached cells were removed, and the adhered cells fixed and stained with crystal violet. Stain eluted from the adhered cells was measured spectrophotometrically as a measure of cell numbers. We found that adhesion of the MDA-MB-468 cells to fibronectin was significantly increased by inhibin (~28% at 1 nM). The effect was dose-dependent, occuring at physiological concentrations of inhibin (0.01 to 10 nM), and was specific as neither serum albumin nor activin (1 nM) altered adhesion of these cells to fibronectin. The effect of inhibin on cell adhesion is independent of a functional TGF-β-Smad signaling pathway since MDA-MB-468 cells have a homozygous deletion in the gene for Smad4 and are not growth inhibited by TGF-β, BMPs and activin. Besides the antagonism of activin signalling through binding to betaglycan, no inhibin-specific signaling pathway has been described. Our results raise the intriguing possibility that inhibin might have actions that are independent of its ability to antagonise activin.

[1] Robinson & Hennighausen, *Development*. 124:2701, 1997. [2] Di Loreto et al, *Eur. J. Endo*. 141:190, 1999. [3] Kalkhoven et al, *Cell Gr. Diff*. 6:1151, 1995. [4] Nissinen et al, *Exp. Cell. Res*. 230:377, 1997

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## 319

### NEOINTIMAL HYPERPLASIA IN THE RAT CAROTID INJURY MODEL IS NOT REDUCED BY LOCAL FOLLISTATIN ADMINISTRATION

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**Background:** Activin A, a member of the transforming growth factor superfamily, and follistatin, its bioneutralising binding protein have been implicated in cardiovascular disease. Previous studies in human vascular tissue have demonstrated that activin A levels, but not follistatin levels, are elevated in atherosclerotic lesions. Other studies in the rat carotid injury model have demonstrated early activin A mRNA expression and subsequently protein in the resulting neointima. The aim of this experiment was to determine whether the presence of activin A is an epiphenomenon or actively involved in neointimal hyperplasia by administering its bioneutralising binding protein follistatin locally at the time of rat carotid injury. **Methods:** Adult male Sprague Dawley rats (398±16g) underwent standard carotid balloon injury. After injury, rats were randomised to either active treatment of 6.2µg follistatin (n=12) or placebo (n=12) administered in 200µl of slow release gel (pluronic gel 25%) placed locally around the injured artery. Animals were sacrificed at 1 week and the carotid arteries were perfusion fixed and embedded in paraffin for morphometric examination using systematic, uniform, random sampling of 6 representative sections spanning 3mm of injured vessel. These results were tested statistically by Generalised Estimating Equations. **Results:** The injured vessels displayed the typical neointimal hyperplasia previously described in this model. On morphometric assessment the neointimal/media ratio of vessels exposed to follistatin treatment was 0.48±0.04 compared with that of placebo 0.42±0.03 (p=0.42). **Conclusion:** Despite previously encouraging studies associating activin A with neointimal hyperplasia, blockade by local administration of follistatin via slow release gel does not inhibit neointimal formation in the rat carotid artery one week after injury. This may demonstrate redundancy in the causative factors that lead to neointimal hyperplasia, or that activin A is not a contributing factor toward neointimal formation.

## 320

### CELLULAR RELEASE OF ACTIVIN A IN ACUTE INFLAMMATORY EVENTS IS PROSTAGLANDIN INDEPENDENT AND MAY REQUIRE DIRECT INTERACTION WITH LIPOPOLYSACCHARIDE (LPS).

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Activin A is a pleiotrophic growth factor with roles in a number of systems including its original characterisation as a reproductive factor. Recently it has been implicated in various non-reproductive processes including the immune system and inflammatory diseases. We have used a model of acute inflammatory challenge of LPS in adult sheep to establish the sequence of activation of activin and other key cytokines (1). Our initial findings identified that activin is released very early into the circulation during inflammatory episodes, preceding the release of key pro-inflammatory cytokines, tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6). The rapid response of activin and its correlation with temperature prompted us to hypothesize that activin is part of the same response mechanism as that of fever. We addressed this premise using three different approaches whereby activin and other cytokines were measured using specific immunoassays (n=5 sheep per treatment). First, to investigate if activin was pyrogenic we injected human recombinant activin A (1 or 5 µg) centrally into the third ventricle of sheep. This failed to elicit changes in temperature or in plasma and CSF concentrations of TNFα and IL-6. Second, we tested if activin increased centrally in concert with systemic increases in temperature and cytokines. LPS administered systemically did not increase activin or TNFα concentrations in cerebrospinal fluid (CSF), whereas IL-6 concentrations in CSF mimicked the systemic response. In the third study, the fever response to LPS was ablated by co-administration of flurbiprofen (2mg/kg), a prostaglandin synthesis inhibitor. LPS response profiles of activin, TNFα and IL-6 were unaffected by the co-treatment with flurbiprofen; basal concentrations were unaffected by flurbiprofen. In conclusion, our studies do not support the hypothesis that activin release during inflammation is directly related to the fever response, indicating a response independent of prostaglandin

stimulation. The early systemic and lack of a central response, suggests activin release requires direct interaction between cells and LPS, as LPS cannot enter the brain due to exclusion by the blood brain barrier. We propose that LPS-responsive cells such as monocytes and vascular endothelial cells are candidate sources for this early release of activin during acute inflammatory challenges.

(1) Jones *et al.* (2000) Endocrinology 141, 1905-1908.

## 321

### EXPRESSION OF MULTIPLE TRANSFORMING GROWTH FACTOR (TGF)- β SUPERFAMILY MEMBERS AND FOLLISTATIN IN THE ANTERIOR PITUITARY

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Pituitary gonadotrophs produce and secrete follicle stimulating hormone (FSH) in response to autocrine/paracrine stimulation by activin. This effect can be blocked by the activin-binding protein, follistatin, which is also synthesised in the pituitary. Inhibin mediates endocrine negative feedback and opposes activin action on FSH. Both inhibin and activin belong to the TGF-β superfamily. Previous studies showed that progesterone (P) can stimulate FSH production and secretion by cultured rat pituitary cells and this effect is blocked by co-administration of follistatin, suggesting the involvement of activin or another member of the TGF-β superfamily.

The aims of this study were 1) to determine pituitary levels of mRNA for activin, inhibin, activin-like factors and follistatin, 2) to investigate the effect of P on these mRNA levels, and 3) to determine whether candidate mediators of the P effect directly stimulate FSH.

RNA obtained from primary rat pituitary cell cultures and from LβT2 mouse gonadotrophs was reverse transcribed and amplified by real-time PCR (LightCycler, Roche) using specific primers targeting candidate members of the TGF-β superfamily. The production and secretion of FSH by the cells were measured using radioimmunoassay.

In addition to inhibin subunits, α, $\beta_A$ and $\beta_B$ subunits other candidate FSH-regulating genes, including BMP-4 and -7, GDF-9 and -9B, were also expressed in the primary rat pituitary cells. However, none was regulated by P (100nM) in the presence of estradiol-17β (4 nM). Expression of follistatin in the rat pituitary was significantly reduced by P treatment. Inhibin α and $\beta_B$, BMP-4, -6 and -7, and GDF-9 and -9B were all expressed by LβT2 gonadotrophs, but were not regulated by P. Although P significantly increased FSH production and secretion in primary pituitary cell cultures, it did not in LβT2 cells, suggesting a paracrine pathway underlies this steroid effect. Neither GDF-9 nor BMP-7 affected FSH levels in the primary cultures. However, in LβT2 cells plated in a closely packed configuration, BMP-7 (3 nM) stimulated FSH secretion, and synergized with activin A (0.5 nM) to increase intracellular FSH levels, effects that were suppressed by follistatin-288 (1 nM).

In conclusion, reduced follistatin may underlie or contribute to the activin-like action of progesterone in the rat pituitary. The study has also identified gonadotrophs as a novel site for mRNA expression of several TGF-β superfamily members with unknown autocrine/paracrine functions. *Supported by the NH&MRC of Australia.*

## 322

### SERUM INHIBIN B & PRO-AC LEVELS WITH EXPERIMENTAL MANIPULATION OF GONADOTROPHIN LEVELS IN NORMAL MEN.

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The hormonal regulation of inhibin B and its free α subunit are unclear. We showed that inhibin B and pro-□C levels rose modestly following acute administration of FSH, hCG or FSH+hCG (ESA 2001 #256). The current study aimed to establish whether these limited responses were modified by the presence of spermatogenic cells using a model of spermatogenic suppression and recovery. Fifteen men received testosterone (T) implants and depot medroxyprogesterone acetate IM (12-week suppression phase) and were then divided into 3 groups receiving 12 weeks of FSH, hCG or FSH+hCG. The acute response of the testis was studied during the first and third weeks of this recovery phase. Serum inhibin B, pro-αC, FSH, LH, T were determined on days 0, 4, 5 and 6

days after single doses of FSH, hCG or FSH+hCG. After 12 weeks of spermatogenic suppression, falls ($p<0.001$) were seen in inhibin B (75+ 5% baseline) and pro-αC (51+ 11% baseline). The incremental response of inhibin B to FSH was similar comparing pre- and post spermatogenic suppression (124+ 9% vs. 141+ 28% baseline, NS), as was the response to FSH+hCG (123+ 12% vs. 119+ 18% baseline, NS). hCG alone had no effect at either time. In contrast, pro-αC levels showed significantly greater stimulation after suppression in the FSH (158+ 33% vs. 225+ 32%, $p<0.05$) and hCG (119+ 17% vs. 279+ 84 %, $p<0.05$) treatment groups. All treatments partially restored spermatogenesis but inhibin B levels either fell with hCG, or returned toward control levels with FSH+/-hCG. No clear relationship was apparent between inhibin B and sperm count. All treatments led to a sustained return of pro-αC toward control levels. We conclude that: (1) both inhibin B and pro-αC are only partially gonadotrophin dependent in normal men. (2) spermatogenic cells do not modify acute inhibin B response to FSH but their loss enhances the gonadotrophic response of pro-αC, and (3) inhibin B is an insensitive index of spermatogenesis in this model of experimental gonadotrophic manipulation in normal men. However the results in this model may differ from other models of testicular damage or genetic impairment in which Sertoli-germ cell interactions may be quite different with unique FSH/inhibin relationships.

**323**

## CLONING AND EXPRESSION OF MOUSE AND RAT HOMOLOGUES OF NOVEL G PROTEIN-COUPLED RECEPTORS, LGR7 AND LGR8; PUTATIVE RECEPTORS FOR RELAXIN AND INSULIN 3

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The peptide hormones relaxin and Insulin 3 (INSL3) are both members of the insulin/IGF superfamily. Relaxin, classically known as a hormone of pregnancy, acts on multiple tissues to induce varying effects primarily via collagen remodelling. INSL3 is expressed in the testicular Leydig cells and mediates testicular decent by inducing development of a specific ligament, the gubernaculum. Recently relaxin was shown to bind two orphan leucine-rich repeat-containing G protein-coupled receptors (LGR), LGR7 and LGR8, causing cAMP activation [1]. Subsequently, LGR8 was shown to be activated by INSL3 (see abstract by Bathgate et al). The LGR family comprises LH, FSH and TSH receptors, 3 orphan receptors, and LGR7 and LGR8. All contain a characteristic extracellular ectodomain with leucine rich repeats. Human LGR7 and LGR8 [1], were used to search the Celera and NCBI genome and EST databases for novel homologues. Particular emphasis was placed on identifying mouse and rat homologues.

We identified a full mouse LGR7 sequence using the Celera genome database and almost complete rat LGR7 and LGR8 sequences in the NCBI high throughput genome database. Furthermore, ESTs corresponding to chicken, pig and zebrafish LGR7 and bovine LGR8 were also discovered. Rat, mouse, pig and bovine sequences were confirmed using RT-PCR and sequencing. Mouse and Rat LGR7 expression was confined to tissues known to possess functional relaxin receptors, such as cervix, cerebral cortex and myometrium, while LGR8 was expressed predominantly in the fetal gubernaculum, the site of INSL3 action.

Human LGR7 exists in two alternately spliced isoforms [1]. We identified mouse and rat LGR7 splice variants of a similar nature, with the spliced region shifted 33 amino acid in the C terminal direction. Both spliced forms were present in all the tissues displaying LGR7 expression. This alternate splicing in the ectodomain of LGR7 may confer changes in ligand specificity or binding. By aligning LGR7 and LGR8 family members we have identified regions of high homology that could also be involved in ligand binding and activation. Overall, the identification of novel homologues of LGR7 and LGR8 will significantly aid in further characterisation of the precise roles and downstream processes involved in relaxin and INSL3's actions.

1. S.Y. Hsu et al, Science 295, 671 (2002)

## 324

### FETAL GROWTH RESTRICTION IMPAIRS INSULIN SENSITIVITY OF GLUCOSE METABOLISM INDEPENDENTLY OF ADIPOSITY AND CIRCULATING FREE FATTY ACIDS IN THE ADULT GUINEA PIG.

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Epidemiological studies show that fetal growth restriction (FGR) is associated with insulin resistance and related diseases, such as type 2 diabetes, in mature adults. The nature and underlying causes of insulin resistance which is "programmed" in utero remain unclear however. The aims of the current study were therefore to determine if FGR in the guinea pig, due to increased litter size, results in adult insulin resistance of glucose metabolism and the tissue and metabolic sites of such resistance. In addition, we examined the extent to which these were associated with factors such as increased adiposity and circulating free fatty acid (FFA) concentrations, which are known determinants of insulin sensitivity in other species. The basal and insulin-stimulated rates of whole body glucose utilisation and production, were measured using concomitant 3-[3H]glucose infusion prior to and during a hyperinsulinaemic euglycaemic clamp (HEC) (insulin infusion rate 7.5mU/min/kg), as was the partitioning of glucose utilisation into glycolysis and storage. A subset of animals had two HEC (7.5 and 30mU/kg/min) to examine the effect of FGR on the dose responsiveness of insulin sensitivity. The basal rate of whole body glucose utilisation, production, or storage did not vary with birth weight (n=19), but correlated positively with relative retroperitoneal and perirenal adiposity ($p<0.05$). Whole body insulin sensitivity of glucose metabolism ($r=0.55$, $p<0.0005$, n=36), glucose utilisation ($r=0.69$, $p<0.005$), production ($r=0.42$, $p<0.05$) and storage ($r=0.60$, $p<0.005$) decreased with decreasing birth weight, were not related to adult adiposity and were predicted by birth weight independently of adult adiposity. The basal rates and the insulin sensitivity of glycolysis did not vary with birth weight. The insulin sensitivity of glucose utilisation ($r=-0.38$, $p<0.05$) and storage ($r=-0.44$, $p<0.03$) correlated negatively with FFA concentrations, and were independently predicted by both birth weight ($p<0.001$) and FFA ($p<0.05$). In conclusion, FGR substantially impairs whole body and peripheral insulin sensitivity of glucose metabolism, including that of glucose storage, in the young adult guinea pig, independently of known contributors to insulin resistance such as adult adiposity and circulating FFA concentrations.

## 325

### AGING AND THE INSULIN RESISTANCE SYNDROME IN THE GUINEA PIG: A ROLE FOR ALTERED BODY COMPOSITION ?

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In humans, aging is associated with an increased risk of developing a cluster of metabolic and cardiovascular abnormalities including insulin resistance, non-insulin dependent diabetes mellitus (NIDDM), dyslipidaemia and hypertension. This cluster of abnormalities has been termed the Insulin Resistance Syndrome (IRS), as insulin resistance is thought to be the primary underlying defect. Aging is also associated with alterations in body composition, in particular increased total fat mass and visceral adiposity. The aim of this study was to determine if the IRS develops with aging in the guinea pig, and whether changes in body fat mass and its distribution, are implicated in this. Vascular catheters were surgically implanted into the carotid artery and jugular vein of weanling (40 days), young adult (4 months) and aged (13 months) guinea pigs under general anaesthesia and strict asepsis. Whole body insulin sensitivity was measured as the steady state glucose infusion rate during a hyperinsulinaemic euglycaemic clamp (HEC) (7.5mU insulin/min/kg). Fasting plasma levels of glucose and free fatty acids and mean resting systolic and diastolic blood pressure (recorded for 2 hours) were also determined. Body composition, including weights of selected adipose depots, were determined at post-mortem. Insulin sensitivity was decreased in aged guinea pigs when compared to weanlings (-58%) and young adults (-26%) ($p<0.05$ for both). Fasting plasma glucose levels were increased in aged guinea pigs when compared to weanlings (+24%) and young adults (+17%) ($p<0.05$ for both). Similarly, fasting plasma free fatty acid levels

were increased in aged guinea pigs when compared to weanlings (+41%) and young adults (+8%) ($p<0.05$ for both). Resting systolic and diastolic blood pressure was increased in aged guinea pigs when compared to weanlings and young adults ($p<0.05$ for both). Visceral adiposity (% body weight) also increased in aged guinea pigs when compared to weanlings (+59) and young adults (+14%) ($p<0.05$ for both). Therefore, it appears that major components of the IRS emerge with increasing age in the guinea pig, as in humans, accompanied by increasing visceral adiposity, which is thought to play a key role in the development of the underlying defect, insulin resistance.

**326**

## SEPARATION OF PROSTAGLANDIN (PG)E2 AND PGF2α FROM THEIR TISSUE METABOLITES USING REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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Prostaglandins are paracrine mediators involved in numerous physiological and pathophysiological processes. They function in a controlled local fashion and are rapidly inactivated in target tissues. $PGE_2$ and $PGF_{2\alpha}$ are metabolised to their inactive 15-keto-derivatives and subsequently to 13,14-dihydro-15-keto-$PGE_2$ and $PGF_{2\alpha}$ (PGEM and PGFM) by 15-hydroxyprostaglandin dehydrogenase and 13,14-prostaglandin reductase, respectively. The activities of these metabolising enzymes are major determinants of bioactive prostaglandin levels. To measure these activities, a fast, simple and robust technique is required to separate $PGE_2$ and $PGF_{2\alpha}$ from their metabolites. Thin-layer chromatography (TLC) is potentially useful for the fast separation of large numbers of samples; therefore, we have conducted systematic studies to establish a TLC procedure that separates 15-keto-$PGE_2$, 15-keto-$PGF_{2\alpha}$, PGEM and PGFM from $PGE_2$ and $PGF_{2\alpha}$. METHODS: Chromatography was performed on RP-18W high-performance TLC plates, using binary mobile phases of organic modifier (methanol, ethanol, acetonitrile, acetone or THF) and water, ranging in composition from 0 to 100% (v/v). The temperature of the TLC chamber was varied from -20°C to 60°C. RESULTS: Retention patterns for the three E-series compounds were similar using binary mobile phases of water with methanol, ethanol, acetone, or THF. Satisfactory separation of the three compounds was not achieved in these systems. Using 100% acetonitrile mobile phase at 20°C, however, $PGE_2$ and $PGF_{2\alpha}$ were separated from each other and from their metabolites. Varying the temperature of the chamber did not significantly improve the separation of metabolites. Van't Hoff plots generated for the three E-series compounds in various mobile phase systems of similar elution strengths were linear, indicating similar retention mechanisms over the temperature range for each compound. CONCLUSIONS: The prostaglandins investigated behaved similarly in a wide variety of TLC systems, highlighting the difficulty in separating them in a practical way. Nevertheless, separation of $PGE_2$ and $PGF_{2\alpha}$ from their metabolites was obtained using the mobile phase of pure acetonitrile. At 20°C, this system robustly separates these prostaglandin compounds within 10 minutes. The procedure is applicable for the fast processing of samples to measure the kinetics of $PGE_2$ and $PGF_{2\alpha}$ metabolism, to determine the activity of 15-hydroxyprostaglandin dehydrogenase, and to pre-purify complex biological samples for the subsequent quantification of individual prostaglandins.

**327**

## SOX13, HIGH SOLT, NUCLEAR LOCALISATION AND INSULIN EXPRESSION

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We have identified the human *SOX13* gene as encoding a pancreatic islet transcription factor[1] and have demonstrated its expression in the nuclei of an islet β cell line. The Sox protein family is characterised by the High Mobility Group (HMG) DNA binding domain, and SOX13 belongs to the D subgroup of SOX proteins, which contain a leucine-zipper motif and glutamine-rich region through which SOX13 can form homodimers[1]. Recently, another member of the D subgroup family, Sox6 was shown to interact with Solt, through leucine-

zipper interactions[2]. The high sequence identity between Sox6 and SOX13 suggests that Solt may be an interacting partner required for SOX13 nuclear transport in β cells.

This study aimed to investigate:

1. The influence of the leucine-zipper-glutamine-rich (LZ-Q) domain on Sox13 function.

2. The effect of Solt on SOX13 nuclear transport.

3. The significance of SOX13 localisation in islet β cells.

Functional studies showed that SOX13 bound poorly to DNA in comparison to a mutant form of SOX13 lacking the LZ-Q domain (SOX13ΔLZ-Q) and in comparision to SOX13ΔLZ-Q, the transactivation activity of intact SOX13 was reduced. The results suggest that SOX13 cannot perform its DNA binding and transactivation functions efficiently when it forms homodimers through the LZ-Q region. When SOX13-GFP and Solt were transiently transfected into COS7 cells cytoplasmic SOX13-GFP became increasingly nuclear in the presence of increasing Solt concentrations. In β cells Solt may be a putative interacting partner of SOX13. In β cells, the subcellular localisation of SOX13 is also likely to influence the production of insulin as nuclear Sox13 correlated with high levels of insulin expression in mouse islets. In summary, Sox13 is implicated in islet β cell function and an interaction with Solt is likely to regulate nuclear localisation to control Sox13 activity.

References:

1. Kasimiotis, H., Myers, M. A., Argentaro, A., Mertin, S., Fida, S., Ferraro, T., Olsson, J., Rowley, M. J., and Harley, V. R. (2000). Sex-determining region Y-related protein SOX13 is a diabetes autoantigen expressed in pancreatic islets. *Diabetes* **49**, 555-61.

2. Yamashita, A., Ito, M., Takamatsu, N., and Shiba, T. (2000) Characterization of Solt, a novel SoxLZ/Sox6 binding protein expressed in adult mouse testis *FEBS Letters* **481**, 147-51.

**328**

## SITE-SPECIFIC EXPRESSSION OF GENES IN THE UPPER AND LOWER SEGMENTS OF THE HUMAN UTERUS DURING PREGNANCY

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Most research on the human uterus has concentrated on samples from the lower segment of the uterus due to practical considerations. This project tests the hypothesis that the fundal (or upper) and lower segments of the human myometrium express different genes during labour for effective parturition and delivery of babies. Paired myometrial tissues from the fundal and lower segments of uteri were obtained from women undergoing Caesarean section at term upon informed consent. Total RNA was extracted, treated with DNase to eliminate genomic DNA, and reverse transcribed into cDNA using SuperScript II (Gibco BRL). Quantitative real-time RT PCR (QRT-RTPCR) was performed using Taqman$^{TM}$ probes in the ABI Prism 7700 Sequence Detection System. Two well-known inflammatory mediators and modulators previously linked with parturition, interleukin-8 (IL-8) and manganese superoxide dismutase (MnSOD) were assayed. The IL-8 and MnSOD assays were multiplexed with 18S ribosomal RNA, to permit simultaneous detection of both the target gene (either IL-8 or MnSOD) and 18S rRNA as the internal reference in the same tube. This allows standardisation of the amount of target to the internal reference gene to control for different amount of cDNA used. The standardised target gene was then compared with an external reference sample, a placental cDNA pool, that was used in every assay. The result of this comparative method of quantification is transformed to an exponential value, $2^{-\Delta\Delta Ct}$, where Ct refers to the cycle threshold, or the cycle when the product was first detected by the software (SDS User Bulletin 2, Applied Biosystems). Standard cycling conditions for QRT-RTPCR were used in all assays (SDS User Bulletin 2). Notably, MnSOD mRNA was found to be differentially distributed between the upper ($0.63 \pm 0.18$, mean ± SEM) and lower ($0.15 \pm 0.05$, $n = 15$, $P = 0.022$) segments of the uterus. In contrast, IL-8 was not ($P$=0.97, n=17). This work shows that there is differential expression of some, but not all genes within the gravid human uterus. These findings indicate the uterus in pregnancy cannot be viewed as a homogeneous organ during labour.

## 329

### REGULATION OF 15-HYDROXYPROSTAGLANDIN DEHYDROGENASE (PGDH) MRNA LEVELS IN THE HUMAN CHORION LAEVE AT TERM.

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Prostaglandins play an essential role in human parturition. PGDH, which converts prostanoids to inactive derivatives, is the key enzyme of the prostaglandin degradation pathway. PGDH expression is high in the chorion laeve, and changing PGDH levels in this membrane may influence intrauterine bioactive prostaglandin concentrations and the timing of labour. Our objectives were to determine the chorionic level of PGDH mRNA before and after term labour and to examine the mechanisms that control the abundance of this mRNA at normal birth. Chorion membranes were isolated following term elective Cesarean section (CS, n=27) and after term spontaneous labour (SL, n=20). PGDH mRNA and PGDH heterogeneous nuclear RNA (hnRNA, the unspliced precursor of mRNA) abundance was determined by real time quantitative RT-PCR. The PCR-primers were directed to an exon-spanning sequence specific to the functional splice variant of PGDH mRNA and to an intron sequence specific to PGDH hnRNA. PGDH mRNA degradation and hnRNA processing rates were measured in the presence of the transcription inhibitor DRB. The dynamics of PGDH mRNA and hnRNA abundance were characterised in tissue incubations for 0-24 h. Results were normalised to □-actin mRNA abundance. PGDH mRNA and PGDH hnRNA levels increased slightly (< 2-fold) but significantly ($p<0.05$) in association with labour. Regression analyses indicated that PGDH hnRNA abundance was a significant predictor of PGDH mRNA abundance in individual tissues after, but not before, labour. PGDH mRNA degradation rate and PGDH hnRNA processing rate did not change with labour in transcriptionally arrested tissues. In tissues maintained in culture for 24 h, PGDH hnRNA abundance decreased by 80% ($p<0.05$) within 18 h, and PGDH mRNA abundance decreased more than in tissues where transcription was blocked. The results suggest that PGDH gene activity in the chorion membrane is maintained by endocrine/paracrine factors and is stimulated with labour at term. Before term labour onset, PGDH mRNA levels are controlled primarily by post-transcriptional mechanisms possibly involving short-lived mRNA-destabilizing factor(s). The significance of these regulations in the control of PGDH activity and in the compartmentalization of uterine prostaglandins during birth remains to be established.

## 330

### CONTROL OF HUMAN PARTURITION INVOLVES CHANGES IN RESPONSIVENESS OF THE MYOMETRIUM TO PROGESTERONE AND ESTROGEN

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Progesterone and estrogen are important regulators of pregnancy and parturition. Progesterone maintains pregnancy whereas estrogen promotes labor and delivery. Consistent with this notion, progesterone levels decrease (progesterone withdrawal) and estrogen levels increase (estrogen activation) before labor onset in many species. However, human parturition occurs without progesterone withdrawal or estrogen activation. Instead, both steroids are elevated through most of pregnancy and during parturition. This has led us to hypothesize that progesterone and estrogen actions in human parturition are controlled by changes in target tissue responsiveness rather than circulating levels. To test this, we examined whether myometrial responsiveness to progesterone (assessed by the extent of HOXA10 expression) and estrogen [assessed by the extent of oxytocin receptor (OTR) and cyclooxygenase type-2 (COX-2) expression] changes in association with the onset of labor. We also examined expression of the progesterone and estrogen receptors (PR-A, PR-B, ERα and ERß). Specimens of myometrium were obtained from women undergoing c-section at term before (n=12) and after the onset of active labor (n=12). Levels of specific mRNAs were determined by real time quantitative RT-PCR. Expression of HOXA10, OTR, COX-2, ERα and PR-A significantly ($P<0.05$) increased in association with active labor. The expression ratio of PR-A to PR-B which is indirectly related to progesterone responsiveness since PR-A is an endogenous suppressor of progesterone action, also increased significantly in laboring specimens. The PR-A/PR-

B expression ratio correlated positively with HOXA10 ($r_s$ = 0.8601; P<0.001) and ERα ($r_s$ = 0.9230; P<0.001) expression. Since progesterone inhibits HOXA10 and ERα expression, these findings indicate that myometrial progesterone responsiveness decreases at parturition and is inversely related to the PR-A/PR-B expression ratio. ERα expression correlated positively with COX-2 ($r_s$ = 0.7483; P<0.001) and OTR ($r_s$ = 0.6643; P<0.05) expression indicating that the increase in ERα expression is directly associated with the activation of contraction-associated genes and estrogen responsiveness. These data also indicate that functional estrogen activation occurs by increased expression of ERα and is linked to functional progesterone withdrawal. Interaction between the myometrial PR and ER systems may coordinate functional progesterone withdrawal with estrogen activation and be critical for controlling human parturition.

**331**

## CRH GENE EXPRESSION IS REPRESSED BY ESTROGEN AND STIMULATED BY THE ESTROGEN-ANTAGONIST ICI 182,780 IN PLACENTA

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Corticotropin-releasing hormone (CRH) and estrogens, produced by placental trophoblasts, have been suggested to play pivotal roles in the control of human parturition. Estrogen has been shown to affect hypothalamic CRH expression. Therefore, we evaluated 17β-estradiol (E2) in the regulation of CRH gene expression in placental cells. In human primary placental culture cells E2 inhibited CRH mRNA expression in a dose-dependent manner, when measured by quantitative RT-PCR. This affect was paralleled by a decrease in CRH protein levels in culture media, as measured by radioimmunoassay. A complete estrogen receptor (ER) antagonist ICI182780 (ICI) not only blocked repression of CRH mRNA levels by E2, but up-regulated CRH mRNA and protein synthesis. An ERα partial agonist and ERβ antagonist, 4-hydroxytamoxifen (OTH), also down-regulated CRH gene expression. Using quantitative RT-PCR, we found that placental trophoblasts express predominantly the ERα form of the receptor. Using a luciferase reporter system linked to CRH promoter DNA sequences, transient transfection assays were conducted in the choriocarcinoma cell line, JEG-3. These experiments demonstrated that when the reporter plasmid was cotransfected with an ERα expression plasmid E2 repressed CRH promoter activity, whereas ICI up-regulated CRH promoter activity in the same experimental system. These studies demonstrate that E2 represses placental CRH gene expression through an ERα mediated mechanism. Estrogen may therefore modulate placental CRH production, influencing the rate of rise of maternal plasma CRH concentrations and potentially the length of gestation.

**332**

## GONADOTROPHIN STIMULATION AND RISK OF MULTIPLE PREGNANCY

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Ovarian stimulation with gonadotrophins is an effective treatment for patients undergoing insemination by the husband or donor (IUI or DI). However, the high risk of multiple pregnancy is a concern. This retrospective study aimed to show that judicious use of gonadotrophin stimulation can result in good pregnancy rates with relatively low risks of multiple pregnancy and cancellation. All patients included in this study have been treated with gonadotrophin stimulation and insemination with sperm from the husband or a donor during the period between 1990 and 1999 in RMU. One thousand nine hundred and sixty four patients had 4411 treatment IUI cycles and 108 patients had 297 treatment DI cycles in this period. Stimulation commenced on day 5 of menses with a starting dose of 37.5 -100 IU of FSH for most women with the aim of encouraging a single large follicle development. Measurement of hormones and ultrasound scan were carried out. With two large follicles (>16 mm), the couple was counselled for the risk of multiple pregnancy and possible cancellation. Cycles with three or

more large follicles were cancelled. The outcomes of the treatment in both IUI and DI are shown in the following table.

| | IUI | DI |
|---|---|---|
| Number of patients | 1964 | 108 |
| Number of treatment cycles | 4411 | 297 |
| Pregnancy rate per patient | 36% | 62% |
| per treatment cycle | 16% | 23% |
| Multiple pregnancy rate | 9% | 6% |
| Cancellation rate | 12% | 8% |

The results suggest that our protocols of gonadotrophin stimulation for IUI and DI resulted in good pregnancy rates with a low risk of multiple pregnancies.

**333**

## GENERATION OF REACTIVE OXYGEN SPECIES BY HUMAN PLACENTAL CELLS

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Oxidative stress occurs when the generation of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defenses, resulting in lipid peroxidation and DNA damage. Excessive oxidative stress is associated with multiple disorders of pregnancy including pre-eclampsia and premature rupture of membranes; both of which can have serious implications for both the mother and fetus. However the source of ROS in these situations remains uncertain. Using sensitive chemiluminescence and spectrophometric techniques, the production of ROS was measured in primary trophoblast cell cultures and three trophoblast cell lines in an attempt to characterise the ROS producing redox systems associated with these cells. We have analysed ROS production in normal and pre-eclampsic trophoblast cells. A sudden increase in the rate of lucigenin-dependent chemiluminescence was observed upon addition of the substrates NADH or NADPH, with the response to NADH being two log orders of magnitude higher than to NADPH. The response to these substrates was sustained for at least 15 min and was reduced by addition of superoxide dismutase (SOD). Furthermore, addition of phorbol 12-myristate 13-acetate (PMA) or the divalent ionophore, A23187, also stimulated the production of ROS as detected by lucigenin- and luminol/peroxidase- chemiluminescent signals. However, in contrast to the NAD[P]H stimulated signal, PMA and A23187 stimulated a progressive increase in the chemiluminescent response rather then a sudden burst. Once again these signals were suppressed by addition of SOD. Preliminary data using trophoblast cells isolated from a pre-eclampsic placenta generated a much greater spontaneous chemiluminescent signal than that seen in 'normal' placenta, suggesting that enhanced ROS generation by trophoblast cells may contribute significantly to the oxidative stress associated with this condition. The level of ROS in pre-eclamptic trophoblasts increased over three days as these cells differentiated into syncytiotrophoblasts. This data suggests that human trophoblasts are able to produce ROS and this may play a role in pathological conditions associated with pregnancy. The addition of SOD and the subsequent reduction in chemiluminescence suggests that superoxide is one species of ROS generated.

**334**

## PLACENTAL RESTRICTION AND PLASMA THYROID HORMONE IN THE NEONATAL LAMB.

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Thyroid hormone (TH), comprising thyroxine (T4) and triiodothyronine (T3), acts directly, as well as indirectly via IGF-I and GH, to promote skeletal muscle and bone growth and maturation. Intrauterine growth restriction (IUGR) is associated with 'catch-up' growth in human infants and in the placentally restricted (PR) lamb. PR increases plasma T4 levels in the first 24 hours following birth, but it is not known if these persist and could

contribute to catch-up growth after IUGR. We hypothesised that placental restriction in the sheep will reduce size at birth, and increase postnatal growth rate, in part due to increased plasma concentrations of TH in the young lamb. Placental and hence fetal growth was restricted by surgical removal of the majority of endometrial caruncles from the uterus prior to pregnancy. Size was measured at birth and subsequently every 5 days after birth and fractional growth rates calculated using absolute growth rate and size at 30 days of age. Plasma total and free T3 and T4 and IGF-I and –II at 30 days of age (fasting) were measured using specific radioimmunoassays. PR reduced size at birth in terms of weight, crown-rump length, tibia and metatarsal lengths, and abdominal circumference ($p<0.05$ for all). PR also increased fractional rates of growth for weight ($p<0.001$), tibia ($p<0.05$), skull length ($p<0.05$) and hind limb and radius/ulna circumferences ($p<0.05$ for both), but did not alter plasma TH concentrations. Fractional growth rate for weight correlated positively with plasma total T3 and free T3 concentrations in PR lambs only ($p<0.05$ for both). Fractional growth rate for tibia length correlated positively with plasma total T4 concentration in PR lambs only ($p<0.01$). Fractional growth rate for weight also correlated positively with plasma IGF-I and IGF-II in all lambs ($p<0.05$). In turn, plasma IGF-I and plasma IGF-II correlated positively with plasma total T3 concentration ($p<0.05$, $p=0.06$ respectively) in PR lambs. These findings suggest that prenatally induced increases in circulating plasma TH may contribute to catch-up growth of soft and skeletal tissue in young lambs following PR and act in part via circulating IGFs. The extent to which the associations of neonatal catch-up growth with the anabolic hormones are casual in nature remains to be determined.

**335**

## BIOMARKER TRAJECTORIES IN PREGNANCY AND THE DETECTION OF PATHOLOGY

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We have demonstrated that for many variables individual women track along predictable trajectories during pregnancy. In particular maternal plasma CRH follows an exponential curve. Deviation from the normal curve is associated with pathology and women in whom CRH increases more rapidly than normal are likely to require indicated caesarian delivery (1). We postulated that by following additional biomarker variables during pregnancy normal population trajectories could be defined against which individual women could be monitored and that this information would identify aberrant trajectories indicative of pathology. To this end, in 256 pregnant women, serial measurements (mean 5.5) of maternal plasma estradiol, estriol, progesterone, CRH, human chorionic gonadotropin, alpha-fetoprotein, alkaline phosphatase, and placental lactogen were made along with ultrasonographic measurements (mean 3.7) of fetal and uterine growth. Tablecurve 2D's built-in library was used to fit a large array of linear and nonlinear equations to data from each individual to obtain a set of parameters that best describe the individual trajectory for each marker through gestation. The individual curves were analysised to produce an optimized population curve; individuals were then assessed for deviation from the population curve and associated pathology. For example, each of the fetal biometry measurements was best fitted by a linear polynomial and a difference was found in the trajectories from women who smoked as compared with nonsmokers. A visualisation program was developed in Matlab (MathWorks Inc.) to enable comparison of trajectories and parameters in multiple dimensions and their relationship to the timing of delivery. By combining these trajectories in a model we may better be able to identify pathological states related to pregnancy and better predict the timing of the onset of parturition for that individual.

1. McGrath, S., et al., Maternal plasma corticotropin-releasing hormone trajectories vary depending on the cause of preterm delivery. Am J Obstet Gynecol, 2002. 186(2): p. 257-60.

## 336

### DOES BULK TRANSFER OF BOVINE BLASTOCYSTS COMPROMISE EARLY EMBRYO DEVELOPMENT?

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The ultimate endpoint of any experimental manipulations involving both *in vitro* produced (IVP) and nuclear transfer bovine embryos is the birth of live healthy calves some nine months later. Furthermore, to achieve statistical significance, sufficient numbers of recipients are required, which adds to the increasing costs of these experiments. We have previously advocated the use of multiple embryo transfer per recipient (bulk embryo transfer) with collection of conceptus material over days 25-27 of gestation to gain more rapid information on the efficacy of these manipulations. Although we have previously used bulk transfers to compare *in vivo* vs *in vitro* embryo development[(1)] based on morphological appearance, there is no data on the effect of bulk transfers on gene expression during earlier development. We describe here the expression of four prominent trophoblast genes from conceptuses generated by either twin or bulk (n = 5 embryos) transfer of IVP grades 1 and 2 bovine blastocysts into each of 8 recipients. A further 28 cows were inseminated artificially (AI) using the same semen as that used in the IVP system. At necropsy on day 18 there were no significant differences in pregnancy rate and embryo recovery among the 3 groups.

Two μg of total RNA extracted from individual conceptuses from AI (n=21), twin (n=9) and bulk transfer (n=13) was loaded in a slotblot apparatus and duplicate blots obtained. One blot was probed with gamma actin as a control and the other was sequentially probed with trophoblast-kunitz-domain protein-1 (TKDP-1), interferon-tau (IFN-τ), H19 and finally cyclooxygenase-2 (COX-2). Between hybridisations, blots were left to decay until radioactivity was undetectable. The intensity of the signal was determined by scanning densitometry of the x-ray films and normalised to control levels. Although there were no significant differences among the treatments in the expression of the four genes, expression in those conceptuses generated by AI was consistently greater. These results, coupled with our early data on embryo development, suggest that bulk embryo transfer with necropsy before day 27 of gestation may indeed expedite the information gained regarding the efficacy of early embryo manipulation.

(1) Pugh, A. et al. Theriogenology 55(1), 314.

## 337

### PURIFICATION AND CHARACTERISATION OF CYTOSOLIC THYROID HORMONE BINDING PROTEIN IN HUMAN PLACENTA

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Placental deiodination is an important factor moderating transplacental transfer of thyroid hormone, however, the mechanisms involved in the control of placental deiodination and transfer of the hormone have not been identified. We postulate that a co-ordinated and regulated system of trophoblastic basal and apical membrane transporters, cytoplasmic binding proteins and Type III deiodinase mediates and modulated thyroid hormone transport across the placenta. Several cytosolic thyroid hormone binding proteins (CTBPs) have previously been characterised in thyroid responsive tissues, but not in placenta. Therefore the aim of this project was to isolate and characterise placental CTBP(s). We postulate that, if present, placental CTBP(s) may be involved in the transport of the hormone within the trophoblast and may contribute to the regulation of trans-placental transfer and deiodination of the hormone. Protein(s) exhibiting thyroid hormone binding activity were isolated from human placental cytosol by gel chromatography, ion-exchange and affinity chromatography and polyacrylamide gel electrophoresis. We assessed binding activity of radioactively-labelled triiodothyronine ($^{125}$I-$T_3$) to placental cytosol following the removal of endogenous $T_3$. We demonstrated in human placental cytosol a $T_3$ binding protein with molecular weight of around 55kD, an apparent $T_3$ dissociation constant ($K_d$) of approximately 3nM, which was activated by the thiol-stabilizing agent DTT, but not by NADPH and NADP. $T_3$ binding was inhibited by D-$T_3$, L-thyroxine (L-$T_4$) and D-thyroxine (D-$T_4$) indicating that CTBP had broad specificity for thyroid hormones and lacked stereospecificity. Our results suggest human placenta has a CTBP for $T_3$, which also interacts with $T_4$.

## 338

### MATHEMATICAL MODELLING OF HUMAN MYOMETRIAL TENSION TRAJECTORIES THROUGHOUT PREGNANCY

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The determinants of parturition in humans remain largely unknown. Investigations in this area have focused on hormonal mechanisms that might determine labour onset. Studies in rats have shown that uterine stretch plays a key role in orchestrating the uterine changes that occur as the uterus becomes active. In human pregnancies, multiple gestations are associated strongly with preterm delivery although the mechanisms are uncertain. It is therefore a strong possibility that uterine stretch may participate in the onset of parturition in humans. The objective of this study is to develop appropriate equations to construct the trajectory of uterine tension for individual pregnancies and to construct a population curve of myometrial tension.

This prospective longitudinal study examines 109 women with low risk pregnancy. Ultrasound examinations were performed at 20, 24, 30 and 36 weeks gestation to measure the myometrial thickness, length, anterior-posterior diameter and transverse diameter of the uterus. Assuming an ellipsoid shape for the uterus a mathematical expression for maximum uterine tension $T_{max}$, was derived, i.e. $T_{max} = \Delta P(a^2b^2)/(wc(a^2+b^2))$, where $\Delta P$ is intrauterine pressure, $w$ is the myometrial thickness, and $2a$ ,$2b$, $2c$ are the ellipsoidal axes lengths. A two-stage method was used to model the trajectories of myometrial tension.

Myometrial tension was modelled and graphed using data from the literature on intrauterine pressure(1) and assuming a uniform myometrial thickness. The individual growth curves and the population growth curve for myometrial tension indicate that myometrial tension increases throughout pregnancy with maximal levels achieved at term. These data are consistent with myometrial tension playing a role in the onset of human labour.

1. Fisk,N.M., Ronderos-Dumit,T.D., Tannirandorn,Y., Nicolini,U., Talbert,D., Normal Amniotic Pressure Throughout Gestation, British J. of Obstetrics & Gynecology, 1992; 99:18-22

## 339

### CYCLICAL INTRAVENOUS PAMIDRONATE IN THE TREATMENT AND PREVENTION OF OSTEOPOROSIS

**Sophie Chan, Liza Nery, Aidan McElduff, Errol Wilmshurst, Greg Fulcher, Bruce Robinson, John Stiel, Jenny Gunton and Phillip Clifton-Bligh.**

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**Objective:** To assess bone mineral density (BMD) before and after cyclical intravenous pamidronate (APD).

**Design:** Retrospective audit of medical records.

**Setting:** Ambulatory care ward of the Northern Metabolic Bone Centre.

**Patients:** 84 consecutive patients at risk of fractures commencing APD between October 1997 and May 2000.

**Intervention:** 56 patients had 30 mg of intravenous APD every 3 months, 22 patients had 60 mg every 6 months and 6 patients had 60 mg every 3 months.

**Outcome measure:** BMD before and after APD.

**Results:** The mean length of treatment and mean total APD dose were 16.8±7.0 months and 186.1±79.5mg respectively. Reasons for using APD were failure to qualify for oral bisphosphonates on the pharmaceutical benefits scheme due to lack of documented minimal trauma fractures (58%), symptomatic gastroeosophageal disease (20%), intolerance of oral biphosphonates (18%) and lack of efficacy of calcitriol (4%). Mean baseline T score at L2-4 spine and femoral neck were –1.54±1.22 and

–2.87±1.19 respectively.

From baseline to after APD treatment, there was a significant increase in L2-4 BMD (0.883±0.175 g/cm$^2$ vs. 0.912±0.176 g/cm$^2$, $p<0.001$, mean increase + 3.5%), in femoral neck BMD (0.667±0.137 g/cm$^2$ vs. 0.680±0.134

g/cm$^2$, p=0.001, mean increase + 2.1%) and in trochanteric BMD (0.549±0.129 g/cm$^2$ vs 0.566±0.132 g/cm$^2$, p<0.001, mean increase + 3.1%). One third of the patients were on oral glucocorticoids at the time of the study and they had a similar increase in BMD compared to patients not on glucocorticoids. Side effects occurred in 7 patients and consisted of a mild flu-like reaction in 4 patients after the first dose.

**Conclusion:** Cyclical APD increases BMD.

**340**

## EXPERIENCE WITH INTRAVENOUS PAMIDRONATE THERAPY FOR THE PROPHYLAXIS AND TREATMENT OF OSTEOPOROSIS.

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Bisphosphonates are widely used in the treatment of osteoporosis and osteopenia. We report 2 years' experience of intravenous pamidronate (30mg 3 monthly) therapy in a diverse range of patients in whom oral bisphosphonates were contraindicated.

Bone mineral density (BMD) was measured by dual energy x-ray absorptiometry (Lunar DPX-IQ).

Thiry-eight patients with at least 12 months BMD data were studied. Baseline lumbar spine (LS) and femoral neck (FN) T scores were -4.9 to +0.94 and –4.5 to –0.52, respectively. Seventeen patients had undergone heart or lung transplantation, 12 were osteopenic/porotic due to glucocorticoid therapy and 9 from other causes. Eight of the transplant patients had their 1year post therapy DEXAs 6-12 months after transplantation, the first 6 months post-transplant being the time when bone loss due to anti-rejection drugs is most rapid.

Table 1: Mean BMD in patients treated with pamidronate.

| Mean BMD (g/cm$^2$) ± SEM | | | |
|---|---|---|---|
| | Baseline | 1 year | 2 years |
| Lung (n=15) or Heart (n=2) Transplant | LS 0.953 ± 0.047<br>FN 0.731 ± 0.024<br>(n=17) | LS 0.948 ± 0.046<br>FN 0.7 ± 0.023<br>(n=17) | LS 0.952 ± 0.069<br>FN 0.693 ± 0.028<br>(n=9) |
| Glucocorticoid | LS 0.884 ± 0.027<br>FN 0.717 ± 0.033<br>(n=12) | LS 0.938 ± 0.031<br>FN 0.729 ± 0.047<br>(n=10) | LS 0.936 ± 0.063<br>FN 0.775 ± 0.072<br>(n=5) |
| Other | LS 0.877 ± 0.060<br>FN 0.693 ± 0.044<br>(n=9) | LS 0.879 ± 0.055<br>FN 0.673 ± 0.03<br>(n=9) | LS 0.927 ± 0.096<br>FN 0.617 ± 0.03<br>(n=5) |
| All | LS 0.913 ± 0.027<br>FN 0.718 ± 0.018<br>(n=38) | LS 0.928 ± 0.027<br>FN 0.701 ± 0.018<br>(n=36) | LS 0.942 ± 0.042<br>FN 0.694 ± 0.027<br>(n=19) |

A significant increase in LS BMD was found after 12 months of pamidronate therapy in the patients with steroid-induced bone loss (p=0.012) but no significant change was seen in any other patient group, even at 6-12 months post-transplant. After 2 years of pamidronate there was an overall increase in lumbar BMD when all patients' results were analysed together (p=0.014). There was, however, no significant change in FN BMD in any patient group during the period of treatment.

These results suggest that pamidronate therapy will maintain (LS, FN) and in some cases improve BMD (LS) in patients for whom a decline in BMD would be expected without treatment and is consistent with data from other centres.

**341**

## UP-REGULATION OF THE VITAMIN D 24-HYDROXYLASE GENE PROMOTER BY 1,25 DIHYDROXYVITAMIN D: RAPID ACTIVATION AND SPECIFIC ROLES OF MAP KINASE PATHWAYS IN THE INDUCTION MECHANISM.

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Levels of 1,25 dihydroxyvitamin D (1,25D) are tightly regulated through the actions of vitamin D 1-hydroxylase and vitamin D 24-hydroxylase which direct the bioactivation and inactivation respectively of the hormone. We are studying the molecular mechanism underlying the induction by 1,25D of the vitamin D 24-hydroxylase gene which codes for the enzyme responsible for preventing accumulation of potentially toxic levels of 1,25D. Two vitamin D responsive elements (VDREs) are present in its promoter and synergy between these sites is responsible for high induction. 1,25D modulates gene expression acting through the vitamin D receptor and VDREs. Recent interest in the non-genomic actions of steroid hormones prompted us to investigate the role of MAP kinase pathways in this induction process. Using expression of promoter-luciferase constructs in transiently transfected cells, the effects of MAP kinase inhibitors and the levels of endogenous MAP kinases ERK1/2 and ERK5 were investigated. 1,25D markedly induces the promoter which is completely abrogated by inhibitors of the ERK1/2 pathway. Inhibitors of the ERK5 pathway lowered promoter activity and ERK5 pathway activity was effected entirely through an Ets-1 binding site located near the proximal VDRE. ERK2 and ERK5 activities were rapidly induced by 1,25D. Using 1,25D-activated ERK2, purified RXR alpha but not VDR is phosphorylated. Purified Ets-1 was phosphorylated by 1,25D-activated ERK5. Immunoprecipitation-western blot analyses of cell lysates have confirmed that ERK5 and ERK2 interact with Ets-1 and RXR alpha respectively. We conclude that the 1,25D induction of the 24-hydroxylase gene promoter is accompanied by activation of MAP kinase pathways critical for induction. Our studies emphasise the role of ERK2 in phosphorylating RXR and of ERK5 in phosphorylating Ets-1. Why these phosphate moieties are important for the functioning of the transcription factors is currently being investigated.

**342**

## CHARACTERISATION OF A STEROID RECEPTOR-SPECIFIC TRANSCRIPTIONAL RESISTANCE IN A GRANULOSA TUMOUR CELL LINE.

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The role of estrogen receptor β (ERβ), the predominant estrogen receptor isoform in granulosa cells, is poorly understood. We have previously reported the expression of ERβ in granulosa cell tumours (GCT) of the ovary (1). In order to understand the function and mechanism of action of ERβ in GCT, we have examined two cell lines, COV434 and KGN (2,3) that express ERβ mRNA. COV434 cells were shown to bind $^{3}H$ $E_2$ with a dissociation constant (*K*d) of 0.7nM, which is similar to that reported in other systems. To examine receptor function, these cells were transfected with a reporter vector containing two copies of the estrogen response element fused upstream of a minimal TATA promoter and luciferase reporter gene ($ERE_2$-TATA-luc). No response was seen despite adequate transfection efficiency as demonstrated by the RSV-βgal transfection control. In order to further define the basis of this lack of a transcriptional response to the endogenous receptor, an ERβ expression plasmid was constructed. Ligand-dependent transactivation at the ERE was observed with this construct in Chinese hamster ovary (CHO) cells. However, this was not observed in the COV434 cells. A general block to transcription was excluded by our previously reported observations that: i) a cAMP response

element is activated by the addition of 8-bromo-cAMP; ii) both heat shock and serum response elements are activated by their appropriate stimuli; and iii) both AP1 and NFκB response elements are constitutively activated in these cells. Conversely, co-transfection of the glucocorticoid receptor with either one of two GRE reporter constructs (both of which are active in CHO cells) again failed to demonstrate ligand-dependent transactivation. Preliminary observations suggests that the same is also true for the KGN cell line. The lack of a ligand-induced steroid receptor transcriptional response in this cell line is intriguing. We are currently exploring the possible mechanisms for this transcriptional block including the possibility that it reflects metabolism or rapid efflux of steroid hormones. Whilst its relevance to GCT *in vivo* is not clear, it is noteworthy that these tumours exhibit a poor response to anti-estrogen therapy.

1) Chu *et al*. *JCEM* 2000. 85(3):p1200 2) van Den Berg-Bakker *et al*. *Int J Cancer* 1993. 53:p613
3) Nishi Y *et al. Endocrinology* 2001. 142:p437

**343**

## INHIBITION OF ANDROGEN SIGNALLING IN PROSTATE CANCER CELLS BY DOMINANT NEGATIVE ANDROGEN RECEPTORS

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There is increasing evidence that the failure of androgen ablation therapy for advanced prostate cancer is not necessarily a result of resistance to androgens, but rather a deregulation of androgen signalling in prostate cancer cells (1, 2). More effective inhibition of the androgen signalling axis, irrespective of the structure, activity or level of expression of the androgen receptor may be a useful approach to the treatment of advanced disease. Here we have designed and constructed variants of the AR that have no intrinsic transactivation activity, and tested them for their ability to inhibit the activity of wildtype AR and mutant ARs identified in human prostate tumours. One dominant negative AR (DNAR) construct, which contained a deletion of the N-terminal transactivation domain, had no intrinsic transactivation activity and was able to inhibit the activity of wtAR by up to 95% when transfected at a 4:1 molar ratio of DNAR to wtAR. Significantly, this DNAR was equally effective in inhibiting the activity of superactive mutant ARs identified in human prostate tumours, in the presence of both classical (dihydrotestosterone) and nonclassical (hydroxyflutamide and progesterone) ligands. The results suggest that DNARs may be particularly useful for the treatment of tumours that have acquired AR mutations that allow activation of the receptor by agents such as hydroxyflutamide. More effective targeting of the androgen signalling axis by molecular agents such as DNARs provides a novel approach for the treatment of advanced prostate cancer, either alone or as an adjunct to the current surgical and hormonal treatment modalities.

(1) Buchanan and Tilley. In: Hormonal Carcinogenesis III, Springer-Verlag, New York, pp 333-341, 2000.
(2) Feldman and Feldman. Nature Rev. Cancer 2: 101-115, 2001.

**344**

## INCREASED ANDROGEN RECEPTOR AND HER2 IMMUNOREACTIVITY IN CLINICALLY LOCALISED PROSTATE CANCER IS ASSOCIATED WITH DISEASE PROGRESSION FOLLOWING RADICAL PROSTATECTOMY

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Recent studies indicate that prostate tumor cells with increased levels of androgen receptor (AR) and or HER2/neu may have a selective growth advantage. HER2/neu may confer a clonal growth advantage by

promoting cancer cell survival via activation of the androgen signaling axis or by induction of Akt-dependent pathways. AR and HER2/neu immunoreactivity was evaluated in archived prostatic tissues obtained from 53 men with clinically organ-confined disease who underwent radical prostatectomy. 40/52 AR positive tumors had strong punctate nuclear AR immunostaining, that was dispersed throughout the nucleus (pattern 1), whilst the remaining tumors (12/52) showed prominent accentuation of AR immunoreactivity around the nuclear membrane that was frequently associated with overt nuclear clearing (pattern 2). Tumors with pattern 1 AR immunostaining had significantly higher percentage AR positive (% AR POS) nuclear area than tumors with pattern 2 immunostaining (medians 77.0% and 51.0%, respectively; $P = 0.001$). In addition, AR immunostaining in all tumors from patients with PSA-relapse (16/16) exhibited pattern 1 AR immunostaining whereas pattern 2 was only observed in tumors of patients who did not progress. HER2 immunoreactivity was localised to both the cell membrane and cytoplasm of tumor cells. Membranous HER2-rich immunostaining was present in 26.4 % (14/53) of tumors, whereas 50.9% (27/53) of tumors had cytoplasmic HER2-rich immunostaining. All tumors with HER2-rich membranous immunostaining had HER2-rich cytoplasmic immunostaining, whereas 24.5% (13/53) had HER2-rich immunostaining in the cytoplasm alone. Membranous HER2 immunostaining did not correlate with PSA-progression nor any of the clinicopathological parameters examined. However cytoplasmic HER2 immunoreactivity was significantly related to Gleason score, pathological stage, seminal vesicle involvement and preoperative serum PSA levels. Percentage AR POS nuclear area and cytoplasmic HER2 within tumor foci were associated with PSA progression in Cox univariate analysis. In Cox multivariate analysis, % AR POS nuclear area and Gleason score were independent predictors of PSA-relapse. Current studies are underway to expand the cohort and to further characterise the specificity of HER2 cytoplasmic immunostaining observed in this study.

**345**

## LACK OF RESPONSE TO THE SYNTHETIC PROGESTIN, MEDROXYPROGESTERONE ACETATE, IN ADVANCED BREAST CANCER IS ASSOCIATED WITH EXPRESSION OF NON-FUNCTIONAL ANDROGEN RECEPTORS

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Increasing evidence suggests that the growth of breast cancer cells is modulated by the androgen signalling axis (ASX), which may be an important target for novel therapeutic agents (1). Until recently, the synthetic progestin medroxyprogesterone acetate (MPA) was extensively used as a second-line hormonal therapy for breast cancer. In previous studies, we found that the level of expression of the androgen receptor (AR) in the primary tumor is the sole predictor of response to MPA therapy (2), suggesting that the action of MPA is mediated in part by high-affinity binding to the AR. We analysed the tumors from these breast cancer patients for AR expression and function, by immunohistochemistry and radio-ligand binding activity, respectively. Patients who did not respond to MPA therapy exhibited a discordance between AR expression and AR function in their primary tumors. Eleven tumors from 51 non-responding patients expressed AR, but showed no detectable ligand binding. DNA sequencing of the ligand binding domain of the AR from these 11 tumors identified two mutations (Met-Thr778 and Met-Val805). In vitro transactivation analysis indicated that these mutations result in non-functional ARs, which have either no ligand binding capacity (Met-Val805) or greatly reduced (Met-Thr778) stability of ligand binding. These studies provide further evidence that AR-mediated pathways are involved in the response of breast tumors to MPA, and emphasize the importance of the ASX as a regulator of breast cancer progression.

(1) Birrell SN et al., J. Mammary Gland Biol. Neoplasia 3: 95-103, 1998.

(2) Birrell SN et al., J. Clin. Oncol. 13: 1572-1577, 1995.

## 346

### RECOVERY OF PITUITARY FUNCTION IN XANTHOMATOUS HYPOPHYSITIS: A CASE REPORT

**Morton Burt, Adrienne Morey and Ken K Y Ho.**

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Inflammatory lesions of the pituitary gland or hypophysitis can mimic pituitary tumours causing headaches, visual impairment and hypopituitarism. Hypophysitis may be primary or secondary to infection or systemic disease. Primary inflammatory lesions which are more common are classified histopathologically into three categories: lymphocytic, granulomatous and xanthomatous(1). Xanthomatous hypophysitis, the least common and first described in 1998, are frequently cystic lesions containing xanthomatous fluid and defined histologically by the presence of lipid rich foamy histiocytes(2). The cause and natural history are unknown. We describe a case of xanthomatous hypophysitis presenting with a mass lesion and reversible pituitary hypofunction. A 29 year old male presented with headaches and severe vomiting against a 3-4 month history of sexual dysfunction. Clinical and biochemical evaluation revealed evidence of cortisol, thyroid hormone and testosterone deficiency. Vomiting resolved immediately with glucocorticoid treatment which induced marked polyuria. Visual field examination revealed bitemporal hemianopia. An MRI scan demonstrated a 15 x 18 mm rim enhancing complex cystic pituitary lesion extending superiorly and compressing the optic chiasm. He proceeded to transphenoidal surgery which revealed a tense cystic lesion containing yellow fluid. Histological examination revealed features consistent with xanthomatous hypophysitis. Post-operative endocrine assessments revealed a normal testosterone (14.5 nmol/L) and peak cortisol of 470 nmol/L, permitting withdrawal from glucocorticoid replacement. This is the first report of recovery of pituitary function after transphenoidal surgery for xanthomatous hypophysitis.

References:

1. Cheung CC, Ezzat S, Smyth HS et al. J Clin Endocrinol Metab. 2001;86:1048-53.
2. Folkerth RD, Lowell Price Jr D, Schwartz M et al. Am J Surg Pathol. 1998;22:736-41.

## 347

### INOPERABLE INTRACARDIAC PHEOCHROMOCYTOMA IN A YOUNG WOMAN WITH SUBSEQUENT PREGNANCY: A CASE REPORT

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We present the case of a non-syndromic intracardiac pheochromocytoma in a young women. Initial evaluation suggested the presence of a mediastinal lesion, confirmed as a functional pheochromocytoma on urinary catecholamine measurements. Subsequent magnetic resonance imaging and coregistration with nuclear medicine scans confirmed the location as intracardiac. Surgical resection was aborted due to extensive free right ventricular wall involvement. Further management of the case has been complicated by the subject's subsequent pregnancy.

Shapiro B., Orringer B. and Gross M., Mediastinal Paragangliomas and Pheochromocytomas, General Thoracic Surgery ( 5th Ed), Vol 2, Ch 184:2333-2355

1. Hamilton B., Francis I., Gross, B., Kjorobkin M., Shapiro B., Shulkin B., Deeb C. and Orringer M. (1997) Intracardiac Paragangliomas ( Pheochromocytomas): Imaging Features, AJR 168: 109-113
2. Jeevanandam V., Oz M., Shapiro B., Barr M., Marboe C. and Rose A. (1995) Surgical Management of Cardiac Pheochromocytoma Resection versus Transplantation, Ann Surg 221(4):415-419

Loh, K., Fitzgerald P., Matthay K., Yeo P. and Price D (1997) The treatment of malignant pheochromocytoma with Iodine-131 metaiodobenzylguanidine ($^{131}$I-MIBG): a comprehensive review of 116 reported patients, J End Inv, 20:648-658

Harper A. a et al, Pheochromocytoma in Pregnancy. Five cases and a review of the literature, Br J Ob Gyn 96,594-606, 1989

Neumann H. et al (2002) Germ-Line Mutations in Nonsyndromic Pheochromocytomas, NEJM 346(19):1450-66

## 348

### PULSED TESTOSTERONE THERAPY: PHARMACOKINETICS AND SAFETY OF INHALED TESTOSTERONE IN POSTMENOPAUSAL WOMEN.

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Background: Testosterone administered by implant (1), or transdermally (2,3), has been shown to increase libido and mood in women. In a recent study, sublingual administration of testosterone was shown to have an acute effect on arousal which peaked at 4½ hours (4) suggesting that acute dosing with testosterone may be a viable therapeutic alternative for the treatment of female sexual dysfunction related to testosterone insufficiency. The lung has a large surface area with a highly permeable blood barrier allowing for rapid and efficient delivery of systemic medications. The AERx System has been developed for the pulmonary delivery of aerosolised liquid formulations of small and large molecules. This system has been used for the pulmonary delivery of insulin , morphine , rhDNase, and other compounds.

Objectives: The purpose of this study was to determine the pharmacokinetics and safety of a single dose of orally inhaled testosterone via the AERx® System, a handheld aerosol delivery system and to predict the dose required to increase plasma testosterone levels to a clinically relevant extent.

Materials and Methods: 12 postmenopausal women on oral oestrogen inhaled a single dose of testosterone (100, 200 or 300 mcg). Pulmonary and cardiovascular adverse events were monitored. Plasma concentrations of sex steroids were measured between 1 and 360 min. Results: At the 300 μg dose, total and free testosterone increased from baseline (mean 0.5+ 0.2 nmol/L (SD), 2.6 + 1.4pmol/L) to Cmax of 62.6 + 20.4 nmol/L and 168.2 + 50.2pmol/L at Tmax 1.3 + 0.5 min and 1.4 + 0.5min respectively at the maximal dose of 300mcg. DHT significantly increased at 60min ($p<0.0002$). All androgen levels returned to baseline by 360 min. Oestradiol and did not vary, but SHBG and albumin fell. No adverse events occurred. Conclusions: The AERx® System delivers aerosolised T and achieves a "pulse" kinetic profile of T with a rapid return to pre-treatment levels. Inhalation of aerosolised T to women offers a potentially more pro re nata approach to manage female androgen insufficiency.

1. Davis SR et al Maturitas 1995;21:227-36.
2. Shifren JL et al. N Eng J Med 2000;343(10):682-8.
3. Goldstat R et al. Proc N.American Menopause Society, N Orleans, USA 2001;
4. Tuiten A et al. Arch Gen Psychiatry 2000;57:149-53.

## 349

### FACTORS UNDERLYING THE RISING INCIDENCE OF PAPILLARY THYROID CARCINOMAIN TASMANIA (1988–1998).

**John R Burgess**

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Background: There has been a significant rise in the incidence of papillary thyroid carcinoma (PTC) in Australia over the past two decades. Possible explanations include a spurious increase in rate due to various forms of ascertainment bias, as well as a true increase in underlying incidence rate resulting from factors such as increased exposure to ionising radiation and changing levels of dietary iodine nutrition. Increased identification of clinically occult thyroid PTC due to greater use of thyroid ultrasound, fine needle biopsy (FNAB) and more detailed histopathological scrutiny of tissue samples, is the most widely postulated source of ascertainment bias. In this report we examine the relationship between thyroid surgical and cytological procedures performed in Tasmania over an 11-year period and spanning a two-fold rise in the incidence of PTC in Tasmania.

Methods: All public and private pathology institutions in Tasmania provided data relating to surgical and FNAB cytological procedures between 1988 and 1998. This information was used to determine time trends for thyroid surgical procedures, the relationship between benign and malignant diagnoses, and the impact of FNAB usage on the rate of PTC diagnosis.

Results: A total of 1940 discrete patient surgical procedures and 1767 FNAB were identified. Between 1988-91 and 1995-98 there was a 68% and 222% increase in surgical and FNAB procedures respectively. The incidence

of PTC increased by 148% during this period. However, the proportion of PTC : thyroidectomies increased from 1:20 to 1:13 over this time. Even after accounting for the increased use of FNAB, there was a 27% absolute increase in the likelihood of diagnosing PTC in any patient undergoing thyroidectomy.

Conclusions: These findings indicate that there has been a rise in PTC incidence which is independent of increase in the use of FNAB or the numbers of patients undergoing thyroid surgery. This may represent either a true increase in underlying PTC incidence or a substantial improvement in the histopathological recognition of occult PTC between 1988 and 1998.

References: Burgess JR. 2002 Temporal trends for thyroid carcinoma in Australia: a rising incidence of papillary thyroid carcinoma (1982 – 1997). Thyroid. 12:141-149.

**350**

## FAMILIAL NEUROHYPOPHYSEAL DIABETES INSIPIDUS (FNDI) ASSOCIATED WITH A MISSENSE MUTATION ENCODING CYS58→ARG IN NEUROPHYSIN II (NPII)

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We report on one daughter from a family in which the father and four children appear to have diabetes insipidus. The proband has confirmed central diabetes insipidus. Symptoms began in early childhood but not infancy. We hypothesised that the family may have familial neurohypophyseal diabetes insipidus (fndi), an autosomal dominant disorder characterised by progressive postnatal deficiency of arginine vasopressin as a result of mutation in the gene that encodes the precursor peptide prepro-avp-neurophysin ii (avp-npii). The avp-npii gene is located in the chromosomal region 20p13 and contains three exons.(1). Mutations that cause fndi have been found within the signal peptide, within the coding sequence for neurophysin ii and within the vasopressin-coding sequence. (2)

Aim: to screen the proband's genomic dna for mutations in the avp-npii gene.

Methods: genomic dna was isolated from the proband's whole blood and pcr products were sequenced. Restriction analysis was performed to verify the sequence results.

Results: the proband was found to have a missense mutation in exon 2 at nucleotide position 1769 (t to c) of the avp- npii gene. The substitution was confirmed by restriction analysis. The mutation was present in only one reading frame, and was present in analysis in both directions. The mutation predicts a cys58→arg within npii, and represents a novel mutation of the avp-npii gene causing fndi.

Discussion: mutations in cysteine residues are believed to make the nacent peptide unstable and prevent appropriate cellular processing. The mutant peptide is believed to accumulate within the vasopressinergic neurons resulting in eventual cell death. This would explain both the slow onset of clinical symptoms and the dominant negative nature of the mutation.

Nfdi is one of the conformational diseases (3)

1. Cell Mol Genet 1985, 11:189-195.
2. Am J Hum Genet 1996, 58: 107-117.
3. N Engl J Med 2002; 346:45-53.

**351**

## REGULATION OF RELAXIN GENE EXPRESSION IN THE CORPUS LUTEUM OF THE TAMMAR WALLABY.

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In the tammar wallaby, a macropodid marsupial, the corpus luteum (CL) produces both progesterone and relaxin, a peptide hormone known to play an important role in remodelling and softening the cervix in late pregnancy. The lifespan of the CL is not extended by the presence of a conceptus in the tammar, and the luteal phase in unmated animals is similar in length to the 26-day gestation. In both pregnant and non-pregnant animals, there is a single CL present on one ovary and a developing follicle on the other ovary throughout the luteal phase. Hence,

the essential difference between the two reproductive cycles is the presence of a conceptus in the pregnant cycle. The aim of this study was to compare relaxin gene expression in the CL of pregnant and non-pregnant tammar wallabies at different stages of the luteal phase, to determine if there is a conceptus influence on ovarian relaxin production. Tissues were obtained from 5 equivalent stages of each cycle (n=4 per stage) and relaxin mRNA levels were measured by real time PCR, using tammar specific relaxin primers and a FAM-labelled probe in an ABI PRISM 7700 Sequence Detector. Relaxin gene expression was significantly ($P<0.01$; ANOVA) upregulated in CLs of pregnant tammars in early and mid luteal stages, but decreased in the late luteal phase, one day before expected births on day 26. Relaxin mRNA levels in non-pregnant animals were also significantly ($P<0.037$) higher throughout the early luteal phase, but decreased in the mid luteal phase on day 20, which is earlier than in pregnant animals. Furthermore, relaxin mRNA levels were significantly ($P<0.05$) lower in non-pregnant animals compared with pregnant animals in early and mid luteal stages. As the yolk sac placenta forms between days 18-19 in pregnant animals, it appeared possible that a placental factor influenced ovarian relaxin production. However, removal of the conceptus on either day 17 (20-somite embryo stage; n=4) or day 20 (fetus and placenta; n=6) of gestation did not alter ovarian relaxin mRNA levels when compared with sham operated controls (n=5). These data suggest that a pregnancy-specific factor influences ovarian relaxin synthesis in the tammar wallaby. However, it is unlikely that this factor is derived from the conceptus.

**352**

## STEROID PRODUCTION BY ADRENALS AND GONADS FROM EMBRYONIC SOUTHERN SNOW SKINKS, NIVEOSCINCUS MICROLEPIDOTUS, DURING THEIR PROTRACTED GESTATION PERIOD.

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In some mammalian species, hormones produced by the embryo(s) at the completion of development trigger the cascade of events that result in parturition; our overall aim is to determine whether a similar mechanism exists in viviparous reptiles. The alpine skink *Niveoscincus microlepidotus* provides a useful model for studies of gestation and parturition in viviparous reptiles as the completion of embryonic development and parturition are temporally separated; ovulation occurs in spring, embryonic development is completed by autumn, but parturition does not occur until the following spring. In this study, we determined how *in vitro* steroid hormone production by embryonic adrenals (progesterone, corticosterone and testosterone) and gonads (oestradiol and testosterone) varied during gestation. Embryonic adrenals were incubated with or without ACTH; embryonic gonads were incubated with or without the steroid precursor pregnenolone. Tissues were incubated for 3 h at either 16 °C or 24 °C (preferred body temperature of pregnant *N. microlepidotus*). Incubation media were analysed for steroid hormones using radioimmunoassay. Low levels of progesterone were produced *in vitro* throughout gestation when embryonic adrenals were incubated with ACTH. *In vitro* corticosterone production by embryonic adrenals occurred throughout gestation; greater production occurred when tissues were incubated at 24 °C, in comparison to production at 16 °C, and when ACTH was included in the incubation media. *In vitro* testosterone production by embryonic adrenals and gonads only occurred in autumn; production at 16 °C was greater than that at 24 °C. Low levels of oestradiol were produced *in vitro* by embryonic gonads in March. Further research is needed to differentiate the activity of various tissues and steroid hormones in the control of embryonic development, sexual differentiation, and the potential regulation of gestation and parturition in *N. microlepidotus* and other viviparous reptiles.

## 353

### DOES HABITAT DETERMINE HORMONAL REGULATION OF METAMORPHOSIS IN AUSTRALIAN ANURANS?

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Several species of North American anurans exhibit accelerated metamorphosis in the face of environmental challenge such as pond drying. Denver (1) demonstrated that thyroid hormone dependant metamorphosis is regulated by CRH. Australian anurans have evolved separately from North American species for 125Myr. Observation of Australian anurans has demonstrated that some species respond to stress with rapid metamorphosis while others do not. We hypothesized that in anurans occupying an ephemeral habitat metamorphosis and thyroid hormone production in tadpoles is regulated by CRH whereas species occupying stable habitats do not exhibit this endocrine control mechanism. To test this hypothesis we have studied two species from the family (Hylidae); the ephemeral pond breeder Litoria chloris (metamorphosis 1-2 months) and the longer-lasting pond dweller Litoria aurea (4-8 months). Brain CRH peptide was extracted from dissected brains at six timepoints through metamorphosis and measured by radioimmunoassay. We detected a steady increase in CRH throughout metamorphosis in Litoria chloris tadpoles (metamorphosis 35d). However, CRH remained low throughout metamorphosis in Litoria aurea (109d). Investigation of more species from each group in this apparent dichotomy of metamorphic strategies is needed to determine whether this difference in brain CRH levels corresponds with the reproductive habitat in which each species evolved.

(1) Robert J Denver (1997). Environmental stress as a developmental cue: corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis, Hormones & Behaviour 31(2):169-79.

## 354

### LOCAL EMBRYO-DERIVED FACTORS INFLUENCE UTERINE OXYTOCIN RECEPTORS IN THE TAMMAR WALLABY.

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Uterine smooth muscle contractions are stimulated by the oxytocin-like peptide, mesotocin (MT) in parturient marsupials. The tammar wallaby is a monovular species of marsupial, with two anatomically separate uteri. The single conceptus is present in one uterus (gravid), whereas the contralateral uterus is nongravid. The unique reproductive tract anatomy of the female marsupial enables local embryo-derived factors that regulate contractile associated proteins to be differentiated from systemic factors. Previous studies showed a marked increase in MT receptor (MTR) gene expression and receptor concentrations in the myometrium of the gravid uterus between Days 22 and 24 of the 26-day gestation. However, the factors that cause this upregulation in MTRs have not been identified. The objective of this study was to determine whether uterine stretch causes the gravid uterus-specific increase in MTRs. The first experiment involved surgical removal of the embryo from the gravid uterus on Day 17 of gestation (n=5). As MTR upregulation occurs on Day 23 of gestation, the animals were euthanized at this stage and myometrial MTR mRNA and receptor concentrations were measured in both uteri, using real-time PCR and a radioreceptor assay ($^{125}$I-OTA) respectively. In a second group of tammars, the embryo was removed on Day 17 and dental rubber ($\approx$ 3ml) was inserted into the gravid uterus to artificially stretch the uterus in the absence of the embryo (n=6). In sham-operated controls (n=5), there was a significant ($P<0.01$: paired t-test) increase in MTR mRNA and receptor concentrations in the gravid uterus compared with the nongravid on Day 23 of gestation. Surgical removal of the embryo effectively eliminated uterine distension and resulted in significantly ($P<0.05$: independent t-test) lower MTR mRNA and receptor concentrations compared with the gravid uterus of sham-operated controls. However, artificial distension of the uterus with dental rubber, in the absence of the embryo, did not cause an increase in MTRs on Day 23. There was no significant ($P>0.05$; independent t-test) difference in MTR mRNA concentrations between the artificially distended uterus and non-distended gravid uterus lacking the embryo. These data demonstrate that local embryo-derived factors regulate MTRs in the gravid

uterus. Furthermore, uterine distension does not appear to be essential for the increase in MTRs observed on Day 23 of gestation.

**355**

## THE EFFECT OF L-CARNITINE ON ENERGY BALANCE IN THE MARSUPIAL SMINTHOPSIS CRASSICAUDATA.

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Free fatty acids (FFAs) may play an important role in regulating energy balance by influencing food intake and the expression of genes associated with energy balance such as leptin and the uncoupling proteins (UCPs). L-carnitine has been shown to increase the specific activity of carnitine palmitolytransferase 1 (CPT1), increasing FFA transport into the mitochondria. The aim of this study was to determine the effect of increasing FFA transport on energy balance in the marsupial Sminthopsis crassicaudata (Sc).

(i). To determine the acute effect of L-Carnitine on food intake, female Sc (n=6/group) maintained on either lab diet (20% fat, 1.01 kcal/g) or mealworms (30% fat, 2.99 kcal/g) were fasted for 24 hours, and then injected (intraperitoneally) IP with either L-carnitine (50, 100 or 200 mg/kg) or 0.9% saline at the onset of the dark phase. Food intake was measured after 0.5, 1, 2, 4, 6 and 24 hours. In lab diet fed animals, L-Carnitine had no effect on food intake, whereas in mealworm fed animals, L-Carnitine, 100 and 200 mg/kg increased food intake ($p=0.047$ and $p=0.014$ respectively).

(ii) Chronic L-Carnitine administration. Female Sc (n=8/group) maintained on either lab diet or mealworms received L-carnitine (200 mg/kg) IP daily for 21 days. Food intake, body weight, tail width and physical activity were measured daily. An intraperitoneal glucose tolerance test was performed at baseline and at day 14. On day 21 animals were sacrificed, plasma FFA and glucose levels were measured. Muscle, liver and fat were collected for the measurement of UCP2/3 and leptin mRNA expression by northern blot analysis. In lab diet fed animals chronic administration of L-carnitine did not affect food intake, body weight or physical activity, plasma glucose, or glucose utilisation, but tail width, a measure of fat storage and plasma FFA levels decreased by 12% ($p = 0.03$) and 57% ($p = 0.037$) respectively. Data analysis is ongoing, but the preliminary data suggest that in Sc increased transport of free fatty acids across the inner mitochondrial membrane modifies energy balance in diet-dependant manner; a decrease in fat mass and plasma free fatty acids does not necessarily lead to an increase in food intake.

**356**

## GROWTH HORMONE TREATMENT OF MEN WITH ABDOMINAL OBESITY REDUCES FAT ACCUMULATION IN THE LIVER.

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The association between fatty liver and abdominal obesity is well known. Since GH treatment reduces visceral fat mass (1) and probably increases the flow of fatty acids in the portal circulation, we hypothesized that GH treatment also influences fat accumulation in the liver. .

**Patients and methods:** Thirty-three healthy men aged 54.7 (range 40.6-67.7) years with serum IGF-1standard deviation score (S-IGF-1 SD score) in the lower quartile and central adiposity were included in an open treatment trial with GH. The dose of GH was adjusted to achieve S-IGF-1 SD score to the upper quartile. Fat accumulation in the liver and abdominal fat was assessed using abdominal CT. Body composition was measured using DXA. Glucose tolerance test (OGTT) and glucose infusion rate (GIR) were measured. Measurements were performed at baseline and repeated after 1 year of treatment.

**Results:** Serum IGF-1 concentration increased from 133.4±6.5 to 263.8±11.3 µg/l ±SEM (S-IFG-1 SD score –0.93±0.12 to 1.83±0.22 SD score). Mean dose of GH after 1 year was 1.27±0.07 IU/day. Liver attenuation increased from 45.2±2.5 to 48.3±2.5 Houncefield units ($p=0.008$). Visceral adipose tissue decreased from 200.1±12.3 to 180.3±19.2 $cm^2$ ($p<0.001$). Fasting P-glucose increased from 5.2 to 5.4 mmol/l ($p=0.044$) while

GIR (p=0.73) and P-glucos 2 h after OGTT (p=0.31) did not change. Changes in liver attenuation correlated to changes in sagittal diameter (r=-0.38 p=0.031), visceral adipose tissue (r=-0.35 p=0.047) and total body fat (r=-0.48 p=0.004).

**Conclusion:** Twelve months of GH treatment decreased fat accumulation in the liver in healthy men with central obesity. This change was associated with a decrease in visceral fat- and total body fat mass.

1. Johannsson G, Mårin P, Lönn L, et al. Growth hormone treatment of abdominally obese men reduces abdominal fat mass, improves glucose and lipoprotein metabolism, and reduces diastolic blood pressure. J Clin Endocrinol Metab. 1997;82:727-734.

**357**

## FUNCTIONAL DIFFERENCE OF SOMATOTROPES FROM AROMATASE KNOCKOUT AND WILD-TYPE MOUSE PITUITARIES

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Available data about the influence of oestrogen on GH levels are controversial with little conclusion. Based on the difference of GH profiles in male (high GH pulse) and female (low GH pulse), an inhibitory effect has been suggested for oestrogen. The GH-deficiency occurring in postmenopausal women suggests however a stimulatory or permissive role of oestrogen in maintaining GH levels. In addition, oestradiol (E2) replacement therapy in postmenopausal women has a positive effect on GH. In this experiment, we investigated pituitary somatotrope function from aromatase knockout (ArKO) mice, where no oestrogen was made due to the knockout of a key enzyme in oestrogen production. Pituitary glands were obtained from 4 groups of female adult mice: 1) wild type (WT) (n=6) 2) WT plus E2 replacement (n=6); 3) ArKO (n=4); 4) ArKO plus E2 replacement (n=4). In E2 replacement, a pellet was implanted to release E2 for 21-days. Expression of GH, GH-secretagogues receptor (GHS-R), GH-releasing hormone receptor (GHRH-R), pituitary-specific transcription factor (Pit-1) and somatostatin receptor (sstR1-5) were measured using semi-quantitative RT-PCR. Pituitary mass and animal weight was also measured. Total RNA was extracted from anterior pituitaries and then reverse transcribed to cDNA. Target genes were amplified with PCR using specific sets of primers. Levels of mRNA were normalised to GAPDH (internal control) before making comparison between groups and ANOVA test was employed for statistical analysis. In ArKO mice, the levels of mRNA encoding for GH, GHS-R, GHRH-R and Pit-1 were low but for sstR1-5 were high. Replacement of E2 increased GH, GHRH-R and Pit-1 mRNA but decreased sst-1, sst-2, sst-4 and sst-5 expression. The pituitary weight, the ratio of pituitary and body weight and amount of pituitary total RNA were increased in both Wt and ArKO mice after E2 treatment. Based on the results, we conclude that somatotropes from ArKO mice have a low expression of GH, GHRH-R, GHS-R and a high expression of sstR, leading to a reduction in GH release. E2 treatment up-regulates GH, Pit-1 and GHRH-R synthesis and decreases sstR synthesis to the levels observed in WT. The function of somatotropes is presumably returned to normal after E2 treatment in ArKO mice. We are now investigating the plasma GH levels in ArKO mice with or without E2 replacement.

**358**

## EVIDENCE THAT INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS HAVE INDEPENDENT AND IGF-I DEPENDENT ENDOCRINE ACTIONS IN PREGNANCY

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Insulin-like growth factors (IGF) are present in blood in association with IGF-binding proteins (IGFBP). Pregnancy and feed restriction alter the concentrations of IGF-I and IGFBP in blood in humans and guinea pigs. The aim of this study was to determine the effects of pregnancy and feed restriction on the relationships between the weights of tissues and the ratio of the concentrations of IGFs and IGFBPs ([IGFs]/[IGFBPs]) in blood. Nulliparous 4 mth old female guinea pigs were fed either ad libitum or 30% less than this for 28d prior to mating and for the first 30d of pregnancy, then 10% less than ad libitum for the next 30d. Dams were killed at day 60 of pregnancy and nonpregnant animals after 88d of the experimental diet for the collection of blood. IGF-I and IGF-

II were measured by RIAs after size exclusion HPLC at pH2.5. IGFBPs were measured by densitometric analysis of western ligand blots. Plasma IGF-I was found to be an independent covariate of pregnant body weight and weights of liver, uterus, fetuses and placentae. Plasma IGFBP-3 was an independent covariate of maternal body composition. The ratio [IGF-I]/[IGFBP-1] was an independent covariate of net maternal body weight and weights of dorsal fat, thymus, soleus muscle and liver. [IGF-I]/[IGFBP-2] was an independent covariate of maternal body weight and weight gain, and weights of carcass, retroperitoneal fat, uterus, fetuses and placentae. In nonpregnant animals IGF-II was related to weights of most tissues. [IGF-II]/[IGFBP-2] was an independent covariate of body composition in nonpregnant animals. Strong positive associations between tissue weights in pregnancy and plasma [IGFBP-3] suggest IGFBP-3 may have IGF independent action on these tissues. Strong positive associations between weights of maternal body composition and conceptus with [IGF-I]/[IGFBP-2] suggest IGFBP-2 inhibits endocrine actions of IGF-I. We propose that pregnancy alters the proportions of IGF-IGFBP complexes in blood. It also appears that actions of IGFBP-2 are IGF dependent whereas IGFBP-3 appears to have IGF independent actions.

**359**

## MDA-MB-468 BREAST CANCER CELLS ADHESION TO FIBRONECTIN IS REGULATED BY INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 (IGFBP-3).

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IGFBP-3, the predominant IGFBP in circulation, is thought to modulate IGF-stimulated signaling by regulating their interactions with the IGF type I receptor. Recent studies, however, have indicated that IGFBP-3 can inhibit cancer cells proliferation in an IGF-independent manner, and may mediate the actions of growth inhibitory factors like TGF-β, retinoic acid, vitamin D, TNF-α, antiestrogens and the tumour suppressor protein p53. We now report that IGFBP-3 (1 μg/ml) can regulate the cell adhesion for two invasive human cell types. In both the DU145 prostate cancer and in MDA-MB-468 breast cancer cell lines, adhesion to fibronectin-coated plates was significantly increased by IGFBP-3. This effect is specific as neither bovine serum albumin nor IGFBP-6 can alter the adhesion of these cells to fibronectin. This effect is extracellular matrix dependent, as IGFBP-3 did not alter cell adhesion to either type V collagen or to vitronectin. Since IGFBP-3 is known to bind to ECM, we next determined whether the effects of IGFBP-3 on cell adhesion correlated with its ability to bind to the extracellular matrix. In a solid phase binding assay using plates coated with either fibronectin, type IV collagen or vitronectin, we found that radiolabelled IGFBP-3 binds to both fibronectin and collagen, but not to vitronectin. These results suggest that IGFBP-3 binding to extracellular matrix is not correlated with its effect on cell adhesion, and that IGFBP-3 effects may involve specific integrin receptors. Interaction of cells with the extracellular matrix regulates a number of important cellular processes including programmed cell death. In this process, FAK, a cytosolic tyrosine kinase that is localized to focal adhesion sites, plays a central role in integrin-mediated signal transduction. Since FAK can stimulate MAPK signaling which can also regulate cell migration, proliferation and survival, we are currently assessing whether IGFBP-3 can alter the activity of FAK and other downstream signaling pathways like MAPKs.

This study is funded by the NH&MRC of Australia

## 360

### PRESENCE OF GROWTH HORMONE SECRETAGOGUES RECEPTOR (GHS-R) AND GHRELIN IN ENDOMETRIAL ADENOCARCINOMA

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The GHS-R and its ligand, ghrelin, are widely expressed in peripheral tissues outside the hypothalamus-pituitary axis and in neoplastic tissues including neuroendocrine, thyroid, intestinal and prostate tumour or cancer cells. The aim of this study was to investigate the cellular origin and the presentation pattern of the GHS-R and ghrelin across pathological grades of endometrial adenocarcinomas. The mRNA levels of the GHS-R and ghrelin across grade 1, 2 and 3 cancer biopsies were determined by reverse transcriptase (RT) polymerase chain reaction (PCR) to identify the presence of mRNA expression for both the GHS-R and ghrelin across the grades of tumours. Tumour biopsies were subjected to immuno-histochemistry for both GHS-R and ghrelin using specific antibodies (Merck Research Lab. USA and Cardiovascular Research Institute, Japan) and immuno-peroxidase detection methods. The intensity of immunostaining was scored semi-quantitatively from (-) negative to (++++) in relation to known positive and negative controls. Tissue was homogenised in guanidium thiocyanate and total RNA extracted using phenol–chloroform. Semi-quantitative PCR was performed after RT reaction. Equal loading was monitored using cyclophillin expression as internal control. All grade 1, 2 and 3 endometrial adenocarcinomas were positive for GHS-R and ghrelin mRNA expression. Specific immunostaining for both the ligand and its receptor was observed across the various grades of tumours. Both GHS-R and ghrelin staining was confined predominantly to epithelial cells in endometrial carcinomas. The intensity of the staining was higher than the highest level observed during the normal menstrual cycle (during the mid-secretory phase) (1). Among tumours, the immunostaining intensity increased with the pathological grade of tumours with the strongest staining in grade 3 tumours ($p<0.01$ grade 1 versus grade 3). It is concluded from above results that the GHS-R and ghrelin appear mainly in epithelial cells in endometrial tumours with increasing immunoreactive intensity along pathological grade of tumours, suggesting a potential role in the development of these tumours. We are currently investigating the function of the ghrelin system in the proliferation and apoptosis of tumour cells in culture. (Supported by NHMRC and Prince Henry's Institute of Medical Research).

(1) ESA-SRB Annual Scientific Meeting Abstract 2001

## 361

### REGULATION OF THE SOCS3 PROMOTER ACTIVITY BY GROWTH HORMONE AND OESTROGEN.

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Members of the Suppressor of Cytokine Signalling (SOCS) family, including SOCS2 and SOCS3, are positively regulated by growth hormone (GH) and play an important role in terminating GH signalling. We have recently demonstrated that oestrogen inhibits GH signalling via the Janus Kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) pathway, indirectly through an inducible factor. The aim of this study was to investigate whether SOCS3 may be involved in this inhibition by oestrogen of GH signalling. The effect of oestrogen and GH was investigated on the promoter activity of the murine SOCS3 gene using luciferase reporter constructs containing the full-length promoter and various truncations. Human embryonic kidney (HEK293) cells stably expressing human GH receptor were transiently transfected with an expression plasmid for human oestrogen receptor-$\alpha$ and the reporter constructs, and treated with GH and 17$\beta$-oestradiol ($E_2$; 1, 10, 100nM) for 24h as appropriate. GH alone (5, 50, 500ng/ml) stimulated the reporter activity in a dose-dependent manner by 2.3±0.1 fold ($P<0.0001$). $E_2$ increased luciferase activity of the full-length SOCS3 promoter (nucleotides -2757 to +929) reporter in a dose-dependent manner by 1.8±0.1 fold at 100nM ($P<0.0001$). Deletion of the 5' region of the promoter from nucleotides –2757 to –854 completely abrogated the $E_2$ effect, while a reporter construct containing nucleotides –2757 to –714 responded to $E_2$ stimulation by 1.9±0.2 fold ($P<0.0001$). Analysis of this

region revealed an incomplete oestrogen response element (**AGGTCA**GAA**AGCCCA**) located at nucleotides –1366 to –1352. $E_2$ at 100nM activated a heterologous promoter tk-luciferase reporter construct containing this element by 1.8±0.1 fold ($P$<0.0001). Co-treatment with GH (50ng/ml) enhanced $E_2$-induced SOCS3 promoter activity by >50% ($P$<0.025). In summary, $E_2$ stimulated murine SOCS3 promoter activity, an effect further enhanced by co-treatment with GH. The stimulatory effect was in part mediated by an oestrogen response element in the promoter. These findings provide the first evidence for steroid hormone regulation of the SOCS3 gene and a potential mechanism for oestrogen inhibition of GH signalling. (Supported by the NHMRC of Australia, Eli Lilly Australia and Vincent Fairfax Family Foundation)

**362**

## THE MOLECULAR EFFECTS OF ANDROGENS ON SKELETAL MUSCLE CELLS IN VITRO

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Androgens exert diverse physiological roles in both males and females. The anabolic effects of androgens on muscle have long been recognized and exploited by athletes, while clinical applications have included treatment of muscle-wasting disorders such as AIDS-wasting syndrome. Despite this clinical use, evidence of a significant effect remains equivocal and the basic biological mechanism of androgen action in muscle is poorly understood. To date, discrepancies between in vitro and in vivo data prevent the establishment of a defined link between physiological observations and the underlying molecular process of androgen-induced muscle hypertrophy. Definition of this pathway will provide significant clinical benefits in the treatment of muscular atrophy associated with disease or trauma.

AIM: We aim to investigate the molecular mechanism of androgen action on proliferation and differentiation of cultured skeletal muscle cells and the regulation of myogenic factors involved in muscle cell hypertrophy.

METHODS: Primary skeletal muscle cells (PP9) and a clonal skeletal muscle cell line (C2C12) were analysed for AR expression by RT-PCR and Northern analysis. PP9 cells were then treated with various androgens and estrogens and assessed by MTT assay to investigate effects on proliferation. Regulation of various myogenic factors by androgens was investigated at different stages of myoblast proliferation and differentiation by Northern analysis.

RESULTS: Two muscle cell systems were chosen for analysis, primary mouse muscle cells and a clonal muscle cell line. The expression of AR was clearly demonstrated in both cultured muscle cell types by RT-PCR. Preliminary data suggests that treatment of PP9 cells with 10nM Testosterone but not its metabolite, 5□-dihydrotestosterone, increased the rate of myoblast proliferation by 35% over a 24hr period compared with ethanol-treated controls. The investigation of myogenic factor regulation by androgens is currently underway.

CONCLUSIONS: Expression of the AR has been clearly demonstrated in proliferating myoblasts of two different skeletal muscle cell culture systems. Preliminary data suggests that androgens may enhance proliferation rate of this primary myoblast culture. Increased proliferation of myoblasts may be the mechanism by which androgens promote hypertrophy. Further study is required to establish if this is a direct effect on myoblasts via the androgen receptor, or through regulation of myogenic factors, or both.

**363**

## CONTROL OF SERTOLI CELL PROLIFERATION AND DIFFERENTIATION

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Sertoli cells provide a supportive framework in which germ cells divide and mature into spermatozoa. Each Sertoli cell is capable of supporting a limited number of germ cells to maturity, hence the number of Sertoli cells present is a critical factor in determining the fertility of an animal. In the rat, Sertoli cells divide during the foetal

and neonatal periods before endocrine signals induce post-mitotic differentiation. The interplay of various other hormonal factors are known to determine the ultimate Sertoli cell complement, but how these signals interact to regulate testis development is poorly understood.

Follicle stimulating hormone (FSH) derived from the pituitary appears to be the primary Sertoli cell mitogen. Data collected from knockout mice indicate that FSH is acting in part by regulating cyclin D2, an intra-cellular cell cycle regulator. Similarly, activin was found to stimulate proliferation in an age dependant manner both alone, and synergistically with FSH. Analysis of this synergy has revealed that it is not due to modulation of FSH or activin receptor expression, rather it is dependant upon cyclin D2. Further to its modulation of proliferation, activin was found to stimulate inhibin expression by the Sertoli cell, suggesting that in vivo it contributes to the testis-pituitary negative feedback loop to suppress FSH.

To address the control of mitotic arrest and differentiation of the Sertoli cell we investigated the action of known and putative Sertoli cell pro-differentiation factors. Thyroid hormone, testosterone and retinoic acid were all found to be capable of suppressing proliferation to similar degrees. Furthermore, these membrane permeable factors modulated the expression of markers of Sertoli cell differentiation such as Gata-4 and Gata-1 and induced cell cycle inhibitors p27 and p21, verifying their pro-differentiation and anti-mitotic functions.

In conclusion, these data indicate that activin plays a key role in maintaining end-stage Sertoli cell proliferation, which is in turn suppressed by the pro-differentiation effects of thyroid hormone, retinoic acid and testosterone. These studies have revealed a number of potential targets for manipulating fertility in experimental animals and livestock.

**364**

## EXPRESSION OF 17HSD3 IN MOUSE PRE-ADIPOCYTES AND THE REGULATION OF ANDROSTENEDIONE TO TESTOSTERONE CONVERSION IN VITRO.

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Obesity and in particular visceral fat is associated with insulin resistance. In human females local conversion of androstenedione to testosterone (A to T) has been demonstrated *in vitro* and *in vivo* through the expression of 17β-hydroxysteroid dehydrogenase type 3 (17HSD3)(1). In addition, it was shown that as women become more obese, their visceral fat becomes more androgenic (2), suggesting a new role for the action of locally produced T in the development of insulin resistance. It is not known if this is a phenomenon specific to humans, or if it occurs across species, making existing animal models applicable to examining this hypothesis. The aim of this work was to demonstrate and characterise androgen production in adipose tissue from both male and female mice.

Subcutaneous (SC) and visceral (Visc) white adipose tissue was obtained from adult female and male BALB/c mice. RNA was isolated from these two sites for analysis of 17HSD expression by RT-PCR. Cells were cultured in Waymouths medium (15% fetal calf serum), with or without dbcAMP or 18β glycyrrhetinic acid (18GA) to examine the regulation of A to T conversion. The cells were exposed to 3.5μM [4-$^{14}$C] androstenedione for 24 hours and steroids were separated by TLC. Analyses were performed using Mann-Whitney U and Wilcoxon Signed Ranks tests.

For the first time conversion of A to T was demonstrated by both male and female pre-adipocytes. There was a trend for the Visc pre-adipocytes to convert more A to T than SC pre-adipocytes (males, $p<0.05$). Like humans, 17HSD3 was expressed in both SC and Visc fat of females, but was inconsistently expressed by males, suggesting the involvement of additional 17HSD isoforms in mice which needs to be further examined. In addition, dbcAMP increased, whereas 18GA decreased A to T conversion.

In conclusion, mouse adipose tissue converts A to T, partially through the expression of 17HSD3 as in humans. Hence it is feasible to utilise mouse models to further examine the role of local T production in insulin resistance where experimentation in humans is limited.

1. Corbould, *et al*. (1998) *JCEM* 83: 187-194.

2. Corbould *et al*. (2001) *Int. J. Obesity* 26:165-175.

# TESTICULAR ACTIONS OF AN ACTIVATING MUTATION OF THE HUMAN FSH RECEPTOR IN TRANSGENIC MICE

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The *in vivo* actions of the only activating mutant FSH receptor (FSHR+) described in humans (1) was examined in transgenic (Tg) FSHR+ mice. We previously showed Tg-FSHR+ promoted FSH-like germ cell *development* in testes of *hpg* mice lacking circulating FSH and LH, and increased testicular FSH binding. In addition, this work suggested FSHR+ may induce local intratesticular androgen production by a paracrine mechanism independent of LH. We now examine the ability of FSHR+ to *maintain* spermatogenesis in adult mice with suppressed gonadotrophins after Zoladex (Z) treatment (3.6 mg depots each 28 days). After 28d-Z, Tg-FSHR+ testes weights were equivalent to untreated controls, whereas Z-treated nonTg testes were significantly reduced, suggesting FSHR+ provided some protection from decreased gonadotrophins. However, Z-treated FSHR+ testes remained non-significantly larger than Z-treated nonTg testes after 28d or 56d Z. Stereological analysis revealed subtle differences between spermatogenic populations in Z-treated FSHR+ and nonTg testes. In 28d-Z males, spermatogonia numbers in FSHR+ testes were higher ($p < 0.05$) than non-Tg testes although this effect was not maintained after 56d-Z. The numbers of pachytene spermatocytes and spermatids in FSHR+ males were also non-significantly higher than in 56d-Z nonTg. FSHR+ expression had no effect of on circulating androgens, and androgen-dependent epididymal weights were reduced to the same extent by Z-treatment in both FSHR+ and nonTg males. Therefore, our Tg model indicates FSHR+ may produce an FSH-like and *androgen-independent* effect in mouse testis despite suppressed gonadotrophin/androgen levels. We conclude that Tg FSHR+ expression exerts a modest protective effect on meiotic and post-meiotic germ cells against gonadotrophin withdrawal. The precise mechanism remains to be further elucidated.

| Tg-FSHR+ | Z | Serum T (nM) | Mean testis wt (mg) | Sertoli cells | Spermatogonia | Pl-Z Spermatocytes | Pachytene spermatocytes | Round spermatids | Elongated spermatids |
|---|---|---|---|---|---|---|---|---|---|
| - | - | 20.0 ± 21.6 | 107.8 ± 9.8 | 4.2 ± 0.7 | 6.1 ± 1.2 | 6.2 ± 2.4 | 20.2 ± 5.7 | 57.9 ± 5.5 | 66.9 ± 10.4 |
| + | - | 16.4 ± 21.3 | 106.5 ± 12.0 | * | * | * | * | * | * |
| - | 28 d | 1.7 ± 1.8 | 88.4 ± 10.7 | 3.7 ± 0.6 | 3.9 ± 1.0 | 9.9 ± 2.8 | 15.5 ± 4.4 | 40.5 ± 11.1 | 61.7 ± 6.9 |
| + | 28 d | 1.5 ± 0.5 | 97.3 ± 11.0 | 4.3 ± 0.7 | 5.0 ± 0.4 | 7.1 ± 3.4 | 20.0 ± 3.4 | 44.1 ± 8.7 | 62.8 ± 11.8 |
| - | 56 d | 0.7 ± 0.8 | 43.7 ± 19.6 | 3.8 ± 0.7 | 2.7 ± 0.4 | 4.1 ± 1.7 | 8.8 ± 2.4 | 16.8 ± 10.6 | 19.4 ± 15.8 |
| + | 56 d | 0.4 ± 0.3 | 57.5 ± 12.5 | 3.1 ± 0.4 | 2.8 ± 0.4 | 4.5 ± 2.3 | 11.2 ± 3.2 | 23.8 ± 9.0 | 32.0 ± 19.3 |

* Data analysis in progress. Cell numbers are mean ± SE x $10^6$ per testis, n = 6-8. We thank Mamdouh Khalil, Fiona Thorn and Adam Koch for technical assistance and AstraZeneca for Zoladex.

(1) Gromoll et al. 1996 J Clin Endo Metab 81, 1367-70.

## 366

### DEVELOPMENT OF TRANSGENIC MICE TO EXPLORE THE FUNCTION OF PHOSPHATIDYLETHANOLAMINE BINDING PROTEINS ON MALE FERTILITY

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The phosphatidylethanolamine binding proteins (pebp) have been implicated in the negative regulation of the MAP kinase pathway through binding of Raf and MEK and the subsequent prevention of phosphorylation of MEK by Raf-1 [1,2]. Recently a male germ cell specific member of this protein family, called pebp-2, was identified in the mouse and localized to the sperm head and tail [3]. Further, previous data has shown that the MAP kinase pathway is activated during spermatogenesis, epididymal maturation and sperm capacitation and hyperactivation. In vivo data on the function of the pebp family of proteins does not exist, however, we hypothesize that the pebps are required for the regulation of the MAP kinase pathway during epididymal maturation and/or the processes of fertilization. To develop an understanding of the function of pebp-2 and pebp-1 in developing sperm, transgenic mice are being generated which over-express the two forms of pebp in a testis specific fashion. In order to produce these mice the pebp-2 and pebp-1 cDNAs were individually linked to the protamine-1 promoter and transgenes were incorporated into the genome of FVB oocytes by microinjection into pronuclei. The protamine-1 promoter was composed of 95bp of 5' UTR and 4.1 kbp of upstream sequence and has previously been shown to result in high levels of testis specific expression. Mice are currently being genotyped and the effect of pebp over-expression on fertility will be assessed using a combination of in vivo and in vitro tests.

[1]. Yeung, K., et al. Suppression of Raf-1 kinase activity and MAP kinase signaling by RKIP. Nature 401, 173-7. (1999).

[2]. Yeung, K., et al. Mechanism of suppression of the Raf/MEK/extracellular signal-regulated kinase pathway by the raf kinase inhibitor protein. Mol Cell Biol 20, 3079-85. (2000).

[3]. Hickox, D., et al. Identification of a novel, testis-specific member of the phosphatidylethanolamine binding protein family - pebp-2. Biol Reprod (In press).

## 367

### A GENOME WIDE SCREEN FOR GENES ESSENTIAL TO MALE FERTILITY USING ENU MUTAGENESIS

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1 in 25 Australian men are affected by infertility and the causal factors in 40% of these cases are unknown. Many of the proteins and their encoding genes and regulatory pathways involved in spermatogenesis are also unknown, creating an absence of specific treatments for infertile men. This highlights the need for the identification of novel genes/proteins that are involved in the production of mature spermatozoa.

N-ethyl-N-nitrosourea (ENU) is a powerful chemical that induces point mutations in spermatogonial stem cells. The mutagenized stem cells repopulate the testis, giving rise to clones of mutagenized sperm. ENU has the highest mutation rate of any mutagen tested in the mouse, with a mutation rate of 0.0015/locus/gamete (Rinchik, 1991). The use of ENU coupled with a controlled breeding program has generated large numbers of mice, some of which are homozygous for recessive mutations affecting spermatogenesis.

The testes of 250 G3 males from 38 ENU treated lines were phenotypically screened to identify defects in spermatogenesis. Infertility was initially based on testis histology. Of the 38 lines screened, 9 had abnormal testis histology, with phenotypes ranging from Sertoli Cell only, hypospermatogenesis and arrest in meiosis and spermiogenesis. Two affected lines were chosen to regenerate and the causal genes are currently being mapped using linkage analysis and searches of the mouse and human genome databases.

Rinchik, E.M. (1991). Chemical mutagenesis and fine structure functional analysis of the mouse genome. Trends in Genetics 7: 15-21.

**368**

## REDUCED N/C INTERACTIONS BUT INCREASED LIGAND INDUCED TRANSCRIPTIONAL ACTIVITY OF AN ANDROGEN RECEPTOR VARIANT CONTAINING A DISRUPTED POLYGLUTAMINE TRACT

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The relative risk of developing prostate cancer is inversely associated with the length of a polyglutamine tract in the androgen receptor (AR) encoded by a polymorphic triplet repeat (CAG) sequence in exon 1 of the AR gene. Functional in vitro analyses have shown that ARs containing shorter polyglutamine tracts have an increased ability to activate AR responsive promoters, providing a rationale for the relationship between CAG repeat length and disease. Studies in our laboratory have identified a human prostate tumour containing a somatic missense AR gene mutation that results in interruption of the polyglutamine tract with two leucine residues. The functional consequences of the polyglutamine disruption were investigated by cotransfection of prostate cancer cell lines with the wild type AR (wtAR) or the polyglutamine variant AR (AR-2xL) and an androgen responsive reporter gene. The AR-2xL exhibited 2-fold greater transactivation activity compared with wtAR while western blot analysis showed an approximate 50% decrease in AR-2xL protein levels. Real time PCR analysis demonstrated that this decrease in AR protein was not due to differences in the steady state levels of wtAR and AR-2xL mRNA. These results suggest the effect of disruption of the polyglutamine tract on AR levels is compensated for by increased activity of the receptor. An intramolecular interaction between the amino and carboxy termini of the AR (N/C interaction), which occurs following ligand binding and serves, in part, to stabilise the receptor-ligand interaction, is reduced in the AR variant. Reduced N/C interactions, which result in altered receptor conformation and exposure of alternative functional and binding motifs, could explain why the variant AR protein has diminished stability while permitting receptor activation via alternative mechanisms, such as increased recruitment of coactivator molecules. Elucidation of the precise mechanisms contributing to increased activity of the AR-2xL variant may explain how variations in repeat length or sequence in normal and malignant prostate cells may facilitate disease progression.