Neonatal Oestrogen Treatment and Effects on the Hypothalamic-Pituitary-Gonadal Axis

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In loving memory
of grandad.

Declaration

I declare that all the experiments detailed in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

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Abstract

Current research has shown that inappropriate exposure of the developing rodent fetus and neonate to exogenous oestrogens can cause long-term deleterious effects in the reproductive tract, hypothalamus, and to some extent the pituitary gland. However, at present there is only limited information about the effects of neonatal oestrogen treatment on the function and morphology of the anterior pituitary gland (and gonadotroph cells in particular). The aim of this thesis was to investigate the effects of neonatal estrogen exposure on the hypothalamic-pituitary-gonadal (HPG) axis, with particular emphasis on determining the basic features of any changes in pituitary gonadotroph function and morphology.

Four main animal studies were undertaken to investigate the effects of neonatal exposure to oestrogens in both marmoset and rat models. In the first study, the emphasis was on the potent synthetic estrogen Diethylstilbestrol (DES), as this has been shown in numerous studies to have significant long-term effects on the HPG axis. Neonatal male rats were treated with a low dose (0.1μg) or a high dose (10μg) of DES. These animals were blood sampled on days 26 (pre-pubertal), 35 (pubertal) and 90 (adult), and plasma LH, FSH, inhibin B, and testosterone levels, the number of gonadotroph cells, LH and FSH mRNA, and numbers of Leydig and Sertoli cells per testis were determined. The results of this study showed that DES had direct effects on the testes (reduction in Sertoli cells and alteration in Leydig cell function), which resulted in a suppression of plasma inhibin B and testosterone levels, and hence altered negative feedback at the level of the pituitary, which resulted in elevated LH and FSH levels (despite no significant increase in LH and FSH mRNA in adulthood).

The second study was composed of two parts. The first part was an *in vivo* study where male and female rats were administered DES neonatally, blood samples were collected in adulthood and were assayed for LH, FSH, inhibin B and testosterone. The results from this study revealed that neonatal DES treatment had differential effects on circulating levels of reproductive hormones in males and females, with male rat plasma hormone levels being more susceptible to disruption with DES than female plasma

hormone levels. The second part of this study was an *in vitro* study, where the pituitaries from these *in vivo* tracked animals were collected and cultured, to investigate the potential effects of neonatal estrogen treatment solely at the level of the pituitary, without any hormonal signals occurring from the gonads. Primary pituitary cells were cultured with various activin and/or GnRH treatments to test whether these hormonal treatments would stimulate LH (and FSH) release from the primary pituitary cells, and to investigate whether the neonatal DES treatments would alter the cells' ability to respond to these treatments. LH and FSH concentrations in the primary pituitary cultures were assayed and there was found to be no significant difference in LH and FSH concentrations between control rats and those treated neonatally with DES.

The third study was a small pilot study which investigated the effects of neonatal treatment with either a GnRH antagonist alone or in combination with DES on adult male rat pituitary function. Both treatment regimes were shown to have significant effects on circulating hormone levels, both *in vivo* and *in vitro*, especially the GnRH antagonist + DES treatment, suggesting that plasma hormone levels were more susceptible to disruption with DES in combination with treatment with a GnRH antagonist.

The last study, was a soy study in marmosets to address health concerns about the possible effects of feeding human infants soy formula milk (SFM), as in North-America 25% of infants are now weaned on SFM. Male Marmoset co-twins were either administered SFM or standard cow's milk formula (SMA) in neonatal life (days 4 to 45), blood samples, pituitaries and testes were collected after the 6-week treatment period. Blood testosterone levels were assayed and pituitary gonadotroph cells and testicular Leydig and Sertoli cells were counted. Plasma testosterone levels were found to be significantly suppressed in the SFM animals and there was a significant elevation in the number of Leydig cells in the SFM marmosets after the 6-week treatment period. There was found to be no significant difference in the number of LH and FSH immunopositive pituitary gonadotroph cells between SMA and SFM fed marmosets. The results from this male marmoset soy study have indicated that SFM suppresses testosterone plasma levels and increases the number of Leydig cells per testis in neonatal life, without

causing any major changes in both pituitary gonadotroph cell number or morphology. The long-term effects of neonatal testosterone suppression and increased Leydig cell numbers are as yet still unclear, but should be clarified when other SFM fed marmosets reach adulthood.

Abbreviations

αGSU	alpha gonadotrophin subunit
17β-HSD	17β-hydroxysteroid dehydrogenase
A	adenine
ACTH	adrenocorticotrophic hormone
AMH	anti-müllerian hormone
AP	anterior pituitary
Arg	arginine
bp	base pairs
BSA	bovine serum albumin
C	cytosine
°C	degrees Celsius
Ca^{2+}	calcium
cDNA	copy deoxyribonucleic acid
CG	chorionic gonadotrophin
CgA	chromogranin A
CgB	chromogranin B
CNS	central nervous system
DAB	3,3'-diaminobenzidine
DES	diethylstilbestrol
DMEM	dulbecco's modified eagle's media
DNA	deoxyribonucleic acid
DTP	guanosine triphosphate
E	oestrogen
ELISA	enzyme-linked immunosorbent assay
ER	oestrogen receptor
ERα	oestrogen receptor alpha
ERβ	oestrogen receptor beta
FCS	fetal calf serum
FSH	follicle stimulating hormone
FSHβ	FSH beta subunit
G	guanine
GAM	goat anti-mouse serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GH	growth hormone

Glu	glutamine
Gly	glycine
GnRH	gonadotrophin releasing hormone
GnRH antag	gonadotrophin releasing hormone antagonist
GnRH-R	GnRH receptor
His	histidine
HPG	hypothalamic-pituitary-gonadal axis
hpg	hypogonadal mouse
HRP	horseradish peroxidase
I	Inhibin B
ICC	immunocytochemistry
Kb	kilobases
Kda	kilodaltons
Leu	leucine
LH	luteinizing hormone
LHβ	LH beta subunit
LHRH	luteinizing hormone releasing hormone
MIS	müllerian inhibiting substance
mRNA	messenger ribonucleic acid
MSH	melanocyte stimulating hormone
OD	optical density
PBS	phosphate buffered saline
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PP	posterior pituitary
PRL	prolactin
Pro	proline
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCT	reverse transcriptase PCR
SAR	swine anti-rabbit serum
s.c.	sub cutaneous
SDS	sodium dodecyl sulphate
Ser	serine

SERM	selective oestrogen receptor modulator
SFM	soy formula milk
SgII	secretogranin II
SHBG	sex hormone-binding globulin
SMA	cow's milk formula
StAR	steroid acute regulatory protein
T	testosterone
T	thymine
TGF-β	transforming growth factor beta
Tm	melting temperature
Trp	tryptophan
TSH	thyroid stimulating hormone
Tyr	tyrosine
UV	ultra-violet

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Chapter one: literature review

1.1 Introduction

Regulation of normal reproductive development and physiology is an extremely complex process involving coordinated interaction of neurotransmitter systems, hypothalamic releasing factors, pituitary hormones, and growth factors. The reproductive system is part of the endocrine system, which contains an elegant feedback system with control centres at the level of the hypothalamus and the pituitary gland, and with target organs such as the testes or ovaries. There are also smaller local feedback loops involving paracrine and autocrine signals at the levels of the pituitary, testes and ovaries, which maintain organ or cell homeostasis. The major players within the hypothalamic-pituitary gonadal axis (HPG) are Gonadotrophin Releasing Hormone (GnRH), Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), inhibin, oestrogen (females) and testosterone (males). GnRH is secreted from hypothalamic neurons in a pulsatile manner and interacts with specific high-affinity GnRH receptors on gonadotroph cells in the anterior pituitary to stimulate the release of the gonadotrophins LH and FSH. In males, LH acts via specific receptors on Leydig cells of the testis to stimulate the production of testosterone. FSH acts on specific receptors on testicular Sertoli cells to stimulate the production of inhibin B. Testosterone exerts negative feedback at the level of the hypothalamus and on LH production by pituitary gonadotrophs, and inhibin B negatively regulates FSH production by pituitary gonadotrophs. In females, GnRH-induced release of gonadotrophins stimulates the secretion of oestradiol and progesterone by the ovary, these then exert negative feedback control over GnRH, FSH and LH secretion. Oestradiol, in addition to exerting negative feedback on LH and FSH secretion, can also exert positive feedback by inducing the preovulatory surge of LH secretion. FSH-induced inhibin secretion from the ovary negatively regulates FSH secretion from the pituitary gonadotroph cells.

The reproductive health of animals in adult life can be affected by several environmental influences acting at different stages of development, resulting in reprogramming of the HPG axis. Numerous studies have shown that oestrogens, administered during the neonatal period of development, can have life-long effects on the function of the HPG axis. The experimental work described in this thesis was performed to gain a greater understanding about the effects of neonatal oestrogen treatment on the HPG axis in both males and females, and to investigate which parts of the HPG axis were most susceptible to oestrogen disruption. This review of the literature will discuss the set-up of the HPG axis, its function in adult life and effects of neonatal oestrogen disruption on feedback along the HPG axis.

1.2 Sexual differentiation into males or females

Sexual determination in mammals is genetically and hormonally controlled. Genetic sex is determined by inheritance of either an X or Y chromosome from the male gamete (Jost *et al.,* 1973). A specific region of the Y chromosome, the testis-determining region Sry, functions dominantly and induces differentiation of the sexually indifferent embryonic gonads into testes. In the absence of Sry, gonads develop as ovaries (Gubbay *et al.,* 1990; Koopman *et al.,* 1990). Sex differentiation is the developmental process that takes place in the reproductive tract of the fetus after the gonad has started to differentiate into a testis or an ovary. In humans, if the gonad is a testis, the first recognisable structural change is the formation at 7-8 weeks of testicular or sex cords composed of Sertoli cells (Gilbert, 1994; Huhtaniemi, 1994). From 8 to 10 weeks, these cells secrete anti-Müllerian hormone or AMH (also called Müllerian inhibitory substance or MIS), a glycoprotein related to the transforming growth factor β (TGF-β) superfamiliy. Its role is to induce regression of the Müllerian ducts (Gilbert, 1994). Germ cells derived from the dorsal endoderm of the yolk sac make a long migration into the gonad in the fourth and fifth weeks (Monk & Martin, 1981). At about 7 weeks,

mesonephric stromal cells invade the gonad and differentiate into Leydig cells (Huhtaniemi, 1994). By 9 weeks the newly formed Leydig cells begin to secrete testosterone (Figure 1.2). The onset of testosterone secretion by the fetal gonad is thought to be regulated by chorionic gonadotrophin (CG) rather than pituitary LH, because LH secretion does not start until 10 weeks and becomes pulsatile at 11 to 12 weeks (Segaloff & Ascoli, 1993; de Roux & Milgrom, 2001). In the normal male fetus, testicular descent begins at 10 weeks and serum testosterone levels peak at 12 to 16 weeks. The final migration of the testis from the abdominal cavity to the scrotum is completed by 25 to 35 weeks (Heyns & Hutson, 1995). Thus, the key factor about masculinisation of the male is that all of the hormones involved originate from the testes. Testes formation does not itself require hormones but all other aspects of masculinisation are dependent upon normal testes function and the consequent production of adequate amounts of hormones (Sharpe, 2001) (Table 1.2).

Hormone	Cellular source	Role/site of action
Anti-müllerian hormone	Sertoli cells	Müllerian duct regression
Androgens (testosterone, androstendione)	Leydig cells and Sertoli cells	Multiple tissues, notably Wolffian ducts and interstitial cells
Oestrogens (oestradiol, oestriol, oestrone)	Sertoli cells and Leydig cells	Multiple tissues, including Wolffian ducts and Sertoli cells

Table 1.2: The production and sites of action of hormones produced by the fetal testes that are thought to play a role in male reproductive development.

Therefore, any disturbance of testicular development or function during early life could have effects on the functioning of the reproductive system. Furthermore, testis cell development (as opposed to testes formation) is itself also dependent on the local action of these same hormones. In contrast to the process of masculinisation, relatively little attention has been given, until recently, to the importance of testes development in fetal

and neonatal life in terms of ensuring future fertility (Sharpe *et al.*, 1999). In the human male, fetal masculinisation and reproductive ability in late puberty are events that are separated by 15 or more years, yet one is dependent on the other. Thus abnormal development of the testes in either fetal or neonatal life could have life-long consequences on reproductive function.

Ovaries differentiate much later than testes. The first germ cells (oocytes) migrate into the ovary from the dorsal endoderm of the yolk sac at 11 to 12 weeks (Ginsburg *et al.*, 1999). The number of oocytes reaches a lifetime maximum of 6 million to 7 million by 20 weeks and declines to 2 million by term (Ginsburg *et al.*, 1999). The fetal ovaries are not thought to play any part in female sex differentiation. They secrete little oestrogen even though follicles begin to develop at about 16 weeks and primordial follicles containing granulosa cells are present by 20 weeks. Like the testis, the ovary descends from the posterior abdominal wall to the pelvis (Heyns & Hutson, 1995).

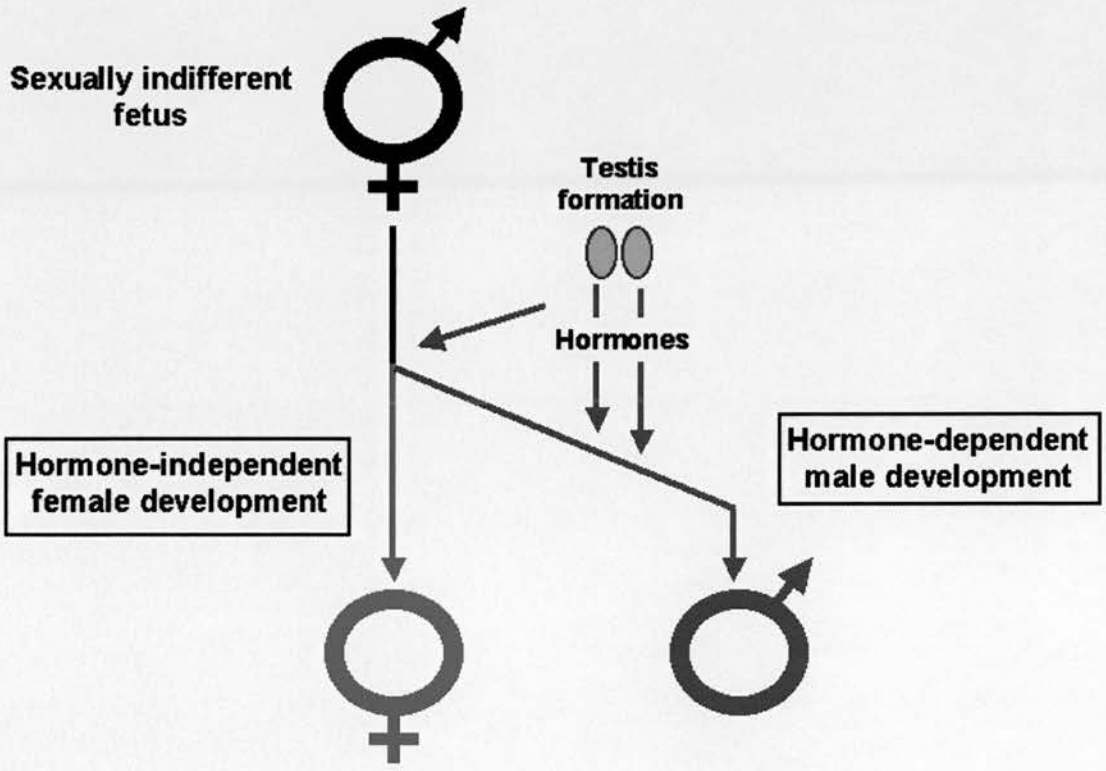


Figure 1.2: Sexual differentiation in mammals. Adapted from Sharpe, 2001.

1.3 The pituitary gland

The pituitary gland is a small oval shaped gland that lies at the base of the brain. It is often referred to as the 'master' endocrine gland as all other endocrine organs depend upon its secretions for stimulation. Together with the hypothalamus, it plays a pivotal role in regulating reproduction (Figure 1.3).

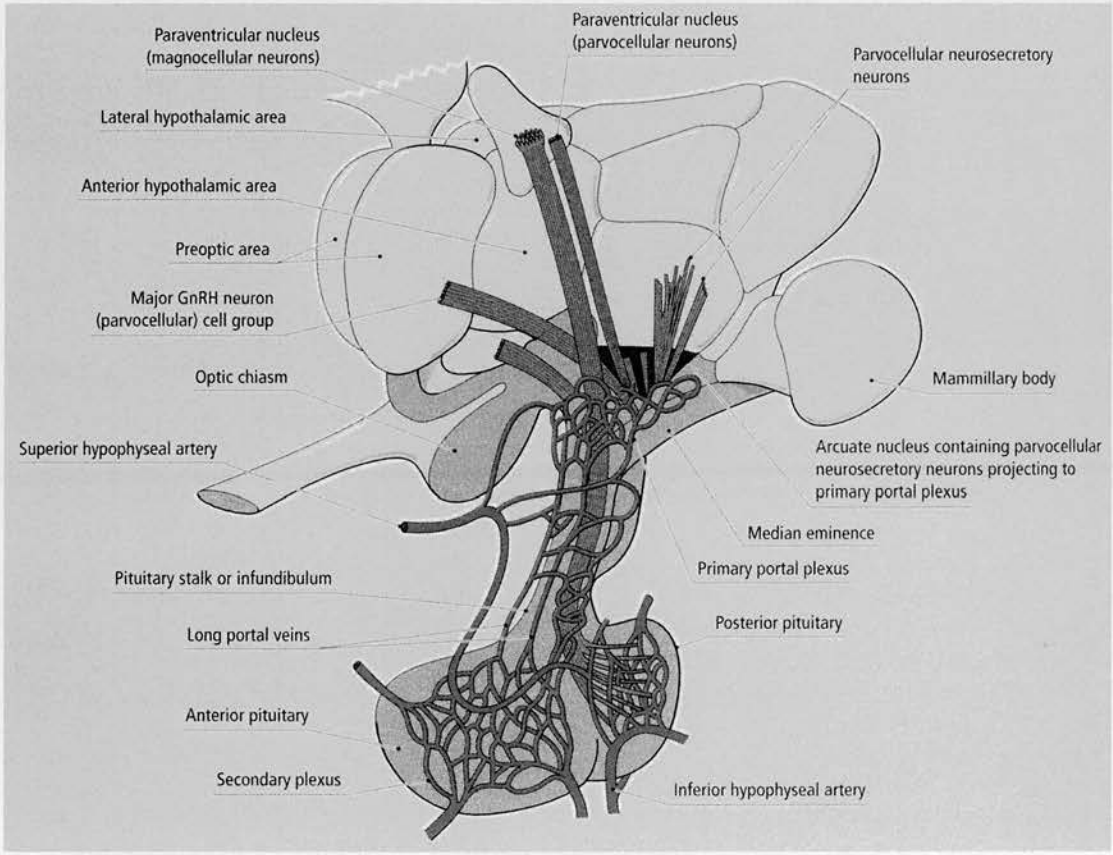


Figure 1.3: A schematic diagram of the hypothalamus and pituitary gland, connected by the pituitary stalk. Adapted from Essential Reproduction, Johnson and Everitt, 2000.

1.3.1 Pituitary gland location and structure

The pituitary gland is located within the skull on the ventral surface of the brain. It lies within the hypophyseal fossa of the sphenoid bone, overlapped by a circular fold of dura mater, the diaphragma sellae, which has a small central opening through which the pituitary stalk, or infundibulum passes. The pituitary has an extremely rich blood supply derived from the internal carotid artery via its superior and inferior hypophyseal branches. Venous drainage is by short vessels which emerge over the surface of the gland and enter neighbouring dural venous sinuses.

The pituitary gland, or hypophysis as it is also known, consists of two major subdivisions, the anterior lobe (or adenohypophysis) and the posterior lobe (or neurohypophysis). The posterior lobe is further divided into the infundibular process and the infundibular stem (or pituitary stalk) which connects to the median eminence. The posterior lobe is made up of neural tissue and is connected to the rest of the brain via the stalk. The infundibular process contains terminals of neurones whose cell bodies reside in the hypothalamus. Thus there is a direct neural link between the posterior pituitary and the brain (Knobil, 1981). The anterior lobe of the pituitary is further subdivided into the pars distalis, pars intermedia, and pars tuberalis. The pars tuberalis surrounds the infundibular stem like a cuff and extends upwards to lie beneath a portion of the median eminence (Table 1.3.1). Unlike the posterior lobe, the anterior pituitary contains no nerve fibres and terminals and so is not in direct neuronal contact with the hypothalamus. Instead it is connected to the brain by a vascular connection, the hypothalamo-hypophyseal-portal system. Most of the blood supplied to the anterior lobe comes from this portal system. In some species, it accounts for over 90% of the total blood supply (Knobil, 1980). In addition to blood flowing down the stalk from the brain to the anterior pituitary, a small proportion of blood flows up the pituitary stalk. This provides a direct vascular link from the anterior pituitary back to the hypothalamus (Bergland & Page, 1978).

Neurohypophysis	Median eminence	Infundibulum
	Infundibular stem	
	Neural lobe (infundibular process)	Posterior lobe
Adenohypophysis	Pars intermedia	
	Pars tuberalis	Anterior lobe
	Pars distalis	

Table 1.3.1: Terminology of the subdivisions of the hypophysis.

The anterior and intermediate lobes of the pituitary gland contain at least six different endocrine cell types, each of them defined by the hormones they produce. These six endocrine cell types include adrenocorticotrophic hormone (ACTH) in corticotrophs, melanocyte stimulating hormone (MSH) in melanotrophs, growth hormone (GH) in somatotrophs, prolactin (PRL) in lactotrophs, thyroid stimulating hormone (TSH) in thyrotrophs and luteinizing hormone (LH) and follicle stimulating hormone (FSH) in gonadotrophs. There is also an embryonic cell type referred to as the rostal tip 'thyrotroph' (Watkins-Chow & Camper, 1998; Sheng & Stern, 1999; Dasen & Rosenfeld, 2001). The anterior lobe also contains folliculostellate cells. These comprise a group of S100-antigen-positive non-endocrine cells whose function remains poorly defined. They are postulated to play an important role as a source of paracrine factors that act locally to modulate pituitary responses to hypothalamic and peripheral signals (Allaerts *et al.*, 1990a; Renner *et al.*, 1996). Electrophysiological and morphological studies have suggested that folliculostellate cells form a functional network of interconnecting cells that facilitate synchronisation and long-term communication among different parts of the pituitary gland (Stojilkovics, 2001). These cells also have been viewed as a pool of progenitor cells that are capable of differentiating into specialised endocrine cells under certain conditions (Inoue *et al.*, 2002). Folliculostelate cells are known to contain annexins. Annexins are phospholipid- and calcium-binding proteins and they are thought to play an essential role in the manifestation of the early

delayed feedback effects of glucocorticoids in the anterior pituitary gland (Ahluwalia *et al.,* 1996; Buckingham & Flower, 1997).

1.3.2 Pituitary gland development

The pituitary gland develops in tandem with the specific hypothalamic nuclei that ultimately regulate homeostatic responses in the mature organism (Watkins-Chow & Camper, 1998; Sheng & Stern, 1999; Dasen *et al.,* 2001). The pituitary gland originates from two embryonic tissues. The anterior and intermediate lobes are derived from the oral ectoderm and the posterior lobe from the neural ectoderm (Schwind, 1928; Kaufman, 1992) (Figure 1.3.2). Three main stages of pituitary gland formation have been defined in the rat (Sheng *et al.,* 1997; Li *et al.,* 1994). The first stage of pituitary organogenesis is the formation of a rudimentary pouch (or placode). This event occurs near embryonic day 8.5 (E8.5) (Kusakabe, 1984). The placode of the pouch appears initially as a thickening of the oral ectoderm at the roof of the presumptive oral cavity. Subsequently, it evolves upwards to form an epithelial bud, the rudimentary pouch. The second stage of organogenesis is the formation of a definitive pouch, also known as Rathke's pouch. During this stage, the rudimentary pouch continues to extend upwards. Meanwhile, the posterior part of the presumptive diencephalon evaginates downwards to form an infundibulum. Throughout development, the neural epithelium and the oral ectoderm destined to form the pituitary are kept in close contact, while migration of mesodermal and neural crest cells into the surrounding space separates the presumptive brain and oral cavities. Thus, a definitive pouch is formed, the main body of which is situated in the brain cavity, with a stalk connecting to the oral cavity (Schwind, 1928; Kaufman, 1992; Camper *et al.,* 1990; Bach *et al.,* 1995). By E12.5, the pouch is completely detached from the oral cavity and the ventral wall of the pouch is proliferating to become the anterior lobe. Thereafter, individual hormone-secreting cells emerge from the pouch in a sequential order and Rathke's pouch is transformed into a

pituitary gland (Rhodes *et al.*, 1994; Voss & Rosenfeld, 1992). The six mature endocrine cell types emerge in a temporally and spatially specific fashion from E12.5 to E17.5, with corticotrophs and rostal tip thyrotrophs emerging first at embryonic day 12.5 and subsequently from E15.5 to E17.5 somatotrophs, lactotrophs, thyrotrophs gonadotrophs and melanotrophs emerging (Figure 1.3.2). There seems to be little or no change in the gonadotroph cell population within the adult pituitary, since cell division of gonadotrophs is almost never observed, even after castration (McNeilly JR, unpublished data). Furthermore, ablation of gonadotrophs in fetal life using the alpha gonadotrophin subunit promoter (Kendall *et al.*, 1991; Seuntjens *et al.*, 1999), the FSHβ promoter (Markkula & Huhtaniemi, 1996) or the LHβ promoter (McNeilly, 2001) to drive ablation agents has been shown to result in a permanent and partial depletion of gonadotroph cells in adulthood. This confirms that there are no progenitor cells within the pituitary gland that can repopulate the gonadotroph pool of cells.

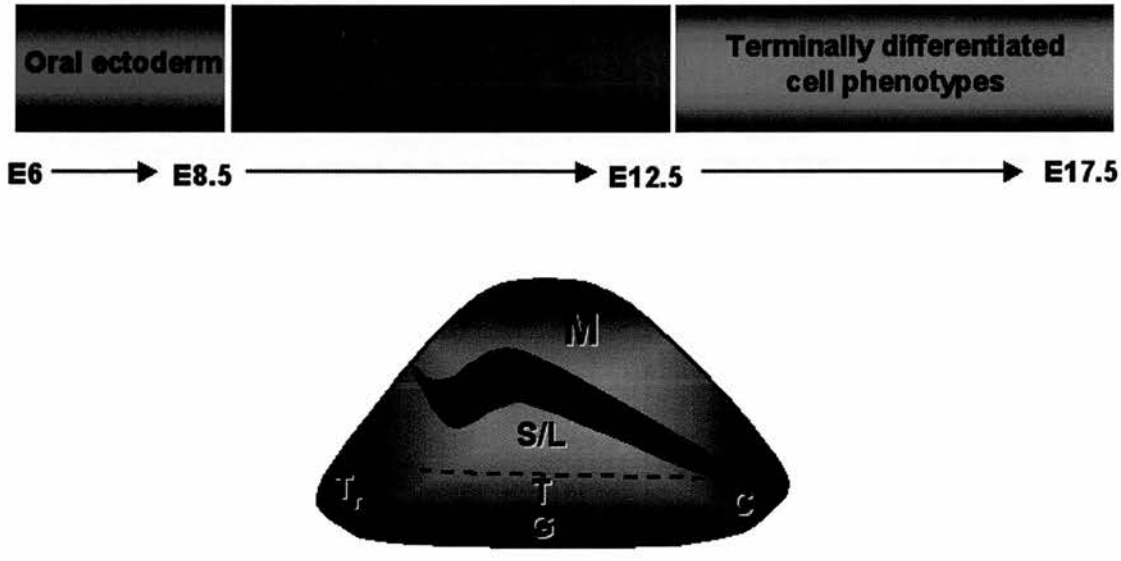


Figure 1.3.2: Timescale of rat pituitary development. Pituitary cell types arise in a temporally and spatially specific manner: E12.5 corticotrophs (C), E12.5 rostral tip thyrotrophs (Tr), and subsequently from E15.5 to E17.5 somatotrophs (S), lactotrophs (L), thyrotrophs (T), gonadotrophs (G) and melanotrophs (M). Adapted from Scully & Rosenfeld, 2002.

In primates, FSH and LH are detectable in the pituitary by embryonic week 10, and their content increases until embryonic week 25-29. The pituitary starts to release gonadotrophins into the general circulation by embryonic week 11-12. Circulating gonadotrophins reach peak levels at midgestation, and subsequently both LH and FSH levels decline during late gestation (Grumbach *et al.,* 1974; Clements *et al.,* 1976). The gonadotrophs in primate fetuses have been shown to respond to GnRH by releasing LH and FSH (Weinstein *et al.,* 1974; Mueller *et al.,* 1981; Matwiji *et al.,* 1987; Dumesic *et al.,* 1991). A sex difference in fetal gonadotrophin levels has been observed during midgestation, where pituitary content and circulating concentrations of LH and FSH in females are higher than those in males (Matwijiw & Faiman, 1989; Winter *et al.,* 1977). Since it has been shown that circulating fetal testosterone levels are high in male fetuses as compared with circulating oestrogen levels in females during midgestation, both the sex difference in gonadotrophin levels and the decrease in gonadotrophin levels towards late gestation in fetuses have been attributed to the development of the negative feedback mechanism by the gonadal steroid hormones from the fetal gonads, as well as from the placenta (Grumbach, 1976). A study in which castration in male rhesus monkeys at E98-104 was shown to increase circulating gonadotrophin levels as high as those in female monkeys at similar ages has supported this notion (Resko *et al.,* 1980). Negative feedback by ovarian steroids is operative in the female primate during late gestation, when oestrogen secretion is elevated (Grumbach *et al.,* 1974). In sheep fetuses during late gestation, LH release has been shown to be pulsatile, and orchidectomy in males, but not ovariectomy in females, has been shown to result in an increase in the pulse amplitude of LH (Mesiano *et al.,* 1991).

1.4 Hypothalamic Gonadotrophin Releasing Hormone (GnRH)

Although the anterior pituitary gland has been known since the mid-1940s to be controlled by chemical signals originating in the central nervous system (Harris, 1948), it was not until 1971 that the gonadotrophin releasing peptide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2 was isolated from hypothalamic extracts (Schally *et al.,* 1971). The hypothalamus is a relatively small area at the base of the brain. It is part of the diencephalon and lies between the midbrain and the forebrain. The hypothalamus has many neurocrine, behavioural and autonomic functions, which include the regulation of sexual and ingestive behaviours, the control of body temperature, and the integration of the cardiovascular and hormonal responses to stress. The gonadotrophin releasing peptide is a decapeptide and is known as luteinizing hormone releasing hormone (LHRH) or gonadotrophin releasing hormone (GnRH). It is synthesised in specialised hypothalamic neurons and packaged into storage granules that travel by axonal transport to the external zone of the median eminence of the pituitary gland. The GnRH molecule is synthesised in specialised hypothalamic neurons as part of a pre-hormone that is enzymatically cleaved at dibasic residues to release the active decapeptide (Seeburg *et al.,* 1987). Although GnRH is resistant to exopeptidases, owing to its blocked N- and C-termini, it is rapidly degraded by endopeptidases and is cleared from the circulation with a half-life of approximately 5 minutes. The amino acid sequence of GnRH is conserved among the mammalian peptides, but at least six isoforms occur in other species (Sherwood *et al.,* 1993). In mammals GnRH is secreted episodically into the capillaries of the hypothalamo-hypophyseal portal system and reaches the anterior pituitary gland, where it binds specifically to receptors on gonadotroph cells and stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Two distinct releasing factors were originally thought to control the secretion of LH and FSH, but the ability of GnRH to stimulate the release of both gonadotrophic hormones and to initiate puberty (Hoffman & Crowley, 1982) has established its

importance as the primary regulator of mammalian gonadotrophin secretion. Although the circulating profiles of LH and FSH are sometimes divergent, this is attributable to factors other than the existence of two releasing hormones. These include differences in the gonadal steroid hormone milieu, the independent regulation of FSH secretion by inhibin and the different circulating half-lives of the two hormones. Also the LH- and FSH-secreting gonadotrophs appear to be differentially regulated by the frequency of the pulsatile stimulation by GnRH (Marshall & Kelch, 1986; Padmanabhan and McNeilly, 2001).

The pulsatile mode of the GnRH secretion reflects the synchronised activity of many of the 1500 or so GnRH neurons that form a network within the hypothalamus. Each burst of GnRH secretion is followed by a prominent pulse of LH release into the systemic circulation. Such LH pulses occur at intervals of 30-120 minutes in various species, and about once per hour in humans. Decreases in the rate of stimulation by GnRH, or sustained treatment with GnRH, are both associated with impaired secretion of both LH and FSH (Knobil, 1989; Millar *et al.,* 1989). The co-ordinated activity of the scattered GnRH neurons is attributed to a neural pacemaker that is termed the GnRH pulse generator (Knobil, 1989). The nature of the pulse generator has yet to be established, but its activity reflects a pattern of electrical activity in the mediobasal hypothalamus that is correlated with episodic increases in circulating LH levels in the rhesus monkey (Knobil, 1981; Sealfon *et al.,* 1997). Thus, the pulsatile nature of GnRH secretion is essential for the physiological maintenance of normal gonadotroph function, and ultimately for normal reproductive capacity (Millar *et al.,* 1987).

1.4.1 GnRH receptor

The actions of GnRH in the anterior pituitary gland are mediated by a calcium-mobilizing seven transmembrane domain receptor that is coupled to phospholipase C-β through $G_{q/11}$ proteins. The GnRH receptor is the smallest of the G protein coupled receptor (GPCR) family, owing to its lack of the C-terminal cytoplasmic tail that is present in all such receptors (King *et al.*, 1997; King & Millar, 1995). Agonist activation of the GnRH receptor is followed by rapid stimulation of phosphoinositide hydrolysis to produce the two second messenger molecules, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The resulting stimulation of calcium mobilisation and influx, and the activation of protein kinase C (PKC), lead to rapid exocytosis of secretory granules from pituitary gonadotrophs and the release of LH and FSH into the circulation (Stojilkovic *et al.*, 1994; Sealfon *et al.*, 1994). The consequences of GnRH-induced activation of IP_3 and calcium signalling pathways in pituitary gonadotrophs include changes in plasma membrane channel activity, increased gonadotrophin synthesis and release, and changes in gene expression (Sealfon *et al.*, 1997).

1.4.2 Differential regulation of gonadotrophins by GnRH

FSH and LH are structurally related glycoproteins composed of a common α-subunit and a hormone specific β-subunit. The subunits are not covalently linked and can be readily dissociated by low pH, or heat treatment (Pierce and Parsons, 1981). Heterodimers formed by recombination of the subunits have the endocrine properties characteristic of the hormone from which their β-subunit is derived (Hertan *et al.*, 1999). The cystine knot disulphide bonds found in both α- and β-subunits are crucial for gonadotrophin activity. This is thought to be because they assume the roles of a hydrophobic core in stabilising the conformation of each subunit (Pierce and Parsons,

1981). The main properties of the gonadotrophins LH and FSH are summarised in Table 1.4.2a.

	LH	FSH
Composition	α-chain of 92 amino acids and two carbohydrate chains β-chain of 121 amino acids and two carbohydrate chains	α-chain of 92 amino acids and two carbohydrate chains β-chain of 111 amino acids and two carbohydrate chains
Molecular weight (kDa)	28	28
Target cells	Males: Leydig cells Females: Thecal cells (antral follicles), Granulosa cells (preovulatory follicles), Luteal cells (corpus luteum)	Males: Sertoli cells Females: Granulosa cells

Table 1.4.2a: Summary of the main properties of the gonadotrophins LH and FSH, including their composition, molecular weights and target cells.

Pituitary gonadotrophs are bihormonal (Liu *et al.,* 1998; Currie & McNeilly, 1995), but under certain physiological and experimental conditions, monohormonal cells have been identified (Childs *et al.,* 1994; Taragnat *et al.,* 1998). Despite the bihormonal nature of gonadotroph cells, they are capable of differentially regulating the secretion of LH and FSH. LH is stored intracellularly and is released mainly in response to pulses of GnRH. This is known as a regulated secretory pathway (Burgess & Kelly, 1987). However, a small quantity of LH can be released via a constitutive pathway (McNeilly *et al.,* 1991). FSH on the other hand, is largely secreted via a 'constitutive-like' pathway, however there is also evidence that FSH may be released by an alternative 'regulated' pathway (Farnworth, 1995) (Figure 1.4.2).

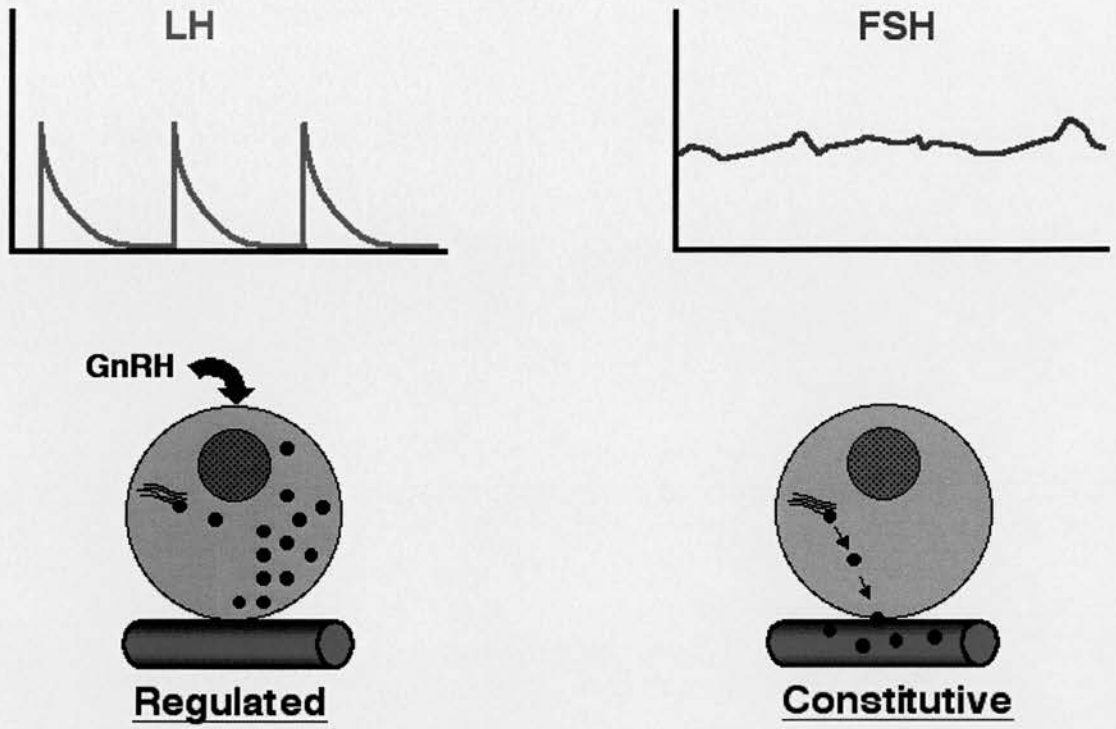


Figure 1.4.2: Differential gonadotrophin secretion. LH is secreted mainly via a regulated pathway, whereas FSH is secreted via a 'constitutive-like' pathway. Adapted from McNeilly *et al.,* 2003.

There are three aspects of differential regulation of LH and FSH which are important to recognise. The first is the requirement to allow the release of FSH and LH in different amounts at the same time, from the same gonadotroph cell. Secondly, LH is released in discrete pulses that are not usually accompanied by release of FSH. Some FSH pulses may be associated with GnRH, and hence LH pulses, but the increase in plasma FSH concentrations often precedes that of either GnRH or LH (Padmanabhan *et al.,* 1997). Thirdly, sufficient LH, in particular, must be stored within the gonadotroph cells to allow enough LH to be released to maintain the preovulatory LH surge. With a half-life in plasma of only approximately 20 minutes (McNeilly *et al.,* 1982), the maintenance of the LH plasma levels during the preovulatory surge has been shown to require the

release of up to 80% of the total pituitary content in sheep pituitaries (Brooks *et al.,* 1993; Currie & McNeilly, 1995; Crawford *et al.,* 2002).

Granins are thought to play a role in regulated secretion of gonadotrophins, as there has been shown to be a correlation between granin regulation and granule morphology in gonadotroph cells (Huttner *et al.,* 1995). In sheep it has been observed that although LH and FSH are co-localised within the same gonadotroph cell, at a gross morphological level they can be seen to be packaged separately (Thomas & Clarke, 1997). At the ultrastructural level, LH has been shown to be packaged in electron-dense core granules (Currie & McNeilly, 1995; Crawford *et al.,* 2002), while FSH appears to be present in less dense granules. Additionally, granules containing an FSH-positive electron-light matrix surrounding an LH-positive electron-dense core have also been observed. Studies in sheep, monitoring the changes in morphology of gonadotrophs after the preovulatory LH surge have clearly illustrated that LH and FSH are mainly packaged in separate granules.

Other studies in the rat (Watanabe *et al.,* 1991), mouse (Crawford *et al.,* 2002) and sheep (Currie *et al.,* 1995) have investigated the role of the granin class of proteins in the organisation of granule formation. These granins are glycoproteins which aggregate at low pH and high salt concentrations, bind calcium, and are closely associated with both LH and FSH. Secretogranin II (SgII) has been shown to be present within the electron-dense core granules associated with LH, and in mice it has been shown that it is these granules that are released in response to a GnRH pulse (Crawford *et al.,* 2002). Chromogranin A (Cg A) is also associated with granules but appears to be associated with FSH in electron light-dense bodies (Wantanabe *et al.,* 1991). The properties of the main granins are summarised in Table 1.4.2b.

Granin	Properties	References
Secretogranin II	86kDa, associates with LH	Wantanabe *et al.*, 1991; Thomas *et al.*, 1998
Chromogranin A	70kDa, co-localises with LH and FSH	Wantanabe *et al.*, 1991
Chromogranin B	100kDa, co-localises with other granins	Pohl *et al.*, 1990; Bassetti *et al.*, 1990

Table 1.4.2b: Summary of the properties of the granins associated with gonadotrophins.

1.5 Male HPG axis in neonatal life

The neonatal period of brain-pituitary-gonadal function is a very important period in development. This period is involved with testicular descent, control of Sertoli cell number, masculinisation of the brain, development of social and sexual behaviour, pubertal maturation of gonadotrophin secretion, the setting of the threshold of testicular-pituitary feedback interactions, and development of the immune system (Figure 1.5a). The need to have a greater understanding of the neonatal period of hypothalamic-pituitary-testicular development has been emphasised by the fact that a range of reproductive disorders such as testicular cancer, hypospadias, and cryptorchidism in males are thought to stem from disruption occurring along the HPG axis during this developmental period. There have also been reports expressing concern about a potential decrease in sperm counts over the past 50 years (Sharpe & Skakkeback,1994), and it has been suggested that potential alterations along the neonatal HPG axis caused by the action of (environmental or synthetic) oestrogenic agents could play a role in this, together with other factors such as changes in life style and diet (Sharpe & Skakkeback,1994).

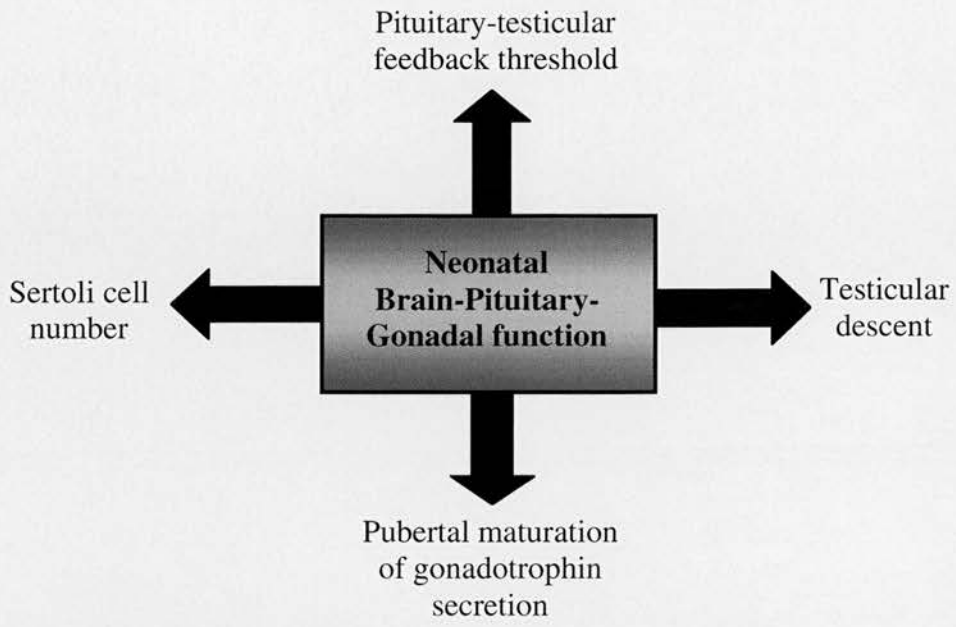


Figure 1.5a: Summary of the role of neonatal brain-pituitary-testicular interactions during neonatal life.

The early postnatal period of male primates is associated with the activation of the hypothalamic-pituitary-testicular axis. Circulating levels of LH and FSH begin to rise in male infants during the second week of postnatal life, reach a peak between 2 and 4 months, and decline thereafter, reaching juvenile levels by one year of age (Forest, 1990). Total testosterone levels rise commensurate with the increase in neonatal LH, reaching peak values that approach the low normal adult male range between 1 and 3 months of age, and then fall in concert with declining LH values to juvenile levels by 6 to 8 months of age (Forest, 1990) (Figure 1.5b). Comparable patterns of neonatal endocrine changes have been reported in the chimpanzee, rhesus monkey, and marmoset (Steiner & Bremner, 1981; Fuller *et al.,* 1982; Mann *et al.,* 1983, 1984; Lunn *et al.,* 1994).

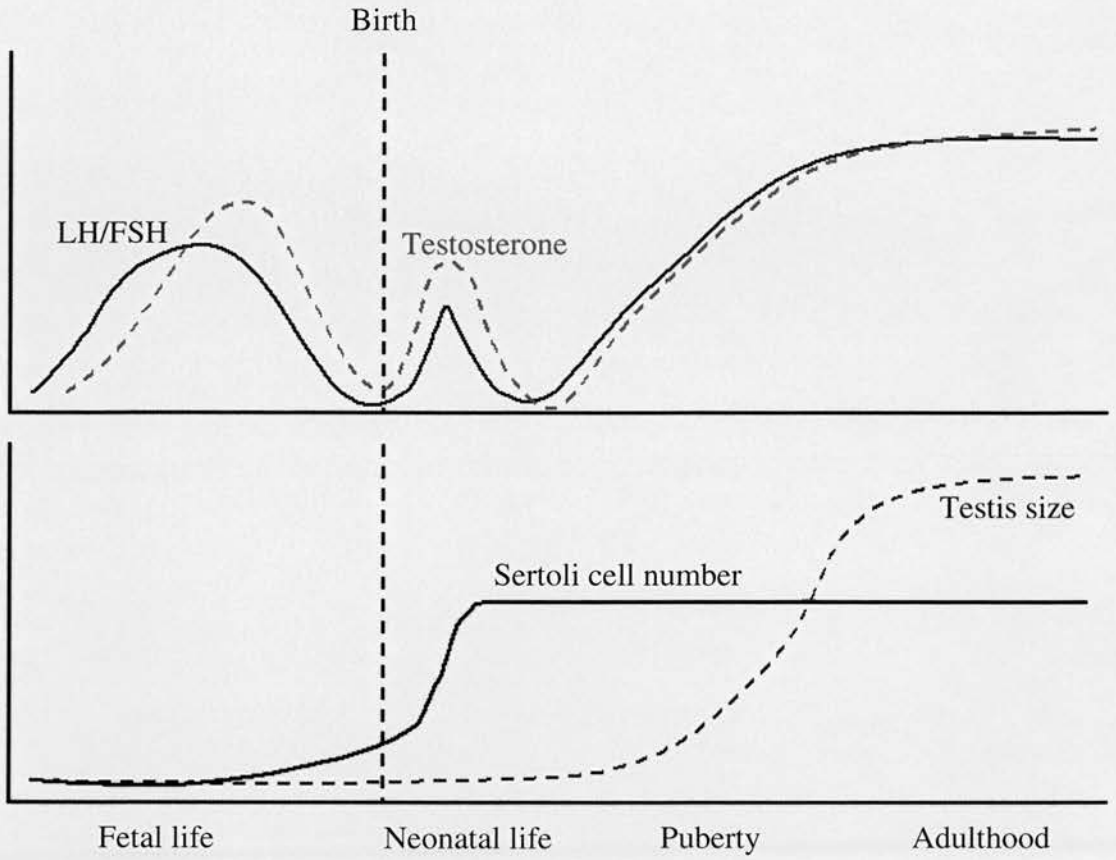


Figure 1.5b: Schematic diagram illustrating the pattern of LH, FSH, and testosterone secretion during development in the primate. The bottom panel shows the marked increase in Sertoli cell number during the neonatal period, while testis size does not increase until the activation of the hypothalamic-pituitary axis at puberty. Adapted from Mann & Sharpe, 1996.

LH secretion is pulsatile in the neonate, and the pituitary-testicular axis of the neonate responds to exogenous GnRH challenge in a similar manner to that of the adult (Plant, 1982; Mann *et al.*, 1984), suggesting that the central nervous system mechanisms controlling this axis may be fully functional during this period. However, unlike the adult, this active period of gonadotrophin and testosterone secretion in the neonate is

transitory and secretion falls to a low level characteristic of the juvenile period before being reactivated at the time of puberty (Mann *et al.,* 1984; Lunn *et al.,* 1994). It remains unclear as to whether the elevation of neonatal testosterone represents a true increase in the availability of bioactive hormone. While total circulating levels of testosterone increase in infants, free testosterone concentrations may not show a comparable change in magnitude (Huhtaniemi *et al.,* 1986) since there is also an elevation of sex hormone-binding globulin (Bolton *et al.,* 1989). Salivary testosterone levels, an index of the free fraction of serum steroid (Riad-Fahmy *et al.,* 1982), actually decline from the day of birth through 6 months of age in infant boys (Huhtaniemi *et al.,* 1986). On the other hand, the free androgen index (ratio of testosterone to sex hormone-binding globulin) is ten times higher in boys than girls (Bolton *et al.,* 1986), and the rise of total serum testosterone is paralleled by a similar pattern of change in unbound testosterone in infant boys (Forest, 1990).

In male rats, the neonatal period shows many similarities with primates, however there are differences. Neonatal rodents are relatively underdeveloped when compared with neonatal primates, however as in primates, the hypothalamo-pituitary-gonadal axis is already established in the new-born male rat (Swerdloff *et al.,* 1971; Grady 1986; Lopez & Negro-Vilar, 1988; Lalau *et al.,* 1990). But, from a developmental point of view, the rat is born with a reproductive system comparable to that observed in humans at 150 days of gestation life (Lalau *et al.,* 1990). In the male rat the serum concentrations of LH fluctuate during postnatal development until adulthood is reached. Two peaks in LH serum concentrations have been observed: the first one starts in the second week, reaches its maximum at approximately 14 days of age and is followed by a rapid decline, whereas the second peak is present during the peripubertal period, between days 40 and 45 (Ojeda & Ramirez,1972; Döhler & Wuttke, 1975; Chiappa & Fink, 1977). In contrast, the hypothalamic content of GnRH increases steeply between days 5 and 9, with a less marked increase rate in hypothalamic GnRH content afterwards, until the adult stage is reached (Chiappa & Fink, 1977). Interestingly, it has been reported that

the infantile female rat is more sensitive to GnRH compared with the neonatal and juvenile rat (Ojeda & Ramirez, 1972), and a corresponding sharp peak in GnRH receptor density in the pituitaries of female infantile rats was also observed (Dalkin *et al.,* 1981). However, during this developmental stage, it was also noted that male pituitaries showed only a discrete increase in GnRH-binding capacity, and the largest receptor density was observed in 30-day old rats (Dalkin *et al.,* 1981). In rodents, there is no prolonged neonatal testosterone rise as is the case for primates, the neonatal testosterone surge is restricted to a matter of hours around the day of birth (Forest, 1990).

Sperm production in adult life is dependent upon neonatal Sertoli cell proliferation. The number of Sertoli cells in the adult testis determines both testis size and daily sperm production. This relationship occurs because each Sertoli cell has a fixed capacity for the number of germ cells that it can support (Orth *et al.,* 1988), though this capacity has been shown to vary amongst species (Sharpe, 1994). Only immature Sertoli cells proliferate, so the final number of Sertoli cells is determined before adulthood (Figure 1.5c). There appear to be fundamental differences between species as to when Sertoli cells proliferate. In rodents, all Sertoli cell proliferation occurs in fetal and neonatal life, whereas in rhesus monkeys proliferation has been shown to occur predominantly in the peripubertal period. However, as more data has been obtained from more species, a general interpretation is now possible (Plant & Marshall, 2001). This indicates that Sertoli cells proliferate during two periods of life, in fetal or neonatal life and in the peripubertal period in all species. In some species (e.g. rhesus monkey), one period may be far more important than the other (Plant & Marshall, 2001), whereas in most species proliferation of Sertoli cells occurs in both periods, although the fetal or neonatal period is thought to be more important. As the number of Sertoli cells determines the number of spermatozoa produced per day, it is vital that the correct number of Sertoli cells is generated. Hormones, in particular FSH, have been shown to be important for generating the correct number of Sertoli cells. But also growth hormone, various paracrine growth factors (Sharpe, 1994; Sharpe *et al.,* 1999) and even LH and

testosterone have been shown to play a role in rhesus monkeys (Ramaswamy *et al.,* 2000). FSH increases the rate of proliferation of Sertoli cells. In contrast, thyroid hormones have been shown to alter the period in which proliferation can occur by regulating the maturation of Sertoli cells. It has been demonstrated that neonatal FSH concentration is very important, as its suppression can reduce the final number of Sertoli cells by approximately 40%, whereas an experimental increase of plasma FSH by injection or by neonatal hemicastration has been shown to increase the number of Sertoli cells by 18 to 49% (Hess *et al.,* 1993; De Franca *et al.,* 1995; Sharpe *et al.,* 2003a).

At around the onset of puberty, Sertoli cells undergo a radical change in their morphology and function, indicating a change from an immature proliferative state to a mature non-proliferative state. Adjacent Sertoli cells start to form tight junctions with each other to create a unique compartment in which meiotic and post-meiotic steps of spermatogenesis can proceed, as well as allowing formation of a fluid-filled lumen. As a result, the germ cells developing in the adluminal compartment become sealed off from direct access to many nutrients and thus they become dependent upon the secretion of such factors by the Sertoli cells (Jégou, 1992; McLaren *et al.,* 1993; Sharpe, 1994).

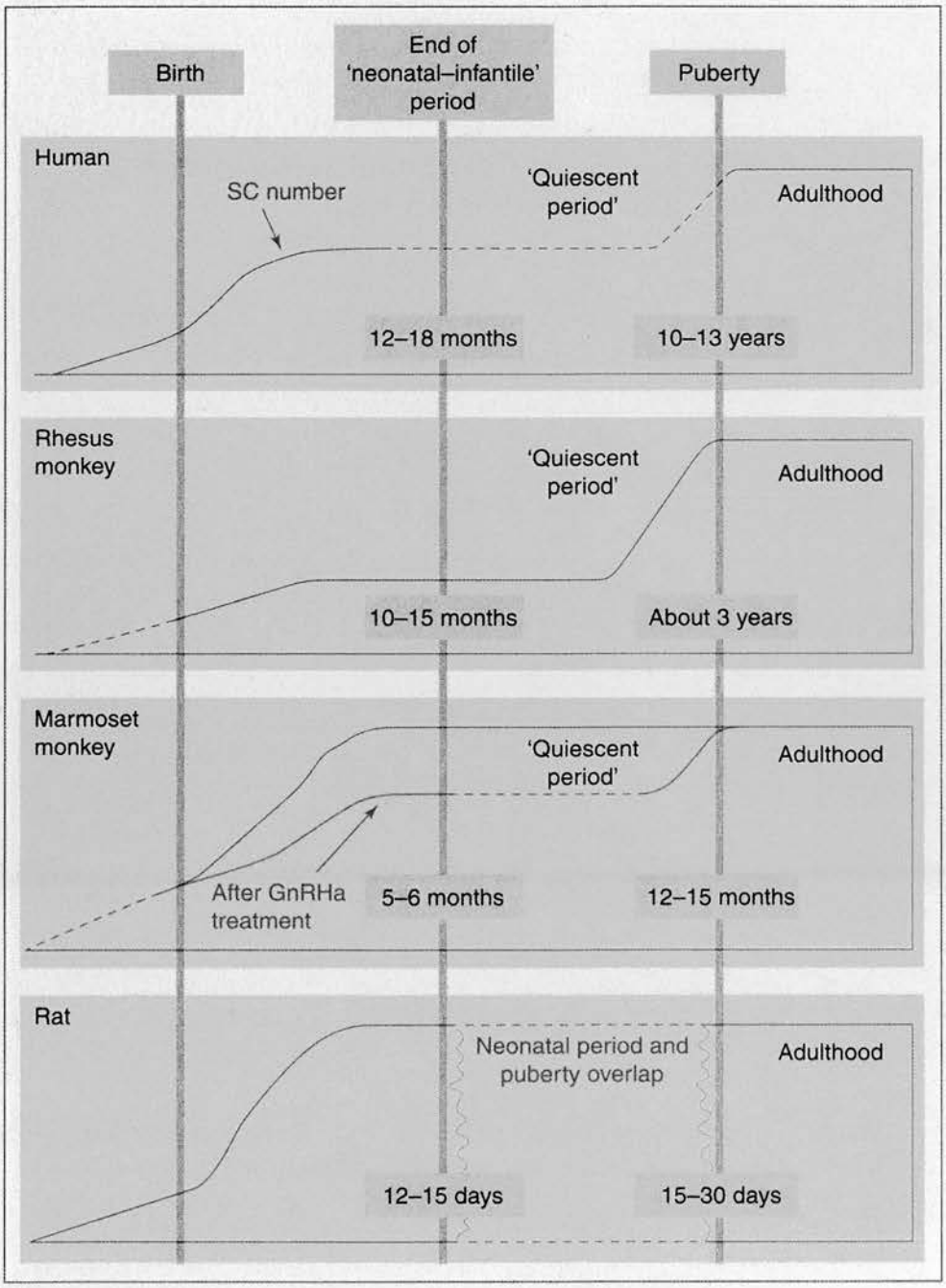


Figure 1.5c: Timing of proliferation of Sertoli cells (SC) in different species. The blue line indicates the change in relative number of SC for that species, where there are gaps in reported data, a dashed blue line is shown. The green line shown in the marmoset panel indicates what happens to the number of SC in animals treated neonatally with a GnRH antagonist to suppress gonadotrophin secretion. From Sharpe *et al.*, 2003a.

1.5.1 Adult male HPG feedback system

The adult male reproductive feedback system consists of the hypothalamus, the anterior pituitary gland and the testes. The testes have two important functions in the adult male: the production of sperm and the synthesis of testosterone. Control of both functions is guided by the central nervous system (CNS) in an elegant endocrine feedback loop with FSH and LH as the key hormonal signals. LH acts primarily on Leydig cells in the testicular interstitium to promote the synthesis of testosterone (T), while FSH acts exclusively on the Sertoli cells and germ cells to facilitate spermatogenesis. FSH and LH secretion from the anterior pituitary is regulated by hormonal signals from both the CNS and the gonads, including testosterone and its metabolites and inhibin (Figure 1.5.1).

Secretion of LH and FSH is under control of GnRH, synthesised in a pulsatile manner in the hypothalamus and carried by the hypothalamo-hypophyseal-portal system to the gonadotroph cells of the anterior pituitary. Binding of GnRH to receptors on the pituitary gonadotrophs causes the release of both FSH and LH. Direct measurement of GnRH in the hypothalamo-hypophyseal-portal blood of sheep has shown that each rise in serum LH is preceded by a GnRH pulse in the portal blood (Clarke & Cummins, 1982). Gonadal hormones can decrease gonadotrophin release both by decreasing GnRH release from the hypothalamus and by affecting the ability of GnRH to stimulate gonadotrophin secretion from the pituitary itself. For example, administration of exogenous testosterone has been shown to lead to a marked slowing in GnRH pulse frequency in men (Matsumoto *et al.,* 1984). Testosterone administration has also been shown to inhibit LH and FSH by a direct pituitary effect (Sheckter *et al.,* 1989).

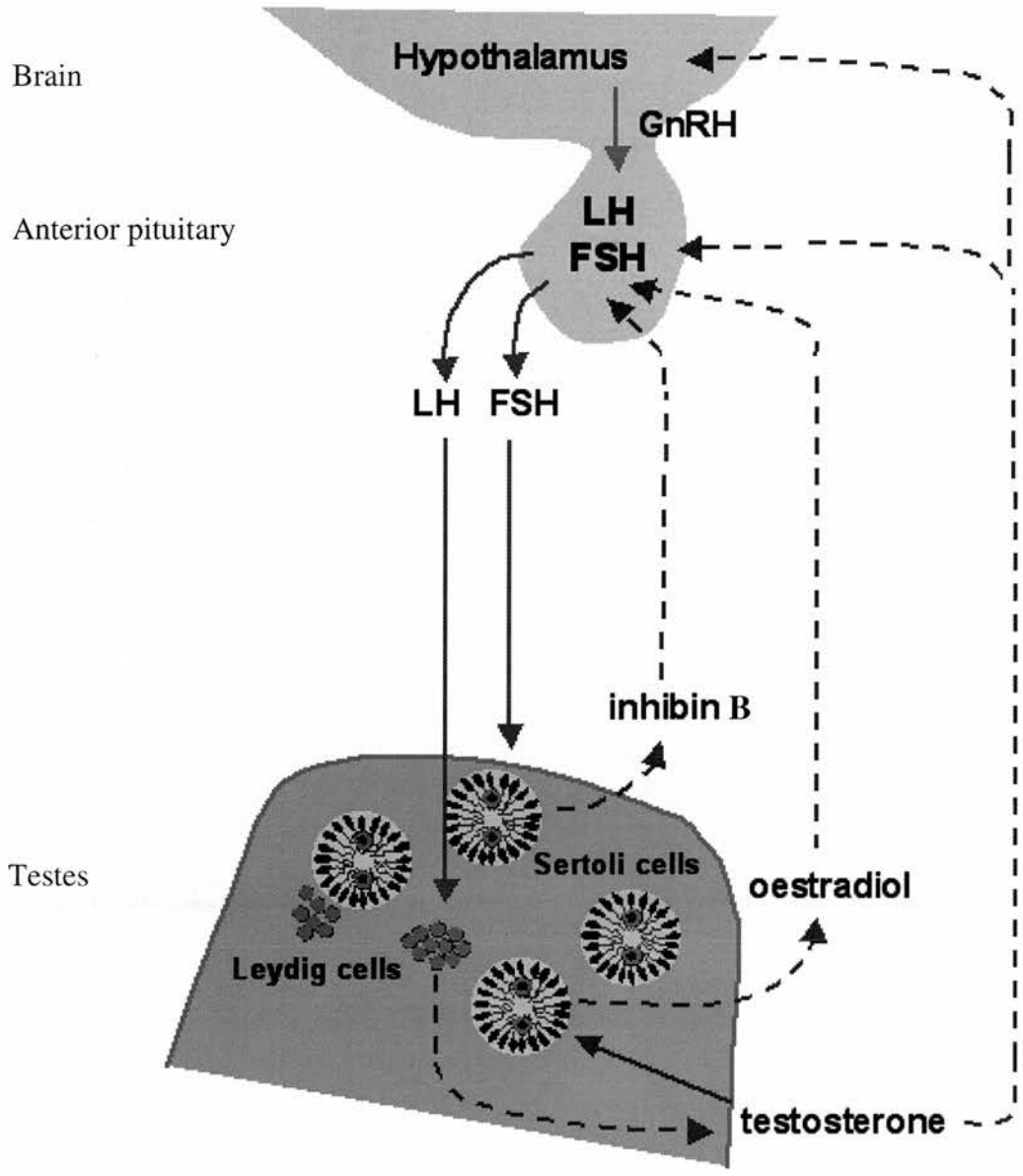


Figure 1.5.1: The hypothalamic-pituitary-gonadal feedback system in adult males. Pulsatile GnRH stimulates LH and FSH (to a smaller extent) release from the anterior pituitary. These stimulate the testicular Leydig cells to secrete testosterone, which feeds back onto the pituitary and hypothalamus, and the testicular Sertoli cells to secrete inhibin which negatively regulates FSH secretion from the pituitary.

The regulation of GnRH release at the hypothalamus is also mediated in part by aromatisation of testosterone to oestradiol (Carreau *et al.,* 1999, Saez, 1994). The biosynthesis of oestrogens from androgens is catalysed by the key enzyme complex named aromatase, composed of a specific form of cytochrome P450 aromatase (P450arom), and a ubiquitous flavoprotein, the NADPH-cytochrome P450 reductase (Simpson *et al.,* 1994; Simpson, 1997). The major protein hormone product of the gonads is inhibin, a 32kDa glycoprotein with two isoforms, termed inhibin A and B. The inhibins, activins and follistatin were isolated on the basis of their ability to modify the secretion of FSH by the pituitary gland (Ling *et al.,* 1985, 1986; Robertson *et al.,* 1985, 1987; Vale *et al.,* 1992; Ueno *et al.,* 1987). Activins are members of the transforming growth factor β (TGFβ) superfamily of growth and differentiation factors. Homo- or heterodimeric activin proteins are formed by linking βA- or βB-subunits through disulphide bonds, and at least three forms of activins with biological activity have been described: activin A (βAβA), activin B (βBβB) and activin AB (βAβB) (MacConell *et al.,* 1999). The isolation of activins followed that of inhibins, which are dimeric proteins composed of an α- and a β-subunit (Gray *et al.,* 2002; de Kretser *et al.,* 2000). Activin is produced in various tissues and stimulates synthesis of FSH by direct action on gonadotroph cells, whereas inhibin is primarily produced by the gonads and acts to counteract the effects of activin by reducing the secretion of FSH (Vale *et al.,* 1999). Inhibin blocks activin effects by competing with activin for receptor binding (Chapman *et al.,* 2001; Gray *et al.,* 2002). Follistatin is a monomeric protein, structurally unrelated to the TGFβ superfamily, that acts primarily by binding to activin and preventing its interaction with its receptor (Michell *et al.,* 1993a; De Winter *et al.,* 1996). Inhibin, activin and follistatin are produced by the ovary and are released into the circulation. However, the activin and inhibin subunits and follistatin are also produced in the pituitary gland itself by the gonadotroph cells and folliculostellate cells in different species, including the rat (Wilson & Handa, 1998; Kogawa *et al.,* 1991), human (Uccella *et al.,* 2000), and sheep (Baratta *et al.,* 2001; Farnworth *et al.,* 1995).

Thus these hormones may have autocrine or paracrine effects on the synthesis and secretion of FSH. Activin exerts its biological effects by interacting with four types of transmembrane receptors (types IA, IB, IIA, and IIB) with protein serine/threonine activity (Attisano & Wrana, 1996). The type II receptors are involved in initial ligand binding, leading to recruitment and phosphorylation of type I receptors by the kinase domain of type II receptors. Once phosphorylated, type I receptors exhibit kinase activity on Smad proteins. Smad-2 and Smad-3 are specific to activin signalling and are phosphorylated by activated activin receptors on serine residues. Phosphorylation of Smad-2 and Smad-3 allows complex formation with Smad-4, a common effector shared by different TGFβ family pathways (Heldin & Ostman, 1996). Once formed, the Smad-2/Smad-4 or Smad-3/Smad-4 complex translocates into the nucleus to activate the transcription of specific target genes. However, it has been suggested that the Smad signalling pathway might not be the sole pathway activated by the activin receptors. Other members of the activin family, such as TGFβ1, have been shown to activate other signalling pathways in addition to Smads, including the kinases extracellular regulated kinase 1/2 (ERK1/2) and phosphatidylinositol 3'-kinase (PI3K) (Vinals & Pouyssegur, 2001). The activin receptor subtypes and the Smad proteins have been characterised in the ovary (Drummond *et al.,* 2002; Van den Hurk & van de Pavert, 2001) and the pituitary, including the gonadotroph-derived LβT2 cell line (Dalkin *et al.,* 1996; Cameron *et al.,* 1994; Pernasetti *et al.,* 2001).

Although inhibins are part of the larger TGFβ superfamily, attempts so far to identify inhibin-specific type I and type II receptors have been unsuccessful (Woodruff,1999). However, inhibins have been shown to bind to activin type II receptors through their β-subunits. This binding does not lead to recruitment or phosphorylation of the type I receptor and thereby provides a mechanism for the inhibins to antagonise the actions of the activins (Attisano *et al.,* 1996; Lebrun & Vale, 1991; Mathews & Vale, 1991; Martens *et al.,* 1997; Xu *et al.,* 1995). However, recently, two proteins were identified

as candidate inhibin receptors: betaglycan and inhibin binding protein/p120 (InhBP/p120) (Lewis *et al.,* 2000; Chong *et al.,* 2000).

The properties of these two proteins and their potential mechanisms for activin antagonism are summarised in Table 1.5.1.

Property	**Betaglycan**	**InhBP/p120**
Expression in inhibin target tissues	Expressed in pituitary, especially in the intermediate lobe. Expressed in gonadotrophs. Also expressed in Leydig cells and in various ovarian cell types. Expression has also been detected in non-reproductive tissues.	Highly expressed in the anterior pituitary. Dynamically regulated in the rat pituitary across the oestrus cycle. More limited tissue distribution than betaglycan, with highest levels of expression observed in reproductive tissues, including rat Leydig cells.
Binds inhibins with high affinity	Binds inhibin A with high affinity and forms a higher affinity complex in the presence of activin type II receptors. Can also bind inhibin B, although the affinity of this association has not yet been reported.	Originally purified through affinity chromatography, but does not bind inhibin A or B in standard receptor binding assays.
Binds inhibins with high specificity	In addition to inhibins, betaglycan binds TGFβ1-3. Does not bind activin A.	Does not bind inhibin A or B and does not bind activin A.
Mechanism for activin antagonism	Forms a high affinity complex with ActRIIA and inhibin A, which blocks activin A binding to ActRIIA and thereby antagonises downstream signalling	Attenuates activin A-stimulated reporter gene activity.

Table 1.5.1: Properties and proposed mechanisms of betaglycan and inhibin binding protein/p120 (InhBP/p120) inhibin receptors. Adapted from Bernard *et al.,* 2002.

1.6 Female HPG axis in neonatal life

In contrast to male primates, where there is a sharp elevation in circulating LH (and FSH to a lesser extent for the first 3 postnatal months) and testosterone levels shortly after birth, lasting approximately 4 to 6 months (Corbier *et al.,* 1990; Faiman & Winter, 1974; Winter *et al.,* 1975), in neonatal females LH levels are only very slightly elevated during the first few months of life. FSH levels on the other hand, are high for the first 5 months (Faiman & Winter, 1974; Winter *et al.,* 1975). A moderate elevation of oestrogen levels has been observed, starting during late gestation and continuing through the neonatal period. After the first six months of life, circulating levels of FSH, LH, and gonadal steroids are all low and the hypothalamo-pituitary-gonadal feedback system enters a quiescent stage until the time of puberty (Figure 1.6) (Faiman & Winter, 1974).

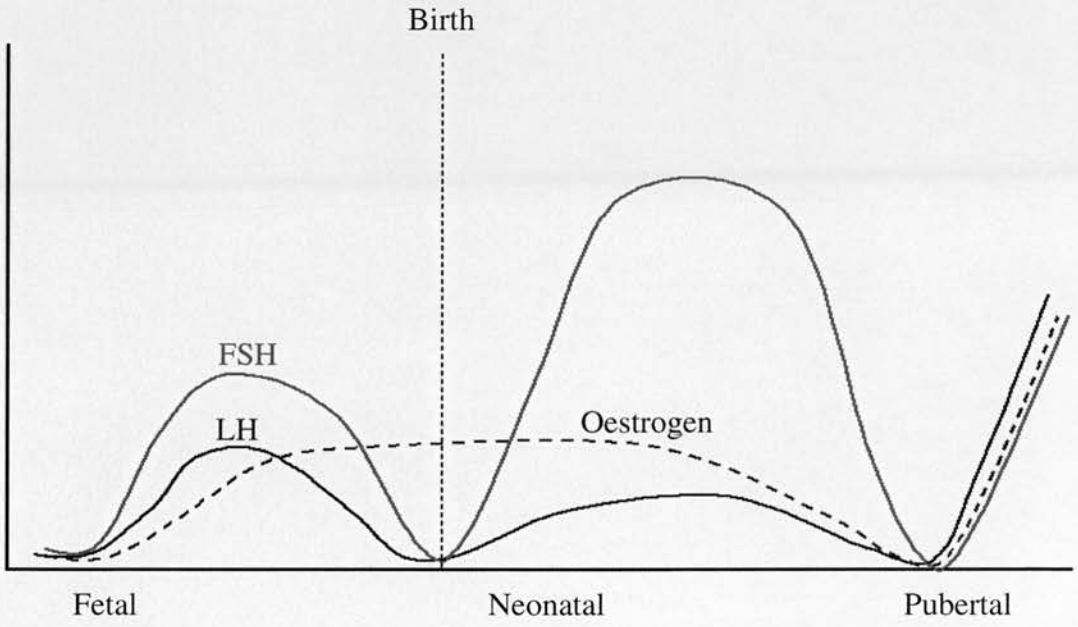


Figure 1.6: Schematic diagram illustrating the pattern of LH, FSH and oestrogen secretion during development in the female primate.

1.6.1 Adult female HPG feedback system

GnRH, LH and FSH control the reproductive cycle in adult females. The reproductive cycle is known as the menstrual cycle in primates and the oestrus cycle in rodents. It can be divided into two phases, follicular and luteal, which are separated by the LH surge (Figures 1.6.1a, b and c). The rodent oestrus cycle is divided into four stages, oestrus, met-oestrus, di-oestrus and pro-oestrus. The rodent oestrus cycle is similar to the menstrual cycle of primates, but there are some differences (Greenwald and Wang, 1994). In rodents, there is a high level of FSH due to reduced circulating levels of inhibin, which produces relatively large follicles. Therefore, the follicles require a shorter period of growth and hence the follicular phase is short, lasting 1 or 2 days (Greenwald and Choudary, 1969). If the ovum remains unfertilised, the corpus luteum degenerates within 2 to 3 days (Marshall, 1984).

Pulsatile GnRH stimulation of pituitary gonadotrophs results in acute secretion of both LH and FSH. Although LH and FSH are both secreted from the same gonadotroph cells in response to pulses of GnRH, the relative plasma concentrations of LH and FSH vary throughout the reproductive cycle, with predominance of FSH in the early follicular phase and LH predominance in both the luteal and late follicular phases. The rise in plasma FSH during the luteal-follicular transition is critical for follicular development and also induces granulosa cell LH receptors and the aromatase enzyme system. Although the exact mechanisms governing the differential secretion of LH and FSH are not entirely clear, at least two mechanisms are involved. Firstly, ovarian secretory products exert selective feedback actions on gonadotroph cells. Oestradiol (Marshall *et al.,* 1983) and the inhibin peptides (Hayes *et al.,* 1998; Vale *et al.,* 1992; Michel *et al.,* 1993b) specifically inhibit FSH secretion, as occurs during the luteal phase. The decrease in plasma levels of both inhibin and oestradiol with the demise of the corpus luteum in the human menstrual cycle is associated with the intermenstrual rise in FSH

which initiates the growth of follicles in the next cycle (Groome *et al.,* 1996). Increases in serum activin A, a peptide hormone that enhances FSH production, is thought to play a role in the rise of FSH during the luteal-follicular transition (Muttukrishna *et al.,* 1996). In addition, oestradiol concentrations above a certain threshold greatly augment LH release, this positive feedback action of oestradiol is integral to the midcycle LH surge. The second mechanism by which differential gonadotrophin secretion occurs involves alterations in GnRH pulse frequency. *In vivo* studies performed in ovariectomised monkeys and sheep have demonstrated that faster frequencies (i.e. once hourly) of pulsatile exogenous GnRH favour LH secretion. However, slow pulse frequencies (i.e. every 3 to 4 hours) favour FSH secretion, such that plasma FSH increases, while LH decreases (Wildt *et al.,* 1981; Clarke *et al.,* 1984). *In vitro and in vivo* studies in a GnRH-deficient rat model have demonstrated that fast GnRH pulses (i.e. every 15 minutes) favoured LH mRNA expression, whereas slow GnRH pulses (i.e. every 120 minutes) resulted in preferential expression of FSH mRNA (Kirk *et al.,* 1994). In this GnRH-deficient rat model, GnRH pulse frequency was also found to regulate FSH production via changes in gonadotroph activin and follistatin. Expression of activin βB was enhanced by slow (every 30 minutes) frequencies of GnRH stimulation. In turn, intragonadotroph activin βB subunit was found to enhance FSH transcription and stabilise FSH mRNA (Dalkin *et al.,* 1999). On the other hand, fast GnRH pulse frequencies were found to augment expression of intragonadotroph follistatin, which acts to reduce production of FSH mRNA by binding to and inactivating activin βB (Kirk *et al.,* 1994). Thus, a regulatory system is present within pituitary gonadotroph cells that effects differential expression of FSH and LH mRNA as a function of the pattern of GnRH pulse stimulation.

Studies utilizing frequent blood sampling and pulse detection analysis have shown that there are changes in the frequency of LH (and by inference GnRH) pulses throughout the menstrual cycle (Reame *et al.,* 1984; Filicori *et al.,* 1986,1984; Backstrom *et al.,* 1982;). LH pulse frequency increases from approximately one pulse per 90 minutes in

the early follicular phase to one pulse every 60 minutes during the midcycle LH surge, and subsequently decreases to one pulse every 3 to 4 hours during the luteal phase. The exact mechanisms controlling alterations in GnRH pulse frequency throughout the menstrual cycle are not fully understood, however, it has been shown that progesterone appears to play a dominant role. Progesterone has been shown to slow GnRH pulse frequency when given to women in the follicular phase and when given to ovariectomised, oestradiol–replaced sheep (Soules *et al.,* 1984; Goodman *et al.,* 1981). Thus ability of progesterone to slow GnRH pulse frequency requires the presence of oestradiol. It has been suggested that this is perhaps a reflection of the ability of oestradiol to induce hypothalamic progesterone receptors (MacLusky and McEwen, 1978; Romano *et al.,* 1989).

Thus, in the primate menstrual cycle, during the luteal phase, oestradiol and progesterone from the corpus luteum slow GnRH pulse frequency to approximately one pulse every 3 to 4 hours. This slow GnRH pulse frequency favours FSH synthesis and, in concert with oestradiol actions and inhibin A to restrain FSH secretion, leads to an increase in FSH stores in pituitary gonadotrophs. In the absence of fertilisation, the corpus luteum regresses in the late luteal phase and oestradiol, progesterone and inhibin A levels decrease. FSH levels begin to increase in the late luteal phase, resulting in the recruitment of ovarian follicles, one of which later develops into a dominant Graafian follicle. Whereas FSH levels exceed that of LH in the first 5 days of the follicular phase, oestradiol and inhibin B from the dominant follicle limit FSH secretion in the later follicular phase. GnRH pulse frequency gradually increases to one pulse every 60 minutes during the late follicular phase (McCartney *et al.,* 2002). This, in combination with the actions of the high levels of oestradiol to augment LH release, triggers the LH surge. Subsequent ovulation and formation of the corpus luteum causes large amounts of oestradiol and progesterone to be secreted, and therefore GnRH pulse frequency is slowed again and hence FSH synthesis is favoured for subsequent release.

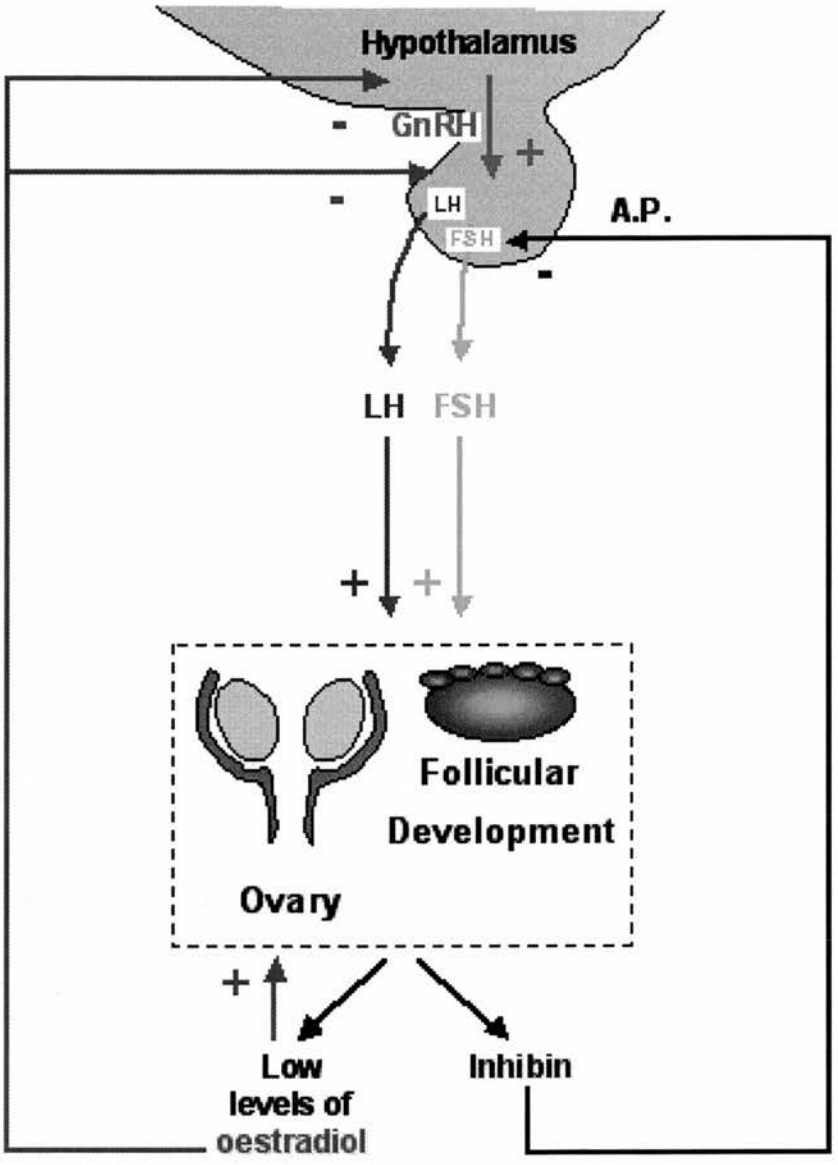


Figure 1.6.1a: Schematic diagram illustrating feedback control of FSH and LH secretion during the follicular phase in primates.

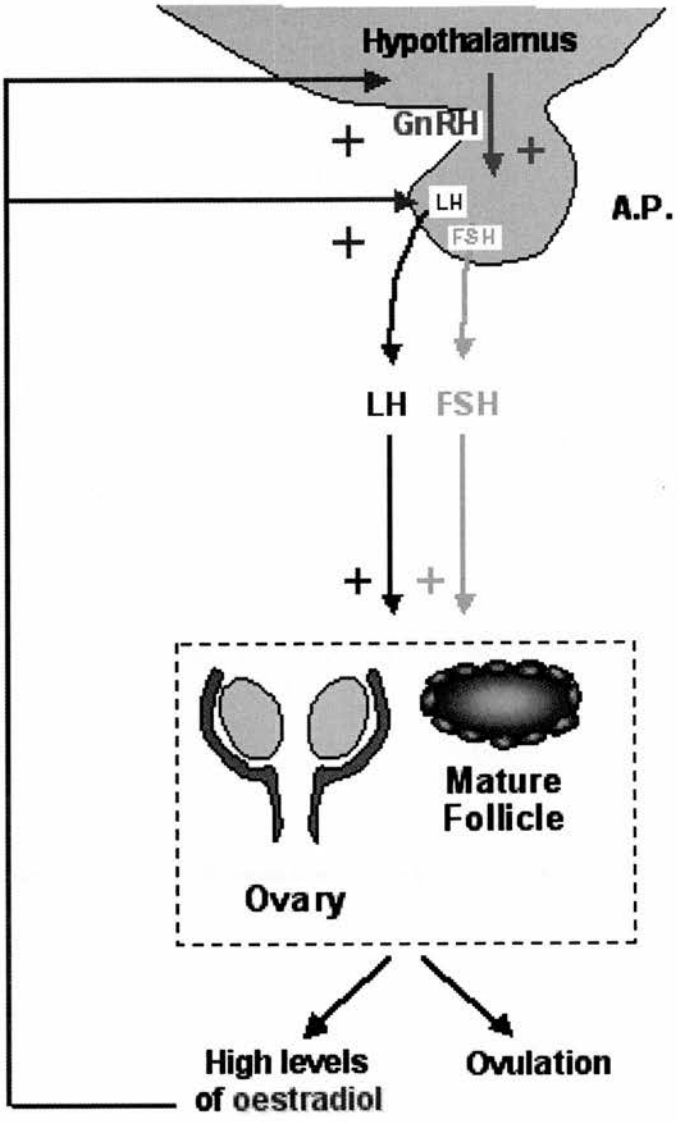


Figure 1.6.1b: Schematic diagram illustrating the control of the LH surge at ovulation in primates.

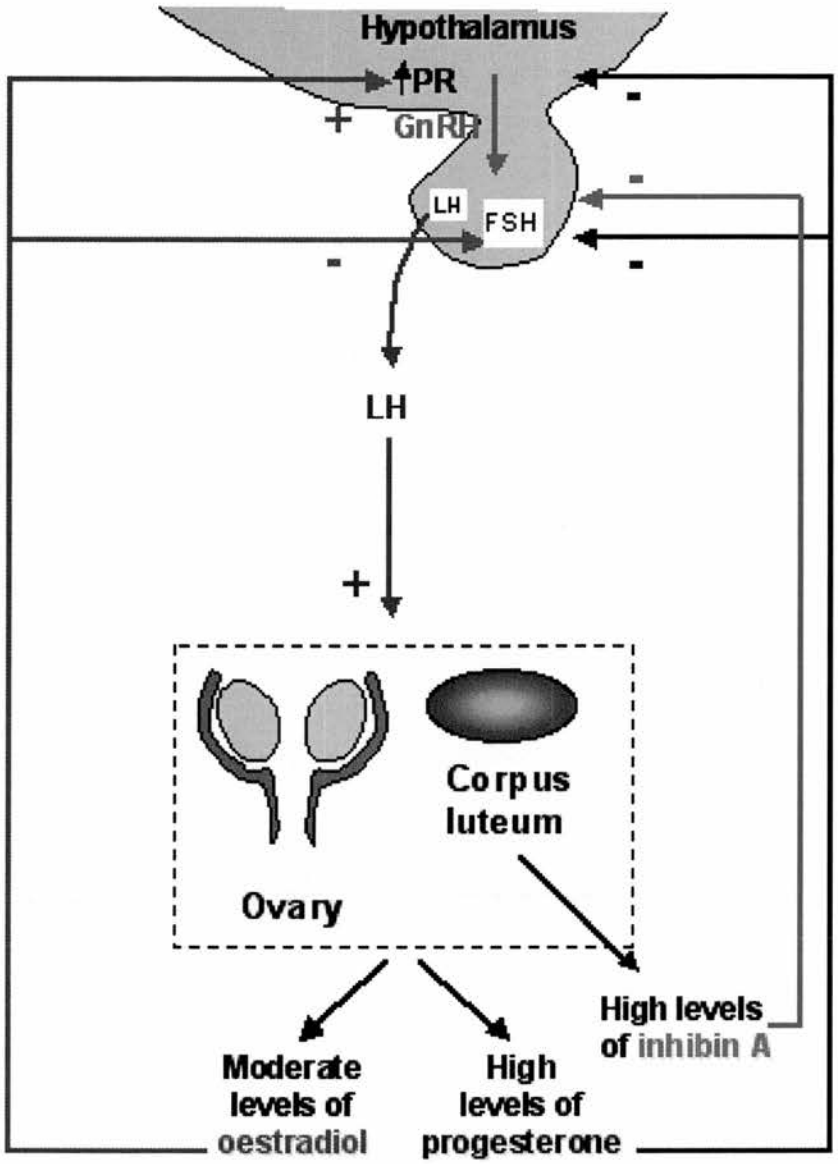


Figure 1.6.1c: Schematic diagram of the feedback control during the luteal phase in primates. PR is progesterone receptor.

1.7 Oestrogen receptor in males and females

More than 40 years ago Jensen and Jacobsen (Jensen & Jacobsen, 1962) came to the conclusion, based on the specific binding of oestradiol-17β in the uterus, that the biological effects of oestrogen had to be mediated by a receptor protein. For 24 years this protein was extensively studied in several laboratories and in 1986, two groups reported the cloning of this oestrogen receptor (ER) (Green *et al.,* 1986; Greene *et al.,* 1986). Until 1995, it was assumed that there was only one ER and that it was responsible for mediating all of the physiological and pharmacological effects of natural and synthetic oestrogens and anti-oestrogens. However, in 1995, a second ER, ERβ was cloned from a rat prostate cDNA library (Kuiper *et al.,* 1996). The former ER is now called ERα. Since then several groups have cloned ERβ from various species (Tchoudakova *et al.,* 1999; Todo *et al.,* 1996; Tremblay *et al.,* 1997) and have identified several ERβ isoforms (Moore *et al.,* 1998; Ogawa *et al.,* 1998; Petersen *et al.,* 1998).

ERα and ERβ belong to the steroid/thyroid hormone superfamily of nuclear receptors, members of which share a common structural architecture (Evans, 1988; Giguere *et al.,* 1988). They are composed of three independent but interacting functional domains: the NH_2-terminal or A/B domain, the C or DNA-binding domain, and the D/E/F or ligand binding domain. Binding of a ligand to ER triggers conformational changes in the receptor and this leads, via a number of events, to changes in the rate of transcription of oestrogen-regulated genes. These events, and the order in which they occur in the overall process, are currently not completely understood, but they include receptor dimerisation, receptor-DNA interaction, recruitment of and interaction with coactivators and other transcription factors, and the formation of a preinitiation complex (Kraus *et al.,* 1995; McKenna *et al.,* 1999; Rachez *et al.,* 1998).

In the pituitary gland, ERα is expressed at high levels in lactotrophs, at somewhat lower levels in gonadotrophs, and at generally low levels in other pituitary cell types (Couse *et al.,* 1999; Scully *et al.,* 1997). Full length ERα is expressed at higher levels in female rats than in males, and oestrogen treatment of females has been shown to result in little change in full-length ERα mRNA levels (Mitchner *et al.,* 1998; Friend *et al.,* 1995). Treatment of neonatal male rats with oestrogen has been shown to permanently decrease both ERα and ERβ mRNAs, suggesting an imprinting effect of steroid (Tena-Sempere *et al.,* 2001). Gender-specific regulation of ERα has also been noted with some hypothalamic peptides, as treatment of males with a GnRH antagonist has been shown to suppress ERα mRNA, while treatment of females showed no effect (Schreihofer *et al.,* 2000).

In the rat testes, both ERα and ERβ are expressed, but the expression profiles of these two receptors are different. In rodent testes, ERα is localised in the nuclei of the Leydig cells (Fisher *et al.,* 1997), while ERβ is localised in germ cells and Sertoli cells, and fetal Leydig cells (Saunders *et al.,* 1997; Van Pelt *et al.,* 1999). In the excurrent ducts of adult male rats, both ERα and ERβ are expressed (Fisher *et al.,* 1998; Hess *et al.,* 1997). In the rat ovary, ERα is expressed primarily in the thecal and interstitial regions, whereas ERβ is primarily expressed in the granulosa cells (Schomberg *et al.,* 1999).

1.8 Endocrine disruption of the HPG axis

A number of synthetic chemicals have been shown to be able to mimic endogenous hormones and bind to oestrogen receptors, and it has been suggested that alterations in the normal pattern of reproductive development seen in some populations of wildlife are linked with neonatal (and/or fetal) exposure to these chemicals (Bicknell *et al.,* 1995; Lemini *et al.,* 1997; Golden *et al.,* 1998). There have been numerous reports of reproductive and developmental abnormalities ranging from snails to humans that have been associated with exposure to environmental hormones (primarily oestrogens). A brief overview of some of the most studied endocrine disrupting chemicals and their observed effects in wildlife and humans are summarised in Table 1.8.

Some of these endocrine disrupting compounds are highly persistent industrial products, such as polychlorinated biphenyls (PCBs) and organochlorine pesticides, such as dichlorodiphenyl trichloroethane (DDT). They can persist for decades and show the characteristic of bioaccumulation within the food chain (Colborn *et al.,* 1993, Kavlock & Ankley, 1996; Bigsby *et al.,* 1999). Others are less persistent such as Bisphenol A, which was first used as the monomer to manufacture polycarbonate plastic and resins, and it is now one of the top 50 chemicals in production in the world (Kirschner, 1996). Dioxins and dioxin-like PCBs are toxic by-products of the manufacture of chlorinated chemicals, and they are also formed during the incineration of chlorinated chemicals if the disposal process is not performed at the required high temperature of 300°C. Because of the range of chemical classes and the number of suspected endocrine disrupting chemicals, these compounds present a complex issue. For the purpose of this thesis, only phytoestrogens and the synthetic estrogen diethylstilbestrol (DES) will be discussed in depth.

Contaminant	Species	Observation	References
DDT	Mammals Humans	Oligospermia, impotence, hypogonadism, decreased libido, reduced sperm counts and motility, menstrual cycle irregularities	Degen & Bolt, 2000
	Birds Japanese quail	Abnormal reproductive behaviour and feather morphology	Bryan *et al.*, 1989
	Gulls	Abnormal development of ovarian tissue and oviducts in male embryos	Fry & Toone, 1981
	Waterbirds	Egg shell thinning, mortality, developmental abnormalities, growth retardation	Safe, 2000
	Reptiles Alligators	Abnormal gonads, altered sex hormone levels	Guillette Jr *et al.*, 1994,1999
PCB's and dioxins	Mammals Seals	Impaired reproductive functions	Tyler *et al.*, 1998
	Mink	Population decline, developmental toxicity, hormonal alterations	Newbold *et al.*, 2000

Table 1.8: Overview of some endocrine disrupting compounds and their effects observed in wildlife and humans.

Contaminant	Species	Observation	References
Bisphenol A	Invertebrates Snails	Masculinisation, malformed oviducts, increased oocyte production	Mizuhashi *et al.*, 2000
Isoflavones and Coumestans	Mammals Cattle	Infertility	Hughes Jr, 1988
	Sheep	Infertility	Hughes Jr, 1988
	Rabbits	Infertility, failure of ovulation, failure of implantation	Hughes Jr, 1988
	Guinea pigs	Infertility	Hughes Jr, 1988

Table 1.8: Continuation of overview of some endocrine disrupting compounds and their effects observed in wildlife and humans.

1.8.1 Diethylstilbestrol (DES) effects on HPG axis

DES is a potent synthetic oestrogen (Figure 1.8.1) that has been thoroughly studied in both humans and animals.

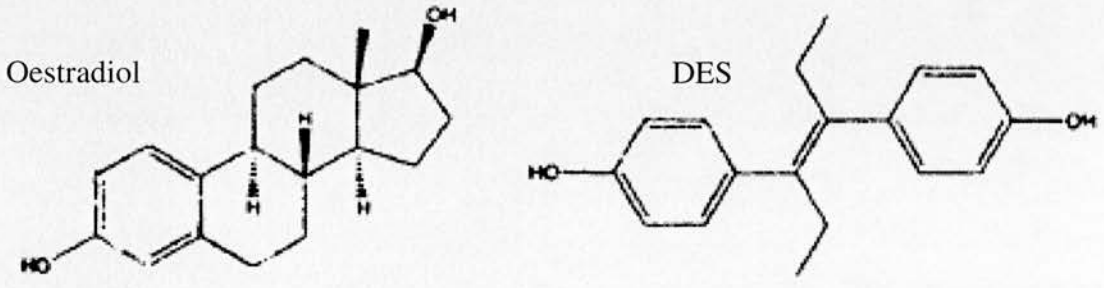


Figure 1.8.1: Diagram illustrating the chemical structures of oestradiol and the synthetic oestrogen DES.

Between 1947 and 1971 over 1,000,000 American women were exposed to DES when their mothers took the drug during pregnancy to prevent miscarriage. Girls exposed as fetuses to DES during the first three months of pregnancy subsequently exhibited changes in the tissue and/or structure of their uterus, cervix, or vagina (Herbst *et al.,* 1971). These changes resulted in later fertility problems and also placed them at risk of developing a rare form of cancer, clear-cell adenocarcinoma of the vagina or cervix, at a young age (Herbst *et al.,* 1971, 1979). DES was also introduced into the environment because of its oestrogenic activity to accelerate the growth of cattle. It was estimated that in 1971 alone as much as 27,000 kilograms of DES were used in livestock feed lots (Metzler, 1981). For the purpose of this thesis, DES was used as a positive control to investigate the effects of neonatal oestrogen exposure on the development and function of the HPG axis, and additionally to gain some insight into the potential mechanisms by which (less potent) oestrogenic endocrine disrupting compounds, such as phytoestrogens, might be exerting their effects.

1.8.1.1 DES disruption of the male HPG axis

The treatment of pregnant females with a high dose of DES has been shown to interfere with the action of Müllerian-inhibiting hormone on Müllerian duct regression in male mice (McLachlan *et al.,* 1975) and humans (Driscoll & Taylor, 1980), such that the utricular remnant in the prostate, which is of Müllerian origin, was enlarged. The exposure of rats and mice to high (microgram per kilogram body weight) doses of DES during prenatal or neonatal life has been shown to interfere with the normal development of the prostate in rodents (Turner *et al.,* 1989; Prins, 1992; Pylkkanen *et al.,* 1993; vom Saal *et al.,* 1997; Gupta, 2000). Squamous metaplasia of the prostatic and coagulating gland and the ductal epithelium in male mice and rats has also been reported after exposure to DES during early life (McLachlan *et al.,* 1975). The exposure of rats and mice to DES during development has also been shown to alter circulating gonadotrophin

levels and gonadal hormone levels (Atanassova *et al.,* 1999), decrease pituitary responsiveness to GnRH and blunt LH secretion in rats (Register *et al.,* 1995; Faber *et al.,* 1993), inhibit Sertoli cell proliferation (Atanassova *et al.,* 1999), suppress Leydig cell development and function (Sharpe *et al.,* 2003); and cause abnormal development of the rete testis (Aceitero *et al.,* 1998; Fisher *et al.,* 1999; McKinnell *et al.,* 2001a), efferent ducts (Fisher *et al.,* 1997), epididymis (Atanassova *et al.,* 2001; McKinnell *et al.,* 2001), vas deferens (Atanassova *et al.,* 2001; McKinnell *et al.,* 2001) and the seminal vesicles (Williams *et al.,* 2001). Neonatal treatment with high, but not with lower doses of DES, has also been shown to suppress expression of the androgen receptor in all of the tissues affected adversely by DES (McKinnell *et al.,* 2001). However, in some studies, some effects of exposure to DES during development were not noticeable prior to the exposed animals reaching old age. For example, Arai and colleagues have shown that the treatment of male rats with DES during the first month after birth did not result in observable malignancies at 6 to 9 months of age. But by 20 months (old age), squamous cell cancer was detected with the involvement of the dorsolateral prostate, coagulating glands, and ejaculatory ducts (Arai *et al.,* 1978).

Other studies with DES have involved the use of very low doses that are considered relevant for predicting the effects of exposure to concentrations of oestrogenic endocrine disruptors encountered by humans and wildlife. The dose issue is important because it has been shown that exposure to high or low doses of DES can result in opposite effects on some of the developing reproductive organs in males (vom Saal *et al.,* 1997; Gupta, 2000). For example, fetal exposure to DES has been shown to alter the number of live pups per litter, the sex ratio of the litters, the anogenital distance of the male and female offspring at birth and the bodyweight of offspring at birth. In most cases it was found that the dose-response relationship was complex, with effects at the highest dose examined being the opposite of the effects seen at the lower doses of DES (vom Saal *et al.,* 1997).

1.8.1.2 DES disruption of the female HPG axis

Exposure to DES during differentiation of the reproductive system has been implicated in the etiology of clear cell vaginal adenocarcinoma in women (Herbst, 1981; Bern, 1992; Mittendorf, 1995). In mice, neonatal exposure to clinically relevant doses of DES has been shown to result in vaginal lesions (Bern *et al.,* 1987). DES has been shown to induce uterine hypoplasia acutely in neonatal rats (Medlock *et al.,* 1988, 1992). Uterine hypoplasia has also been observed in adult women exposed as fetuses to DES (Mittendorf, 1995). The same treatment has been shown to inhibit uterine gland differentiation in human fetal uteri transplanted into DES-treated nude mice, and these studies have suggested that the DES-induced effects might not be due to changes in the brain-pituitary-gonadal axis (Cunha *et al.,* 1987). The effects of DES that are dependent on the presence of ovaries (ovarian-dependent) as opposed to independent of the presence of ovaries have been examined in mice (Iguchi & Takasugi, 1987). DES has been found to lower uterine oestrogen receptor levels in adulthood when administered during postnatal days 1 to 5 in rats (Medlock *et al.,* 1992). However, in adult females, changes in oestrogen receptors induced by exposure to DES have been shown to be reversible, whereas changes in oestrogen receptors induced by exposure to DES during early uterine development have been shown to be permanent and irreversible (Bern *et al.,* 1987; Iguchi *et al.,* 1988). Changes in uterine epithelial as well as myometrial morphology and function have been observed following treatment with DES during prenatal (Wordinger *et al.,* 1991) or early neonatal (Brody & Cunha, 1989) life in rodents, and these changes are thought to be mediated by DES binding to oestrogen receptors in the mesenchyme (Stumpf *et al.,* 1980).

DES exposure during development has also been shown to alter the immune system in mice (Ways *et al.,* 1980; Blair *et al.,* 1987) and humans, which have shown evidence of a hyperactive immune response (Ways *et al.,* 1987) and an increased incidence of autoimmune diseases (Noller *et al.,* 1988).

1.8.2 Phytoestrogen effects on HPG axis

Not all oestrogenic chemicals are synthetic, there are also some natural chemicals present in the environment that can mimic the action of oestrogens such as phytoestrogens (Kang *et al.,* 2001; Tyler *et al.,* 1998). The phytoestrogens are ubiquitous within the plant kingdom, being synthesised in plants from phenylpropanoids and simple phenols (Rolfe, 1988). Phytoestrogens are a diverse group of polyphenolic non-steroidal plant compounds that bind to oestrogen receptors, and exert their characteristics of endogenous steroidal oestrogens. Based on their chemical structure, phytoestrogens can be classified into four main groups, i.e. isoflavonoids, flavonoids, stilbenes, and lignans. Isoflavones are the most studied group of phytoestrogens and are found almost exclusively in the family Leguminosae (King & Young, 1999). A large number of isoflavones has been identified from plants, with daidzein (Denis *et al.,* 1999) and genistein (Lee *et al.,* 1991) being the principal isoflavones. They occur in plants as the inactive glycosides daidzin (Potter *et al.,* 1998) and genistin (Bakhit *et al.,* 1994), and their respective 4'-methyl ether derivatives, formononetin and biochanin A.

A great number of isoflavonoids have been tested in a competition binding assay to assess their relative binding affinities (Branham *et al.,* 2002). The oestrogen receptor relative binding affinities of the isoflavonoids tested were found to decrease in the following order : 17β-oestradiol (control) > coumestrol > genistein > equol > daidzein > biochanin A. Isoflavones have been found to have a relatively greater binding affinity for ERβ than for ERα, but are 10^2 to 10^5 times less active than steroidal oestrogens. They are, however, frequently present in the human body in much higher quantities than endogenously produced oestrogens (Kuiper *et al.,* 1997,1998; Morito *et al.,* 2001; Schmitt & Stopper, 2001). Some isoflavones are able to inhibit several key enzymes in oestrogen and androgen biosynthesis, including 5α-reductase (Evans *et al.,* 1995), 17β-hydroxysteroid oxidoreductase (Mäkelä *et al.,* 1998), and aromatase (Adlercreutz *et al.,* 1993), and can stimulate the synthesis of sex hormone-binding globulins (SHBG)

(Mousavi & Adlercreutz, 1993). Isoflavones have also been reported to have selective oestrogen receptor modulator (SERM) activity, in that they have both weak oestrogenic and anti-oestrogenic properties (Whitten *et al.,* 1992; Park *et al.,* 2000).

Early developmental exposure to oestrogenic compounds is known to cause reproductive tract abnormalities, decreases in reproductive organ weights, alter neonatal gonadal hormone levels, and to potentially compromise sperm production and sperm quality in experimental animals, and possibly wildlife populations and humans (Jensen *et al.,* 1995; Toppari *et al.,* 1996; Tyler *et al.,* 1998; Sharpe *et al.,* 2002). These findings have raised concern over exposure to oestrogenic isoflavonoids and other exogenous weak oestrogens in our food and water supplies. Because of the weak oestrogenic activity of genistein and other isoflavones, there has been considerable debate over the possible risk and/or health benefits of isoflavone consumption during the sensitive stages of fetal and infant development (Lamartiniere *et al.,* 1998; Bouker & Hilakivi-Clarke, 1993; Sharpe *et al.,* 2002). In the United States, 25% of infants are now fed solely with soy formula milk after birth (Klein, 1998; Lonnerdal, 1994), and there are concerns that the high isoflavonoid content of soy formula milk might exert adverse effects on the developing infant (Irvine *et al.,* 1995; 1998, Setchell *et al.,* 1997, 1998). Central to the debate on the safety of soy formulas are reports of adverse health effects of isoflavones on animal reproductive systems. One of the most well-known complications of isoflavones are the reproductive disorders observed in sheep that graze on clover with a high isoflavone content, often referred to as clover disease (Adams, 1990). This clover disease was caused by extremely high concentrations of equol, a potent oestrogenic isoflavone, on the mature reproductive system of seasonal breeding ruminant animals at a critical endocrinological period when disruption of complex and usually well-orchestrated hormonal events caused infertility.

Millions of American infants have been fed soy formula over the past three decades. Several studies so far have demonstrated that soy formula supports normal growth and development in term infants (Formon *et al.,* 1971; Businco *et al.,* 1992; Churella *et al.,*

1994; Kohler *et al.,* 1984; Graham *et al.,* 1970). When growth was studied over the first year of life, body weight gains and body length of infants were virtually the same whether the infants were fed soy formula milk or cow's milk based formula or breastfed (Lasekan *et al.,* 1999). More recently, a study was undertaken on 811 young adults between 20 and 34 years of age who were fed either cow's milk formula or soy formula milk as infants, to determine the long-term health consequences of early soy intake (Strom *et al.,* 2001). The study found that there were no significant differences in growth, development, puberty, reproductive function, or pregnancy outcomes (Strom *et al.,* 2001). However, it must be taken into account that the number of subjects was low and that the subjects were too young to determine the risk of developing chronic diseases that occur later in life.

Because of obvious ethical and safety reasons, data on the fetal plasma isoflavone concentrations are essentially non-existent, and the data on infant plasma concentrations are extremely limited. However, there are two studies that have provided some insight into the likely isoflavone exposure throughout the life cycle. Adlercreutz *et al.,* have demonstrated that there are significant concentrations of isoflavonoids transported from the mother to the fetus in Japanese women who consumed their usual diet rich in isoflavone sources, including soy foods (Aldercreutz *et al.,* 1999). Setchell *et al.,* has reported the plasma isoflavone concentrations of infants fed breast milk, cow's milk formula or soy formula milk, as well as the approximate plasma oestradiol concentrations of women and infants (Setchell *et al.,* 1997), however, the number of subjects was limited in both studies. The plasma concentrations obtained from these studies are summarised in Table 1.8.2.

Parameter	Approximate plasma concentrations	
	Oestradiol (nmol/L)	**Isoflavones (nmol/L)**
Maternal	148	233
Cord blood	148	299
Cow's milk formula fed neonate	0.00002	25
Soy formula milk fed neonate	-	7000
Breast milk fed neonate	-	25

Table 1.8.2: Approximate plasma concentrations of oestradiol and isoflavones in Japanese women and infants. Based on data from Setchell *et al.,* 1997 and Adlercreutz *et al.,* 1999.

The mean total maternal plasma isoflavonoid concentration of Japanese women who consumed their normal diets was found to be approximately 233nmol/L (range 19-744nmol/L) and the mean concentration of isoflavonoids in cord blood was 299nmol/L (range 58-831nmol/L) (Setchell *et al.,* 1997; Adlercreutz *et al.,* 1999). This suggests that isoflavonoid phytoestrogens could be transferred from the mother to the developing fetus. Although the oestrogenic potency of isoflavones is estimated to be lower than that of oestradiol, the plasma isoflavone concentrations are approximately 50 to 100 times higher in infants fed soy formula milk than the oestradiol levels achieved by women during pregnancy (Setchell *et al.,* 1997; Adlercreutz *et al.,* 1999).

As yet, no human data support the toxicity of soy foods, especially regarding reproductive competency. In countries in which soy has been consumed at the greatest daily intake for centuries, the population has increased at normal rates (Adlercreutz *et al.,* 1999). It has been shown that women who consume soy foods that result in high circulating concentrations of isoflavones are capable of conceiving, taking the pregnancy to term, delivering normal infants, and lactating normally (Lasekan *et al.,* 1999). Because these women consumed soy foods before pregnancy, and continued eating soy during pregnancy and during lactation, it has been suggested that soy foods, including

soy formula milk, do not seem to have great adverse effects on early human development or later reproductive function (Setchell *et al.,* 1997; Adlercreutz *et al.,* 1999). However, it must be taken into consideration that no large long-term studies, sampling different ethnic groups of the general population, have been performed as yet.

1.9 Aims of this thesis

The experimental work undertaken in this thesis focuses mainly on investigating the potential short-term and long-term effects of neonatal oestrogen treatment on the function of the hypothalamic-pituitary-gonadal axis. The main focal point of these studies was the anterior pituitary gland, in particular investigating whether pituitary function was affected by neonatal oestrogen treatment and if pituitary function could be re-programmed on a permanent basis. Both *in vivo* and *in vitro* approaches were undertaken to explore the neonatal oestrogen effects on both male and female pituitary function, and comparisons were made for oestrogen-induced effects in both males and females. The potent synthetic oestrogen, DES, was used during these studies as a positive control for neonatal oestrogen disruption of the HPG axis, as DES has been shown extensively to cause long-term effects along multiple facets of the HPG axis in both males and females. The information gathered from these DES studies then provided a valuable link to the next set of studies undertaken, which investigated the potential effects of less potent environmental oestrogens, namely phytoestrogens, on the HPG axis. These phytoestrogen experiments were undertaken to address health concerns of feeding human infants soy formula milk, which is rich in phytoestrogens.

The experimental chapters will be presented as follows: chapter 3 examined whether treatment of DES in infant rats caused any significant short-term or long-term changes in pituitary function. Potential pituitary changes were evaluated by measuring the number of immunopositive gonadotrophs and the mRNA levels for each of the gonadotrophins,

and by assaying plasma hormone levels. Data obtained from the testes of these animals collected by Richard Sharpe's laboratory, were included to shed light upon potential altered feedback along the pituitary-testicular axis. Chapter 4 involved setting up a primary pituitary cell culture system from individual rat pituitaries to explore the potential effects of neonatal DES treatment on pituitary function *in vitro*, without feedback occurring from the testes and hypothalamus. Both adult male and female pituitaries were cultured from rats that were tracked *in vivo*, by measuring circulating pituitary and gonadal hormone levels. Cultured pituitary cells from these *in vivo*-tracked neonatally DES-treated rats were administered GnRH and/or activin treatments and gonadotrophin output was measured. DES-induced effects on male and female pituitaries were compared and contrasted. Chapter 5 investigated the effects of neonatal treatment with a long-acting GnRH antagonist on its own or in combination with neonatal DES treatment on pituitary function. This treatment regime was included to investigate the potential effects of DES on GnRH, and hence by removing GnRH it was tested whether DES would still have effects at the level of the pituitary gland. Chapter 6 addressed health concerns of feeding human infants phytoestrogen-rich soy formula milk. Pituitary function was investigated in both rats and marmoset monkeys. Finally, chapter 7 summarised the main findings and conclusions of the experimental work and suggested several avenues which might lead further to the present understanding of neonatal oestrogen disruption of the HPG axis.

Chapter two: general materials and methods

2.1 Animals and treatments

Both marmoset and rat studies were performed according to the Animal Scientific Procedures Act (1986) under Project Licence approval by the UK Home Office. The marmoset study was also approved by the local Ethical Reviews Process (ERP) for studies in primates, and the rat studies were also approved by the University of Edinburgh ERP.

2.1.2 Wistar rats

Wistar rats bred at the MFAA (University of Edinburgh Animal House) were kept under standard conditions and maintained on a standard diet (Rat breeding diet, SDS, Dundee, UK) that contains soy meal. Beginning on postnatal day 2, groups of three to six rats were subjected to one of the following treatments administered by subcutaneous injection: (a) diethylstilbestrol (DES) (Sigma Chemical Co., Poole, Dorset, UK) at a dose of 10μg or 0.1μg in 20μl corn oil on days 2,4,6,8,10 and 12; (b) 10mg/kg of a long-acting GnRH antagonist (GnRHa) (Antarelix; Europeptides, Argenteuil, France) in 20 μl 5% mannitol on days 2 and 6; (c) combined treatment of DES 0.1μg (as in treatment a) with GnRHa (as in treatment (b)); (d) s.c. injection of 20μl corn oil alone (control). During the various rat studies a number of male and female rat blood samples were taken at various stages of development, ranging from days 18,25,35 to day 90 (prepubertal, pubertal, adolescent, adulthood). Rats were anesthetized with flurothane and blood samples were taken with a heparinized syringe either from the tail vein during treatment or directly from the heart, for blood samples collected shortly before animals were killed. Blood samples were centrifuged at 3000rpm for 30 minutes at 4°C for plasma separation and the plasma was stored at –20°C until used for assay. Rats that

had terminal blood samples taken were killed using flurothane and cervical dislocation. Rats that did not have terminal blood samples taken were killed using CO_2 and cervical dislocation. Specific treatments are detailed further in each individual chapter of this thesis.

2.1.3 Marmosets

Captive-bred common marmosets *(Callithrix jacchus)* were maintained in a colony that has been self-sustained since 1973. There were a total of 30 newborn male marmosets, of which 26 were 13 pairs of male co-twins. Only co-twins were used in the soy study detailed in chapter six (one SMA-fed, one SFM-fed), as marmosets tend to show considerable between-animal variability but co-twins tend to be highly comparable. This enabled pair-wise evaluation of data and also minimized the use of animals. Treatment of infant marmosets commenced at day 4 or 5 of age. During the daytime (approximately 8 hours on weekdays and approximately 2 hours at weekends), the infant marmosets were separated from their mothers and left in the care of their fathers in the family cage. Normally it is the fathers or older siblings that care for the infant marmosets (apart from feeding) and this daytime separation from the mothers caused no obvious problems or distress for either infants or mothers. When the mother was absent from the family cage, the infant marmosets were removed at set intervals and were hand-fed with either standard (cow's milk-based) formula milk (SMA Gold; SMA nutrition, Taplow, Berkshire,UK) or with SFM (soy-based milk) formula (Wysoy; SMA nutrition) from day 4 or 5 until day 35 to 45. The formula powders were added to tapwater and heated and diluted according to manufacturer's guidelines. The suspension was administered to the infant in a 1ml syringe with a soft silicon rubber tube fitted to the end and the infant was allowed to drink as much milk as it wanted to. Each animal was weighed prior to feeding and the amount of formula milk taken was recorded. Infants were fed 3 to 4 times during weekdays and 1 to 2 times at weekends. After the last

formula feed of the day, the infant was returned to its mother and she was allowed to breastfeed them until the following morning.

Approximately half-way through the study (day 18-20), a 0.2ml blood sample was obtained from the femoral vein of each infant using a 1ml syringe fitted with a 27G needle. Animals were not sedated for this procedure as it caused them very minimal discomfort. On day 35-45, seven sets of co-twins were killed via i.p. injection of an overdose of sodium pentobarbitone (Euthatal; Rhone Merieux Ltd, Harlow, Essex, UK). Immediately after death, a terminal blood sample was taken by cardiac puncture with a heparinized syringe and the plasma was separated by centrifugation and stored at –20°C until used for assay. Blood samples were also collected on day 35-45 from the remaining 16 animals (8 SMA-fed, 8 SFM-fed), including six sets of co-twins, but their treatments were then discontinued and the animals were returned to their cages. These marmosets are currently growing to adulthood so that the possibility of any long-term effects to the SFM-exposure can be investigated.

2.2 Tissue collection and processing

2.2.1 Rat tissue

For the *in vivo* rat studies, anterior pituitaries were carefully removed and placed either directly into liquid nitrogen for subsequent messenger RNA (mRNA) analysis or were immersion fixed in Bouin's for 6 hours at room temperature before being processed, for subsequent immunocytochemical analysis. The Bouin's fixed tissue was transferred into 70% ethanol before being processed for 17.5 hours in an automated processor and embedded in paraffin wax.

For the *in vitro* rat primary pituitary cell culture, both the rats and the surrounding bench area were first swabbed with 70% ethanol and anterior pituitaries were removed using autoclaved surgical instruments. Each surgical instrument was cleaned thoroughly with

70% ethanol before use on each rat to minimize the risk of transferring an infection into the cell culture system. Anterior pituitaries were placed in ice-cold Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM HAM F-12) (Sigma, UK) supplemented with 10% fetal calf serum, 50U/ml streptomycin, 50U/ml penicillin and 5ml L-glutamine (Becton Dickinson Labware, Oxon, UK) for transportation back to the cell culture facility.

2.2.2 Marmoset tissue

Marmoset anterior pituitaries were dissected free of connective tissue and immersion-fixed for 5.5 hours in Bouin's fluid before being processed in an automated Leica TP-1050 processor and embedded in paraffin wax, for subsequent morphological evaluation and cell quantification studies.

2.3 Molecular biology

Unless otherwise stated, general chemicals were purchased from Merck-BDH (UK), Roche (UK) or Sigma-Aldrich (UK), and were Analar grade. All water used in reaction mixtures was double distilled and deionised before autoclaving.

2.3.1 Guidelines for use of RNA

RNA molecules are easily digested by RNases, therefore a number of strict guidelines were followed when handling RNA. Gloves and separate RNA-designated labcoats were worn at all times, RNase-free Gilson pipettes and RNase-free pipette tips containing filters (Promega, UK) were used for the transfer of all solutions and RNase-free water treated with 0.1% diethyl pyrocarbonate (DEPC) (Sigma,UK) was used to make up reaction mixtures.

2.3.2 RNA extraction from anterior pituitary tissue

RNA was extracted from rat anterior pituitary tissue in accordance with the RNAzol-B method. This method is based on the unique property of RNAzol-B, which promotes formation of complexes of RNA with guanidinium and water molecules and abolishes hydrophilic interactions of DNA and proteins. In effect, DNA and proteins are efficiently removed from the aqueous phase, while RNA remains in this phase during the sample extraction with RNAzol-B. Unless otherwise stated, tissues were kept on ice at all times and all reactions were carried out on ice. Firstly the pituitary tissue samples were homogenized with RNAzol-B (2ml per 100mg of tissue) with a motor homogenizer (Techmate Ltd. UK). 0.2ml of chloroform per 2ml of homogenate was added to the samples, the samples were covered tightly and shaken vigorously for 15 seconds and were then left on ice for 5 minutes. The cell suspension was subsequently centrifuged at 12000g for 15 minutes at 4°C. After addition of chloroform and centrifugation, the homogenate formed two phases: the lower blue phenol-chloroform phase and the colourless upper aqueous phase, proteins and DNA were in the interphase and organic phase. The aqueous phase, containing the RNA, was transferred to a fresh tube and an equal volume of isopropanol was added, before storing the samples overnight at –70°C. The following day, samples were centrifuged for 15 minutes at 12000g at 4°C. The RNA precipitate (which was invisible before centrifugation) formed a small white-yellow pellet at the bottom of the tube. The supernatant was removed and the RNA pellet was washed once with 75% ethanol (0.8ml ethanol per 50-100µg DNA) by vortexing vigorously and subsequent centrifugation for 8 minutes at 7500g at 4°C. At the end of the procedure, the pellet was dried for 10-15 minutes on the bench, taking care not to overdry the pellet as this greatly decreases its solubility. The RNA pellet was then dissolved in 0.1% sodium dodecyl sulphate (SDS) (Sigma, UK) by vigorous vortexing for at least 20 minutes. The amount and purity of the DNA was measured on

a spectrophotometer and required a 260/280 ratio of 1.8 or higher for further use. RNA samples were kept at –70°C for long-term storage.

2.3.3 DNase treatment of pituitary samples

RNA was DNase treated using the Ambion DNA-free kit. This kit is specifically designed to remove contaminating DNA from RNA preparations and to subsequently remove the DNase and divalent cations from the sample. Using DNA-free, contaminating DNA is removed to levels below the limit of detection by routine PCR.

To the RNA, 0.1 volume of 10 x DNase I Buffer and 1µl of DNase I was added. The samples were mixed gently and incubated at 37°C for 20-30 minutes. The DNase Inactivation Reagent was resuspended by flicking and gently vortexing the tube. Subsequently, 5µl of the Inactivation Reagent slurry was added to each sample, taking care to insert the pipette tip well below the surface and observing that the aliquot withdrawn was completely white without a significant amount of clear fluid. This ensured the efficient removal of DNase and divalent cations. To prevent the Inactivation Reagent from precipitating out, it was mixed briefly before addition to each sample. Thereafter the samples were incubated at room temperature for 2 minutes, the tubes were flicked once during incubation to re-disperse the DNase Inactivation Reagent. Finally the samples were centrifuged at 13000g for 2 minutes. The RNA solution was removed from the pelleted DNase Inactivation Reagent and stored at –70°C.

2.3.4 Spectrophotometrical analysis of nucleic acids

After DNase treatment the quantity and quality of the nucleic acids was assessed using a spectrophotometer. The optical density (OD) of the samples was measured at 2 distinct wavelengths, 260 and 280nm, in the ultraviolet region. Nucleic acids were detected at 260nm, whereas proteins and complex carbohydrates were detected at 280nm. High quality preparations possess a 260:280 ratio of approximately 2 for RNA and 1.8 for DNA.

2.3.5 Design of oligonucleotide primers

The Genejockey software programme (P.Taylor, MRC) was used to design oligonucleotide primers and they were manufactured by Genosys (UK). The primers were generally 18-20 base-pairs in length and had a melting temperature (Tm) in the range of 55-65°C. The following empirical formula was used to determine the appropriate Tm:

Tm = [(G+C) x 4C] + [(A+T) x 2C] (where G is guanine, C is cytosine, A is adenine, and T is thymine.)

The RNADRAW (Microsoft, UK) software programme was used to check that there was no secondary structure formation (i.e. hairpin loop formation and intramolecular annealing) within the primers. Any primer sequences containing secondary structures were discarded. Since there is over 95% amino acid sequence homology between mouse and rat for LHβ, FSHβ, GAPDH, αGSU and GnRH receptor (GnRHr), mouse primers were used for all Reverse Transcriptase Polymerase Chain Reactions (PCR). However, for Real-Time PCR specific rat primers were designed as this method requires 100% homology between primer sequence and amino acid sequence for efficient product amplification.

2.3.6 Reverse Transcriptase Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction process is a widely used technique for rapid and efficient amplification of cDNA reverse transcribed from RNA to analyse gene expression. The GeneAmp RNA PCR kit from Perkin Elmer (Applied Biosystems, USA) was used for both Reverse Transcriptase PCR and Real-Time PCR. This kit performs the reverse transcription of RNA to cDNA using cloned Murine Leukemia Virus (MuLV) Reverse Transcriptase for subsequent amplification with AmpliTaq DNA Polymerase, all in a single reaction tube. MuLV Reverse Transcriptase is an RNA-dependent DNA polymerase that uses single stranded RNA as a template in the presence of a primer to synthesise a complementary DNA strand and it is suitable for synthesis of first strand cDNA. Before starting the Reverse Transcriptase step in the PCR, each RNA sample (which had been DNase treated previously) was diluted to 50ng/µl with distilled water. Subsequently 6µl of the 50ng/µl stock for each sample was added to the (x1) mastermix (Table 2.3.6a) to make 30µl cDNA. The mastermix volume was adapted according to the number of RNA samples present. The appropriate non-enzyme control was also carried out, where a mastermix was prepared in which the MuLV was replaced with distilled water.

Reaction components	Quantity
10 x Buffer	3µl
Magnesium Chloride	6.6µl
NTP	1.5µl
Oligo dT	1.5µl
RNase Inhibitor	0.6µl
Murine Leukemia Virus Reverse Transcriptase (MuLV)	0.75µl
Distilled water	5.55µl
TOTAL VOLUME	24µl

Table 2.3.6a: Volumes of reaction components used for the RT-PCR reaction mixture.

The mastermix contents were mixed by carefully pipetting up and down and they were transferred to PCR tubes in 24µl aliquots for each sample, to which 6µl of RNA of each sample was added. The Reverse Transcriptase step was completed by running the samples for 45 minutes on the following heat-cycle programme (Table 2.3.6b) on the PCR machine:

Step	**Incubation**	**RT**	**RT inactivation**
TIME	70 minutes	30 minutes	5 minutes
TEMPERATURE	25°C	48°C	95°C

Table 2.3.6b: Incubation, reverse transcriptase (RT) and RT inactivation steps used during the RT-PCR reaction.

Subsequently the prepared cDNA was cut with the appropriate primers (Table 2.3.6c) in the following mastermix:

Reaction components	**LHβ, FSHβ 6µM primers**	**GAPDH, αGSU, GnRHr 10µM primers**
Distilled water	15.72µl	16.55µl
10 x Buffer	2.5µl	2.5µl
NTP	0.5µl	0.5µl
Magnesium Chloride	1.5µl	1.5µl
Forward primer	1.04µl	0.625µl
Reverse Primer	1.04µl	0.625µl
Taq polymerase	0.2µl	0.2µl
TOTAL VOLUME	22.5µl	22.5µl

Table 2.3.6c: Reaction mixture components and the primers used for the RT-PCR reaction.

Thereafter, each sample from the reaction mix was put through the following reaction cycles (Table 2.3.6d) for 45 minutes:

Hot start	95°C	15 minutes
Denaturing	95°C	15 seconds
Annealing	59°C	15 seconds
Extension	72°C 72°C	30 seconds 10 minutes

Table 2.3.6d: Reaction cycles used during the RT-PCR reaction.

2.3.7 Agarose gel electrophoresis

RNA molecules were separated out by agarose gel electrophoresis. A 2% agarose gel was prepared containing 0.89M Tris Borate, 0.0025M ethylenediaminetetraacetate (EDTA) and 2.5µl Ethidium Bromide (Sigma,UK) and was microwaved until the agarose had completely dissolved. 3µl Orange G (Sigma,UK) was added to each sample before loading onto gel, and samples were electrophoresed for approximately 1.5 hours.

2.3.8 Quantitative Real-Time PCR

The quantitative Real-Time PCR technique was used for mRNA detection as there was generally insufficient RNA present in the pituitary samples to run Northern Blots. The selection of specific PCR products during a Taqman reaction is dependent upon there being present within the reaction a third oligonucleotide. As depicted below, the third oligonucleotide has two fluorescent dye labels attached to it. One is a quencher dye, TAMARA, whilst the other is a reporter dye, which can be one of several available, e.g. FAM (5-carboxyfluorescein) and TET (tetrachloro-6-carboxyfluorescein). Whilst intact, in solution, the presence of the quencher results in a reduced level of detectable

fluorescence due to energy transfer between reporter and quencher dye. The probe is designed to anneal specifically to the amplicon sequence defined by the PCR primers. During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. As Taq polymerase extends from the primers, it will encounter the probe bound to one of the template strands. Initially this results in displacement of the 5'-end of the probe (including the reporter dye), giving rise to a Y-shaped structure. At the same time, the specific endonuclease activity of the Taq polymerase cleaves the 5' end of the probe, releasing a 3-6 base oligonucleotide containing the reporter dye still attached. The remainder of the probe is then continually cleaved until the resulting probe fragment has a melting temperature (Tm) below that of the extension reaction. This oligonucleotide fragment is displaced from the target and the polymerisation of the strand continues.

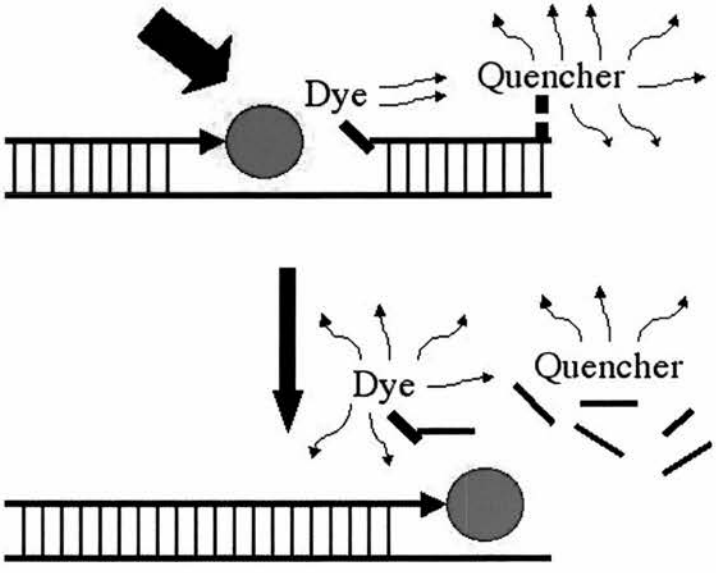


Figure 2.3.8a: Schematic diagram of the Taqman Real-Time PCR reaction, showing the probe annealing between the forward and reverse primer sites.

The 5'-3' nucleolytic activity of Taq cleaves the probe between the reporter and the quencher dyes only if this probe hybridises to the target, i.e. Taq does not digest free probe (Figure 2.3.8a). This process occurs in every cycle of PCR and does not interfere with the exponential accumulation of the product. The separation of the reporter dye from the quencher dye results in an increase in detectable fluorescence of the reporter. This increase is measured and is directly proportional to the target amplification during PCR. Because the probe is in excess, every copy of the template made generates a cleaved probe which has active reporter fluorescence (Figure 2.3.8b).

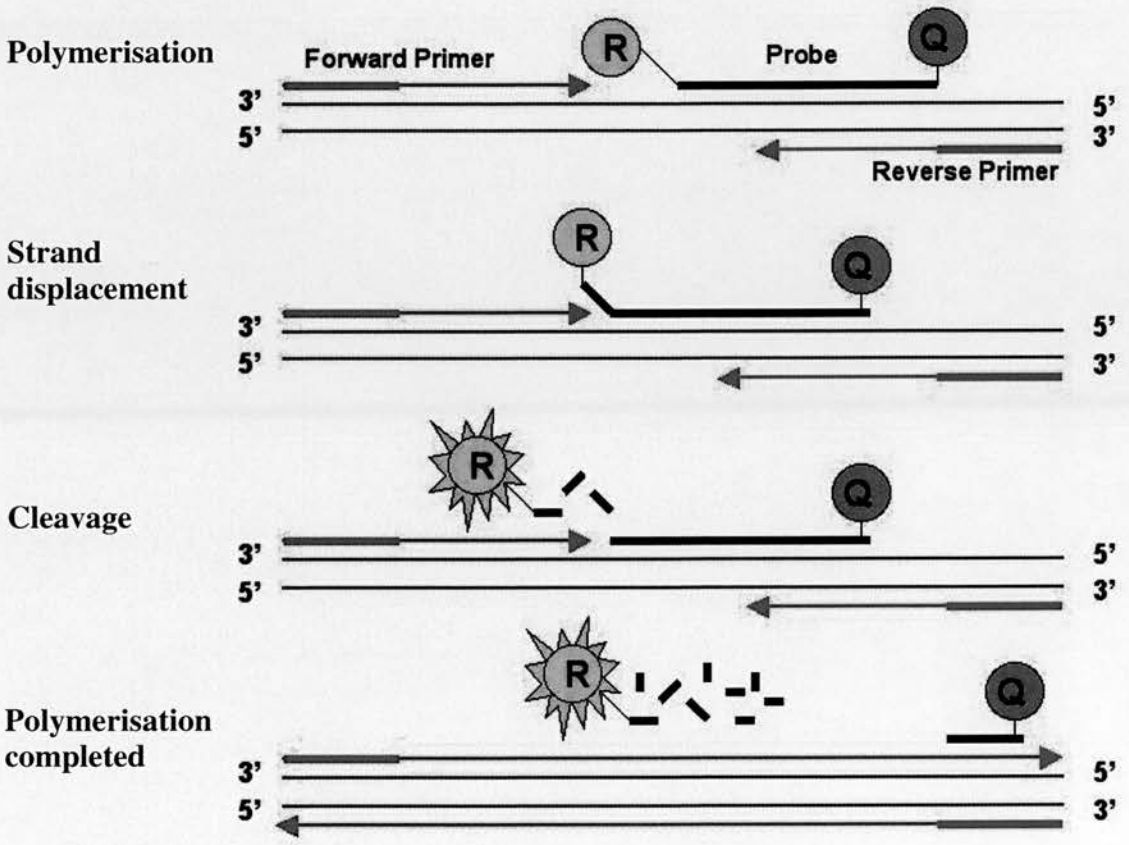


Figure 2.3.8b: Schematic diagram showing the polymerisation, strand displacement, cleavage, and final polymerisation completion steps in the Taqman Real-Time PCR reaction.

2.3.9 Primers and probes design for Real-Time PCR

Primers and probes were designed using Primer Express software (PE Biosystems, Warrington, Cheshire, UK) and synthesised by Biosource. Ribosomal 18S primers and probes were from a Taqman Ribosomal RNA Control Reagents kit (VIC labelled probe; PE biosystems). Forward primers, reverse primers and probes were designed for rat LHβ and FSHβ. Whilst designing the primers and probes several important features were taken into account which are summarized in Table 2.3.9.

<table>
<tr><th>Primer</th><th>Probe</th><th>Amplicon</th></tr>
<tr><td>Melting temperature (Tm) 58-60°C</td><td>Tm higher than Primer Tm</td><td>50-150 base pairs in length</td></tr>
<tr><td>20-80% GC</td><td>20-80% GC</td><td rowspan="5">As close to the probe as possible without overlapping</td></tr>
<tr><td>Length 9-40 base pairs</td><td>Length 9-40 bases</td></tr>
<tr><td>< 2°C difference in Tm between the forward and reverse primers</td><td>No G on the 5' end</td></tr>
<tr><td rowspan="2">No more than 2 G's or C's in last 5 bases at 3' end</td><td>< 4 contiguous G's</td></tr>
<tr><td>Must not have more G's than C's</td></tr>
</table>

Table 2.3.9: Summary of the properties of primers and probes for Real-Time PCR.

The sequence for the rat FSHβ primers and probe were: forward, 5'-CGGTGAAATGAAAGAATAAGGAACA-3' (7771.13kDa, annealing temperature 62°C), reverse 5'-TTTTGGATATCATGGTCCTTCAAG-3' (7314.72kDa, annealing temperature 60°C) and probe, 5'-FAM-TGGACATTGCCATTCACCCACCCTT-TAMARA-3' (7478.81kDa, annealing temperature 64°C). The primers and probe were

validated using a Real-Time amplification plot from which the comparative C_T was plotted against the log of the RNA (Figures 2.3.9a and b). The gradient of this graph needed to be between –0.1 and 0.1 to ensure validation, the gradient for FSHβ primers and probes was 0.093.

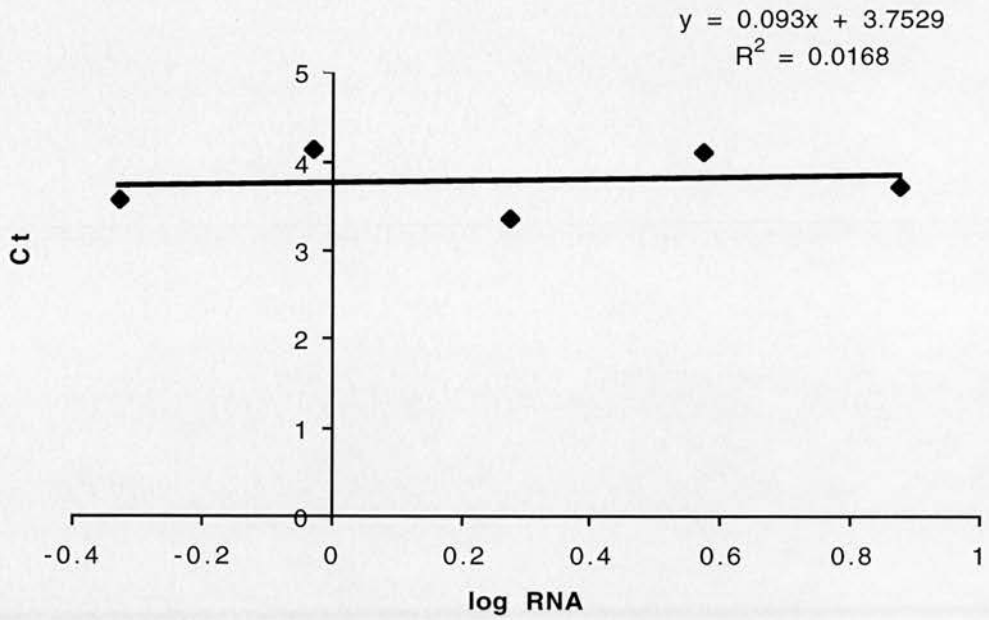


Figure 2.3.9a: Validation of the FSHβ primers and probes for the Real-Time PCR reaction. Ct is the cycle threshold.

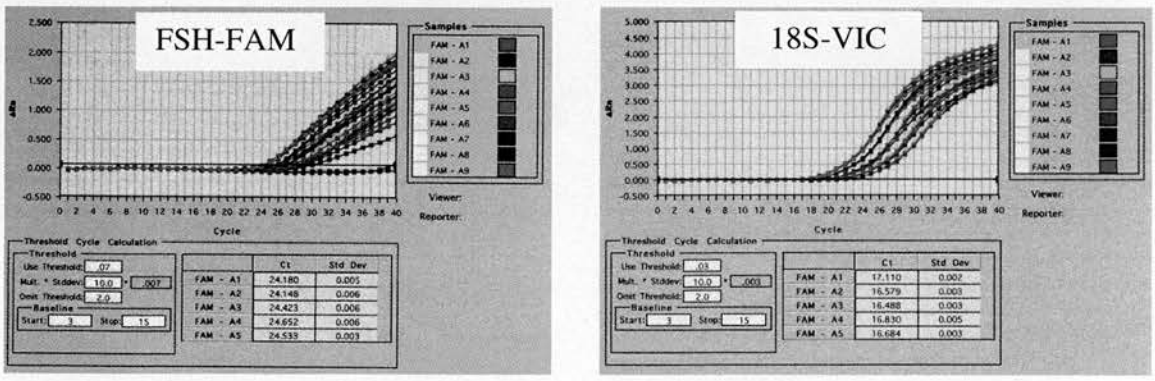


Figure 2.3.9b: Reaction cycles obtained for the validation of FSH primers and probes.

Numerous sequences for rat LHβ primers and probes were tried and tested, but since the LHβ sequence is rich in G's and C's at the 3'-end there were some initial problems validating the primers and probes. The most successful sequences for the rat LHβ primers and probe were found to be: forward, 5'-ACTGTCCTAGCATGGTTCGAGTACT-3' (7613.91kDa, annealing temperature 68°C), reverse 5'-AGCTCACGGTAGGTGCACACT-3' (6397.15kDa, annealing temperature 60°C) and probe, 5'-FAM-CTGCCTTGCCTCCCGTGCCTC-TAMARA-3' (6226.96kDa, annealing temperature 66°C). The LHβ primers and probe were validated for Real-Time PCR using the standard curve method, where a standard curve is drawn for the LHβ calibrator samples and for the internal 18S controls, and samples are read off the standard curve (Figures 2.3.9c,d,e). This is another common method used for validating primers and probes.

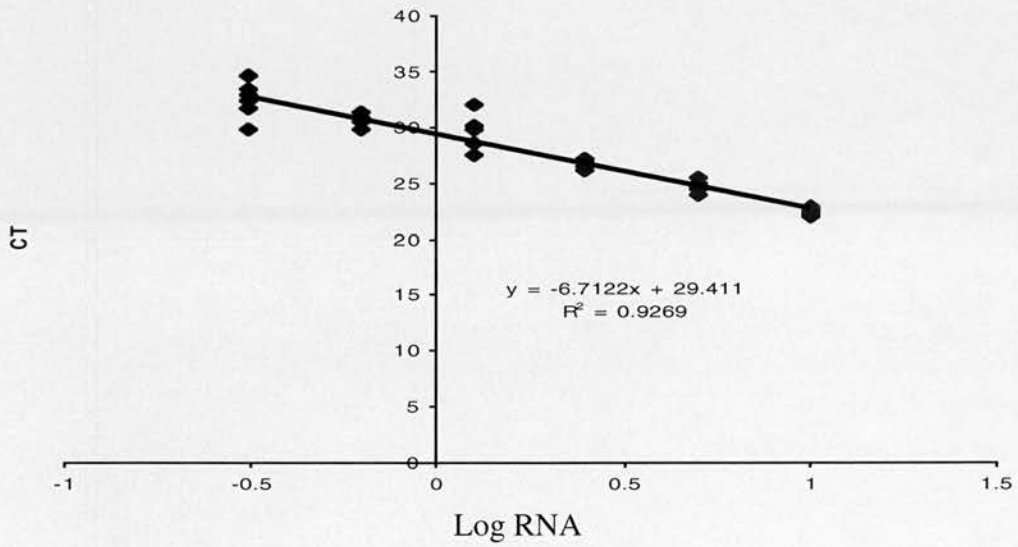


Figure 2.3.9c: Validation of the LHβ primers and probes for the Real-Time PCR reaction using the standard curve method. Standard curve for LHβ calibrator samples. The copy number of each gene is extrapolated on the basis of a standard curve. When the known concentrations (expressed in logarithmic form) of target gene are plotted against the corresponding cycle threshold (Ct), the result is a line representing the linear correlation between the two parameters. The equation describing this relationship is used to extrapolate the gene copy number in experimental samples.

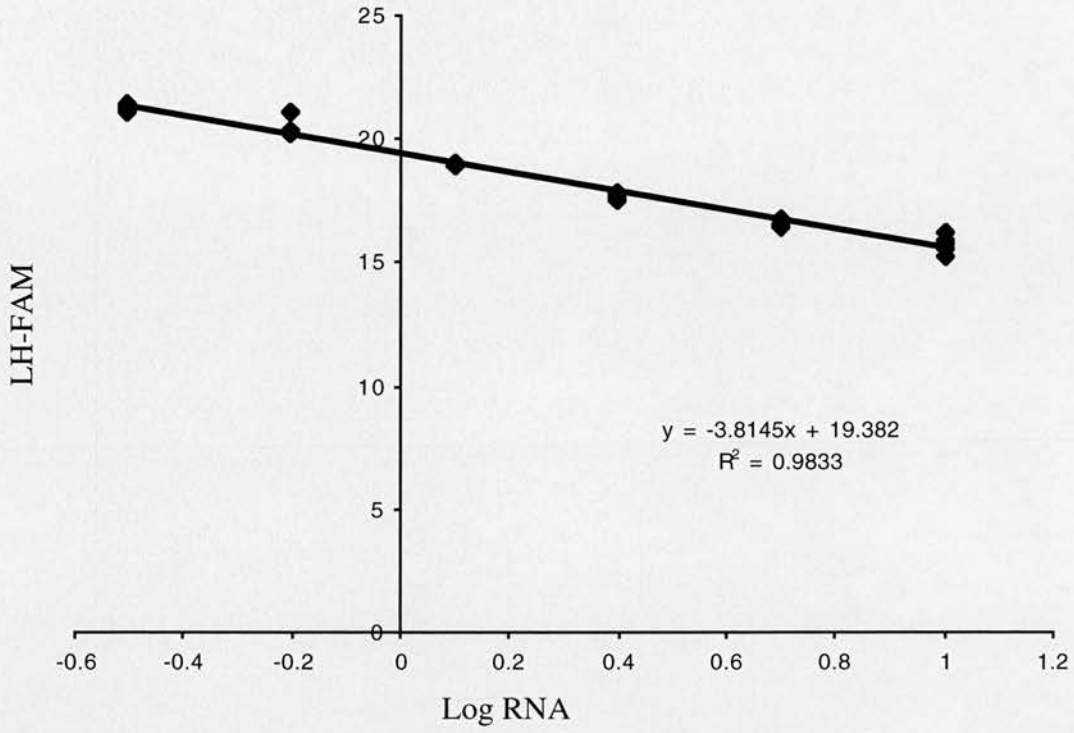


Figure 2.3.9d: Validation of the LHβ primers and probes for the Real-Time PCR reaction using the standard curve method. Standard curve for 18S internal controls for LHβ calibrator samples.

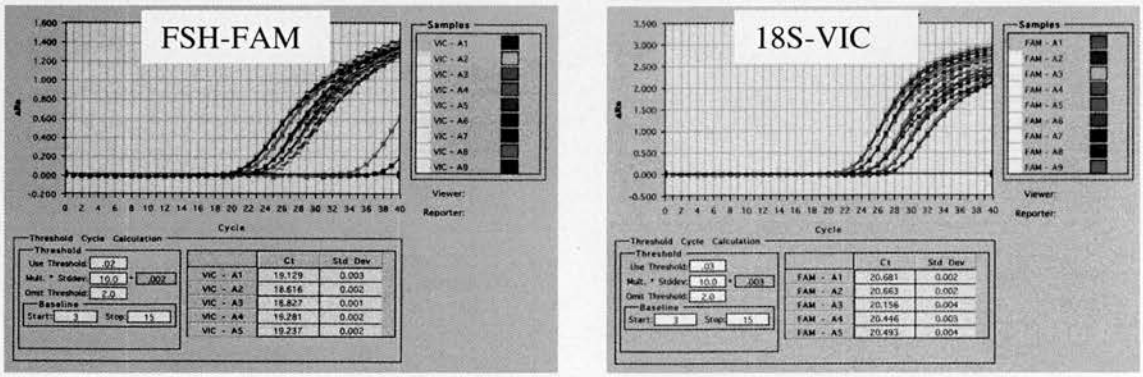


Figure 2.3.9e: Reaction cycles obtained for the validation of LH primers and probes.

2.3.10 Real-Time PCR reaction and analysis

The cDNA was prepared as described previously, with the following modifications. 6μM of the reverse and forward primers, 4μM of the probe and ribosomal 18S forward and reverse primers were added to the mastermix and the PCR annealing temperatures were also adapted appropriately. To measure cDNA expression, duplicate 24μl samples were placed in a PCR plate, a no template control (containing water) was also included in duplicate. Wells were sealed with optical caps and the PCR reaction was carried out using an ABI Prism 7700 (Applied Biosystems). Data were analysed and processed using Sequence Detector version 1.6.3 (Applied Biosystems) as instructed by the manufacturer. Briefly, the software calculates the reaction cycle number at which the fluorescence reaches a determined level for both 18S control and LHβ/FSHβ. By comparing this to an internal positive control, relative expression can be determined. Results are expressed as relative expression to an internal positive standard included in all reactions.

2.4 Radioimmunoassay (RIA)

This assay is based upon competition of ^{125}I-peptide and peptide (either standard or unknown) binding to the limited quantity of antibodies specific for peptide in each reaction mixture. As the quantity of standard or unknown in the reaction increases, the amount of ^{125}I-peptide able to bind to the antibody is decreased. By measuring the amount of ^{125}I-peptide bound as a function of the concentration of peptide in standard reaction mixtures, it is possible to construct a standard curve from which the concentration of peptide in unknown samples can be determined. The antibody-peptide complexes can be detected using a gammacounter.

The concentrations of LH and FSH in rat plasma samples, cell culture media and intracellular protein extracts were measured by RIA using reagents supplied by Dr A Parlow (NIDDK, Torrance, CA, USA), with all samples from each experiment being assayed in duplicate.

2.4.1 Iodination of antigens

Antigens were iodinated in the hotlab, where the local Health and Safety Rules and Radiation Protection Safety were strictly adhered to. All monitoring records and all isotope usage and disposal records were completed after iodination. A pre-iodination sweep check of the bench areas using a Geiger counter was always carried out and any contamination found was reported to the Area Radiation Protection Officer. All contaminated beakers and flasks were soaked in Decon detergent (Sigma, UK) after use. ^{125}I-LH and ^{125}I-FSH antigens were separated from unreacted ^{125}I using a PD 10 Column (Amersham Pharmacia Biotech, UK). Firstly, the PD 10 Column was prepared by cutting off the end of the Column with scissors, which allowed the stabilizing solution to drip out into a flask. The Column was subsequently washed with 2 washes of 5ml 1% BSA/PBS buffer and 1ml of the buffer was loaded above the level of the Column packing. The Column was then clamped off so that it was ready to load with the iodinated preparation. The iodinated preparation was prepared in the hotlab designated fume hood, using the Iodogen method. 500µCi of Na ^{125}I in 5µl was added into the Iodogen tube followed by 100µl of 5µg rat LH (rLH) or rat FSH (rFSH). After vortexing, the reaction was allowed to proceed for 60 seconds when 400µl of 0.1M PBS buffer was added. The reaction mixture was then loaded into the PD10 column and 0.5ml fractions were collected using a Gilson model 201 fraction collector. ^{125}I-rLH or ^{125}I-rFSH were eluted in 5 to 7 ml of PBS buffer. Peak fractions were pooled and stored in a lead-lined container at -20°C.

2.4.2 LH and FSH rat plasma and cell culture media RIA

Unless otherwise stated, 50µl of sample was diluted in 100µl assay buffer (0.05M phosphate buffer pH7.5 with 0.45M NaCl, 0.05% Tween and 0.1% BSA; Sigma Aldrich, UK) using a Microlab automatic pipettor and added to 3ml plastic test tubes. 50µl of primary antibody was subsequently added to each tube, mixed and incubated overnight at 4°C. The appropriate tracer was diluted in assay buffer to give 5000-7000 counts per 20 seconds and was added to each tube and incubated overnight at 4°C. Following addition of the tracer, 50µl of the secondary antisera and 50µl of normal serum were added to the tubes, mixed and incubated overnight at 4°C (Table 2.4.2). Finally, the immunocomplexes were separated by addition of 1ml 0.9% saline, 4% PEG 6000 and 0.2% Triton X-100 and centrifugation at 3000rpm in a Sorvall Omnispin R centrifuge (Dupont, UK) for 30 minutes at 4°C. Supernatants were removed and tubes were left to dry at room temperature. Radioactivity was measured using a 1261 Multigamma counter (Wallac, UK) and data were analysed using AssayZap (P.Taylor, MRC).

AssayZap plots a standard curve using a 4 parameter fit which uses the Bo and NSB values to estimate the upper and lower limits of the curve and then finds the best estimated fit through the standard points. The programme then adjusts this fit by weighting individual points according to how closely they agree with the estimated fit, effectively ignoring outlying points. The programme keeps a record of quality controls and provides a constantly updated measure of the inter-assay coefficient of variation, as well as calculating the intra-assay coefficient of variation for each of the quality controls.

Sample	Buffer	Primary antibody	Tracer	Secondary antibody
Total counts	-	-	50µl	-
NSB	150µl	-	50µl	50µl
Bo	100µl	50µl	50µl	50µl
Standards/Sample	100µl	50µl	50µl	50µl

Table 2.4.2: Summary of the volumes of buffer and primary and secondary antibody used for LH and FSH RIA. The limits of detection for LH and FSH RIA were 0.60ng/ml to 80.0ng/ml, and the inter- and intra-assay coefficients of variation were <10%.

2.4.3 RIA sera and dilutions

The antisera dilutions used for LH and FSH RIA are outlined in Table 2.4.3.

Assay	Primary antisera	Final Dilution primary antisera	Secondary antisera source	Secondary antisera dilutions	
				NRS	DARS
rLH	NIDDK rLH-S-11	1:600,000	SAPU	1:400	1:16
rFSH	NIDDK rFSH-S-11	1:100,000	SAPU	1:400	1:16

Table 2.4.3: Summary of antibody dilutions used for LH and FSH RIA. Antibodies were obtained from the Scottish Antibody Production Unit (SAPU), Carluke, Scotland.

Many of the cell culture plasma samples did not read on the standard curve. LH levels were typically higher than the highest value on the standard curve and FSH levels were typically much lower than the lowest value on the standard curve. Therefore LH cell culture samples were diluted 1:12 for assaying. FSH samples were initially freeze-dried overnight and re-suspended in assay buffer in order to make the samples more concentrated, after which they read on the standard curve. However, since this method was very elaborate and time-consuming a series of dilutions were tried and tested, of which a 3-fold increase in sample concentration was found to lie within the appropriate range of the standard curve.

2.4.4 Marmoset and rat plasma testosterone Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of testosterone in plasma were measured using an enzyme-linked immunosorbent assay (ELISA) adapted from an earlier radioimmunoassay method (Corker and Davidson, 1978). Plasma, to which was added trace amounts of [3H] testosterone (Amersham International, Little Chalfont, Bucks, UK) was extracted twice with 10 vols hexane:ether (4:1, v/v) and the organic phase dried down under N_2 at 55°C. The efficiency of extraction averaged 75%. The second antibody was immobilized to an ELISA plate by addition of 100µl acid-purified donkey anti-goat/sheep IgG (250-350 mg/ml) diluted in 0.1 mol/l sodium carbonate buffer, pH 9.6. The plate was sealed and incubated overnight at 4°C. The wells were then washed twice with 0.1% Tween-20 and incubated for 10 minutes at room temperature with 0.2 ml of the same solution to block non-specific binding sites. Samples, in duplicate, (50µl) were assayed after dilution in 0.1 mol/l PBS, pH 7.4 containing 0.1% gelatin (Sigma,UK) and incubated overnight at 4°C with 50µl sheep anti-testosterone-3-cmo-bovine serum albumin (BSA) diluted 1:100 000 plus 50µl testosterone-3-cmo labelled with 1:20 000 horse-radish peroxidase (Amdex; Amersham Pharmacia Biotech, St Albans, Herts, UK). The plate was washed

several times with 0.1% Tween-20 before addition of 0.2 ml substrate (5 mmol/l *O*-phenylenediamine; Sigma) and 0.03% hydrogen peroxide diluted in 0.1 mol/l citrate/phosphate, pH 5.0 to each well. The plate was then incubated in the dark for 10-30 minutes until the colour reaction was optimal. The reaction was stopped by addition of 50µl 2mol/l sulphuric acid to each well and the optical density then read at 492 nm in a plate reader. The limit of detection was 12pg/ml and the inter- and intra-assay coefficients of variation were <15%.

2.4.5 Marmoset and rat plasma inhibin B ELISA

All incubations (except after addition of amplifier) took place at room temperature in an airtight box, with the Immuno Plate always covered by a plate sealer (Life Technologies, UK). 50µl of antibody in coating buffer (4.24g Na_2CO_3, 4.04g $NaHCO_3$, 1000ml deionised water adjusted to pH 9.6 with hydrochloric acid, Sigma UK) was added to each well of the NUNC Immuno Plate (Life Technologies, UK) and incubated overnight. The excess coating antibody was subsequently removed and the plate was washed with washing solution (302g Tris, 450g NaCl, 25ml Tween, 2000ml deionised water, Sigma UK) five times. 100µl of the standard or sample and 50µl SDS were added to polypropylene microtubes (Sigma,UK) and were left to float in boiling water for 3 minutes. After allowing the tubes to cool for 10 minutes, 50µl of 6% hydrogen peroxide solution was added and incubated for 30 minutes, before adding 100µl of assay buffer (1.21g Tris, 0.9g NaCl, 10g Bovine Serum Albumin (BSA), 5.0ml Triton X-100, 100ml deionised water, Sigma UK). 100µl of sample and standard was added in duplicate to the plate and incubated overnight. The following day the plates were washed 10 times with washing solution before adding 50µl of the secondary antibody coupled to Alkaline Phosphatase (Sigma, UK) for 2 hours. Subsequently the plate was washed again 10 times with washing solution before adding substrate solution for 2 hours. Then 50µl of Amplifier Solution was added and incubated until colour in the top

standard had an optical density reading of 2.0-2.5 (usually 40-45 minutes). Finally 50µl of 0.5M HCl stopping solution was added to stop the reaction and the plate was read on a VICTOR plate reader at 490nm. The limit of detection was 60pg/ml and the inter- and intra-assay coefficients of variation were <15%.

2.5 Immunocytochemistry

In order for an immunocytochemical reaction to be viewed under a microscope, a component of the reaction must carry a label. The first label to be attached to an antibody was a coloured dye but the resulting intensity was too low for visualisation. Nearly all labels that have been used subsequently require additional steps to enhance them to a point of visibility. Fluorescent compounds, the first practical labels, require excitation with light of a specific wavelength to make them emit visible light. Enzymes must react with a substrate and chromogen to produce a visible deposit. Radioactive labels require autoradiographic development. Biotin must itself be labelled or reacted with labelled avidin. Labelling with an enzyme requires an additional large molecule such as glutaraldehyde to cross-link the enzyme to the antibody, unless an antigen-antibody reaction is used. A radioactive label may be conjugated to an antibody via another molecule or through direct labelling, e.g. with ^{125}I as described for LH and FSH RIA (Section 2.4.2). Antibodies are easily labelled with biotin and kits for doing this are readily available commercially.

2.5.1 Tissue fixation

The purpose of fixation is to preserve all components of a tissue sample in their true situation, without diffusion. In addition, the tissue must be protected from osmotic damage (swelling and shrinking). For immunological purposes the antigen to be localised must be made insoluble, yet remain available for reaction with an applied

antibody. Choice of fixative is very important for successful immunostaining and needs to be adapted for the antigen molecule to be localised, taking into consideration the structure of the antigen molecule and the mechanism of action of particular fixatives.

All rat and marmoset tissues were fixed with Bouin's solution. This fixative denatures proteins by destroying the hydrophobic bonds which hold together the tertiary conformation of the protein molecule. The primary and secondary structures are left intact, so the amino acid sequences acting as antigenic sites remain available for their antibodies. Bouin's is an acidic fixative (70% saturated aqueous picric acid, 10% commercial formalin, 5% acetic acid) and therefore fixes rapidly because of the preponderance of the active $^{+}CH_2(OH)$ form of the formaldehyde. Bouin's-fixed material was processed using paraffin, as antigens in paraffin blocks (and in sections stored for a long duration at room temperature) retain their antigenicity indefinitely. Bouin's fixative has been proven to be excellent for preserving the antigenicity of small peptides (Stefanini *et al.,* 1967).

2.5.2 Heat-mediated antigen retrieval

It has been shown (Shi *et al.,*1991) that some antigens previously unreactive in Bouins'-fixed paraffin-embedded tissue could be 'retrieved' by heating sections in a solution of a heavy metal salt in a microwave oven without deleterious effects on the structure of the tissue. Subsequently, it was shown that the rather toxic heavy metal salts could be replaced by simple buffers such as citrate buffer at pH6.0. It was shown that heat, rather than microwaves, is important in the retrieval process, since boiling the sections in a pressure cooker (Norton *et al.,* 1994) or autoclaving them (Bankfalvi *et al.,* 1994) in the buffer solution achieved the same effect. It has been suggested (Morgan *et al.,* 1994) that heating provides the energy not only to rupture the bonds formed by the fixative

with the protein antigen, freeing some antigens, but also to release tissue-bound calcium ions which contribute to tighter bonds with the fixative.

Pressure cooking was only used for antigen retrieval of ERα. Slides were dewaxed and rehydrated before being added to a Tefal Clypso pressure cooker containing 2 L of citrate buffer pH6.0. This was set to pressure setting 2 and brought to the boil, until at full pressure, which was indicated by steady hissing. After 5 minutes the pressure cooker was removed from the heat and the pressure valve was opened to release pressure. When the red pressure indicator valve had dropped, the slides were left to stand in the buffer for 20 minutes. Subsequently cold tap water was added to the pressure cooker to cool the slides. Once slides were sufficiently cooled, they were ready for use in the next phase of immunocytochemistry.

2.5.3 LH and FSH 3,3'-Diaminobenzidine (DAB) immunostaining

DAB immunostaining is one of the most commonly used staining methods (Figure 2.5.3a). It produces a dark brown insoluble precipitate at the site of reaction, using hydrogen peroxide as the substrate and DAB as the chromogen. The tetrahydrochloride of DAB is used as it is more soluble than the free base. This method is one of the most sensitive available and provides a permanent preparation with good contrast. The simplified mechanism of peroxidase reaction with DAB and H_2O_2 is shown in Figure 2.5.3b.

Prior to DAB staining it is important to block all endogenous peroxidase. Peroxidase is an enzyme present in peroxisomes and suppression of its activity is carried out at any stage before application of the peroxidase-linked reagent, usually with excess of the enzyme's substrate, hydrogen peroxide, in methanol.

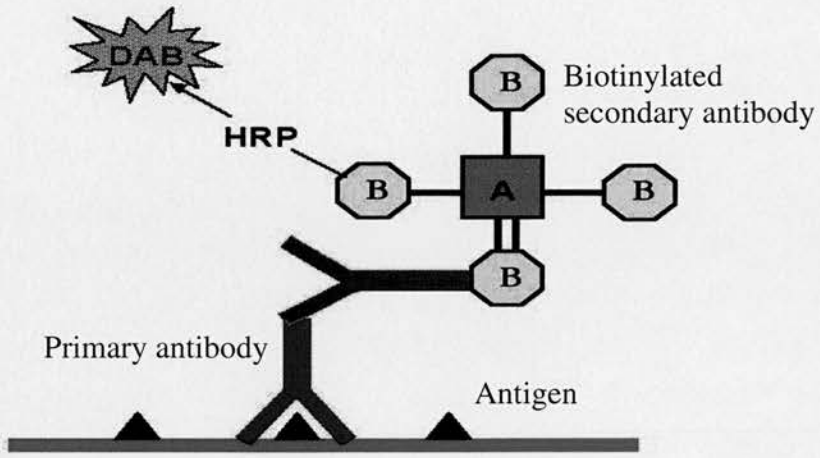


Figure 2.5.3a: Schematic diagram of DAB immunostaining showing the biotinylated antibody (A-B complex) reacting with Horse Radish Peroxidase (HRP) and subsequently forming a brown precipitate by reacting with DAB.

Peroxidase + H_2O_2 (substrate)

↓

Peroxidase/ H_2O_2 complex
(with oxidised haem prosthetic group)

↓

Diaminobenzidine (electron donor)

↓

DAB/peroxidase
(with oxidised haem group)

↓

Oxidised DAB + peroxidase

↓

Polymerised insoluble brown precipitate
(chelates osmium-electron dense product)

Figure 2.5.3b: The simplified mechanism of peroxidase reaction with DAB and H_2O_2, to form a brown coloured precipitate.

Unless otherwise stated, all incubations were performed at room temperature. Sections were cut at 5µm and floated onto slides coated with either 2% 3-aminopropyltriethoxy-silane (Sigma) or poly-lysine (BDH Chemicals, Poole, Dorset, UK) and dried overnight at 50°C. The slides were dewaxed, rehydrated and endogenous peroxidase was blocked using 3% (v/v) hydrogen peroxide in methanol. After washing in water, the sections were washed twice (5 minutes each) in Tris-buffered saline (0.05 mol/l TBS, pH7.4 and 0.85% NaCl). For FSH, slides were then blocked for 30 minutes with normal swine serum (NSS; Diagnostics Scotland, Carluke, UK) diluted 1:5 in TBS containing 5% BSA; for LH, slides were blocked with normal rabbit serum (NRS) diluted 1:5 in TBS with 5% BSA. The primary antibodies were diluted in the appropriate blocking solution (FSH 1:1000; M91 polyclonal anti-human FSH antibody, gift from Dr S Lynch, Birmingham, Crawford *et al.,* 2002) (LH 1:5000; 518B7, monoclonal anti-bovine LH, from Dr J Roser, Department of Animal Science, UCCA, Matteri *et al.,* 1987) and 100µl was added to each slide before incubation at 4°C overnight in a light-proof humidity chamber. The slides were then washed in TBS (2 x 5 minutes) before incubation for 30 minutes with a biotinylated second antibody, namely swine anti-rabbit (Dako, Ely, UK) for FSH or rabbit anti-mouse (Dako,UK) for LH, diluted 1:500 in the appropriate blocking serum. The slides were developed until the colour reached the required intensity in the control sections, and the reaction was then stopped by immersing the slides in distilled water. The slides were counterstained with haematoxylin before being dehydrated by immersion in a graded series of ethanols and then being cleared in xylene. A coverslip was fixed over the sections using Pertex mounting medium (Cell Path, Hemel Hempstead, UK).

2.5.4 LH and FSH dual fluorescent staining

Sections were washed in histoclear for 10 minutes before being hydrated in decreasing concentrations of ethanol and washed thoroughly for 5 minutes in tap water. Then sections were washed in Phosphate Buffered Saline (PBS, Sigma UK) for 2 x 5 minutes before being incubated in blocking solution (1ml Normal Goat Serum, 1ml Normal Swine Serum, 8ml PBS, 0.5g BSA, Sigma UK) for 1 hour at room temperature. The blocking solution was carefully removed with a paper towel and the primary antibodies diluted in blocking solution (LH 1:50, FSH 1:200) were added to each section and incubated at 4°C overnight. The following day, the slides were washed twice in PBS before being incubated with the secondary antibody diluted in PBS (LH 1:20 goat-anti-mouse fluorescein isothiocyanate, (GAM-FITC, Dako); FSH 1:20 swine-anti-rabbit tetramethyl rhodamine isothiocyanate (SAR-TRITC, Dako)), for 2 hours at room temperature under dark conditions. Subsequently, the secondary antibody was removed by washing the slides in PBS for 2 x 5 minutes, before mounting the slides in permafluor (Sigma, UK) ready for viewing under the confocal microscope.

2.5.5 Aromatase or oestrogen receptor α (ERα) DAB staining

Pituitary tissue sections were cleared in histoclear for approximately 10 minutes and hydrated in decreasing concentrations of ethanol and washed briefly in tap water. The sections were then incubated with 3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase. After a wash in water, the slides were transferred into Tris buffered saline (TBS, 0.05M Tris pH 7.4, 0.8M saline) for 5 minutes. The sections were blocked in normal rabbit serum (NRS, Diagnostics Scotland, Carluke, UK) diluted 1:4 in TBS containing 5% bovine serum albumin (BSA, Roche Diagnostics Ltd, Lewes, UK) for 30 minutes at room temperature. The purified IgG fraction of the monoclonal raised aromatase (0.5mg/ml, Turner *et al.,* 2002) was diluted 1:50 in NRS/TBS/BSA,

and the monoclonal mouse ERα (Novocastra, UK) was diluted 1:20 in NRS/TBS/BSA. The sections were incubated with the antibody overnight at 4°C. A rabbit anti-mouse biotinylated secondary antibody (DAKO, Cambridge, UK) was diluted 1:500 in NRS/TBS/BSA and incubated with the tissue sections for 1 hour at room temperature. Bound antibodies were visualised by incubating the sections with ABC-HRP complex (DAKO) followed by incubation with 3,3'-diaminobenzidine (DAB, Sigma). Sections were counterstained with haematoxylin and mounted.

2.5.6 Negative and positive controls

In order to check that the tissue sample was immunostained specifically, a negative control was performed for every batch of samples stained. This was done by substituting the primary antiserum with non-immune serum. A positive control sample, known to contain the antigen in question, was also included every time an immunostain was performed.

2.5.7 Digital photomicroscopy

Tissue sections were examined using an Olympus Provis microscope (Olympus Optical, London, UK) and images were captured using a digital camera, Kodak DCS330 (Eastman Kodak, Rochester, NY, USA). Captured images were stored on a G4 Macintosh (Apple Macintosh, Apple Computer, Cupertino, CA) and montages were compiled using Photoshop 5.0 (Adobe Systems Inc., Mountain View, CA).

2.5.8 Quantification of immunostaining for LH or FSH DAB staining

Quantification of immunopositive cells was done manually by examining immunostained sections on a Zeiss Axioskop light microscope using a x40 objective.

The number of immunopositive cells was determined by placing a 21mm diameter graticule over a randomly selected area of the anterior pituitary. Within the graticule, the number of immunopositive (nucleated) cells (brown) was counted and the total of non-immunopositive cells (blue) was also counted, and the percentage of immunopositive cells was calculated. Only cells with a nucleus visible were counted. This was done for 4 randomly selected areas within the anterior pituitary.

2.5.9 Confocal microscopy for fluorescent staining

Immunofluorescence was analysed using a Zeiss Axiovert 100M microscope and LSM510 scanning module with oil immersion x40, x63 and x100 objectives (Zeiss, UK). Scans were usually performed at 2048x2048 pixels resolution and data was analysed using a Dell Optiplex Gxi computer (Zeiss, UK). Secondary antisera were either conjugated with FITC or TRITC, which allowed antigen binding to be visualised. The excitation and emission spectra for FITC (488nm) and TRITC (535nm) are distinct which allowed co-localisation studies to be carried out. FITC excitation was carried out using an argon laser whereas TRITC excitation was accomplished with a HeliumNeon (HeNe) laser.

2.6 Static primary rat pituitary cell culture

2.6.1 Preparation of anterior pituitaries for culture

Unless otherwise stated, Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM, Dulbecco's Modified Eagle's Medium, Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride and Dulbecco's Phosphate Buffered Saline with calcium chloride and magnesium chloride were heated to 37°C in a hot water bath prior to adding them to the cells. All primary pituitary cells were plated onto

12-well plates coated with Matrigel (Becton Dickinson Labware, Oxford, Oxon, UK) diluted 1:29 in Dulbecco's Modified Eagle's Medium (Sigma, UK). All cell culture was carried out in a Biomat-2 class II microbiological safety cabinet which complies with BS5726-1992 standards and cells were incubated in a 37°C, 5% CO_2 Gallenkamp incubator.

Various methods were tried and tested for preparation of anterior pituitary cell culture. The most successful method is described directly below, other methods tested and some methods frequently used by other laboratories are outlined in Table 2.6.1.

The pituitary cells from the rat anterior pituitaries were mechanically dispersed using a 0.8mm x 40mm syringe needle which fractionated the tissue into smaller cell clumps. These cell clumps were further dispersed by first passing them 2 times through a 10ml syringe with a 0.8mm x 40mm needle attached and then by passing them another 3 times through a 10ml syringe with a 0.6mm x 30mm needle attached to it. Subsequently the cell suspension was centrifuged at 1000g for 15 minutes at 4°C on a Centaur 2 centrifuge. The supernatant was aspirated off to remove all the cell debris. The cell pellet was resuspended in 12 ml of Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (Sigma, UK) supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 U/ml streptomycin and 10ml L-glutamine (Becton Dickinson Labware, Oxford, Oxon, UK). Pellet resuspension was obtained by passing pellet and media four times through a 10ml syringe with a 0.8mm x 40mm needle attached to it. Making sure to mix the cell suspension well, the cells were plated out onto 12-well plates coated with Matrigel (Becton Dickinson Labware, Oxford, Oxon, UK).

Reference	Culture medium	Method of cell dispersion	Cell attachment	Number of cells used
Krsmanovic *et al.*, 2000	DMEM/Ham's F-12 (1:1 vol/vol) with L-glutamate, high glucose (4.5mg/ml) and 10% heat inactivated FCS	Enzymatic, trypsin	Preswollen Cytodex-2 beads (perfusion culture)	2×10^7
Sugihara *et al.*, 1999	DMEM with 0.1% BSA, penicillin (100 units/ml), fungizone (2.5µg/ml) and 10% FCS	Enzymatic, 0.3% collagenase, 0.1% hyaluronidase and DNase 1 (10µg/ml)	Poly-L-lysine coated 24-well plates	2-3 x 10^5 cells/well
Wassen *et al.*, 2000	Calcium- and Magnesium-free Hank's Balanced Salt Solution with 10g/l human serum albumin, penicillin (10^5 U/l), amphotericin B (0.5mg/l) and sodium bicarbonate (0.4g/l)	Enzymatic, dispase (2.4 x 10^3 U/l)	Uncoated 24-well plates (took 2 days for cells to attach)	5 x 10^5 cells/well
Farnworth P, direct communication	DMEM-F12 with L-glutamine, penicillin, streptomycin, 10% FCS	Enzymatic, Trypsin and DNase	Uncoated 48-well plates	-
Martin B	DMEM-F12 with L-glutamine, penicillin, streptomycin, FCS	Mechanical, scalpel blade Enzymatic, varying times (2-20 minutes) and concentrations trypsin/EDTA	Uncoated 12-well and 24-well plates	3-5 x 10^5-10^6 cells/well

Table 2.6.1: Summary of pituitary cell dispersion methods used by other laboratories.

2.6.2 Viable cell counts

Viable primary pituitary cells were assessed using the dye exclusion method, and counted using a haemocytometer (Sigma, UK) after staining with 0.4% Trypan Blue (Sigma,UK). A balanced salt solution was prepared by adding 100µl of the cell suspension to 400µl sterile Dulbecco's Phosphate Buffered Saline with calcium chloride and magnesium chloride (Sigma, UK). Subsequently, 0.5 ml of 0.4% Trypan Blue solution (w/v) was transferred to a test tube, also to which 0.3ml of Dulbecco's Phosphate Buffered Saline with calcium chloride and magnesium chloride (Sigma, UK) and 0.2 ml of the cell suspension was added. The suspension was mixed well and was allowed to stand for 5-10 minutes on ice. With the cover-slip in place, a Pasteur pipette was used to transfer 20µl of the Trypan Blue-cell suspension mixture to one of the chambers on the haemocytometer. The edge of the cover-slip was touched carefully with the pipette tip to allow the chamber to fill by capillary action. The loaded haemocytometer was viewed using an inverted Olympus CK2 microscope (Olympus, Japan). All the cells in the 1mm corner squares were counted. Non-viable cells stained blue, whereas viable cells didn't stain. The number of viable cells was counted. Each square of the haemocytometer represents a total volume of $0.1mm^3$ or 10^{-4} cm^3, since $1cm^3$ is equivalent to approximately 1ml, the subsequent viable cell concentration per ml was determined by the following calculation:

CELLS PER ML = the average viable cell count per square x dilution factor x 10^4

2.6.3 Protein content extraction

Total protein was extracted from the primary pituitary cells using 100mM sodium carbonate. The cell medium was aspirated off the cells and cells were washed once in Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride (Sigma, UK). Subsequently the cells were trypsinized by adding 500µl trypsin (Sigma, UK) to each well of the 12-well plate. The plates were placed in the incubator (37°C, 5% CO_2) for 5 minutes to aid trypsinization. Once the cells were detached from the plate, an equal volume (500µl) of Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Mixture (Sigma, UK) was added to quench the trypsin. The trypsin-media cell suspension was collected from each well and transferred to 2ml Eppendorf tubes. All tubes were centrifuged at 2000rpm for 5 minutes at 4°C in an Eppendorf centrifuge. The supernatant was removed and 100µl sodium carbonate was added to each tube. The samples were left to stabilize at room temperature before putting them through 3 freeze-thaw cycles, by incubating them in a dry ice-ethanol bath for 5 minutes and by then placing them in a 37°C waterbath for 5 minutes. Lysates were then centrifuged at 13000 rpm for 20 minutes at 4°C in an Eppendorf centrifuge to remove cell debris. The supernatant was transferred to new Eppendorf tubes and stored at –20°C.

2.6.4 Protein content quantification

Following Bradford's method (Bradford, 1976) total protein concentrations in cell lysates were determined. A 200-fold dilution of the lysate was made in a total of 1ml sterile water, which was vortexed briefly. 200µl of protein dye (Bio-Rad, UK) was added to the diluted sample and mixed by inverting 5 times. 6 protein standards containing 25, 12.5, 6.25, 3.125, 1.56 and 0.78µg/ml bovine serum albumin (BSA, Sigma, UK) were treated similarly. The optical density (OD) was measured at 595nm after the samples were incubated at room temperature for 5 minutes.

2.6.5 RNA extraction from cells

RNA was extracted from primary pituitary cells (cultured on 12-well plates) using RNAzol-B (AMS Biotechnology, Witney), in accordance with the manufacturer's guidelines. The medium was aspirated off the cells and the cells were washed once with Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride (Sigma, UK). Unless otherwise stated, all reactions were carried out on ice. For efficient RNA extraction, 3 wells were pooled together. Thus, 0.33ml of RNAzol-B was added to each of the 3 wells, making a total of approximately 1ml. The contents of each of the 3 wells was pooled together into one Eppendorf tube. Subsequently 100µl chloroform was added to each Eppendorf and was shaken vigorously for 15 seconds. The samples were centrifuged at 12200rpm for 15 minutes at 4°C in an Eppendorf 5417R centrifuge and the supernatants were removed and transferred to new Eppendorfs. An equal volume (approximately 600µl) of ice-cold isopropanol was added to each Eppendorf, the contents were mixed by inverting tubes twice and were stored at –70°C overnight. Thereafter, the samples were centrifuged at 12200g for 15 minutes at 4°C on an Eppendorf 5417R centrifuge and the supernatants were removed and transferred to new Eppendorfs. 800µl of ice-cold 70% ethanol was added to each tube and vortexed vigorously. The samples were centrifuged on an Eppendorf 5417R centrifuge at 7500g for 8 minutes at 4°C and as much as possible of the ethanol was aspirated off taking care not to dislodge the pellet formed. Any ethanol left in the tubes was left to evaporate off on the bench. 5µl of 0.1% SDS (Sigma,UK) was added to each sample and the pellets were resuspended by vortexing vigorously for at least 2 hours, or until the pellet was completely dissolved. The purity of the RNA preparations was assessed by spectrophotometrical analysis.

2.6.6 Continuous culture of hypothalamic GT1-7 cells

GnRH-expressing GT1-7 cells were grown in monolayers on 75cm^2 cell culture flasks in Dulbecco's Modified Eagle's Medium (Sigma, UK) supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 U/ml streptomycin and 10ml L-glutamine (Becton Dickinson Labware, Oxford, Oxon, UK), maintained at 37°C in an atmosphere of 5% CO_2. They were grown to approximately 60% confluency. The growth medium was removed and replaced every 2-3 days. When cells reached maximum confluency (80%) they were trypsinized as follows. The medium was aspirated off and the cells were washed with Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride (Sigma, UK). Approximately 3ml of x1 trypsin (Sigma, UK) was added to the cells and incubated at 37°C, 5% CO_2 until the cells detached from the plate. The trypsin was quenched with 3ml Dulbecco's Modified Eagle's Medium (Sigma, UK) and the trypsin-cell suspension was aspirated off the plate and transferred to a 10ml sterilin tube. The cells were centrifuged on a Centaur 2 centrifuge at 1500g for 5 minutes at room temperature, the supernatant was removed and the pellet was resuspended in 10ml media. A 1:100 dilution of the cell suspension was made with PBS and the cells were counted on a haemocytometer.

2.6.7 Immunofluorescent staining of primary pituitary cells

Cells were grown on 8-well chamber slides coated with Matrigel before fluorescent staining. The cell culture medium was removed and the cells were washed with PBS containing calcium and magnesium. Subsequently 200µl of ice-cold methanol was added to each well and was incubated at –20°C for 10 minutes. The methanol was aspirated off and the cells were washed twice in PBS containing calcium and magnesium. Then 200µl immunoblock solution, which contained PBS without calcium and magnesium, 1% BSA, 10% FCS, penicillin, streptomycin and 0.2% NP-40, was

added to each well and incubated for 30 minutes at room temperature. The immunoblock-NP-40 solution was then aspirated off and the cells were washed twice with 300µl PBS containing calcium and magnesium. After that 200µl of immunoblock solution without NP-40 was added to each well and incubated for one hour at room temperature. Then the primary antibody (at a dilution of 1:50 for LH and FSH) was added to each well and was incubated overnight at 4°C (Table 2.6.7). After primary antibody incubation, the immunoblock-antibody solution was aspirated off and each well was washed twice with PBS containing calcium and magnesium. Then the desired amount of secondary antibody was added in 200µl of immunoblock to each well and was incubated in dark conditions for one hour at room temperature. Subsequently the secondary antibody-immunoblock solution was aspirated off and each well was washed twice with PBS containing calcium and magnesium. The top of the wells was removed, to reveal a flat microscope slide with the cells attached to it, and placed in PBS containing calcium and magnesium. The slide was removed from PBS and was mounted onto a 22 x 64 mm coverslip with permafluor.

Primary antibody and source	**Primary antibody dilution**	**Secondary antibody and source**	**Secondary antibody dilution**
LH (518B7 monoclonal bovine anti-LH)	1:50	TRITC (DAKO, UK)	1:100
FSH (M91 polyclonal rabbit anti-human FSH)	1:50	FITC (DAKO, UK)	1:100

Table 2.6.7: Summary of the primary and secondary antibody dilutions used for fluorescent staining of primary cultured pituitary cells.

Chapter three: effects of neonatal oestrogen treatment on male rat pituitary function

3.1 Introduction

The primary purpose of this long-term study was to investigate whether neonatal oestrogen disruption, using the potent synthetic oestrogen DES, in male rodents could permanently re-programme pituitary gland function. There is a substantial amount of evidence in the literature that shows that exposure of male rodents to high levels of exogenous oestrogens results in major morphological and functional abnormalities of the HPG axis, including the testis and reproductive tract (Arai *et al.,* 1983; Newbold *et al.,* 1985; Fisher *et al.,* 1998, Khan *et al.,* 1998; Sharpe *et al.,* 1998). Many of these effects have also been shown to have life-long consequences (Khan *et al.,* 1998; Atanassova *et al.,* 2000). Furthermore, studies on the sons of women who were exposed to DES during pregnancy have shown that a similar range of abnormalities can also be induced in human males exposed to high levels of exogenous oestrogens in utero (Stillman, 1982; Toppari *et al.,* 1996). However, at this present time, the exact mechanisms underlying the induction of these reproductive abnormalities are unclear. One mechanism that has been proposed so far has suggested that DES-induced effects on the testis are indirect, and result from suppression of gonadotrophin secretion by the pituitary gland during DES treatment (Brown-Grant *et al.,* 1975; Bellido *et al.,* 1990).

The purpose for performing this study was based on recent findings by Richard Sharpe's laboratory (Atanassova *et al.,* 1999), which showed that neonatal treatment of male rats with DES had permanent effects on the HPG axis in male rats. Atanassova *et al.,* reported that neonatal treatment with 10μg of DES significantly reduced testis weight, germ cell volume, the number of Sertoli cells per testis (but no change in Leydig cells),

plasma inhibin B concentrations and plasma testosterone concentrations and significantly elevated plasma FSH levels in adulthood (Table 3.1) (Atanassova *et al.*, 1999). These effects, although to a much lesser extent, were also obtained for neonatal rats treated with a lower dose (0.1µg) of DES.

Parameter	Effect	Statistical significance
Testis weight (mg)	reduced	$p<0.001$
Germ cell volume/testis (mm^3)	reduced	$p<0.001$
Sertoli cells/testis (10^6)	reduced	$p<0.001$
Plasma FSH (ng/ml)	elevated	$p<0.001$
Plasma inhibin B	reduced	$p<0.001$
Leydig cells/testis (10^6)	no change	-
Plasma LH (ng/ml)	no change	-
Plasma testosterone	reduced	$p<0.001$

Table 3.1: Summary of effects observed by Atanassova *et al.*, on testis and circulating reproductive hormone levels in adult male rats treated neonatally with 10µg of DES. Based on data from Atanassova *et al.*, 1999.

In light of these findings, this *in vivo* DES study was performed to clarify whether a) DES was having potential effects directly at the pituitary gland, or via altered negative feedback from the testes to the pituitary gland, and b) whether any potential DES-induced pituitary effects were transient or permanent.

To address these questions, male rats were injected during neonatal life (days 2,4,6,8,10 and 12) with either a low dose (0.1µg) or a high dose (10µg) of DES. Blood samples were collected on day 26 (early puberty), day 35 (mid puberty) and on day 90 (adulthood), and were assayed for gonadotrophins (LH and FSH) and inhibin B and testosterone. The animals were either killed shortly after the DES was administered, at 18 days of age, or in adulthood, at 90 days of age, and both the pituitary and the testes were collected for immunohistochemical analysis. The pituitaries were stained for LH and FSH and the number of immunopositive cells was counted either at day 18 or at day

90 for both DES treatment groups. The mRNA levels for the gonadotrophins after treatment were measured by Real-Time PCR. Testes data were collected and produced by Richard Sharpe's laboratory in parallel to my pituitary analyses. The testes were weighed, stained for Leydig and Sertoli cells and the number of immunopositive cells was counted for each. This enabled potential effects observed at the level of the pituitary gland to be related to corresponding testes data and vice versa.

3.1.2 Summary of analysis methods

The following approach was taken to investigate the short-term and long-term effects of neonatal DES treatment on male rat pituitary gland function:

- Immunocytochemistry for LH and FSH in the anterior pituitary at day 18 (shortly after DES treatment) and day 90 (adulthood). Enumeration of LH and FSH immunopositive cells at day 18 and day 90.

- Measurement of plasma hormone levels of FSH, LH, inhibin B and testosterone at days 26, 35 and 90.

- Real-Time PCR analysis of mRNA levels of LH and FSH at days 18 and 90.

- Presentation of adult rat testis data, including testis weight, morphology, and number of Leydig and Sertoli cells present per testis.

3.2 Animals and neonatal treatments

Male Wistar rats were bred in the University of Edinburgh MFAA and all-male litters of 8-12 pups were generated by cross-fostering pups on the first days of birth. They were maintained under controlled conditions at 22-23°C ambient temperature, 55-60% relative humidity, 12L:12D cycle, and were supplied with a standard diet (rat and mouse breeding diet; SDS, Dundee, UK) that contains soya meal. Male rats were injected s.c. with either a low dose (0.1µg in 20µl corn oil) of DES (Sigma, UK), or a high dose (10µg in 20µl corn oil) of DES and control rats were injected s.c. with 20µl corn oil only, on days 2,4,6,8,10 and 12 (neonatal period).

Rats were injected on alternating days as DES is long-acting. Animals were anaesthetized with flurothane and blood samples were collected from the tail vein at day 26 (early puberty), 35 (mid puberty) and 90 (adulthood), as shown in Figure 3.2, and were assayed for LH, FSH, inhibin B and testosterone by RIA and ELISA respectively. The methods for these assays can be found in Chapter 2. Rats were killed at day 90 by CO_2 inhalation followed by cervical dislocation. The whole pituitary was removed and the right testis was dissected out and weighed. The pituitaries and testes were subsequently fixed for 5 hours in Bouin's fixative, after which the tissues were transferred into 70% ethanol before being processed for 17.5 hours in an automated TP1050 processor (Leica Corp., Deerfield, IL, USA) and embedded in paraffin wax. Sections of 5µm thickness were cut, floated onto slides coated with 3% 3-aminopropyltriethoxysilane (Sigma, UK), and dried overnight at 50°C before being used for immunohistochemical analysis and cell enumeration.

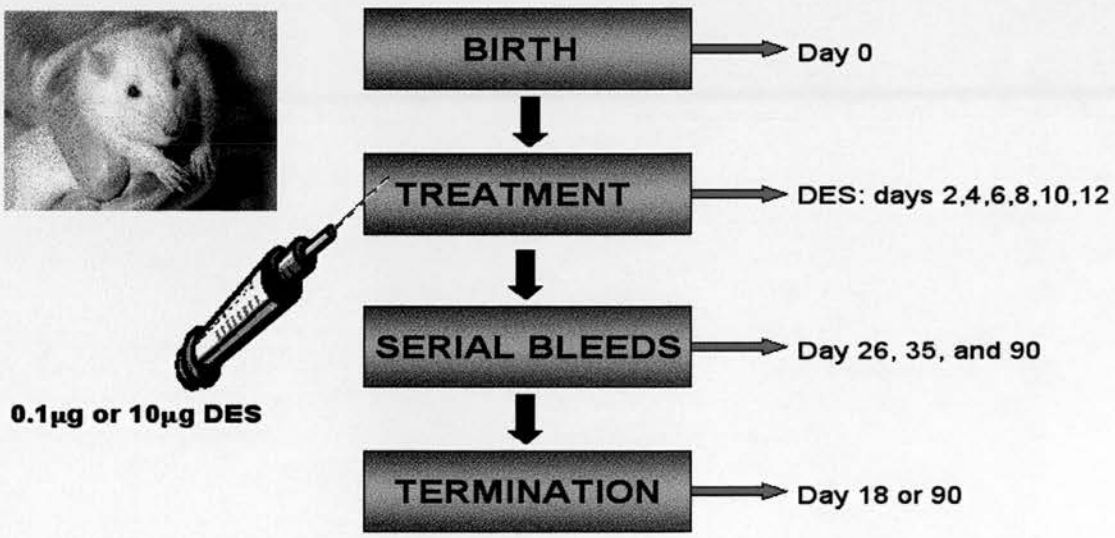


Figure 3.2: Schematic outline for the neonatal DES treatment experiment, showing the neonatal period during which rats were injected with DES and the days on which blood samples were collected.

3.2.1 Specific methods

Pituitary sections were stained for LH and FSH. The method used for immunolocalisation of LH and FSH in the rat pituitary is described in detail in Chapter 2. A brief summary of the antibody dilutions and the methods of detection are summarised in Table 3.2.1.

Primary antibody + source	Dilution	Blocking solution	Secondary antibody + source	Detection method
LH (518B7) Monoclonal mouse raised against bovine (Gift from Dr JF Roser, Univ. of California Davis, USA)	1:5000	Normal Rabbit Serum (NRS, Scottish Antibody Production Unit, Carluke, Scotland) diluted 1:5 in TBS with 5% BSA	Biotinylated rabbit anti-mouse (DAKO,UK) 1:500	Avidin-biotin conjugated horseradish peroxidase (ABC-HRP, DAKO, UK) and diaminobenzidine (DAB, DAKO, UK)
FSH (M94) Polyclonal rabbit antibody raised against human (Gift from Dr S Lynch, Birmingham, UK)	1:1000	Normal Swine Serum (NSS, Scottish Antibody Production Unit, Carluke, Scotland) diluted 1:5 in TBS with 5% BSA	Biotinylated swine anti-rabbit (DAKO,UK) 1:500	Avidin-biotin conjugated horseradish peroxidase (ABC-HRP, DAKO, UK) and diaminobenzidine (DAB, DAKO, UK)

Table 3.2.1: Details of reagents used for immunocytochemistry for LH and FSH.

For the enumeration of LH and FSH immunopositive cells in the anterior pituitary, a 21mm diameter graticule was used, as shown in Figure 3.2.1.

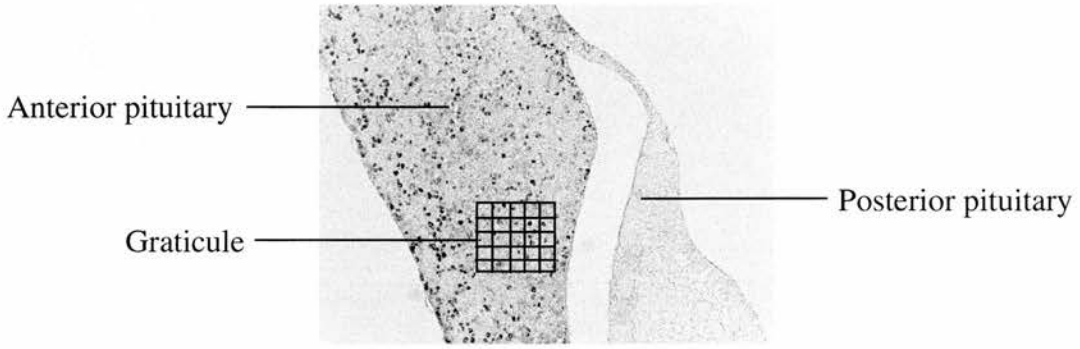


Figure 3.2.1: Method of quantification of gonadotroph cell numbers in the anterior pituitary.

The number of immunopositive cells was determined by placing the 21mm graticule over a randomly selected area in the anterior pituitary. As there was no automated stage available for the microscope, the graticule area was selected at random manually, by moving the graticule to a different area in the anterior pituitary without looking down the microscope. Within the graticule, all the brown immunopositive cells (i.e. LH or FSH immunopositive cells) and all the blue non-immunopositive cells were counted. Only cells with a nucleus visible were counted. This was done for 5 randomly selected areas of the pituitary and every fifth consecutive section of the pituitary gland. The percentage of LH and FSH immunopositive cells and the ratio of LH to FSH cells could then be determined for each rat and the results were plotted in Excel (Microsoft, UK).

3.3.1 Effect of DES treatment on plasma LH levels

Neonatal treatment with DES had significant age-dependent effects on plasma LH levels (Figure 3.3.1). Treatment with either 0.1μg DES ($p=0.0023$) or 10μg DES ($p=0.011$) elevated plasma LH concentrations significantly in adulthood (day 90). LH levels were suppressed slightly for both DES doses at days 26 and 35 of age, however this suppression was small and not statistically significant

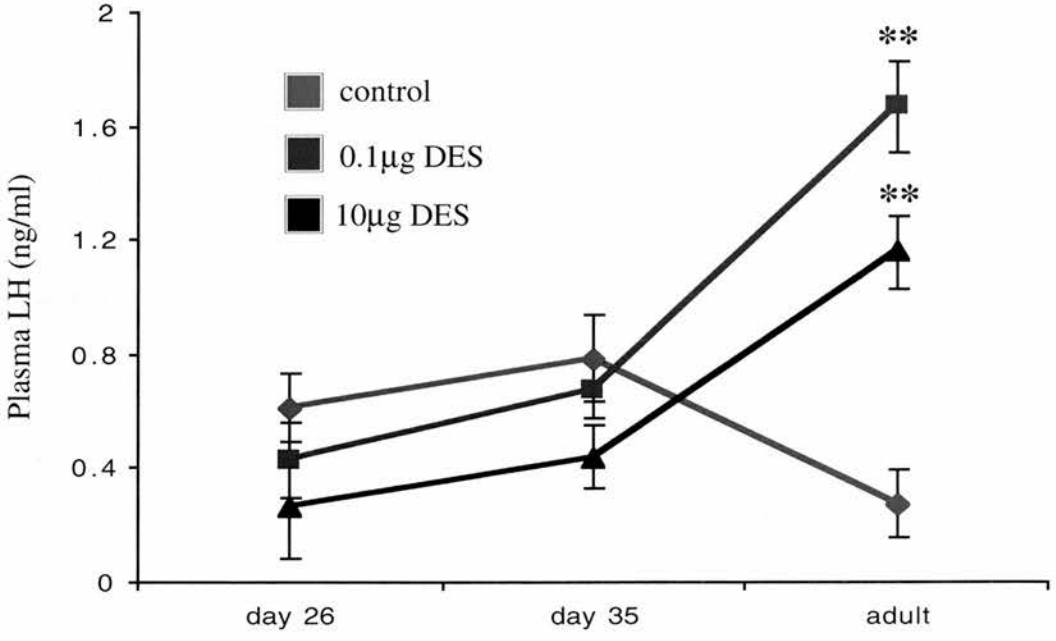


Figure 3.3.1: Plasma concentrations of LH at days 26, 35 and 90 of age, in male rats treated neonatally with either 0.1μg or 10μg DES. Values shown are means ± SEM for 4 rats per group. ** $p<0.01$, in comparison with respective controls.

3.3.2 Effect of DES treatment on plasma FSH levels

Neonatal treatment with DES had significant effects on FSH levels, which were both age- and dose dependent (Figure 3.2.2). Treatment with 10µg, but not with 0.1µg DES, suppressed plasma FSH levels significantly ($p<0.01$) at 26 days of age. Both treatments resulted in elevation of plasma FSH levels at days 35 and 90, though this was only statistically significant for 0.1µg DES at day 90 ($p=0.0415$).

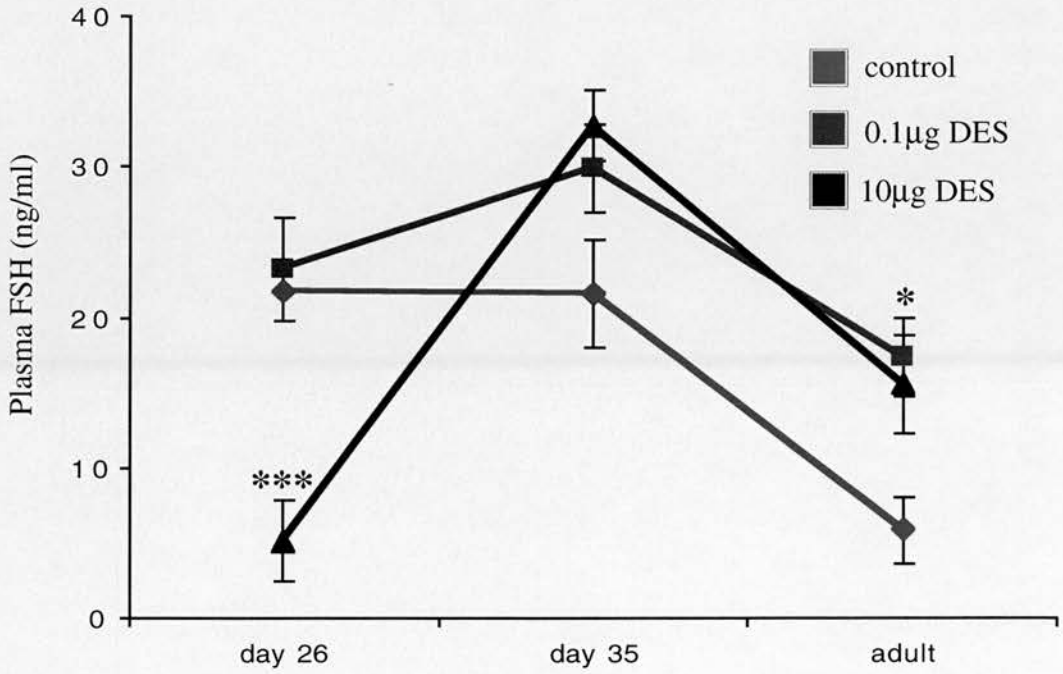


Figure 3.3.2: Plasma FSH concentrations at days 26, 35 and day 90 of age, in male rats treated neonatally with either 0.1µg or 10µg DES. Values shown are means ± SEM for 4-8 rats per group. * $p<0.05$, *** $p<0.01$, in comparison to respective controls.

3.3.3 Effect of DES treatment on plasma testosterone levels

Plasma testosterone levels (Figure 3.3.3) were significantly suppressed in adulthood in a dose-dependent manner (0.1µg DES p=0.025; 10µg DES p=0.0008). As the plasma LH levels at day 90 (Figure 3.3.1) for these animals were significantly elevated, this suggests that neonatal DES treatment could have had an effect on the function of the testicular Leydig cells.

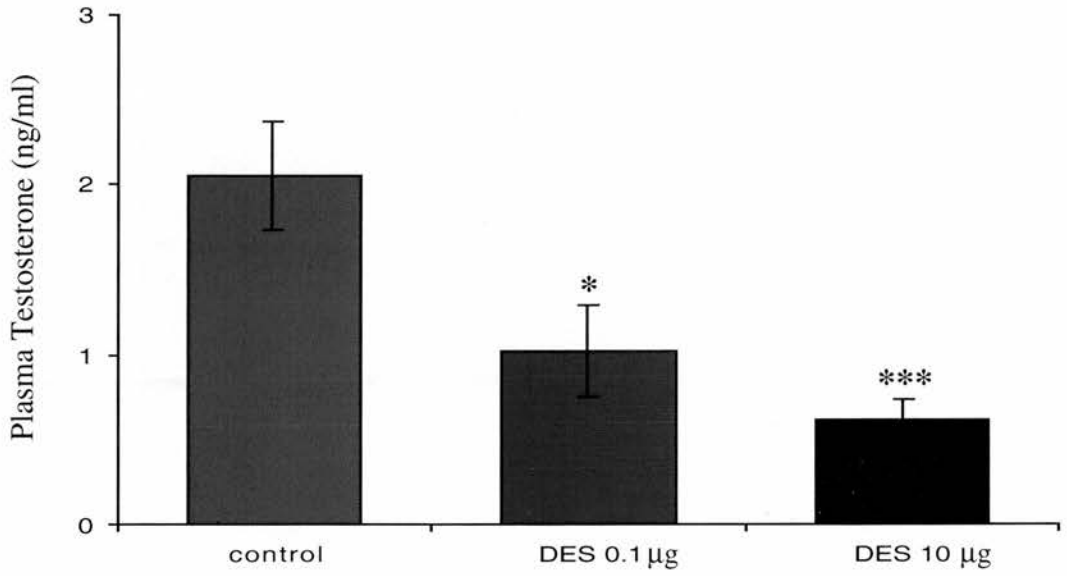


Figure 3.3.3: Plasma testosterone concentrations in adult rats treated neonatally with 0.1µg or 10µg DES. Values are means ± SEM. For control n=8, for 0.1µg DES n=10, for 10µg DES n=8. * $p<0.05$, *** $p<0.001$, in comparison to respective controls.

3.3.4 Effect of DES treatment on plasma inhibin B levels

Plasma inhibin B levels (Figure 3.3.4) were significantly suppressed at day 90 for both 0.1µg (p=0.0062) or 10µg (p=0.05) DES treated rats. This corresponds with the significantly elevated FSH levels (Figure 3.2.2) observed for these animals, as plasma inhibin B negatively regulates plasma FSH levels.

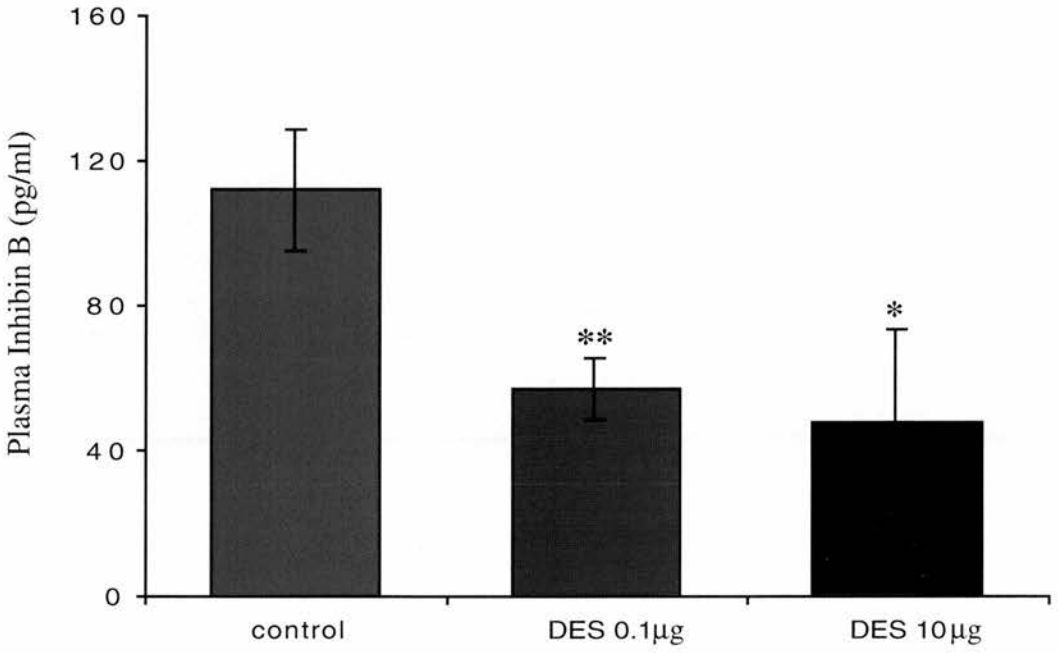


Figure 3.3.4: Plasma inhibin B concentrations in adult rats treated neonatally with 0.1µg or 10µg DES. Values are means ± SEM. For control n=8, for 0.1µg DES n=9, for 10µg DES n=4. * $p<0.05$, ** $p<0.01$, in comparison with respective controls.

3.3.5 Short-term effect of DES treatment on the number of LH and FSH immunopositive cells in the pituitary gland

The number of LH and FSH immunopositive cells in the pituitary gland was counted at 18 days of age, for rats treated neonatally with either the low or high dose of DES (Figure 3.3.5a). There was a significant reduction in the percentage of FSH immunopositive cells ($p=0.181$) for the high dose DES treated rats. There was also a slight reduction in FSH immunopositive cells for the low dose DES treated rats but this reduction was not statistically significant. There was no significant change in the percentage of LH immunostained cells for both doses of DES, though values were consistently lower in the DES treated animals.

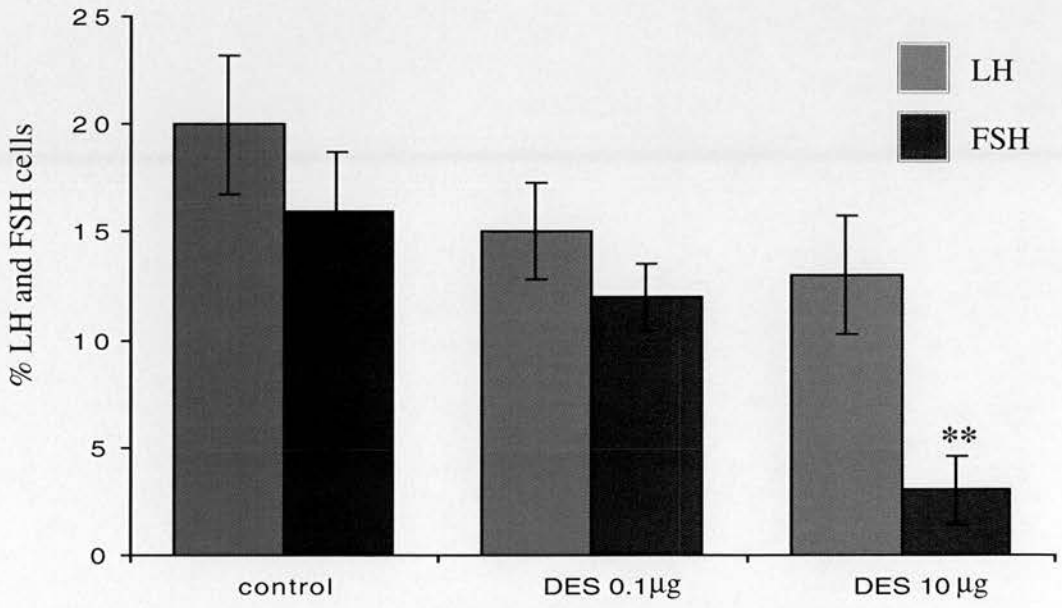


Figure 3.3.5a: Number of LH and FSH immunopositive gonadotrophs at day 18, after neonatal treatment with either the low dose or high dose of DES. Values are means ± SEM. For control n=6, for 0.1µg DES n=4 and for 10µg DES n=4. ** $p<0.01$, in comparison with respective control values.

Figure 3.3.5b demonstrates the significant suppression of FSH immunopositive staining for the high dose DES treated rats at day 18. Interestingly, though LH immunopositive staining was lower than in controls, this was nowhere near as dramatic as for FSH.

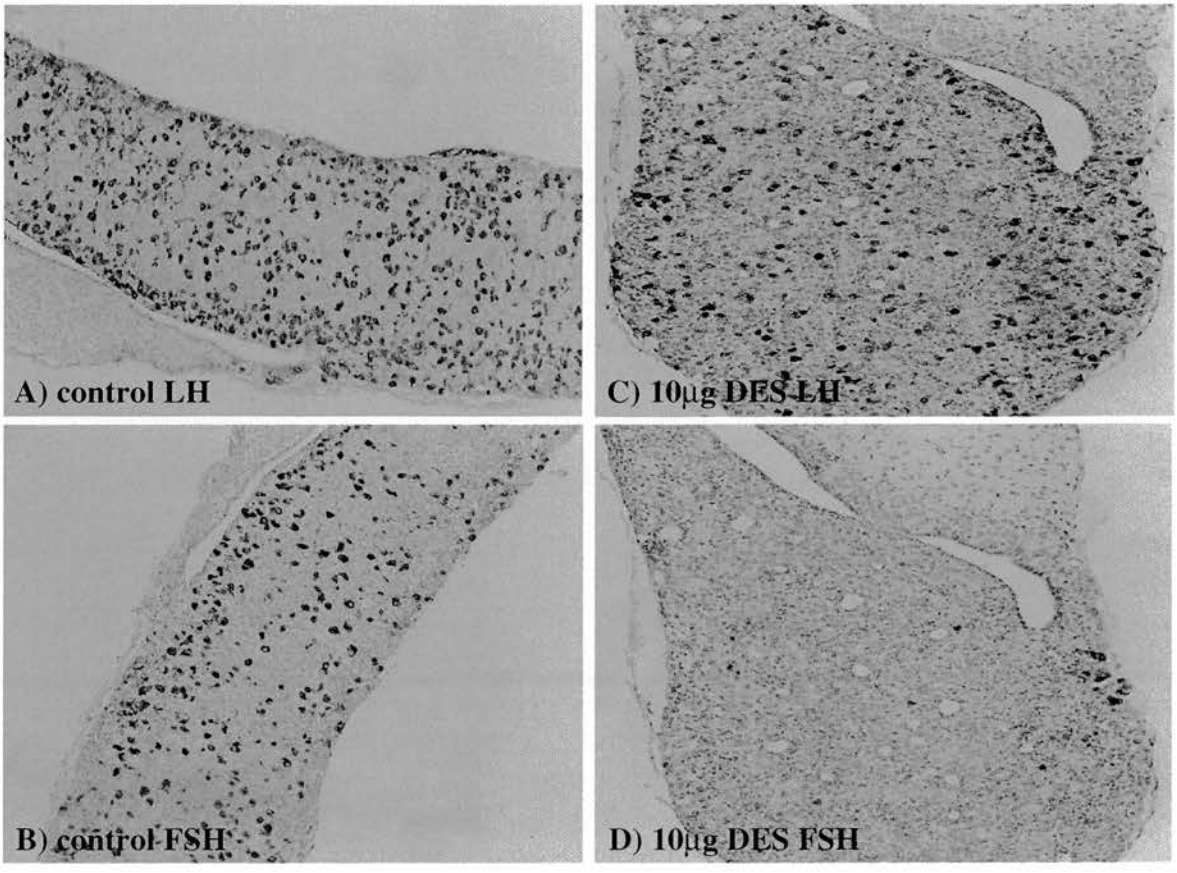


Figure 3.3.5b: Immunocytochemistry for LH and FSH in the anterior pituitary gland of control rats and rats treated neonatally with 10µg (high dose) DES. Note the significant suppression of FSH immunopositive staining at day 18 for the high dose DES treated rats (D) when compared with control (B), whereas a more marginal effect was evident for LH (compare C with A).

3.3.6 Long-term effect of DES treatment on the number of LH and FSH immunopositive cells in the pituitary gland

In adult rats treated neonatally with either the low or high dose of DES, there was no difference in the percentage of LH (0.1µg DES p=0.169, 10µg DES p=0.177) and FSH (0.1µg DES p=0.778, 10µg DES p=0.113) immunopositive cells in the anterior pituitary gland when compared to controls (Figure 3.3.6a). Figure 3.3.6b also demonstrates this by showing the immunostaining for LH and FSH in controls and rats administered the high dose of DES neonatally. Since in adulthood there was no longer any suppression of FSH immunopositive staining (as observed at day 18), this implies that this DES-induced FSH reduction was acute and did not persist to adulthood.

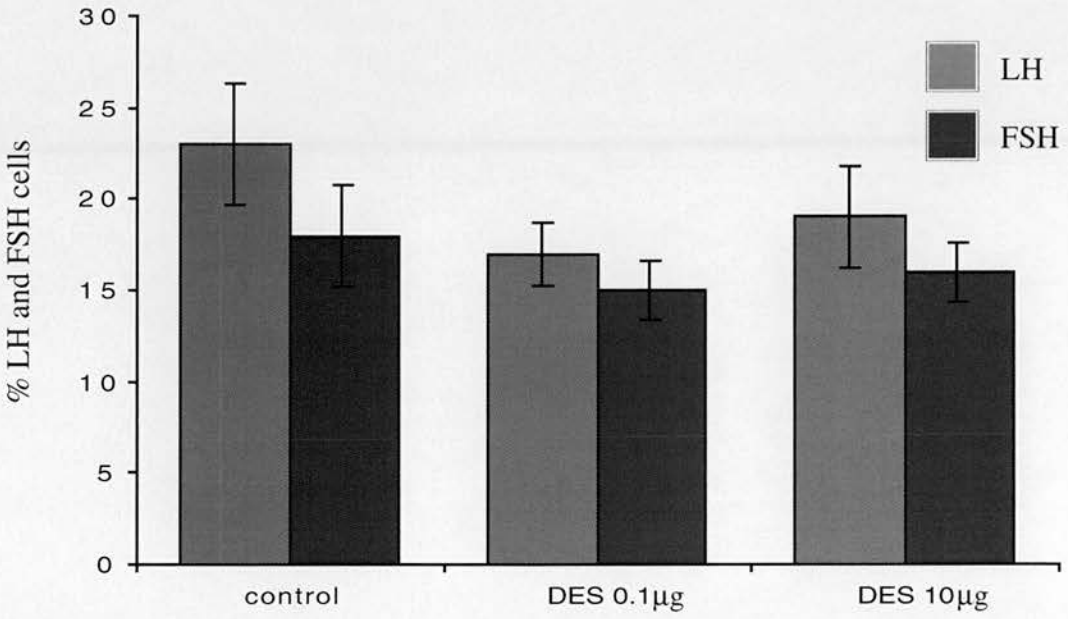


Figure 3.3.6a: Mean percentage of LH and FSH immunopositive cells at day 90, after neonatal treatment with DES. Values are means ± SEM. For control n=3, for low dose DES n=3 and for high dose DES n=3.

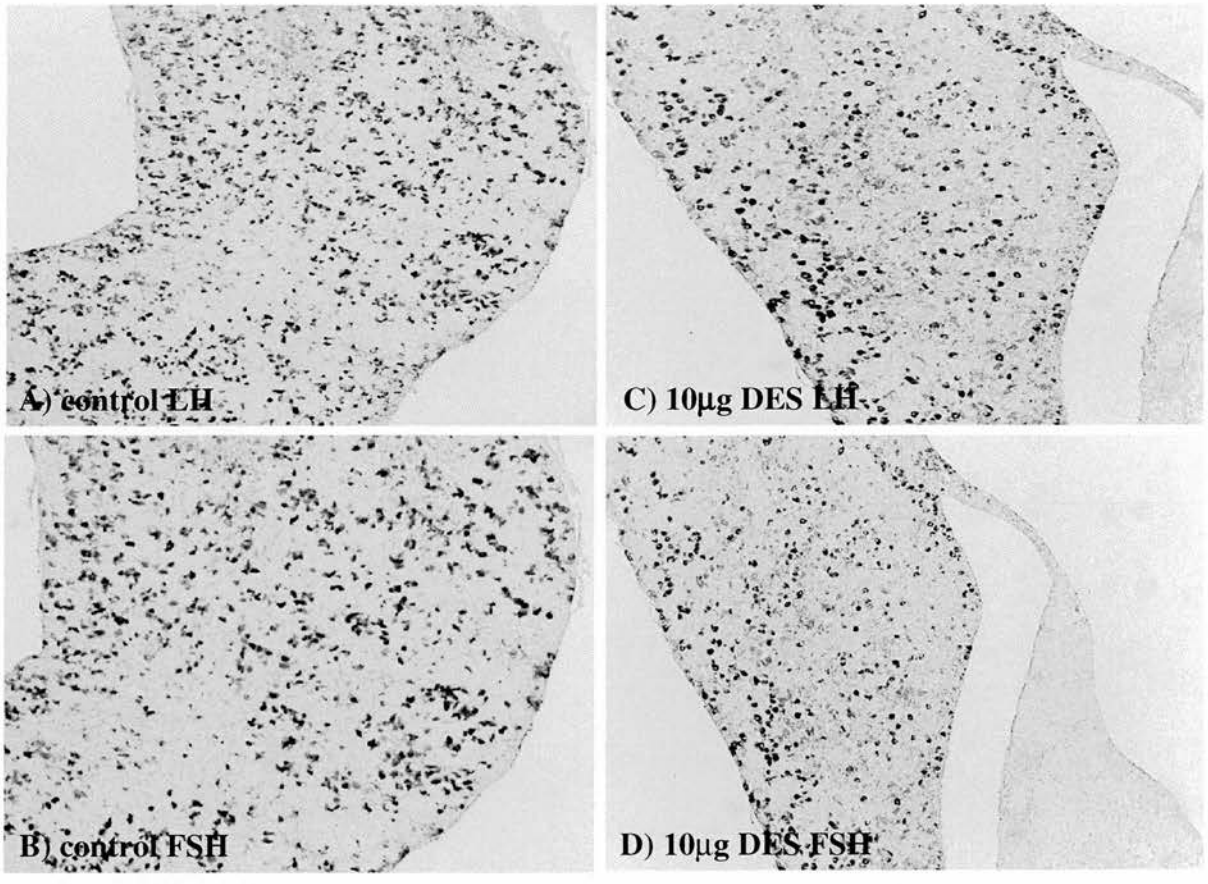


Figure 3.3.6b: Immunocytochemistry for LH and FSH in adult pituitary gonadotrophs for control rats and rats injected neonatally with high dose DES. Note there was no significant difference in immunopositive staining in adulthood (day 90) for both LH (compare A to C) and FSH (compare B to D) for high dose DES neonatally treated animals.

3.3.7 Effect of neonatal DES treatment on LH and FSH mRNA levels at day 18 in the anterior pituitary gland

Real-Time PCR analysis of mRNA levels for LH and FSH in pituitaries from rats aged 18 days revealed a dramatic significant reduction in FSH mRNA levels (p=0.009) in animals treated with 10µg DES, but there was no significant change in LH mRNA levels (Figure 3.3.7a). The reduction in FSH mRNA correlated well with the significant reduction in FSH immunopositive cells observed at day 18 (Figures 3.3.6a and b), and possibly also explains the marked suppression of plasma FSH levels at day 26 (Figure 3.3.2). There was no significant change in either LH or FSH mRNA in rats treated with 0.1µg DES.

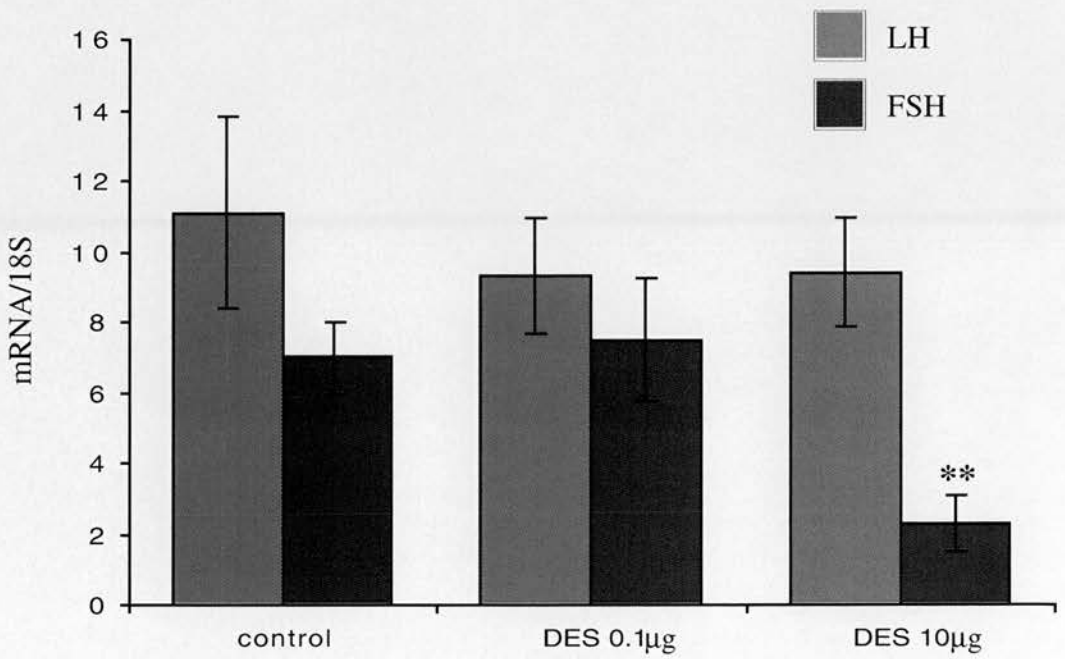


Figure 3.3.7: Messenger RNA levels in 18 day old rats treated neonatally with 0.1µg or 10µg DES. Values are means ± SEM. For control n=4, for low dose DES n=2 and for high dose DES n=2. ** p<0.01, in comparison with respective control values.

3.3.8 Effect of neonatal DES treatment on LH and FSH mRNA levels at day 90 in the anterior pituitary gland

Real-Time PCR analysis of mRNA levels for LH and FSH in pituitaries for rats aged 90 days revealed that there was no significant difference with controls (Figure 3.3.8). This correlates well with the immunostaining for LH and FSH cells observed for these rats (Figures 3.3.6a and b), as there was no significant change in the number of immunopositive cells. This suggests that DES only had a short-term effect on FSH mRNA levels.

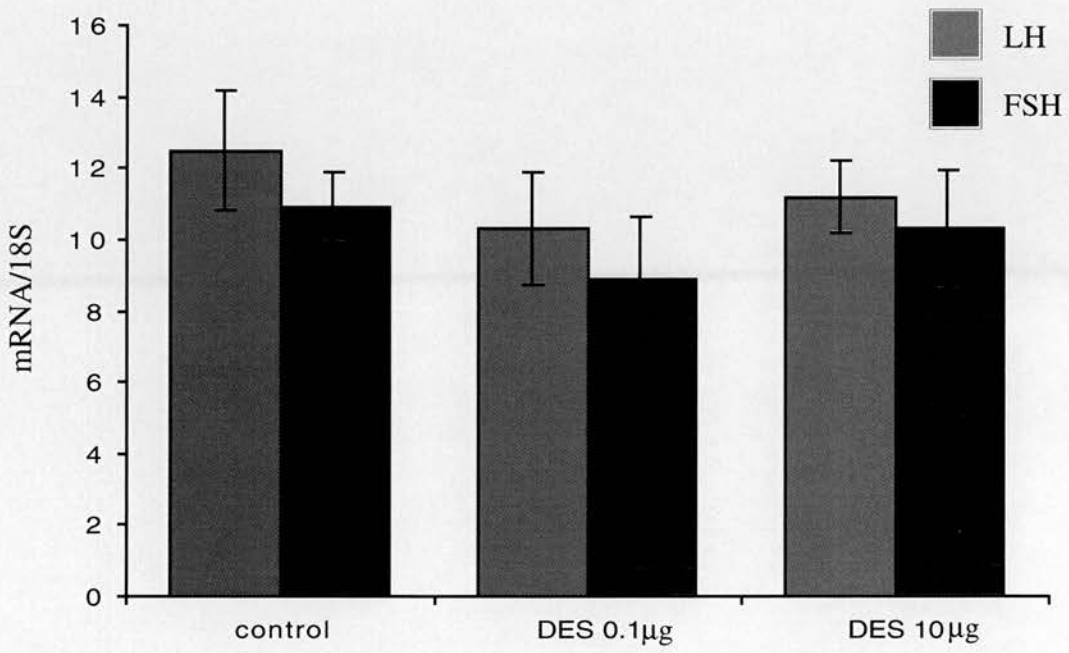


Figure 3.3.8: Messenger RNA levels for 90 day old rats treated neonatally with either a low or high dose of DES. There was no significant difference in LH and FSH mRNA levels for both 0.1µg DES (LH p= 0.786 and FSH p=0.864) and 10µg DES (LH p=0.831 and FSH p=0.612) treated rats. Values shown are the means ± SEM. For control n=8, for low dose DES n=10 and for high dose DES n=6.

3.3.9 Effect of neonatal DES treatment on testicular morphology and function in adulthood

Adult testis weight was dose-dependently reduced in rats treated neonatally with DES (Table 3.3.9). This implies that DES may have permanently altered some or all of the cellular components of the testis, and this has been explored in parallel studies that have been published (Atanassova *et al.*, 1999; Sharpe *et al.*, 2003b). These findings are summarised below to aid interpretation of the present findings on LH, FSH and pituitary function.

Parameter	control	0.1µg DES	10µg DES
Testis weight (mg) *(This study)*	1818 ± 79.6 n=10	1561 ± 43.2 n=17 (p<0.001)	907 ± 98.1 n=11 (p<0.001)
Testis weight (mg) *(Atanassova et al., 1999)*	2000 ± 85	1500 ± 44 (p<0.001)	1000 ± 96 (p<0.001)
Leydig cells/testis (10^6) *(Atanassova et al., 1999)*	32.3 ± 6.6	32.3 ± 5.4	27.0 ± 6.0
Sertoli cells/testis (10^6) *(Atanassova et al., 1999)*	45 ± 7.7	36 ± 6.3 (p<0.01)	28 ± 4.1 (p<0.001)

Table 3.3.9: Summary of testis weight, and Leydig and Sertoli cell number per testis for adult rats treated neonatally with DES. Values are the means ± SEM. For data from Atanassova *et al.*, controls n=12 and DES treated n=5 (Atanassova *et al.*, 1999).

As the testis weights for the DES-treated rats were significantly reduced both for the animals used during my study and for the Atanassova *et al.*, study, it can be assumed that the Leydig and Sertoli cell numbers per testis for my study showed the same pattern; i.e. that there was a significant reduction in the number of Sertoli cells, but no change in the number of Leydig cells per testis.

In adult rats treated neonatally with DES, there was no significant change in Leydig cell number per testis (Table 3.3.9 and Figure 3.3.9). Thus the pronounced reduction in blood testosterone levels reported earlier in this chapter (Figure 3.3.3) was not due to any decrease in the number of Leydig cells in the testis. In contrast, rats treated neonatally with DES exhibited a significant and dose-dependent reduction in the number of Sertoli cells per testis (Table 3.3.9). This change correlated with the reduced concentrations of inhibin B in plasma that were found in similarly treated rats, and suggests that the fall in inhibin B levels was due to a reduction in Sertoli cell number per testis.

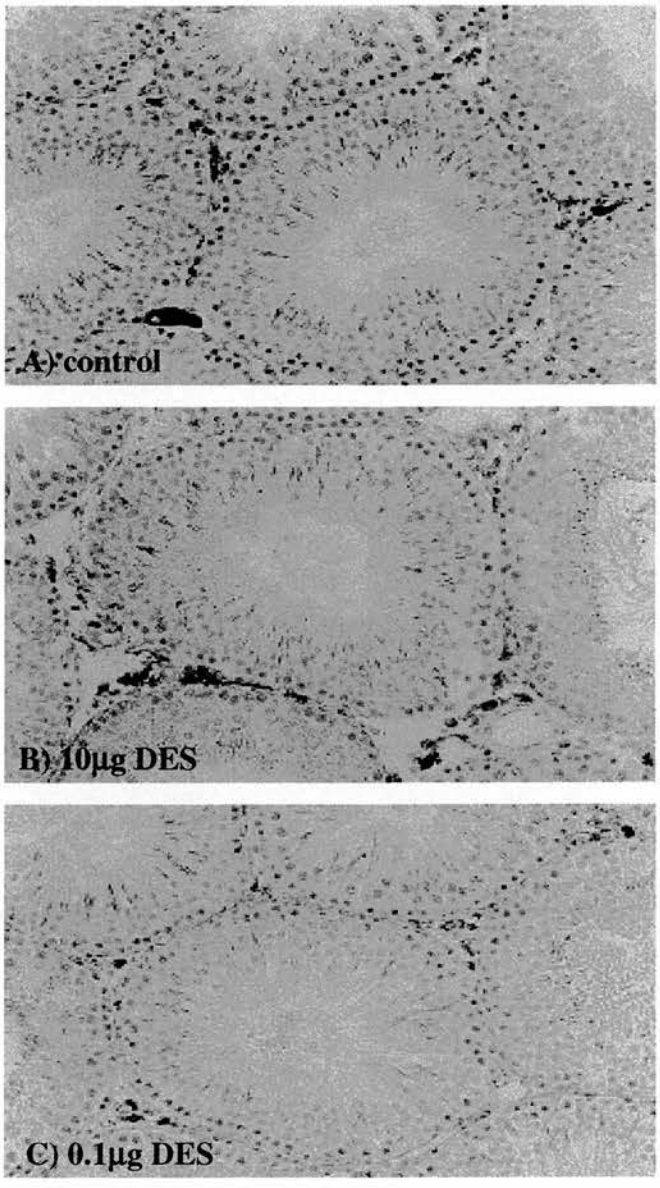


Figure 3.3.9: Immunoexpression of the Leydig cell marker 3β-HSD (brown staining) in the adult testis following neonatal treatment with vehicle (control, A) or with 10µg DES (B) or 0.1µg DES (C). From Sharpe *et al.,* 2003.

3.3.10 Summary of the changes in the hypothalamic-pituitary-gonadal axis observed in adulthood in male rats treated neonatally with DES

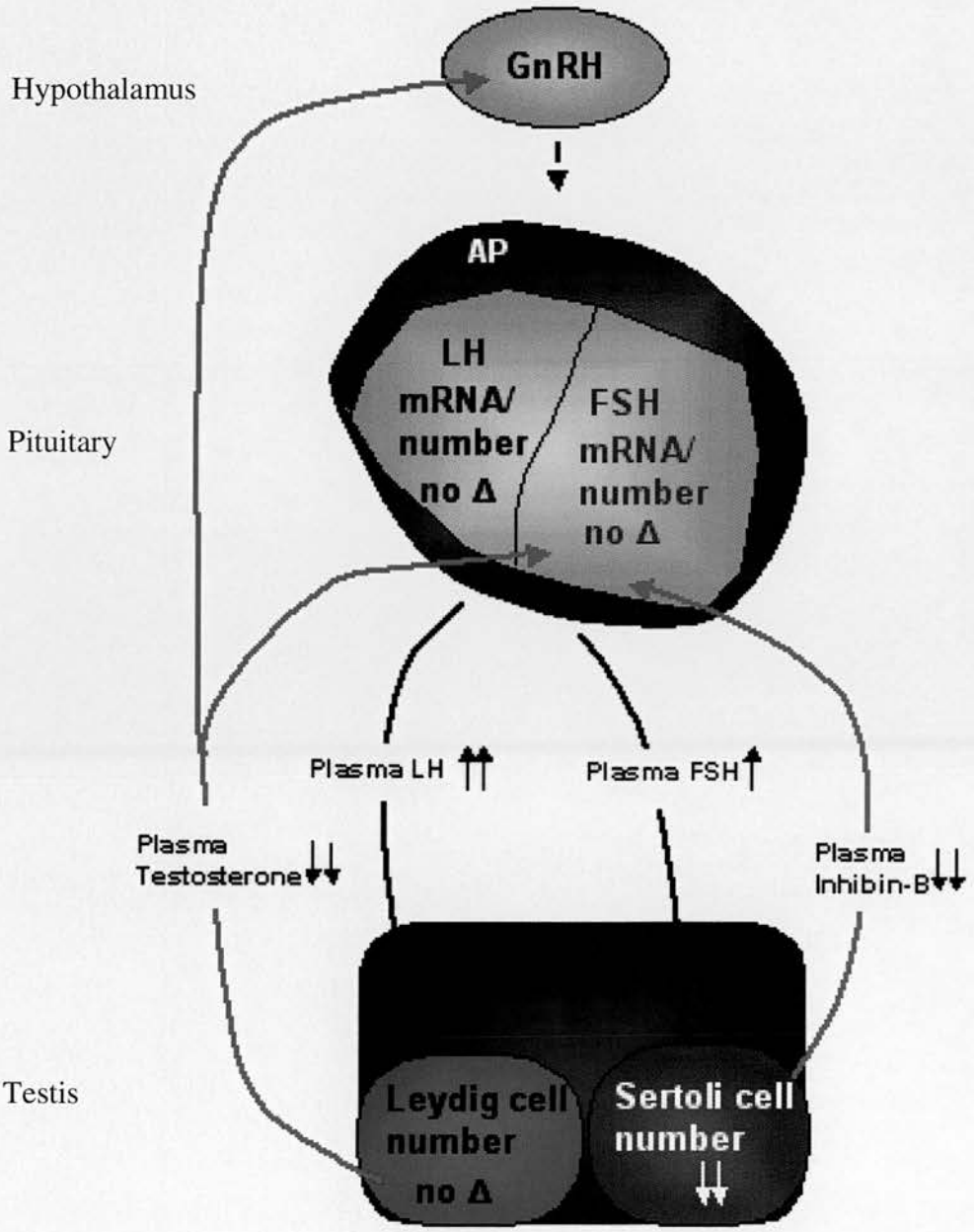


Figure 3.3.10: Summary of DES-induced effects on the function of the rodent HPG axis in adulthood. AP is anterior pituitary and Δ is change.

3.4 Discussion

The primary aim of this long-term study was to determine whether neonatal treatment with the potent oestrogen, DES, would induce changes in pituitary gland function, and whether potential pituitary effects would be long-lasting. There have been numerous reports in the literature showing that neonatal treatment of male rats with high doses of DES can induce a range of developmental abnormalities, including inhibition of Sertoli cell proliferation (Atanassova *et al.,* 1999), suppression of Leydig cell development and function (Sharpe *et al.,* 2003b), and abnormal development of the rete testis (Aceitero *et al.,* 1998; Fisher *et al.,* 1999; McKinnell *et al.,* 2001b), efferent ducts (Fisher *et al.,* 1997, 1999), epididymis (Atanassova *et al.,* 2001; McKinnell *et al.,* 2001b), vas deferens (Atanassova *et al.,* 2001; McKinnell *et al.,* 2001b), seminal vesicles (Williams *et al.,* 2000, 2001) and the prostate (Prins and Birch, 1995). However, in contrast, studies using lower (but still physiologically substantial) doses of DES have failed to induce most of the gross structural changes in the testis, caused by administering a high dose of DES (Atanassova *et al.,* 2001; Rivas *et al.,* 2002). Coincident with the DES induction of these testicular abnormalities, neonatal treatment with high, but not low, doses of DES have also been shown to grossly suppress expression of the androgen receptor in all of the testicular tissues affected adversely by DES treatment (McKinnell *et al.,* 2001b).

However, until now, it was unclear whether neonatal DES treatment in rodents had any direct effects on the morphology and function of the anterior pituitary gland, and more specifically on the pituitary gonadotroph cells. The results from this study showed that shortly after the neonatal DES treatment, at 18 days of age, DES significantly reduced the number of FSH-immunopositive gonadotroph cells ($p<0.01$) in the pituitary. This significant change was only induced by the high dose (10μg) of DES and not by the low dose (0.1μg) of DES. On the other hand, there was found to be no significant change in LH-immunopositive staining at 18 days of age, for both doses of DES. As the vast

majority of gonadotroph cells are bi-hormonal (i.e. contain both LH and FSH) (Liu *et al.,* 1998; Currie and McNeilly, 1995) and there was no significant change in the total number of cells present within the anterior pituitary gland, this suggests that neonatal 10μg DES treatment depleted FSH from the gonadotroph cells, without abolishing the entire gonadotroph. The mRNA analysis of these 18 day old rat pituitaries revealed that there was a significant reduction in FSH mRNA levels for these rats ($p<0.01$), but there was no significant change in LH mRNA levels. Plasma hormone levels measured shortly after the treatment period, at days 26 (early puberty) and 35 (mid puberty) of age, showed that circulating LH levels were slightly suppressed for both DES doses at days 26 and 35, although this was not statistically significant. FSH concentrations on the other hand, were significantly suppressed ($p<0.001$) for the 10μg DES treated animals at 26 days of age. Both DES treatments resulted in an elevation of plasma FSH at 35 days of age, but this change was not statistically significant.

In adulthood, at 90 days of age, there was no longer a significant suppression of FSH-immunopositive staining or suppression of FSH mRNA levels, suggesting that the neonatal DES treatment did not have any significant long-lasting effects on FSH-immunopositive staining or on FSH mRNA levels in the anterior pituitary. However, circulating plasma levels of LH were significantly elevated for both doses of DES ($p<0.01$) at day 90, and both doses caused an elevation in plasma FSH levels, although this was only statistically significant for the 0.1μg DES treated rats ($p<0.05$).

LH acts primarily on the testicular Leydig cells to promote the synthesis of testosterone, and subsequently testosterone exerts negative feedback on hypothalamic GnRH release and on the pituitary (Matsumoto *et al.,* 1984; Shecter *et al.,* 1989). In adulthood, neonatal DES treatment caused a marked reduction in circulating testosterone levels for both doses of DES (0.1μg DES $p<0.05$ and 10μg DES $p<0.001$). Interestingly, there was no significant DES-induced change on the number of Leydig cells present per testis.

This suggests that the DES-induced suppressed testosterone concentrations in adulthood were caused by a lack of the Leydig cells to produce and secrete testosterone, and not due to a reduction in the total number of Leydig cells present per rat testis. Since testosterone exerts negative feedback at the level of the pituitary and hypothalamus, and as testosterone levels were suppressed after DES treatment, this caused altered feedback to both the hypothalamus and pituitary and as a result plasma LH levels were elevated in adulthood ($p<0.01$). FSH on the other hand, acts primarily on testicular Sertoli cells to promote the synthesis of inhibin B, which subsequently negatively regulates FSH production and secretion from the pituitary gland (Jensen *et al.,* 1997; Nachtigall *et al.,* 1996). At day 90, there was a significant increase in circulating FSH levels for both doses of DES treatment ($p<0.05$). Neonatal DES treatment also induced a significant reduction in the number of Sertoli cells per testis (0.1µg DES $p<0.01$ and 10µg DES $p<0.001$) and a significant suppression of plasma inhibin B levels (0.1µg DES $p<0.01$ and 10µg DES $p<0.05$). This suggests that the suppressed inhibin B levels were due to a reduction in the number of Sertoli cells per testis, this resulted in a significant increase in plasma FSH levels, as inhibin B negatively regulates FSH production and secretion from the pituitary gland.

The hormones FSH and testosterone play important roles in spermatogenesis, although in nonseasonal animals testosterone is thought to be the most important regulatory factor (Sharpe, 1994). In addition, the most important factor that determines the ceiling of sperm production and output in all mammals is the number of Sertoli cells per testis (Sharpe, 1994). Sperm production in adult life is dependent upon neonatal Sertoli cell proliferation. The number of Sertoli cells in the adult testis determines both testis size and daily sperm production. This relationship occurs because each Sertoli cell has a fixed capacity for the number of germ cells that it can support (Orth *et al.,* 1988). As the number of Sertoli cells per testis determines the number of spermatozoa produced per day, it is vital that the correct number of Sertoli cells is generated in neonatal life

(Sharpe, 1994). It has been demonstrated that the neonatal FSH concentration is very important as FSH increases the rate of Sertoli cell proliferation (Hess *et al.,* 1993; De Franca *et al.,* 1995; Sharpe *et al.,* 2003a). Thus, as plasma FSH levels were significantly reduced at day 26 for the DES-treated rats, this suggests that this caused a decrease in Sertoli cell proliferation during the neonatal period and hence resulted in there being a lower number of mature Sertoli cells present per testis in adulthood for these treated animals (Figure 3.3). The reduced number of Sertoli cells in adulthood subsequently caused the suppressed circulating inhibin B levels and hence altered negative feedback on the pituitary gland, resulting in permanently elevated concentrations of FSH.

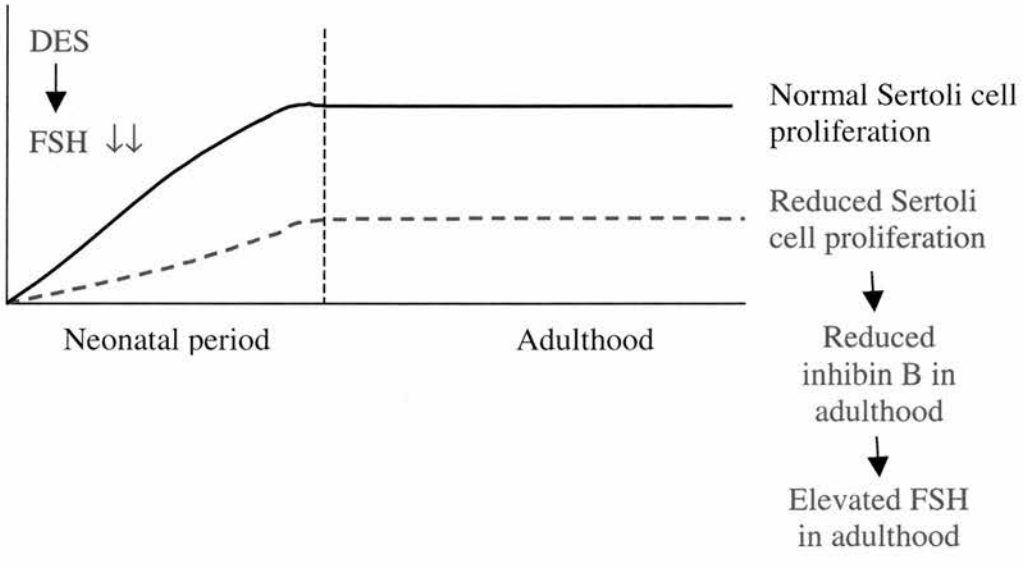


Figure 3.4: Summary of neonatal DES effects on adult Sertoli cell number, and circulating plasma inhibin B and FSH levels.

In conclusion, neonatal DES treatment has been shown to have direct effects on the function of the pituitary gonadotrophs during the neonatal period, by significantly reducing the number of FSH-immunopositive cells and by significantly suppressing FSH mRNA levels and plasma FSH concentrations at day 18. This resulted in reduced proliferation of testicular Sertoli cells during the neonatal period and permanently

lowered the number of Sertoli cells per testis in adulthood, and hence caused a life-long suppression of plasma inhibin B levels and a permanent elevation of plasma FSH levels. In adulthood however, the pituitary gonadotrophs appeared to recover from this neonatal DES insult, as there was no longer a suppression of FSH immunopositive staining and FSH mRNA levels. But, plasma inhibin levels remained suppressed and plasma FSH levels remained elevated in adulthood due to a permanent alteration in Sertoli cell number. DES also appeared to have direct effects on testicular function as neonatal DES treatment caused a permanent reduction in the ability of the Leydig cells to produce and secrete testosterone. To shed more light on DES-induced effects directly at the pituitary gland, in the next chapter an *in vitro* system will be set-up to examine DES-induced pituitary effects without any feedback occurring from the testis.

Chapter four: comparison of effects of neonatal oestrogen treatments on responsiveness of adult male and female rat pituitaries

4.1 Introduction

The experiments described in this chapter are concerned with investigating potential alterations to adult male and female rat pituitary function, both *in vivo* and *in vitro*, after neonatal exposure to the synthetic estrogen, DES. This chapter builds upon results described in the previous chapter, where the short- and long-term *in vivo* effects of DES on the male HPG axis were investigated. In females, neonatal DES exposure has been shown to have a number of significant effects along the HPG axis, including vaginal lesions and clear cell vaginal adenocarcinoma (Herbst, 1981; Mittendorf, 1995; Bern *et al.,* 1987), uterine hypoplasia (Mittendorf, 1995), disruption of ovarian steroidogenesis (Nagai *et al.,* 2003), decreased uterine oestrogen receptor (Medlock *et al.,* 1992), and decreased pituitary responsiveness to GnRH (Register *et al.,* 1995; Faber *et al.,* 1993). Similarly, in males, neonatal DES treatment has also been shown to have significant effects on the HPG axis including, suppression of Leydig cell development and function (Sharpe *et al.,* 2003b), inhibition of Sertoli cell proliferation in early life (Atanassova *et al.,* 1999), abnormal prostate development (Turner *et al.,* 1989; Gupta, 2000), abnormal development of the rete testis (Fisher *et al.,* 1999), alteration of circulating gonadotrophin levels and gonadal hormone levels (Atanassova *et al.,* 1999) and decreased pituitary responsiveness to GnRH (Register *et al.,* 1995; Faber *et al.,* 1993).

This chapter focuses on comparing and contrasting between neonatal DES-induced effects on the adult male and adult female HPG axes. Two major long-term studies are described in this chapter; the first study investigated the effects of neonatal DES treatment on both the adult male and female HPG axes, with particular emphasis on

pituitary gonadotrophins and feedback along the HPG axis. The second study involved setting up a primary pituitary cell culture system from the adult male and female rats treated neonatally with DES. The purpose for this was to investigate to a greater extent any possible DES-induced effects directly at the level of the pituitary, without any feedback occurring from the gonads. Therefore, the neonatally DES treated male and female rats were killed in adulthood and the pituitaries were removed and cultured. The primary pituitary cells were assessed for their phenotypic characteristics and their functional capacity.

Hormones and growth factors produced by the hypothalamus, pituitary and gonads maintain a normal reproductive cycle and regulate genital, gonadal, and sexual development in both males and females. For example, in the female, growth and maturation of follicles and ovulation depend upon endocrine, paracrine, and autocrine acting hormones (de Ketser *et al.,* 2000; Pezzani *et al.,* 2001). FSH and LH are both involved in the activation and regulation of the reproductive axis. Hormones produced by the gonads regulate FSH and LH synthesis and secretion in a feedback loop. One such gonadal hormone that inhibits FSH synthesis by the pituitary is inhibin (Burger *et al.,* 1998; Hayes *et al.,* 1998). In females, the ovary is the major source of inhibin synthesis. Granulosa cells in developing follicles, or lutein cells in the corpus luteum, secrete inhibin in response to gonadotrophins and various other factors (Knight and Glister, 2001). In females, it has been established that inhibin B is secreted from developing preantral and small antral follicles from the beginning of the follicular phase, with a peak following the FSH increase and a progressive fall during the luteal phase (Groome *et al.,* 1996). In males, inhibin B is produced by testicular Sertoli cells and the other gonadal hormone, testosterone, is produced by Leydig cells (Matsumoto *et al.,* 1984; Sharpe, 1994).

For the *in vivo* part of this study, neonatal male and female rats were administered DES from days 2 to 12. The males received either a low dose (0.1μg) or a high dose (10μg)

of DES, whereas the female animals were administered a low dose of DES only (0.1μg), as there were insufficient female animals available for treatment. In adulthood, blood samples were collected and basal levels of the gonadotrophins and gonadal hormones (inhibin B and testosterone for males, inhibin B for females) were measured. Thereafter, to test whether neonatal DES treatment affected pituitary responsiveness to GnRH, the rats were administered a 30 minute GnRH pulse, and gonadotrophin hormone and gonadal hormone levels were measured in response to GnRH.

For the *in vitro* part of this study, the intention was to set up a primary cell culture system using just one pituitary gland, as this would have enabled us to track pituitary responsiveness after DES treatment for each rat individually both *in vivo* and *in vitro*. As there tends to be considerable variability in gonadotrophin levels between animals, this would have reduced that variability considerably. However, it was found that one pituitary gland did not yield a sufficient amount of cells to perform experiments with, and hence rats were randomly paired from each treatment group and two pituitary glands were used for cell culture. As it has been shown that stimulation of the gonadotroph cell line LβT2 or primary pituitary cells with GnRH treatment and/or activin treatment can stimulate the release of gonadotrophin hormones (Turgeon *et al.*, 1996; Nicol *et al.*, 2002; Okada *et al.*, 2003), it was tested whether neonatal DES treatment would alter the responsiveness of the primary pituitary cells to these treatments. GnRH was either administered manually, or by co-culturing primary pituitary cells with hypothalamic GnRH-producing GT1-7 cells, as this has been shown to stimulate gonadotrophin production from primary pituitary cells in culture (Krsmanovic *et al.*, 2000).

Thus, an *in vivo* and an *in vitro* approach was used in both male and female rats to test whether neonatal DES treatment altered pituitary function on a permanent basis, and to investigate whether neonatal DES treatment had differential effects on male and female pituitary function.

4.1.2 Summary of approaches used to investigate effects of neonatal DES treatment on male and female rat pituitary function

In vivo rat study: Investigation into the chronic effects of neonatal DES treatments on the hypothalamic-pituitary-gonadal axis, and in particular pituitary gonadotroph function. In this study the primary goals were:

- Determination of adult (day 90) basal LH, FSH (by RIA), inhibin B and testosterone (for males only) (by ELISA) concentrations.

- Assessment of changes in plasma FSH, LH, inhibin B and testosterone, in response to an acute GnRH dose challenge to adult rats (100ng GnRH for 30 minutes).

In vitro rat study: Investigation into the effects of neonatal DES treatments on adult primary pituitary cell culture responsiveness. This study involved:

- Setting up primary pituitary cell culture and maintaining cells in culture for 7-10 days. Immunofluorescent staining for LH and FSH in adult pituitary cells in culture to investigate whether bi-hormonal nature of gonadotrophs was maintained.

- Measurement of mRNA for LH, FSH, and GnRH I receptor for pituitary cells in culture.

- Assaying basal LH and FSH output from pituitary cells in culture from day 1 to 10.

- Administering a range of GnRH pulses (various doses and time-points) to the pituitary cells in culture and measuring LH and FSH output in response to each GnRH pulse.

- Treatment of pituitary cells with activin, GnRH or GnRH with activin and measurement of LH and FSH secretion in response to these treatments.

- Co-culturing primary pituitary cells with hypothalamic GnRH-secreting cells (GT1-7 cells) and measurement of LH and FSH output from pituitary cells in culture.

- Extraction of pituitary LH and FSH cell content and Western Blot analysis of GnRH I receptor in whole cell lysates in both primary pituitary cells and LβT2 cells (control).

4.2 *In vivo* male and female rat study: long-term effects of neonatal DES treatments on the hypothalamic-pituitary-gonadal axis

In this first *in vivo* study, male rats were injected neonatally with either a low (0.1µg) or a high (10µg) dose of the potent synthetic estrogen, DES. Female rats were injected with just a low dose of DES, as unfortunately insufficient female animals were available to inject also with the high dose of DES. In adulthood, when the rats were approximately 90 days old, any DES-induced effects on the hypothalamic-pituitary-gonadal (HPG) axis were evaluated by measuring plasma levels of gonadotrophins (LH and FSH) and the gonadal hormones (inhibin B and testosterone). Subsequently, any DES-induced effects on pituitary responsiveness to hypothalamic GnRH was tested by measuring gonadotrophin output in response to a 30 minute 100ng GnRH pulse. After these measurements were taken, pituitary glands were removed and were sub-cultured for a number of days to evaluate the effects of these neonatal DES treatments solely on the function of pituitary gland, isolating them from any feedback from the gonads or the hypothalamus.

4.2.1 Animals and neonatal treatments

Neonatal male rats were injected s.c. on days 2 ,4 ,6 ,8 ,10 and 12 with either 0.1µg DES in 20µl corn oil, or 10µg DES in 20µl corn oil. Neonatal female rats were also injected s.c. on days 2,4,6,8,10 and 12, but with just the low dose (0.1µg) of DES. Control neonatal rats were injected with just 20µl corn oil. When these rats reached adulthood (approximately day 90), they were anaesthetized with flurothane and blood samples were collected from the tail vein with a heparinized syringe. Plasma was separated from these blood samples by centrifugation at 3000rpm for 30 minutes at 4°C, before storage at -20°C. Subsequently samples were assayed for LH, FSH, testosterone and inhibin B. Approximately 4 weeks later, these adult animals were injected s.c. with 100µg GnRH

in 200µl sterile Phosphate Buffered Saline (PBS, Sigma UK). Thirty minutes after injection, blood samples were collected from these animals and were assayed for LH, FSH, inhibin B and testosterone. Three weeks after the GnRH injections, the rats were killed by suffocation with CO_2, followed by cervical dislocation after which the pituitaries were removed for primary pituitary cell culture.

4.2.2 Specific methods

Blood plasma levels of LH and FSH were measured by RIA, as described in detail in Chapter 2 of this thesis. Briefly, 50µl of sample was diluted in 100µl of assay buffer (0.05M phosphate buffer pH7.5, 0.45M NaCl, 0.05% Tween, 0.1% BSA; Sigma UK) using an automatic pipettor and added to plastic test tubes. Subsequently, 50µl of primary antibody (NIDDK rLH-S-11 at 1:600 000 final dilution or NIDDK rFSH-S-11 at 1:100 000 final dilution) was added to each tube and incubated overnight at 4°C. LH or FSH tracer diluted in assay buffer to give between 15000-21000 counts per minute (cpm), was added to each tube and incubated overnight at 4°C. Then, 50µl of the secondary antisera and 50µl of normal serum (normal rabbit serum 1:400 and donkey anti-rabbit serum 1:16, Scottish Antibody Production Unit, Carluke, Scotland) were added to the tubes, mixed and incubated overnight at 4°C. Finally, immunocomplexes were separated by addition of 1ml 0.9% saline, 4% PEG 6000 and 0.2% Triton-X (Sigma, UK) and centrifugation at 3000rpm for 30 minutes. Supernatants were removed and tubes were left to dry at room temperature prior to measuring radioactivity using a 1261 Multigamma counter.

Testosterone and inhibin B were measured using ELISA, the methods for this are described in detail in Chapter 2.

4.3.1 Effect of DES on plasma FSH levels before and after a GnRH challenge in adult male rats

Basal FSH levels for male adult rats treated neonatally with either 0.1µg or 10µg of DES were significantly elevated in a dose-dependent manner (Figure 4.3.1a). The high dose DES treated rats showed a more significant elevation (p=0.0039) in adult plasma FSH concentrations than the low dose DES neonatally treated rats (p=0.05). This confirms that DES-induced FSH elevation in adult males is a robust effect as the same observations were made for the male *in vivo* DES study, described in Chapter 3.

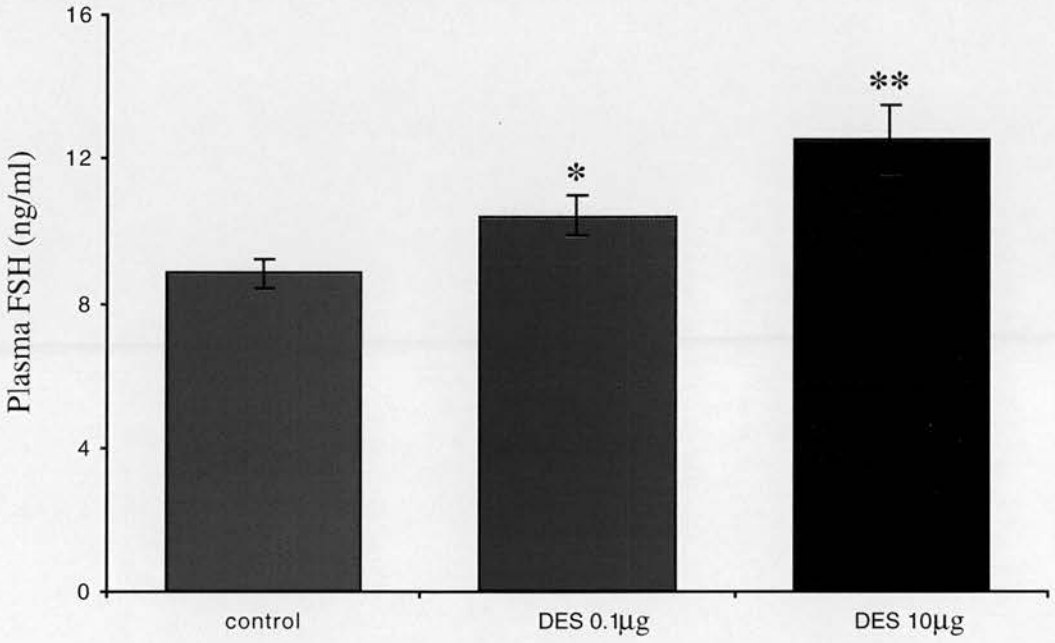


Figure 4.3.1a: Mean basal FSH concentrations for adult male rats treated neonatally with either 0.1µg or 10µg DES. Values are the means ± SEM. For controls n=7, low dose DES n=12, and high dose DES n=8. * $p<0.05$ and ** $p<0.01$, in comparison with respective controls.

When FSH concentrations were measured for these male rats after a 30 minute GnRH challenge was administered (Figure 4.3.1b) there was a very small increase in FSH levels for both male control and treated rats, however this increase was statistically not significant. Since FSH secretion from pituitary gonadotrophs is not under direct control of GnRH, this explains why there is only a nominal, statistically insignificant, increase in adult plasma FSH levels in response to the 30 minute GnRH stimulus. Plasma FSH levels after the GnRH challenge still showed the same basic pattern however, in that FSH levels were elevated for the neonatally DES treated rats, especially for the high dose DES treated rats ($p=0.023$). This again confirms that neonatal DES treatment has potent and long-lasting effects on male adult rat FSH plasma levels, yet does not alter the animal's response to GnRH challenges with respect to circulating FSH levels.

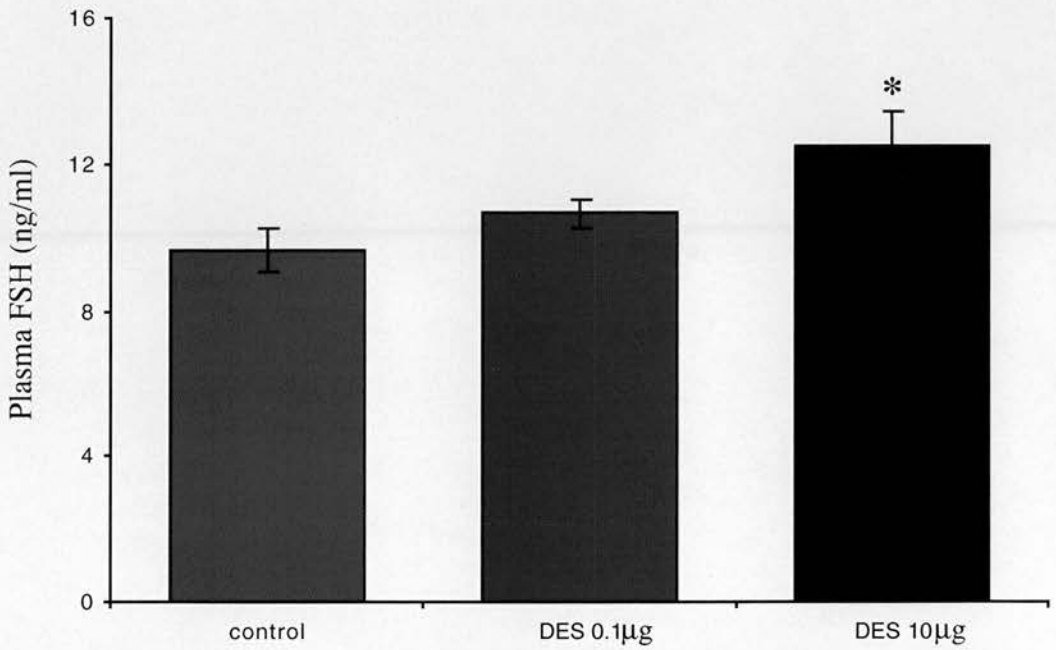


Figure 4.3.1b: Mean FSH concentrations after a GnRH challenge, for adult male rats treated neonatally with DES. Values are the means ± SEM. For controls n=7, low dose DES n=12, and high dose DES n=8. * $p<0.05$, in comparison with respective controls.

4.3.2 Effect of DES on plasma FSH levels before and after a GnRH challenge in adult female rats

There was no significant difference (p=0.4206) in FSH levels between adult control females and adult females treated neonatally with DES (Figure 4.3.2a). As neonatally DES-treated male rats showed a significant elevation in adult FSH levels (Figure 4.3.1a), this suggests that neonatal DES treatment may have differential effects on adult male and adult female gonadotrophin concentrations, with the male FSH levels being more susceptible to alteration by DES treatment than the female FSH levels.

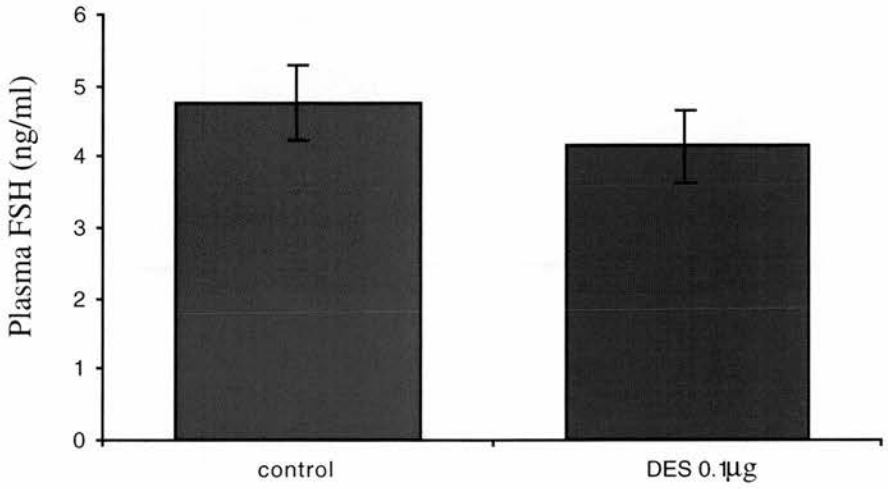


Figure 4.3.2a: Mean basal FSH concentrations in adult female rats treated neonatally with 0.1µg DES. Values are means ± SEM. For controls n=6, low dose DES n=5.

When FSH levels for these adult females were measured after a 30 minute 100ng GnRH pulse (Figure 4.3.2b), there was no significant increase in FSH concentrations in response to the GnRH pulse. This corresponds with the literature reviewed in Chapter 1, and with observations made in the male rat, in that FSH secretion is not under direct control of GnRH and hence no dramatic elevation in FSH was expected in response to GnRH. Plasma FSH levels after the GnRH challenge still showed the same basic pattern however, in that there was no significant change in FSH levels between control females and females treated neonatally with 0.1µg of DES (p=0.1302). However, the mean level of FSH for the DES treated females was generally lower than in control animals, again in contrast to the effects of DES on males. This suggests that DES treatment could have differential effects on male and female plasma FSH levels.

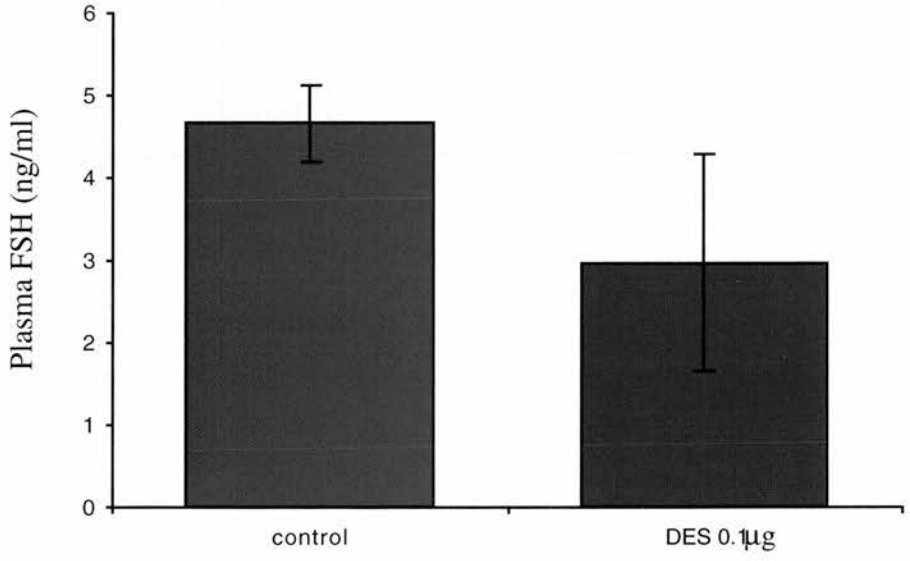


Figure 4.3.2b: Mean FSH concentrations after a 30 minute GnRH pulse in adult female rats treated neonatally with 0.1µg DES. Values are means ± SEM. For controls n=6, low dose DES n=5.

4.3.3 Effect of DES on plasma LH levels before and after a GnRH challenge in adult male rats

Mean basal plasma LH levels for male adult rats treated neonatally with either 0.1µg or 10µg DES (Figure 4.3.3a), were elevated significantly for both doses of DES, with the high dose of DES (p=0.0001) treated rats showing a greater elevation in LH levels than the low dose (p=0.032) treated rats.

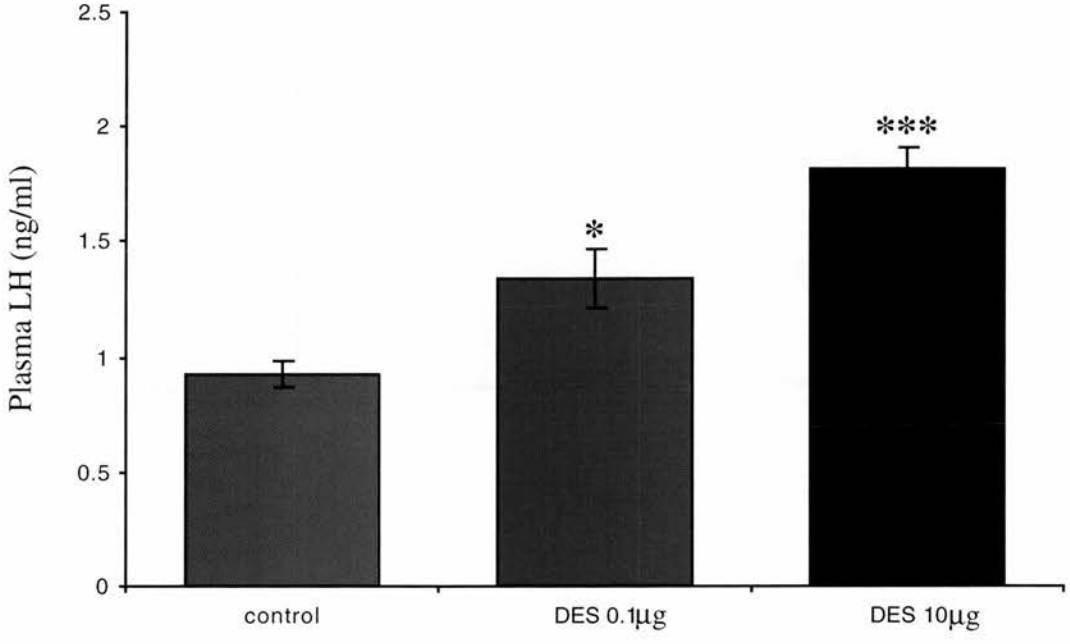


Figure 4.3.3a: Mean basal concentrations of LH for male adult rats treated neonatally with DES. Values are the means ± SEM. For controls n=7, for low dose DES n=12, and for high dose DES n=8. * $p<0.05$ and *** $p<0.001$, in comparison with respective controls.

When LH concentrations were measured for these male rats after a 30 minute GnRH challenge was administered (Figure 4.3.3b), there was a significant increase in LH levels for both male control and DES treated animals ($p<0.001$). This observation corresponds with the literature reviewed in Chapter 1, in that LH production and secretion from pituitary gonadotrophs, in contrast to FSH, is directly regulated by GnRH. Hence, when there is a GnRH pulse, LH will be released from the gonadotroph cells in response to that GnRH pulse. The adult male plasma LH levels after the 30 minute GnRH stimulation showed the same trend as the male basal LH levels, in that LH concentrations were significantly elevated for both 0.1µg ($p=0.0329$) and 10µg DES ($p=0.0001$) treated rats. Thus, the DES treated male animals still retained a normal physiological response to GnRH pulses.

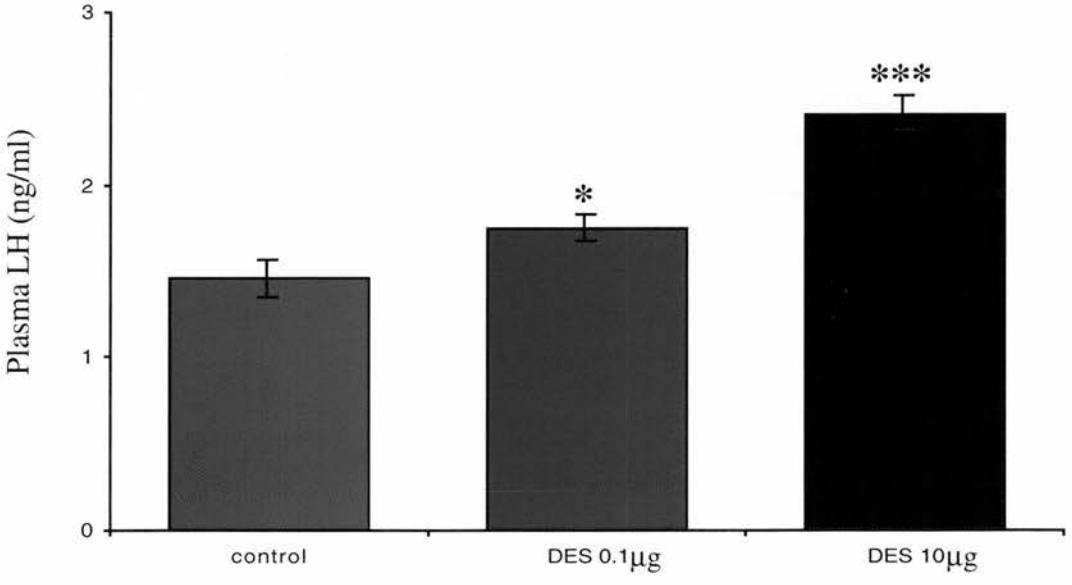


Figure 4.3.3b: Mean concentrations of LH after a GnRH challenge, for adult male rats treated neonatally with DES. Values are the means ± SEM. For controls n=7, for low dose DES n=12, and for high dose DES n=8. * $p<0.05$ and *** $p<0.001$, in comparison with respective controls.

4.3.4 Effect of DES on LH plasma levels before and after a GnRH challenge in adult female rats

There was no significant increase in adult LH levels between control females and females treated neonatally with DES (p=0.800) (Figure 4.3.4a). This is in contrast to what was observed in males (Figure 4.3.3a), as in adult males there was a significant elevation in LH levels after neonatal treatment with DES. Therefore it appears that female gonadotrophin levels are less susceptible to disruption by neonatal DES treatment than male gonadotrophin levels. However, it is important to note the fairly high SEM on the DES treated female levels for LH.

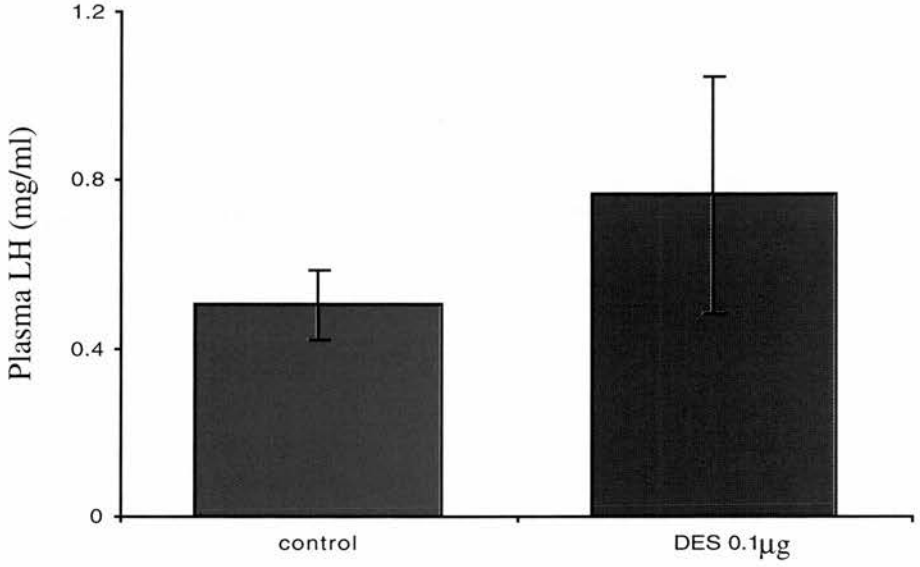


Figure 4.3.4a: Mean basal LH concentrations in adult female rats treated neonatally with 0.1µg DES. Values are means ± SEM. For controls n=6, low dose DES n=5.

When LH concentrations were measured after a 30 minute GnRH pulse for the adult female rats treated neonatally with the low dose of DES, there was a significant increase in LH levels for both the control and DES treated rats ($p=0.008$), as shown in Figure 4.3.4b. This corresponds with the literature reviewed in Chapter 1, in that LH secretion (unlike FSH secretion) is directly controlled by GnRH pulses. Therefore, neonatal DES treatment appears to have not affected LH release in response to the GnRH pulse. Plasma LH levels after the GnRH challenge still showed the same basic pattern however, in that there was no significant change in LH levels between control rats and rats treated neonatally with DES.

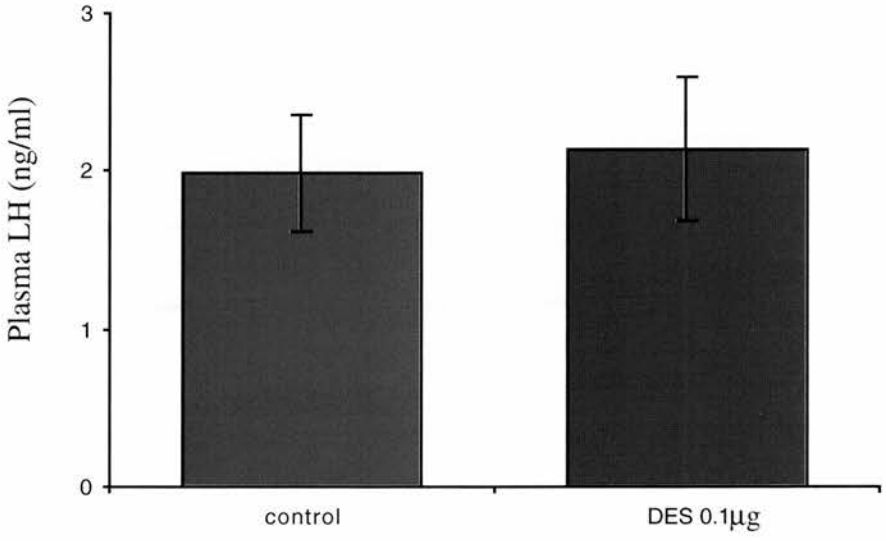


Figure 4.3.4b: Mean LH levels after a 30 minute GnRH pulse for adult female rats treated neonatally with 0.1µg of DES. Values are means ± SEM. For controls n=6, low dose DES n=5.

4.3.5 Summary of the percentage changes in gonadotrophins induced by a GnRH challenge

Treatment	FSH		LH	
	Male	Female	Male	Female
Control	9% ↑	0.6% ↓	67% ↑ p<0.001	296% ↑ p<0.001
0.1µg DES	2.3% ↑	28% ↓	30% ↑ p<0.01	181% ↑ p<0.01
10µg DES	0%	-	32% ↑ p<0.01	-

Table 4.3.5: Summary of the average percentage increases or decreases in LH and FSH concentrations after a 30 minute 100ng GnRH pulse, compared to mean basal LH and FSH concentrations.

In control animals, basal concentrations of FSH and LH were significantly lower in females compared to males, with adult female basal gonadotrophin levels being almost 50% lower than adult male gonadotrophin levels. After a 30 minute GnRH challenge, the male rats showed a 9% elevation in FSH levels and a 67% elevation in LH levels in response to the GnRH dose. This corresponds well with the literature reviewed in Chapter 1, in that LH production and secretion is directly regulated by GnRH, whereas FSH production and secretion is not directly controlled by GnRH. The female rats on the other hand, showed no significant elevation in FSH levels in response to the acute GnRH challenge (a slight 0.6% decrease even), but LH concentrations were elevated significantly in response to the GnRH pulse (296%). This elevation in LH levels in response to the acute GnRH challenge was much larger in the female control rats (296%) than in the male control rats (67%), almost 4-fold higher in females than in males.

In the 0.1μg DES treated male animals, basal concentrations of FSH ($p<0.05$) and LH ($p<0.05$) were significantly elevated compared to male control rats. However, in the 0.1μg DES treated female rats, there was no significant change in basal gonadotrophin levels compared to controls. In females, there was a small increase in basal LH levels compared to controls and a slight decrease in basal FSH levels compared to controls, but these differences were small and not statistically significant. After a 30 minute GnRH challenge, in the male DES treated animals, there was a 2.3% elevation in FSH levels and a 30% elevation in LH levels in response to the acute GnRH pulse. The increases in LH and FSH concentrations in 0.1μg DES treated male animals in response to the GnRH pulse were almost 50% lower than the GnRH-induced increases observed in LH and FSH levels in control male rats. This indicates that neonatal treatment with DES alters the GnRH-responsiveness of these male rats in adulthood, diminishing their ability to respond to GnRH and release LH (and FSH to a small extent) into the circulation. After an acute GnRH challenge in the DES treated female rats, there was a 28% reduction in FSH levels and a 181% elevation in LH levels in response to the GnRH. This, as for males, corresponds with the literature reviewed in Chapter 1, in that production and secretion of LH is directly controlled by GnRH. However, the increase in LH secretion in response to GnRH was much lower in the DES treated females than in the female control animals. Thus, as for the male rats treated with DES, neonatal DES treatment reduced the capacity of GnRH to mediate LH release in adult females.

No comparisons between male and female animals could be made for the 10μg DES treated animals as only male rats were treated with a high dose of DES, due to a shortage in the number of female animals available. Similarly to the 0.1μg treated males, there was a significant elevation in adult basal gonadotrophin levels (LH $p<0.001$; FSH $p<0.01$) for the 10μg DES treated rats. They showed a comparable impaired response to the GnRH challenge as for 0.1μg dose treated rats, in that there was approximately a 50% reduction in LH levels secreted in response to GnRH compared to the control males.

4.3.6 Effect of DES on plasma testosterone levels before and after a GnRH challenge in adult male rats

Basal testosterone levels were suppressed for both the 10μg and 0.1μg DES treated rats, with the lower dose showing the greatest significant reduction in plasma testosterone levels (p=0.0003). There was a relatively large SEM for the 10μg DES treated rats (Figure 4.3.6a), which is probably the reason why adult plasma testosterone levels were reduced but not statistically significantly. Interestingly, these testosterone levels did not show a direct correlation with the LH levels measured for these rats (Figure 4.3.6b). Basal adult LH concentrations were dramatically elevated, thus it would have been expected that testosterone concentrations would have risen accordingly. Therefore, this finding suggests that there has been a definite alteration in the males' HPG axis, which confirms the observations made at the level of the testis in Chapter 3.

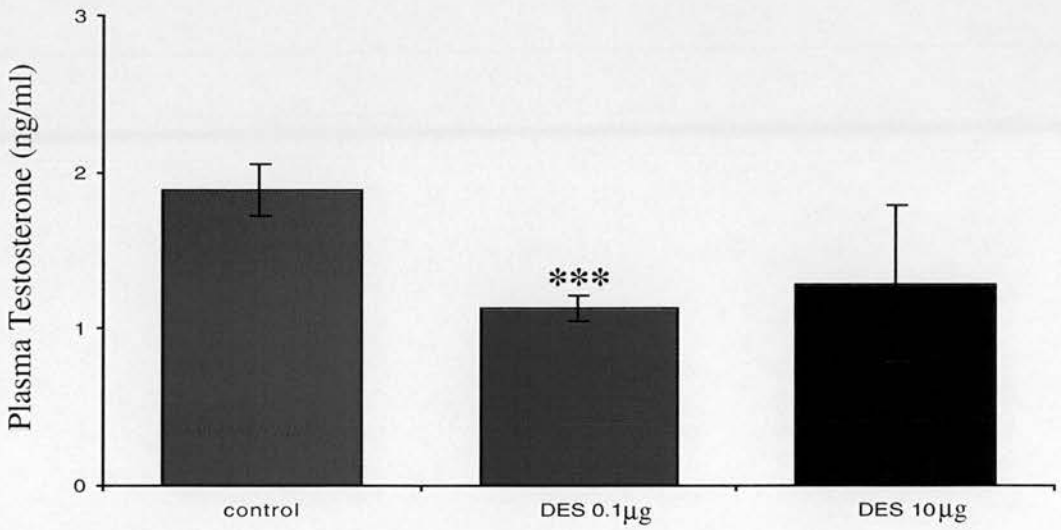


Figure 4.3.6a: Mean basal testosterone levels for adult male rats treated neonatally with DES. Values are means ± SEM. For controls n=7, low dose DES n=12, and high dose DES n=8. *** p<0.001, in comparison with respective controls.

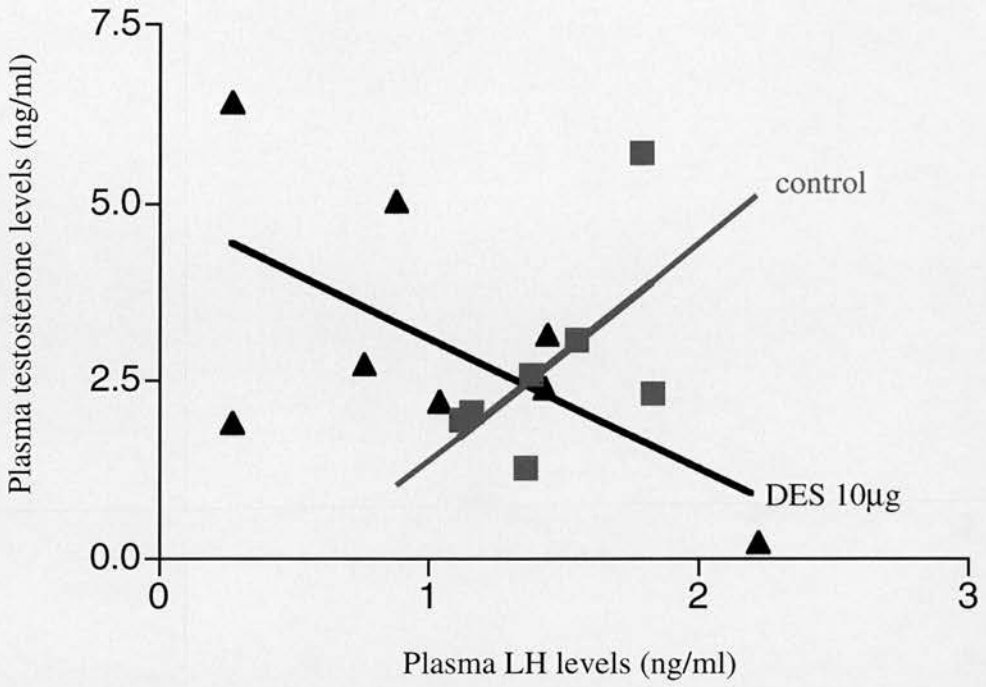


Figure 4.3.6b: Graphical representation of the direct correlation between plasma LH and plasma testosterone levels for adult male control rats, and the inverse relationship between plasma LH and testosterone levels for adult male rats treated neonatally with 10µg DES. For controls n=7 and for 10µg DES n=8.

After a 30 minute stimulation with GnRH, testosterone levels were still suppressed for both the 0.1µg (p=0.526) and the 10µg (p=0.747) DES treated rats, but not statistically significantly (Figure 4.3.6c). However, testosterone concentrations after the 30 minute GnRH pulse were significantly higher than the basal testosterone levels for both the 0.1µg (p=0.0001) and 10µg (p=0.05) DES treated rats. This corresponds with the LH levels measured for these rats after the GnRH pulse (Figure 4.3.3b). LH levels rose in response to the GnRH pulse and testosterone levels have risen accordingly. This suggests that although DES has a long-lasting effect on plasma testosterone levels, it does not appear to affect the general qualitative ability of the testicular Leydig cells to

produce and secrete testosterone in response to hypothalamic GnRH and pituitary LH. However it must be noted that the degree of GnRH-induced testosterone release was lower than that in control animals, thus the DES treated animals could still respond to GnRH yet the magnitude of the testosterone response was not as great as in controls (0.1µg DES $p<0.05$ and 10µg DES $p<0.01$).

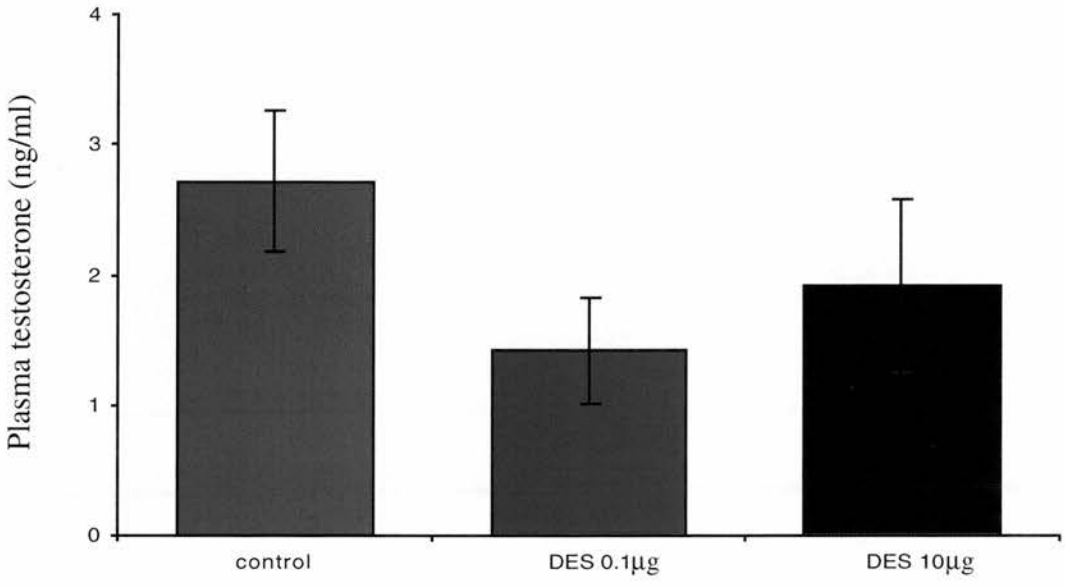


Figure 4.3.6c: Mean testosterone levels after a GnRH challenge for adult male rats treated neonatally DES. Values are means ± SEM. For controls n=7, low dose DES n=12, and high dose DES n=8.

4.3.7 Effect of DES on plasma inhibin B levels before and after a GnRH challenge in adult male rats

Basal inhibin B levels were dramatically suppressed for both the 10μg (p=0.024) and 0.1μg (p=0.0086) DES treated male rats (Figure 4.3.7a). This effect corresponds with the plasma FSH concentrations measured for these animals (Figure 4.3.1a), as these were found to be significantly elevated in adulthood. This suggests that neonatal DES treatment had long-lasting effects on inhibin B levels in male rats.

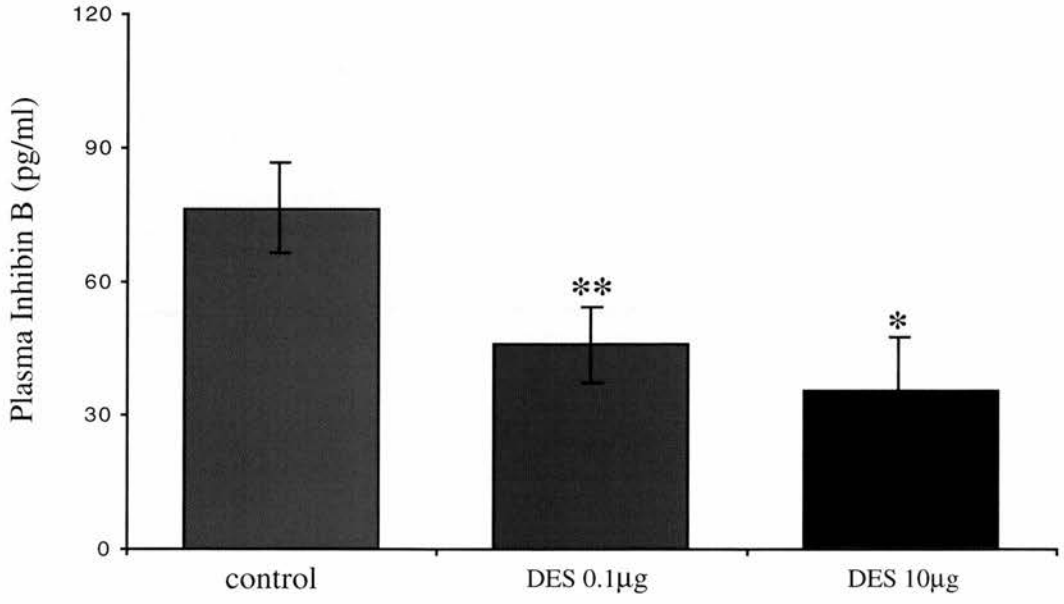


Figure 4.3.7a: Plasma inhibin B concentrations in adult male rats treated neonatally with DES. Values are the means ± SEM. For controls n=7, for low dose DES n=12, and for high dose DES n=8. * $p<0.05$ and ** $p<0.01$, in comparison with respective controls.

After a 30 minute stimulation with GnRH, inhibin B levels were still dramatically and statistically significantly suppressed for both the 0.1µg (p=0.0001) and 10µg (p=0.0001) DES treated rats (Figure 4.3.7b). There was no significant change in inhibin B levels in response to the 30 minute GnRH stimulation.

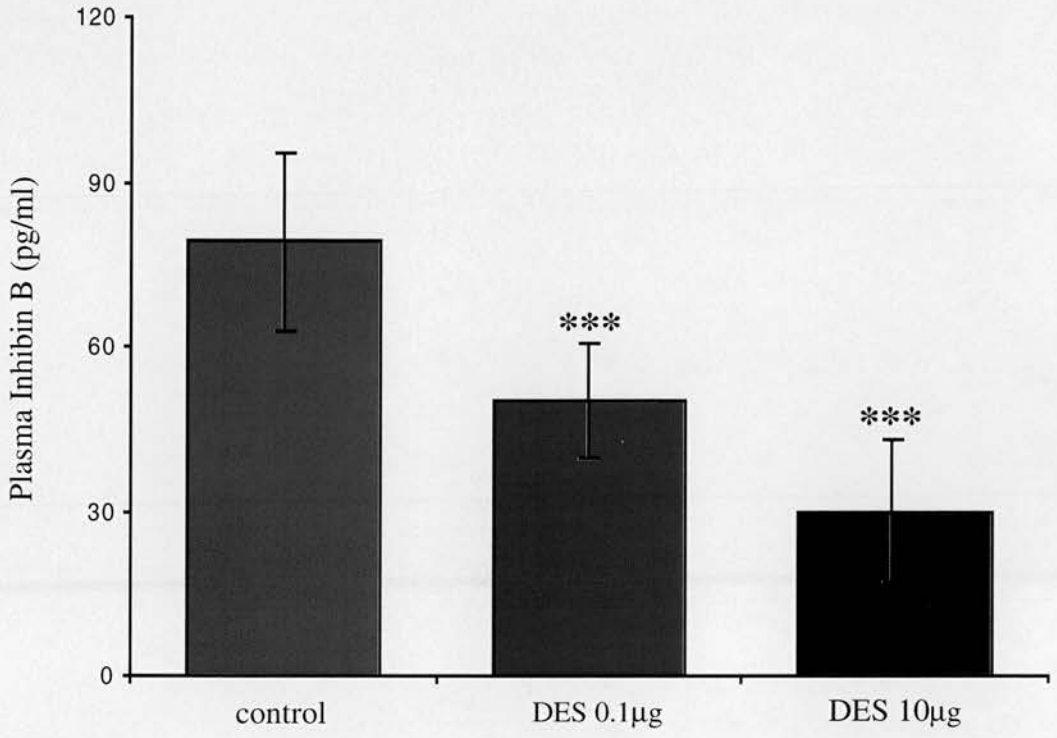


Figure 4.3.7b: Mean inhibin B levels after a GnRH challenge in adult male rats treated neonatally with DES. Values are means ± SEM. For controls n=7, low dose DES n=12, and high dose DES n=8. *** p<0.001, in comparison with respective controls.

4.3.8 Effect of DES on plasma inhibin B levels before and after a GnRH challenge in adult female rats

There was a small increase in inhibin B levels in DES treated female rats (Figure 4.3.8a), however this elevation was small and was not statistically significant (p=0.063). This suggests that neonatal DES treatment had not caused any long-term effects on inhibin B levels in the adult female rat. Surprisingly, this observation is the opposite of the effects observed in male rats (Figure 4.3.7a). In DES treated males, there was a significant suppression in inhibin B levels in adulthood ($p<0.01$ for 0.1µg and $p<0.05$ for 10µg DES). This suggests that male levels of inhibin B are more susceptible to DES-induced alterations than female plasma inhibin B levels.

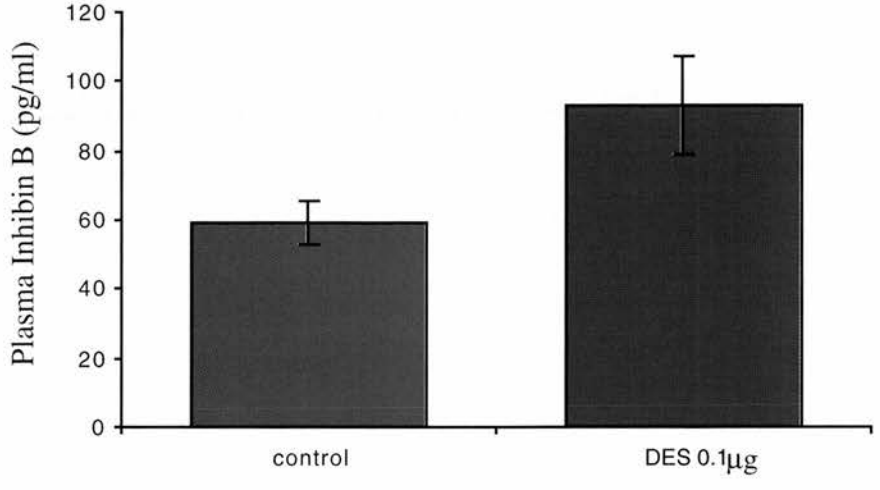


Figure 4.3.8a: Plasma inhibin B concentrations in adult female rats treated neonatally with 0.1µg DES. Values are means ± SEM. For controls n=6, low dose DES n=5.

Inhibin B concentrations were measured after a 30 minute 100ng GnRH pulse (Figure 4.3.8b). There was a significant increase in inhibin B levels for adult control females ($p=0.01$), but there was no change in plasma inhibin B levels in response to GnRH for DES treated females.

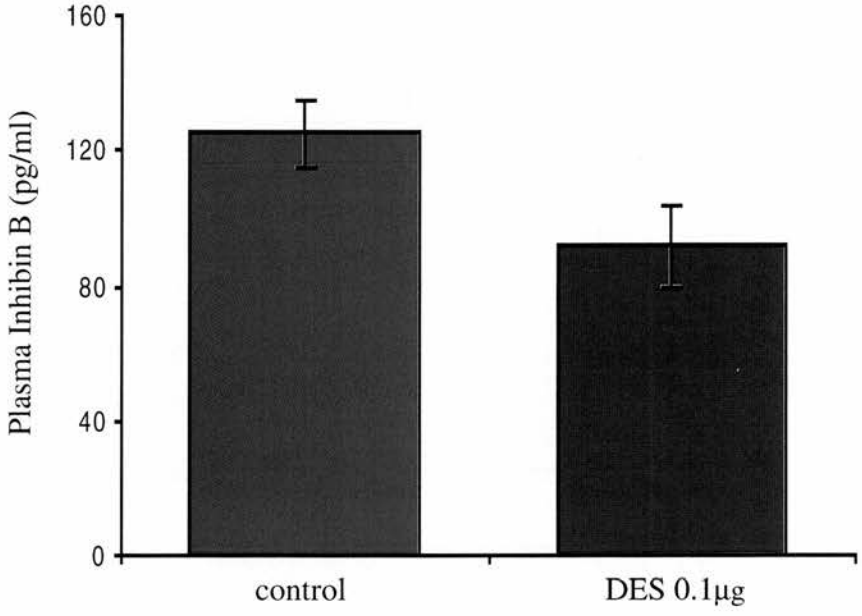


Figure 4.3.8b: Plasma inhibin B concentrations after a 30 minute GnRH challenge in adult female rats treated neonatally with 0.1µg DES. Values are means ± SEM. For controls n=6, low dose DES n=5.

4.3.9 Summary of the percentage changes in plasma testosterone and inhibin B levels induced by a GnRH challenge

Treatment	Inhibin B		Testosterone
	Male	Female	Male
Control	1% ↑	112% ↑ p<0.001	45% ↑ p<0.01
0.1µg DES	9% ↑	1.3% ↓	25% ↑ p<0.05
10µg DES	2% ↓	-	48% ↑ p<0.01

Table 4.3.9: Summary of the average percentage increases or decreases in inhibin B and testosterone concentrations after a 30 minute 100ng GnRH pulse, compared to mean basal inhibin B and testosterone concentrations.

In adult control animals, basal inhibin B levels were higher for control male rats than control female rats. After a 30 minute GnRH challenge, control female rats showed a much greater GnRH-induced increase in plasma inhibin B than control males. Control females showed a 112% elevation in inhibin B levels in response GnRH whereas the control males only showed a 1% elevation in inhibin B levels in response to the GnRH challenge.

In the 0.1µg DES treated males, basal concentrations of inhibin B ($p=0.032$) and testosterone ($p=0.0086$) were significantly suppressed compared to male control rats. The female 0.1µg DES treated rats on the other hand, showed no suppression of plasma inhibin B. On the contrary, inhibin B levels were elevated slightly in the low dose treated female rats, however these increases were only small and not statistically significant. This suggests that neonatal DES treatment could have differential effects on circulating levels of inhibin B in males and females. After a GnRH challenge, there was

a 1.3% decrease in plasma inhibin B levels for the DES treated females in response to GnRH, compared to control females where there was a 112% GnRH-induced elevation in plasma inhibin B levels. In male DES treated animals however there was a 9% elevation in inhibin B levels after a GnRH pulse, compared to only a 1% increase in GnRH-induced inhibin B release in control males. This suggests that DES treatment impairs the capacity of GnRH to mediate the release of inhibin B in female rats. Testosterone levels after a GnRH pulse were elevated by 25% for the DES treated males, compared to a 45% elevation for control male rats. This suggests that neonatal DES treatment in male rats caused a significant reduction in the rats' capacity to release testosterone in response to GnRH.

No comparison could be made between males and females for effects observed for the 10μg DES treatment regime, as there were no female animals available for treatment. In the males treated with 10μg of DES, basal inhibin B ($p=0.0001$) and testosterone ($p=0.02$) levels were significantly suppressed, compared to control males. Unlike in the 0.1μg DES treated males, there was no reduction in the rats' capacity to secrete testosterone in response to a GnRH challenge.

4.3.10 Summary of effects of DES treatments on adult plasma hormone levels

Hormone measured	Control males		Control females	
	Basal concentration	Concentration after a GnRH pulse	Basal concentration	Concentration after a GnRH pulse
FSH (ng/ml)	8.8 ± 0.97	9.6 ± 0.72 (9%↑)	4.7 ± 0.527	4.67 ± 0.46 (0.6%↓)
Inhibin B (pg/ml)	76.3 ± 8.32	77.2 ± 9.45 (1%↑)	59.1 ± 6.55	125.3 ± 9.84 (112%↑)
LH (ng/ml)	0.9 ± 0.019	1.5 ± 0.17 (67%↑)	0.50 ± 0.084	1.98 ± 0.367 (296%↑)
Testosterone (ng/ml)	1.9 ± 0.13	2.75 ± 0.37 (45%↑)	-	-

Table 4.3.10a: Mean concentrations of FSH, inhibin B, LH and testosterone before and after a 30 minute GnRH pulse for control male and female rats. Values are the means ± SEM.

Hormone measured	0.1µg DES males		0.1µg DES females	
	Basal concentration	Concentration after a GnRH pulse	Basal concentration	Concentration after a GnRH pulse
FSH (ng/ml)	10.4 ± 0.99 (p=0.05)	10.64 ± 0.78 (p=0.193) (2.3%↑)	4.148 ± 0.517	2.97 ± 1.33 (28%↓)
Inhibin B (pg/ml)	45.8 ± 8.5 (p=0.032)	50.2 ± 10.4 (0.0329) (9%↑)	93.1 ± 14.37	91.82 ± 11.9 (1.3%↓)
LH (ng/ml)	1.34 ± 0.29 (p=0.0003)	1.75 ± 1.12 (p=0.526) (30%↑)	0.76 ± 0.279	2.14 ± 0.459 (181%↑)
Testosterone (ng/ml)	1.12 ± 0.19 (p=0.0086)	1.41 ± 1.21 (p=0.0001) (25%↑)	-	-

Table 4.3.10b: Mean concentrations of FSH, inhibin B, LH and testosterone before and after a 30 minute GnRH pulse for male and female rats treated neonatally with 0.1µg DES. Values are the means ± SEM.

Hormone measured	10µg DES males	
	Basal concentration	Concentration after a GnRH pulse
FSH (ng/ml)	12.5 ± 1.26 (p=0.003)	12.5 ± 1.32 (p=0.023) (0%)
Inhibin B (pg/ml)	30.7 ± 12.0 (p=0.0001)	29.8 ± 12.9 (0.0001) (2%↓)
LH (ng/ml)	1.81 ± 1.29 (p=0.302)	2.4 ± 1.17 (p=0.747) (32%↑)
Testosterone (ng/ml)	1.29 ± 1.09 (p=0.0243)	1.91 ± 1.47 (p=0.0001) (48%↑)

Table 4.3.10c: Mean concentrations of FSH, inhibin B, LH and testosterone before and after a 30 minute GnRH pulse for male rats treated neonatally with 10µg DES. Values are the means ± SEM.

4.4 *In vitro* study: determination of effects of neonatal DES treatments on adult rat pituitary primary cell culture

The aim of this *in vitro* study was to investigate the potential long-term effects of the neonatal DES treatments upon pituitary cells isolated from any external systems, *i.e.* hormonal signals from the gonads and hypothalamus. This was achieved by investigating the tissue excision and cell sub-culture conditions required for an efficient maintenance of anterior pituitary cells in a temporary primary tissue culture system. This would enable us to evaluate directly if there were any chronic effects of neonatal DES treatment on adult pituitary cells in culture conditions, separated from non-pituitary hormonal feedback mechanisms.

As there was no specific pituitary primary cell culture method established at this present time within this Unit, numerous modifications of methodology for pituitary cell dispersal and culture were made, as outlined in section 4.4.1. The first aim of this study was to demonstrate that a viable anterior pituitary cell sub-culture primary population could be generated from tissue removed from a single animal, as this would allow us to examine each rat individually both *in vivo* and *in vitro*. This would enable us to eliminate any potential animal-based variability as each rat typically can display divergent basal levels of gonadotrophins and hence any small DES-induced effects could be missed due to inter-animal variability. Despite numerous attempts we have demonstrated that typically one anterior pituitary gland failed to elicit sufficient tissue to result in enough viable cells (bearing in mind that only 10% of pituitary cells are gonadotrophs) to produce and secrete sufficient amounts of LH and FSH that were within the detection limits of the gonadotrophin RIA. However upon modification of the general protocol, by randomly pairing rats from each treatment group, we were able to recover sufficient primary tissue-viable cells from which gonadotrophin hormones could be measured. Employing this experimental paradigm we were able to retrieve

viable pituitary gonadotrophs that demonstrated similar morphology to gonadotroph cell lines (e.g. LβT2 cells) in sub-culture and could be shown to contain gonadotrophin hormones. Another important reason for minimizing the number of pituitaries used for cell culture was the often limited number of suitable experimental animals. One primary factor involved in this was connected with the early treatment regimes, i.e. with neonatal treatments there was a high mortality rate of pups injected with compounds. It is therefore evident that there were multiple technical problems to surmount, however with the modifications made in the tissue excision and sub-culture techniques, it was found to be possible to adequately produce primary culture tissue from only two adult rat anterior pituitary glands.

As it has been shown previously that GnRH and activin (either separately or combined) treatments can stimulate murine LβT2 cells (a gonadotroph cell line established from male murine pituitary) to produce and secrete LH and FSH (Pernasetti *et al.,* 2001; Liu *et al.,* 2002; Nicol *et al.,* 2002), it was decided to test whether this too was the case for primary pituitary cells and whether the neonatal DES treatments would interfere with pituitary responsiveness to GnRH and/or activin. Activin (and its counterpart inhibin) is a member of the transforming growth factor-β family and it plays an important role in the modulation of hormone secretion from pituitary gonadotrophs. Activin stimulates the synthesis of FSH (Ling *et al.,* 1986) whereas inhibin represses the synthesis of FSH in the pituitary (Carroll *et al.,* 1989; Vale *et al.,* 1992). Activin is secreted by the gonads and within the pituitary gland, where it acts as an autocrine and/or paracrine regulator of the reproductive axis (Mathews, 1994).

4.4.1 Cell culture methodologies

Adult rats were sacrificed as previously described in the general methods section (Chapter 2). Before individual whole anterior pituitaries were removed using autoclaved surgical instruments, both the rats and the surrounding bench area were swabbed thoroughly with 70% ethanol. Each surgical instrument was soaked in 70% ethanol before use on each rat to minimise the risk of transferring an infection into the cell culture system. The pituitaries were placed in ice-cold (4°C) tissue culture media for transportation back to the cell culture facility. All processing took place in a laminar flow sterile tissue culture hood and dedicated primary culture room.

Initially primary cell cultures were maintained in 12-well plates for up to a week. However, after the first 2 days LH and FSH levels were barely detectable by RIA. Hence it was decided to only culture cells short-term and use assay media from the first day of culture for comparisons between controls and treated. Basal LH and FSH output from these cells was measured to investigate whether the neonatal DES treatment would have any long-term effect on the function of primary pituitary cells in culture.

4.4.2 Methodologies for primary pituitary cell culture set-up

The numerous methods tested for pituitary cell dispersion and cell culture are summarized in Table 4.4.2.

Culture medium	Method of cell dispersion	Cell attachment
DMEM-F12 supplemented with L-glutamine, penicillin, streptomycin, FCS	Enzymatic: 20-60mg trypsin (with or without heating at 37°C for 5 minutes)	Uncoated 12-well and 24-well plates
	Enzymatic: 20-60mg deoxyribonuclease (DNase)	12-well plates coated with Matrigel
	Enzymatic: 20mg trypsin and 20mg DNase, heated at 37°C for 5-10 minutes	12-well plates coated with poly-L-lysine
	Enzymatic and Mechanical cell dispersion: Using scalpel blade to chop up pituitaries + trypsin and/or DNase.	
	Mechanical: 9-12 gauge needles	

Table 4.4.2: Summary of methods attempted to set up primary pituitary cell culture.

The final pituitary cell culture method found to successfully isolate and maintain the largest amount of viable cells will be detailed in section 4.4.2.3 of this chapter.

4.4.2.1 Pituitary gland volume and eventual cell retrieval

As part of the preliminary studies of the pituitary primary cell culture system the relative effects of the number of initial whole pituitaries upon the eventual total cell retrieval in sub-culture was assessed. The eventual number of viable cells retrieved upon day 1 from the specific number of pituitary gland was measured using a Trypan Blue dye to differentiate between viable and non-viable cells. As can be seen from Table 4.4.2.1, it was evident that when the initial amount of pituitary tissue used was high, *i.e.* 4-5 pituitaries, a much greater viable cell yield was attained. Presumably this may be due to an effect in which during the mechanical dispersal process large pieces of tissue would initially show signs of deterioration at their edges while the cells at the core of the tissue remained relatively healthy. It would have been ideal if 5 to 6 pituitaries for each primary pituitary cell culture could have been used, however due to the lack of animals available, especially animals treated neonatally with specific compounds such as DES, two pituitaries were used for each primary pituitary cell culture. However it was found that with only two pituitaries a sufficient amount of cells could be retrieved with which sufficient experiments could be performed.

Number of pituitaries	Males	Females
	Number of viable cells/ml	Number of viable cells/ml
1	4×10^5	6×10^5
2	8×10^5	13×10^5
4	29×10^5	36×10^5
5	37×10^5	46×10^5

Table 4.4.2.1: Average number of viable adult male and female rat pituitary cells isolated from 1-5 pituitaries.

An interesting phenomenon noted during the set-up of this primary pituitary cell culture system was that there was always a higher number of viable cells isolated from female rat pituitaries than from male pituitaries (Table 4.4.2.1). There were usually

approximately 25% more viable cells isolated from female pituitaries than from male pituitaries. The reason for this difference is unclear as there was no significant variation in size between male and female rat pituitaries (visual assessment of pituitaries showed that male rat pituitaries tended to be slightly larger than female pituitaries, but not significantly) and the male and female pituitaries were processed in exactly the same manner. However, this difference in the number of cells isolated from male and female rat pituitaries did not affect the primary pituitary cell culture as the same number of cells was plated out (onto 12-well plates) for both male and female rats, thus a direct comparison could be made between them.

4.4.2.2 Effect of temperature upon pituitary cell retrieval

Since it was found that two whole pituitary glands supplied a sufficient number of pituitary cells in sub-culture for experimentation, it was then investigated whether treating the cells/tissues post animal excision at 4°C would cause any significant cell mortality. Therefore two pituitaries were transported back to the cell culture facility either at body core temperature (37°C) or at 4°C. It was noted that there was not any appreciable loss of cell viability by transferring the tissue to 4°C. As can be seen in Table 4.4.2.2, the dispersal and isolation at body core temperature resulted in retrieval of less viable cells than when processed at 4°C. Thus it appears that reducing tissue temperature increased the amount of retrieved tissue after removal from rat body core temperature, possibly this was due to a reduction in cell death mediated by excessive calcium influx inducing excitotoxic apoptosis.

Temperature	Number of pituitaries	Males	Females
		Number of viable cells/ml	Number of viable cells/ml
Ice (4°C)	2	7×10^5	12×10^5
Room temperature	2	5×10^5	7×10^5

Table 4.4.2.2: Comparison of storage methods for transporting isolated pituitaries back to the cell culture facility.

4.4.2.3 Final method used for pituitary cell culture and treatments administered to cells

The method found to yield the highest number of viable cells post excision was the following. Two whole pituitaries, stored on ice, were mechanically dispersed using syringes with 9-12 gauge needles attached. Cell clumps were passed through each syringe 2 times in total. Subsequently, the cell suspension was centrifuged at 1000g for 15 minutes at 4°C. The supernatant was aspirated off to remove cell debris and the cell pellet was resuspended in 12ml of DMEM-F12. Adequate pellet resuspension was obtained by passing the pellet and media 5 times through a syringe with 15 gauge needle attached. Viable primary cultured anterior pituitary cells from two whole pituitaries were counted (as detailed in Chapter 2) and then equally distributed between each well of a 12-well Matrigel coated culture plate. The cells were found to adhere to Matrigel coated plates more rapidly than to Poly-L-lysine coated plates or uncoated plates. Matrigel basement membrane matrix is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin and nidogen. It also contains TGF-beta, fibroblast growth factor and tissue plasminogen activator. Matrigel has been shown to be very effective for the attachment and differentiation of many cell types, including Sertoli cells

and vascular endothelial cells. The pituitary cells were allowed to attach overnight to the artificial cell matrix before any hormonal culture addition was made. All cells were maintained in fetal calf serum-containing Dulbecco's modified Eagles medium, at 37°C and 5% CO_2. Cells were left overnight to attach to the plates and stabilize. After overnight culture, medium was removed and replaced with either fresh DMEM-F12 medium (controls), medium containing 20ng or 50ng activin, medium containing 10nM GnRH or 100nM GnRH, for 30 minutes, or medium containing 20ng or 50ng activin + 10nM or 100nM GnRH, for 30 minutes. The media was removed after 30 minutes and was replaced with either DMEM-F12, or with DMEM-F12 + activin for the activin treated cells. At the end of the day, media was collected from each well and was stored at -20°C for subsequent LH and FSH RIA. The treatments and doses administered to the cells are summarised in Table 4.4.2.3.

Hormonal treatment	Dose administered	Duration of treatment
Activin	20ng	Continuously in culture medium. Culture medium changed daily.
	50ng	
GnRH	10nM	One GnRH pulse daily, for 30 minutes.
	100nM	
Activin + GnRH	20ng + 10nM	Activin continuously in culture medium + one GnRH pulse daily, for 30 minutes. Culture medium changed daily.
	50ng + 100nM	

Table 4.4.2.3: Outline of hormonal treatments, doses and duration of treatment administration.

All these treatments were administered to cells from control animals. As the 10nM GnRH + 20ng of activin dose showed the best response in the cells (see Sections 4.4.3.1 and 4.4.3.2), all cells obtained from DES treated animals were administered these doses.

4.4.2.4 Maintenance of primary cultured pituitary cells

The adult pituitary cells in culture were examined and photographed using a digital camera (Kodak DCS330, Kodak USA) on days 2, 4 and 6 of culture, as depicted in Figure 4.4.2.4a. On day 2 of culture, the cells were small and relatively round in appearance. However after 4 days of culture many cells were more elongated and there tended to be clusters of cells. After 6 days in culture, the cells were much larger and more elongated and a heterogenous cell population was visible, with some cells being oval shaped whereas others were elongated and tended to form clusters.

Mature gonadotroph cells are typically known to be bi-hormonal (Liu *et al.,* 1988, Currie and McNeilly 1995; Thomas and Clarke 1997). Thus, to investigate whether the pituitary cells obtained in primary culture contained adequate numbers of gonadotroph cells and to test whether these gonadotrophs had maintained their bi-hormonal nature (i.e. contained both LH and FSH) in culture, the cultured pituitary cells were immunostained on day 6 of culture for LH and FSH using TRITC and FITC fluorescent markers. The methods used for immunofluorescent staining for LH and FSH in gonadotrophs is detailed in Chapter 2.

Figure 4.4.2.4b depicts immunofluorescent staining for LH and FSH *in vivo*, in an adult rat pituitary. Panel A shows fluorescent staining for LH, panel B shows staining for FSH whereas panel C shows dual immunofluorescent staining for both LH and FSH, demonstrating that both hormones are co-expressed within the same gonadotroph cell.

Figure 4.4.2.4c shows immunofluorescent staining at day 6 for adult primary cultured pituitary cells. Panels A and C are phase-contrast pictures showing the gonadotroph cell morphology. Panels B and E show immunofluorescent staining for LH and panels D and F depict cells stained for FSH. Panel G shows a gonadotroph cell dual stained for LH and FSH. This confirms that the bi-hormonal nature of gonadotroph cells is maintained in culture and can be maintained for up to 6 days in culture.

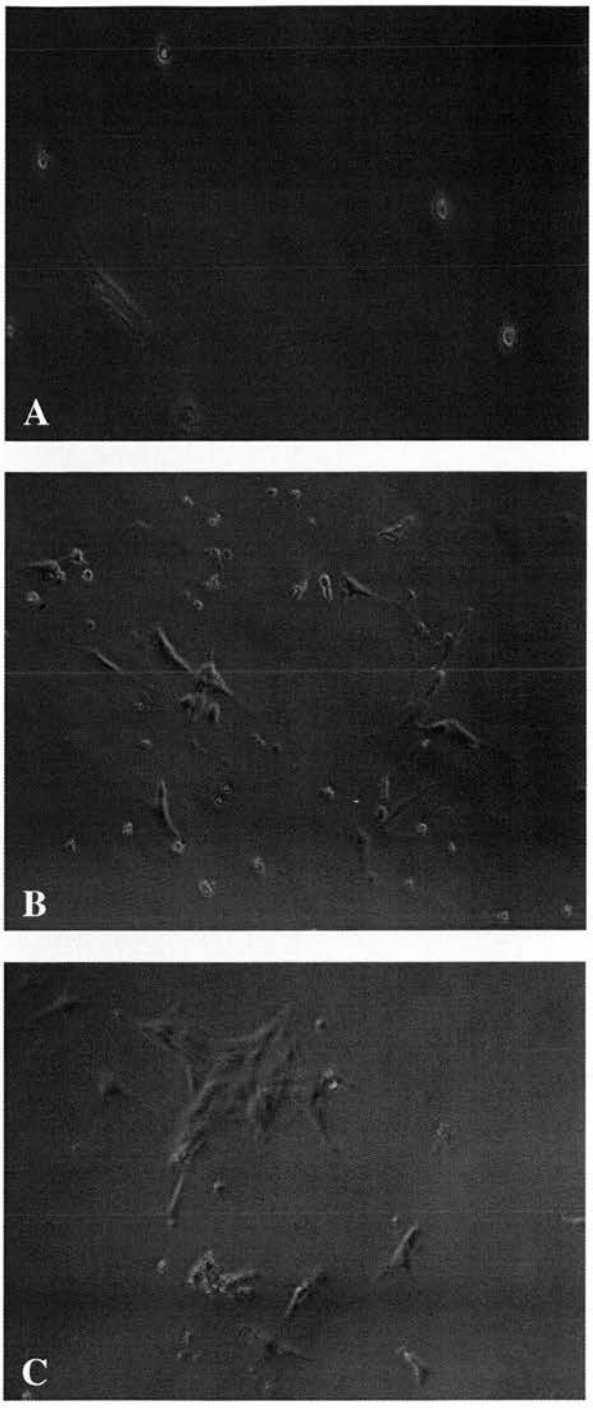


Figure 4.4.2.4a: Pituitary cell growth and morphology on day 2 (A), day 4 (B) and day 6 (C) of culture.

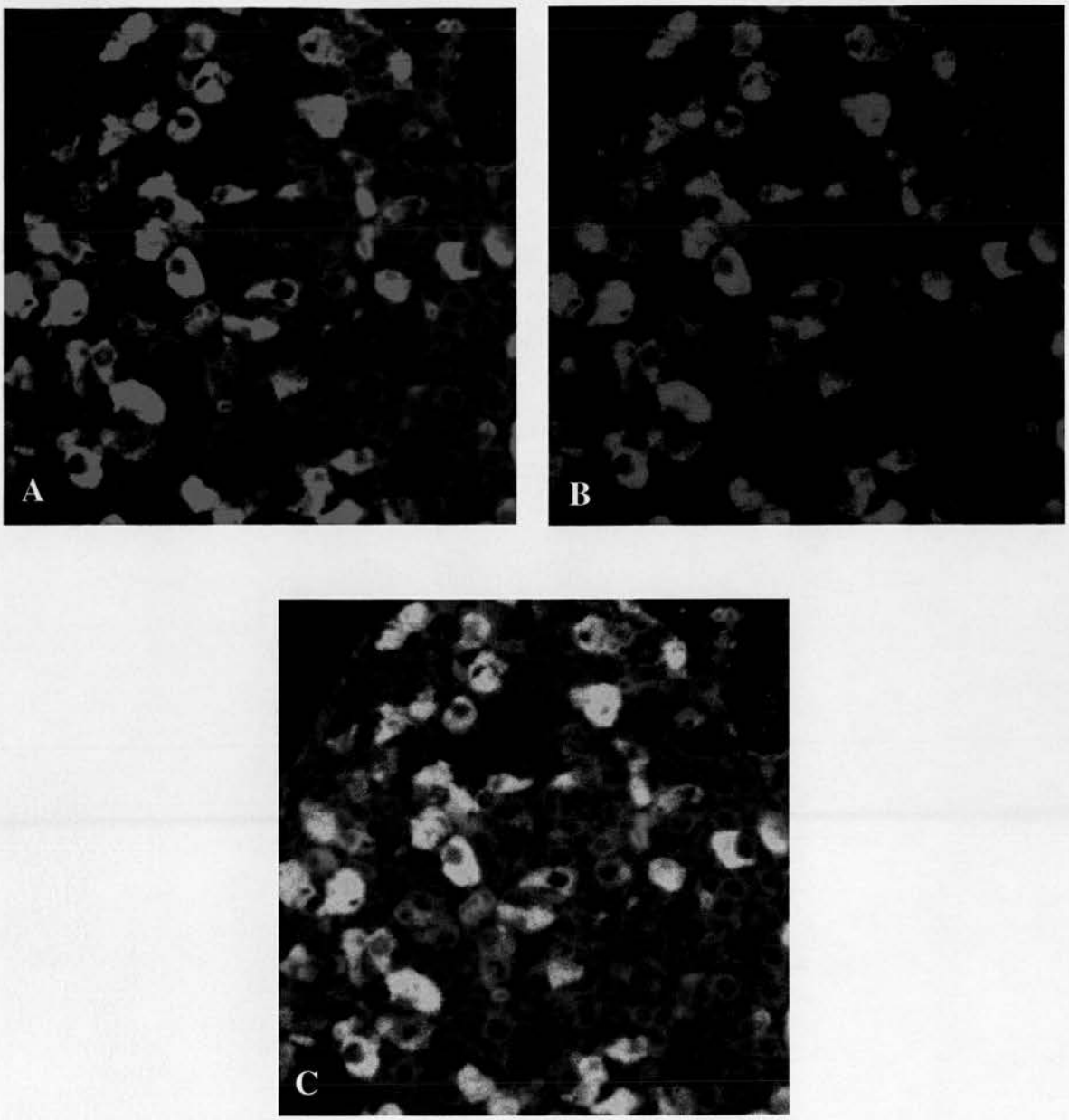


Figure 4.4.2.4b: Immunofluorescent staining in an adult rat pituitary, A depicts single staining for LH, B shows single staining for FSH and C shows dual staining for both LH and FSH. Thus *in vivo*, LH and FSH are co-localised in gonadotrophs.

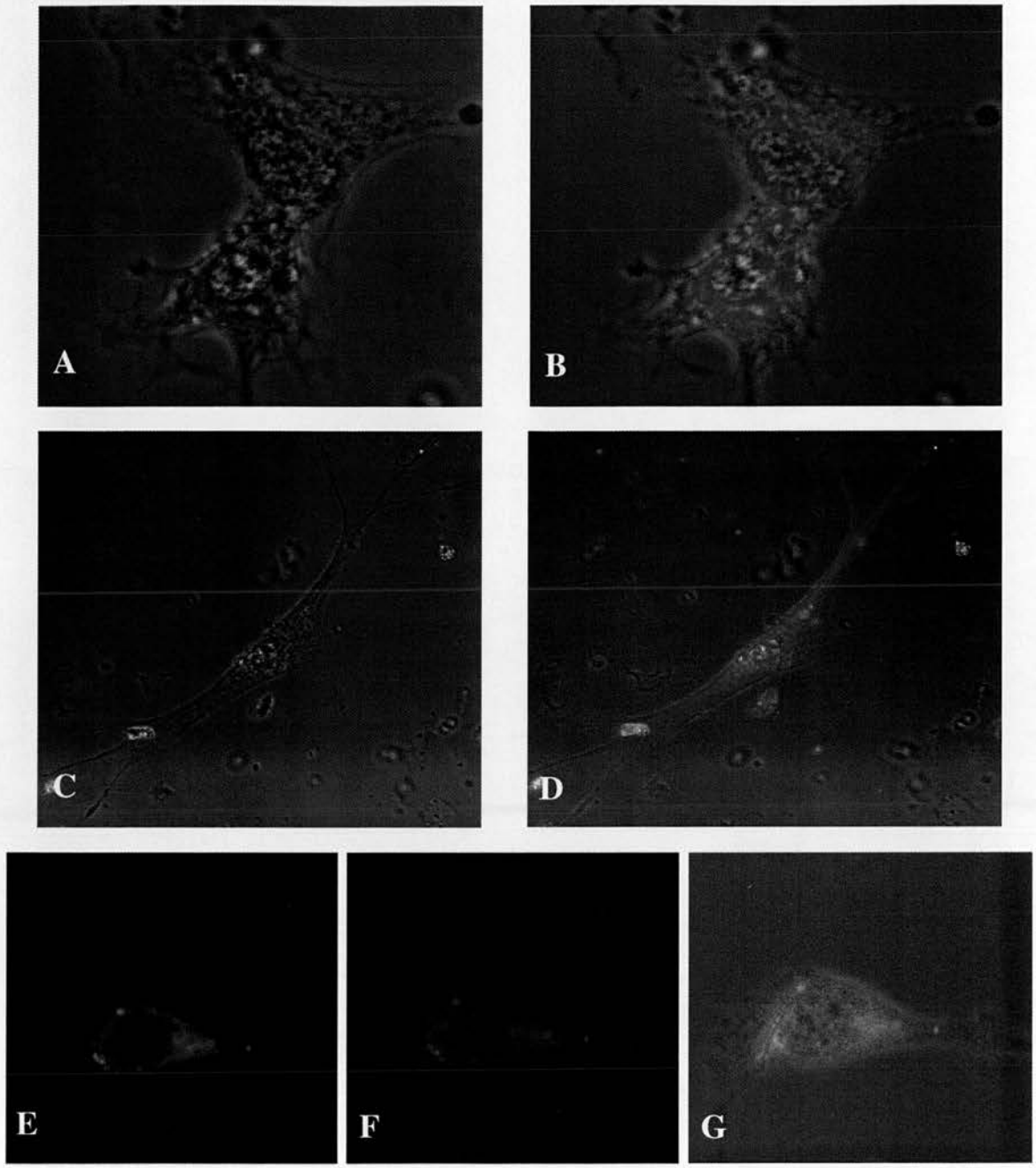


Figure 4.4.2.4c: Immunofluorescent staining in cultured pituitary cells (day 6 of culture). Panels A and C are phase-contrast pictures to depict cell morphology, panels B and E show immunofluorescent staining for LH, D and F show staining for FSH and panel G shows dual staining for LH and FSH. This confirms that gonadotrophs maintain their bi-hormonal nature after 6 days of culture.

4.4.3 Measurement of LH and FSH from cells

The concentrations of LH and FSH in the cell culture media were measured by RIA, as described in detail in Chapter 2. However, since most of the cell culture samples were initially not within the detection limits of the RIA, the protocol had to be altered to some extent. LH values were typically higher than the highest value on the standard curve, whereas FSH levels were typically much lower than the lowest value on the standard curve. Numerous dilutions were tested for the LH samples, including a 1:6 and a 1:24 dilution of the sample in assay buffer. Eventually a 1:12 dilution was found to be most suitable as all the values read on the standard curve. As many samples were small in volume (0.5ml), a different approach was first tried for FSH. This involved freeze-drying the cell culture medium samples overnight and re-suspending the pellets in assay buffer to reconstitute the samples. This made the samples more concentrated and hence the values were within the detection limit of the assay. However, this method was elaborate and time consuming, as only a small number of samples could be freeze-dried at one time and each sample had to be reconstituted with assay buffer. Thus this approach was not feasible for use with a large number of samples to be assayed. Hence, a series of dilutions was tried and tested, including a 1.5-, 2-, and 3-fold increase in sample concentration. The 3-fold more concentrated samples were found to lie within the detection limits of the RIA and hence this dilution was applied to all the FSH samples.

Culture media collected from day 1 to day 10 from control rat cells, isolated from two pituitaries, were assayed for LH and FSH. This is depicted in Figures 4.4.3.1 and 4.4.3.2.

4.4.3.1 LH levels in cell culture medium

LH concentrations were measured for cells isolated from 2 adult male or female pituitaries maintained in culture for 10 days (Figure 4.4.3.1a). Media was collected and changed daily and was assayed for LH by RIA. On the first day of culture LH levels were in the range of 25ng/ml. However on day 2 of culture LH levels had almost halved, to approximately 14ng/ml. After 3 days in culture, the LH levels were at the lower end of the detection limit of the RIA. As LH is mainly stored within gonadotrophs and produced and secreted mainly in response to GnRH (Burgess and Kelly, 1987), this suggests that the gonadotroph cells were emptying their LH stores on day one, hence the high concentration of LH in the culture medium. However, as LH levels were very low after 3 days in culture, this could suggest that no LH was being synthesised de novo by the gonadotrophs once they were removed from the donor animal. This observation was made for primary pituitary cells cultured from both adult male and adult female rats.

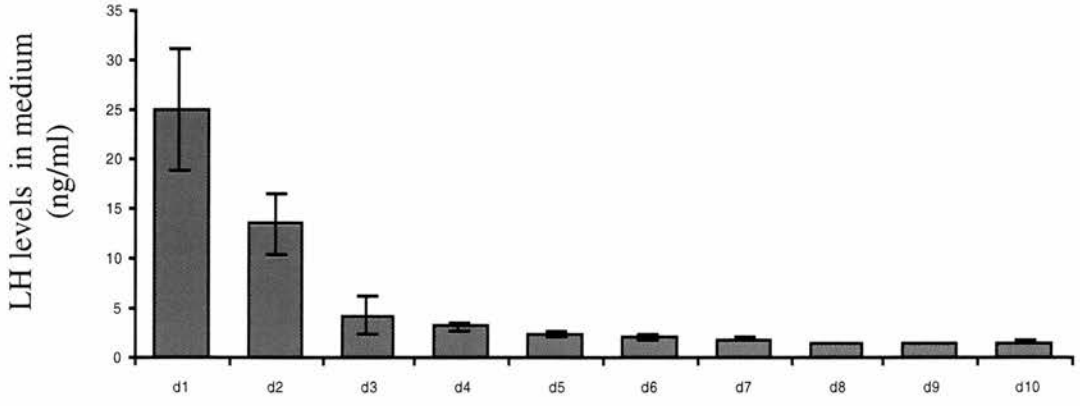


Figure 4.4.3.1a: Mean LH concentrations for primary pituitary cells isolated from 2 male control adult pituitaries (day 90), cultured for 10 days. Values are means ± SEM.

Male primary pituitary cells were also treated with activin and/or GnRH to test whether these treatments would stimulate LH production and secretion. However, these treatments were not found to significantly stimulate LH secretion from these primary pituitary cells (Figures 4.4.3.1b-e).

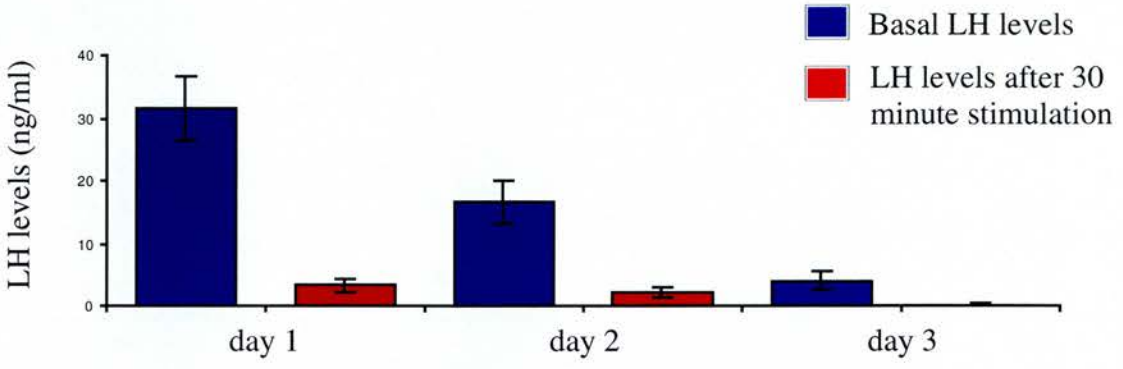


Figure 4.4.3.1b: Mean LH concentrations for male primary pituitary cells isolated from 2 control adult pituitaries, cultured for 3 days and stimulated daily for 30 minutes with 10nM GnRH. Values are the means ± SEM.

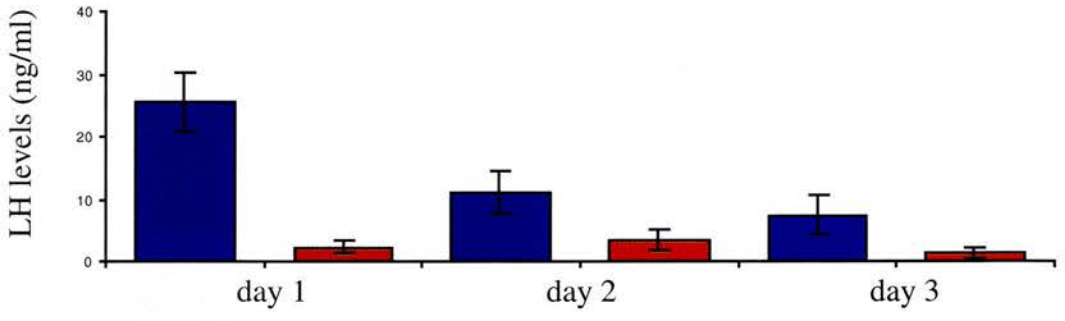


Figure 4.4.3.1c: Mean LH concentrations for male primary pituitary cells isolated from 2 control adult pituitaries, cultured for 3 days and stimulated daily for 30 minutes with 10nM GnRH +20ng activin. Values are the means ± SEM.

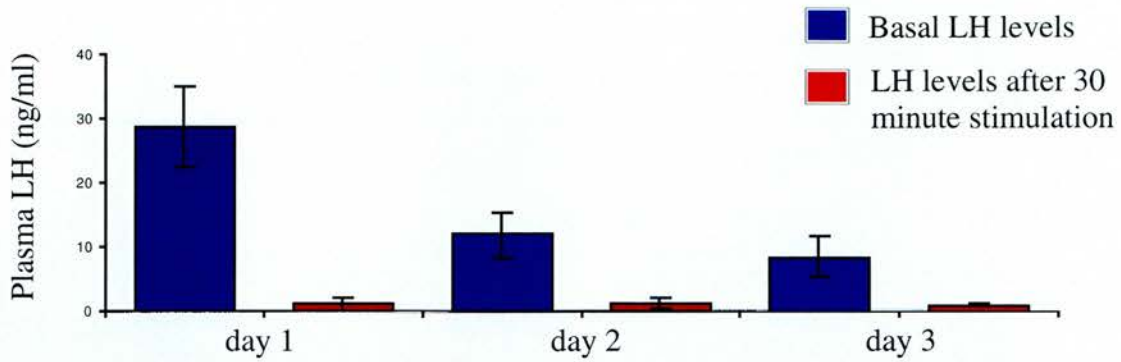


Figure 4.4.3.1d: Mean LH concentrations for male primary pituitary cells isolated from 2 control adult pituitaries, cultured for 3 days and stimulated daily for 30 minutes with 100nM GnRH. Values are the means ± SEM.

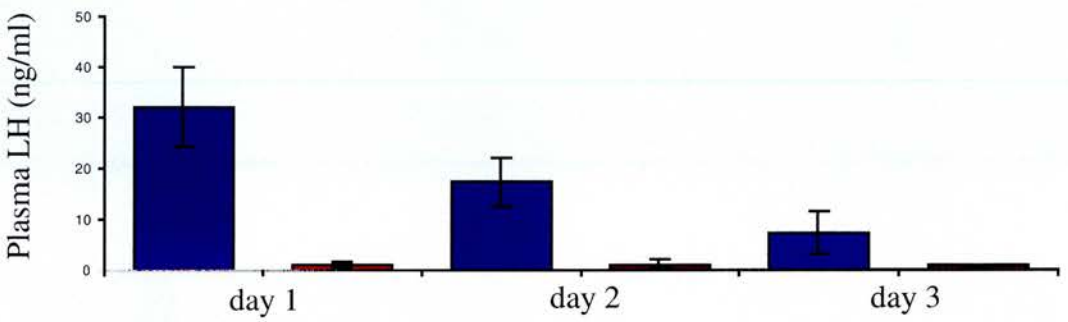


Figure 4.4.3.1e: Mean LH concentrations for male primary pituitary cells isolated from 2 control adult pituitaries, cultured for 3 days and stimulated daily for 30 minutes with 100nM GnRH + 50ng activin. Values are the means ± SEM.

4.4.3.2 FSH levels in cell culture medium

FSH concentrations were measured for cells isolated from 2 adult male or female pituitaries maintained in culture for 10 days (Figure 4.4.3.2a). Media was collected and changed daily and was assayed for FSH by RIA. On the first day of culture FSH levels were in the range of 7ng/ml. This was significantly lower than the LH levels measured for these cells. Similarly to LH concentrations, as from day 3 media FSH concentrations were at the lower end of the detection limit of the RIA. This suggests that there was no steady state release of FSH. It is known that FSH is largely secreted from gonadotrophs via a constitutive pathway, this means that FSH is not stored within gonadotrophs, but is secreted from the cell in a continuous manner. FSH release is also not directly controlled by GnRH (Childs *et al.,* 1994; Farnworth 1995). This observation was made for primary pituitary cells isolated from both adult male and adult female rat pituitaries.

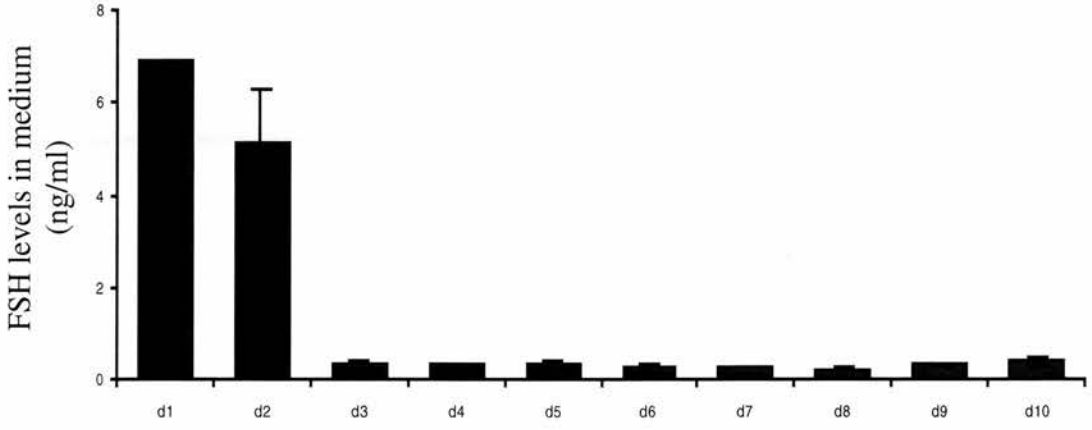


Figure 4.4.3.2a: Mean FSH concentrations for primary pituitary cells isolated from 2 male control adult pituitaries (day 90), cultured for 10 days. Values are means ± SEM.

The FSH levels in the media were measured for these cells after the activin and GnRH treatments. Similarly for LH, these hormonal treatments were not found to significantly stimulate the production and release of FSH from the primary pituitary cells.

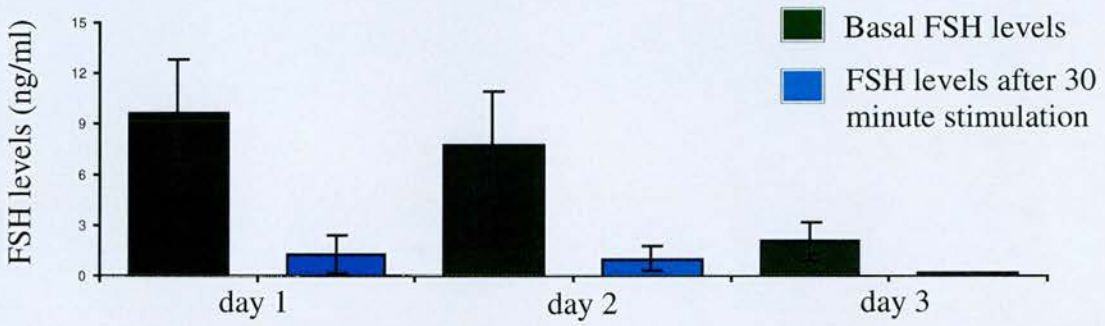


Figure 4.4.3.2b: Mean FSH concentrations for male primary pituitary cells isolated from 2 control adult pituitaries, cultured for 3 days and stimulated daily for 30 minutes with 10nM GnRH. Values are the means ± SEM.

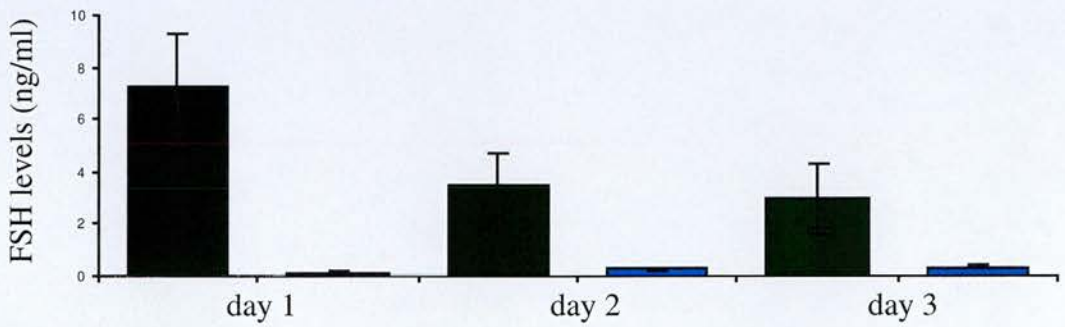


Figure 4.4.3.2c: Mean FSH concentrations for male primary pituitary cells isolated from 2 control adult pituitaries, cultured for 3 days and stimulated daily for 30 minutes with 10nM GnRH + 20ng activin. Values are the means ± SEM.

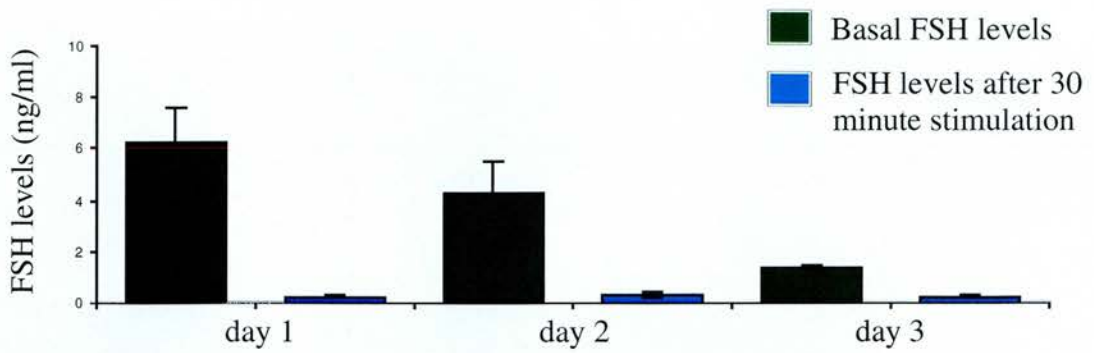


Figure 4.4.3.2d: Mean FSH concentrations for male primary pituitary cells isolated from 2 control adult pituitaries, cultured for 3 days and stimulated daily for 30 minutes with 100nM GnRH. Values are the means ± SEM.

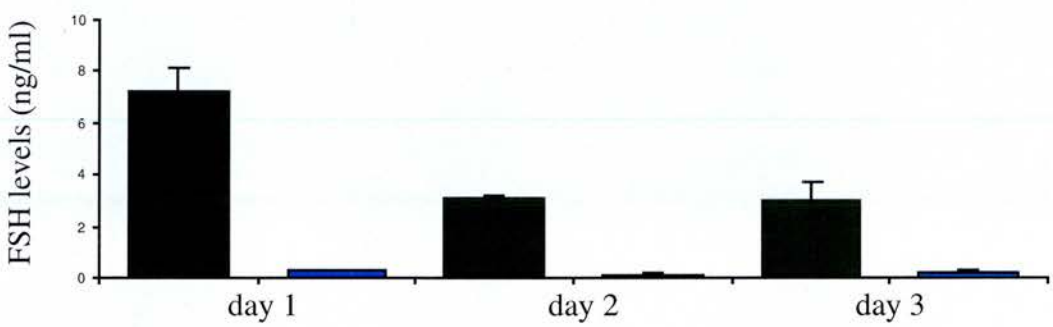


Figure 4.4.3.2e: Mean FSH concentrations for male primary pituitary cells isolated from 2 control adult pituitaries, cultured for 3 days and stimulated daily for 30 minutes with 100nM GnRH + 50ng activin. Values are the means ± SEM.

4.4.3.3 LH and FSH mRNA in primary pituitary cells

Reverse transcriptase PCR was used to determine mRNA levels for LH, FSH and GnRH receptor I, for primary pituitary cells obtained from 2 male or 2 female rat pituitaries and cultured for 6 days (Figures 4.4.3.3a and b), without the addition of any hormonal treatments. The methods used for PCR are outlined in Chapter 2. After 6 days in culture mRNA could still be detected for LH, FSH and GnRH receptor I in primary pituitary cells from both males and females. Primary pituitary cells cultured from female pituitaries tended to have more mRNA for LH and GnRH receptor type I than primary pituitary cells cultured from adult male pituitaries.

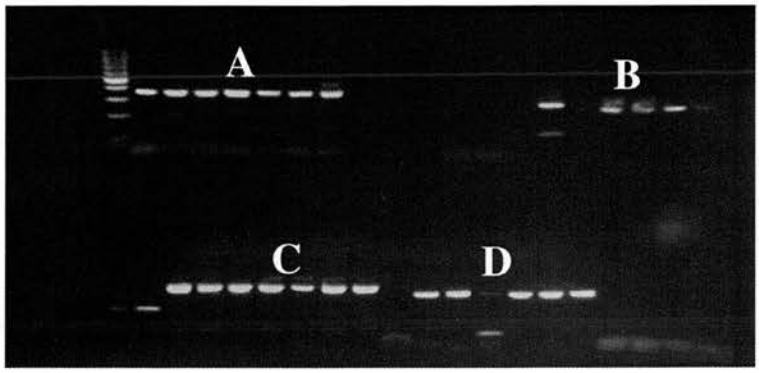


Figure 4.4.3.3a: mRNA in male primary pituitary cells for GAPDH (A), FSH (28kDA)(B), LH (28kDa) (C) and GnRH receptor I (38kDa) (D).

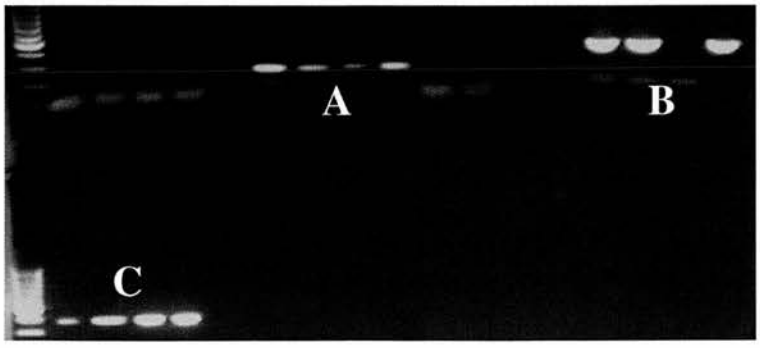


Figure 4.4.3.3b: mRNA in female primary pituitary cells for FSH (28kDa)(A), LH (28kDa) (B), and GnRH receptor I (38kDa) (C).

4.4.4 LH and FSH levels in primary pituitary cells cultured from rats treated with DES neonatally

LH and FSH concentrations were assayed on day 1 of culture for both male and female adult control pituitary cells and cells from adult animals treated neonatally with DES. The cell culture medium was collected from the cells after they had been in culture for 23.5 hours and was replaced with fresh media. Subsequently, a 30 minute 10nM GnRH pulse was administered to the cells and that media too was collected for subsequent gonadotrophin RIA. Both the basal LH/FSH levels (after 23.5 hours of culture) and LH/FSH levels after a 30 minute 10nM GnRH pulse were measured by RIA. After 23.5 hours in culture, there was no significant difference in LH levels between cells from control rats and cells from DES treated rats, even with treatment with activin or GnRH or with activin co-stimulation with GnRH. This was the case for both male and female primary pituitary cells (Figures 4.4.4 a and c). However, basal LH levels were almost 4-fold higher in cells isolated from male pituitaries (for both control pituitaries and DES treated) than in cells isolated from female pituitaries. After an acute stimulation for 30 minutes with 10nM GnRH, low levels of LH were secreted in that period of time (approximately 12ng/ml for males and 9ng/ml for females) in response to GnRH, but there was no significant difference between the various treatment groups in their response to a GnRH pulse.

When the basal measurements for the other major gonadotrophin, FSH, were made from the same series of experiments it was also noted that despite the presence of viable cells, immunoreactive for both gonadotrophin hormones (and highly characteristic of functional gonadotrophs), there was also no significant effect for the cells isolated from control rats and cells isolated from DES treated rats, even with treatment with activin or GnRH or with activin co-stimulation with GnRH. This was the case for both male and female primary pituitary cells (Figures 4.4.4b and d). However, basal FSH levels were

significantly higher in cells isolated from male rats (for both control and DES treated rats) than in cells isolated from female rats. Similarly to LH, after a 30 minute GnRH pulse there was a small quantity of FSH produced but there was no significant difference between the different treatment groups.

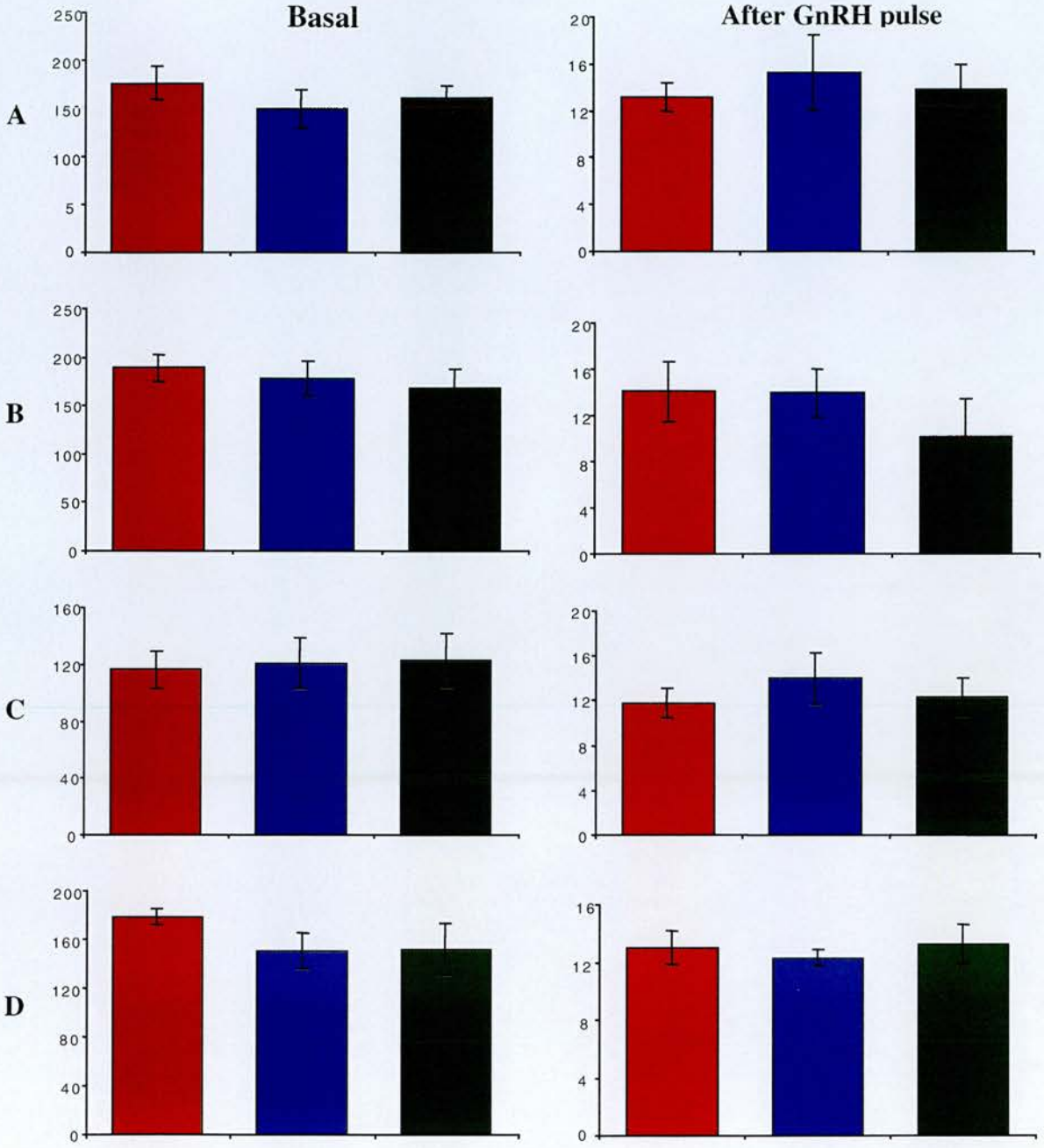


Figure 4.4.4.a: LH levels in male primary pituitary cells before and after a 30 minute 10nM GnRH challenge. Values are the means ± SEM. A=control, B=activin, C=GnRH, D=activin + GnRH. Red bars are control, blue 0.1µg DES and green bars 10µg DES. Units are ng/ml.

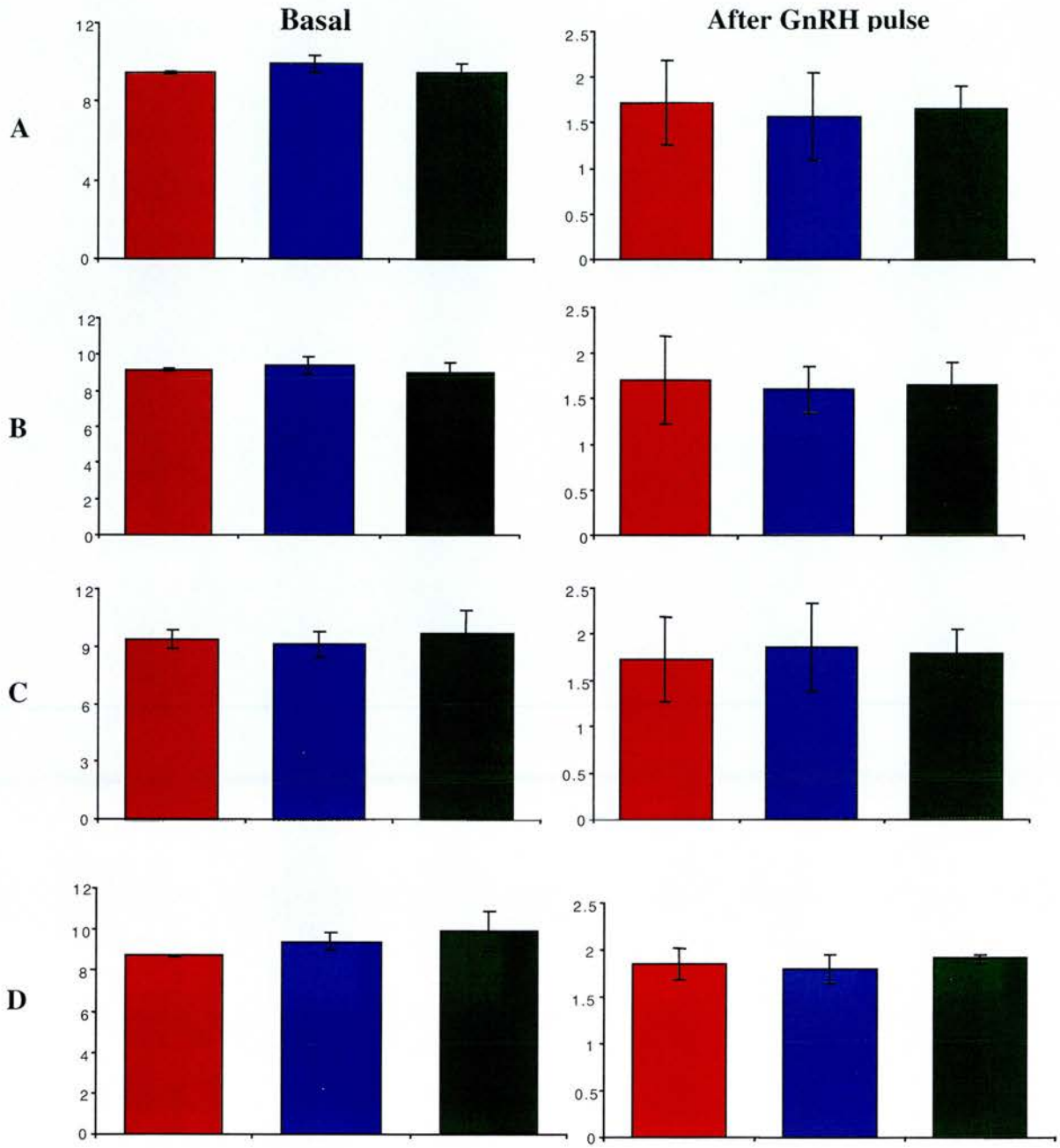


Figure 4.4.4b: FSH levels in male primary pituitary cells before and after a 30 minute 10nM GnRH challenge. Values are the means ± SEM. A=control, B=activin, C=GnRH, D=activin + GnRH. Red bars are control, blue 0.1µg DES and green bars 10µg DES. Units are ng/ml.

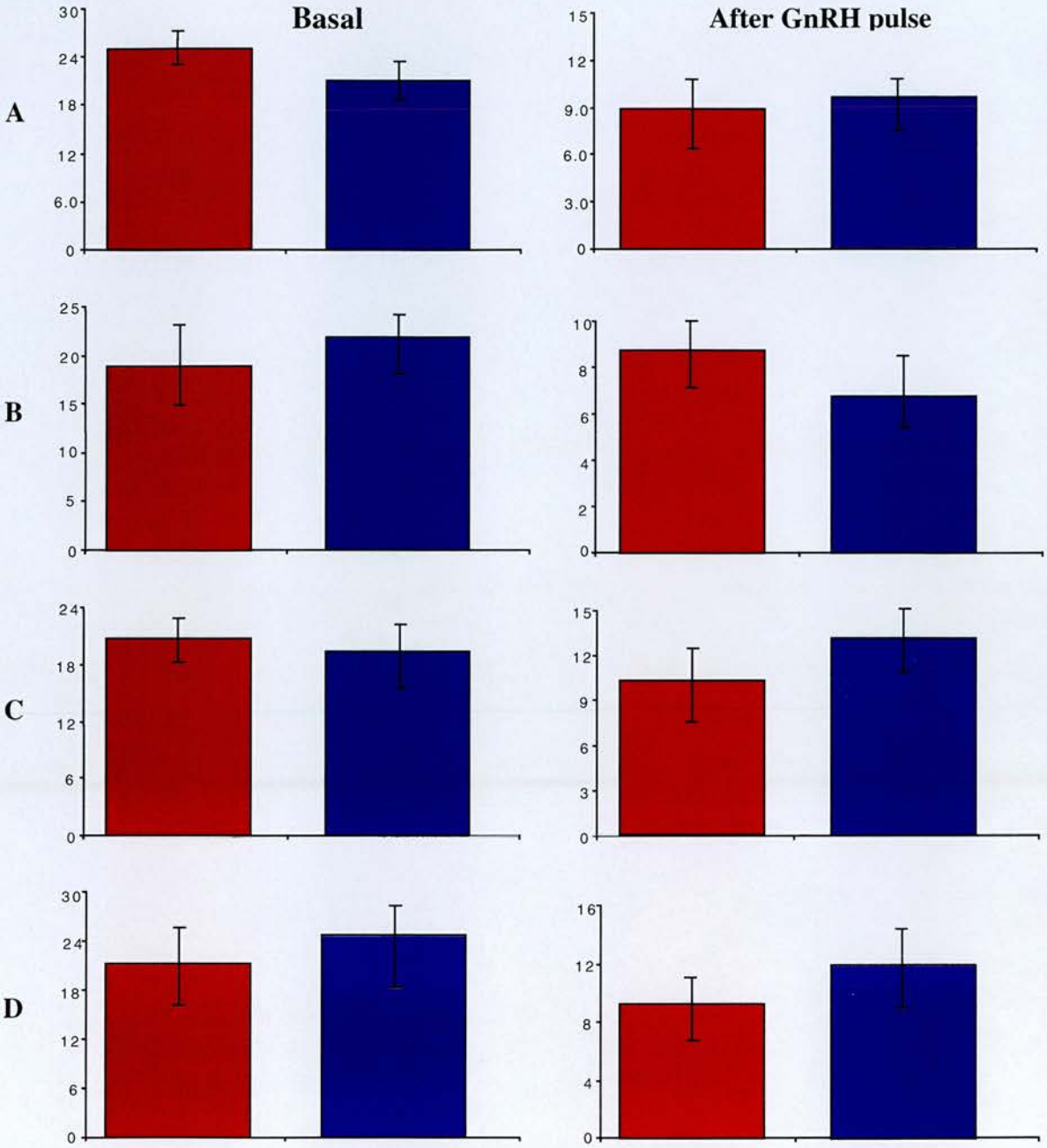


Figure 4.4.4.c: LH levels in female primary pituitary cells before and after a 30 minute 10nM GnRH challenge. Values are the means ± SEM. A=control, B=activin, C=GnRH, D=activin + GnRH. Red bars are control, blue 0.1µg DES. Units are ng/ml.

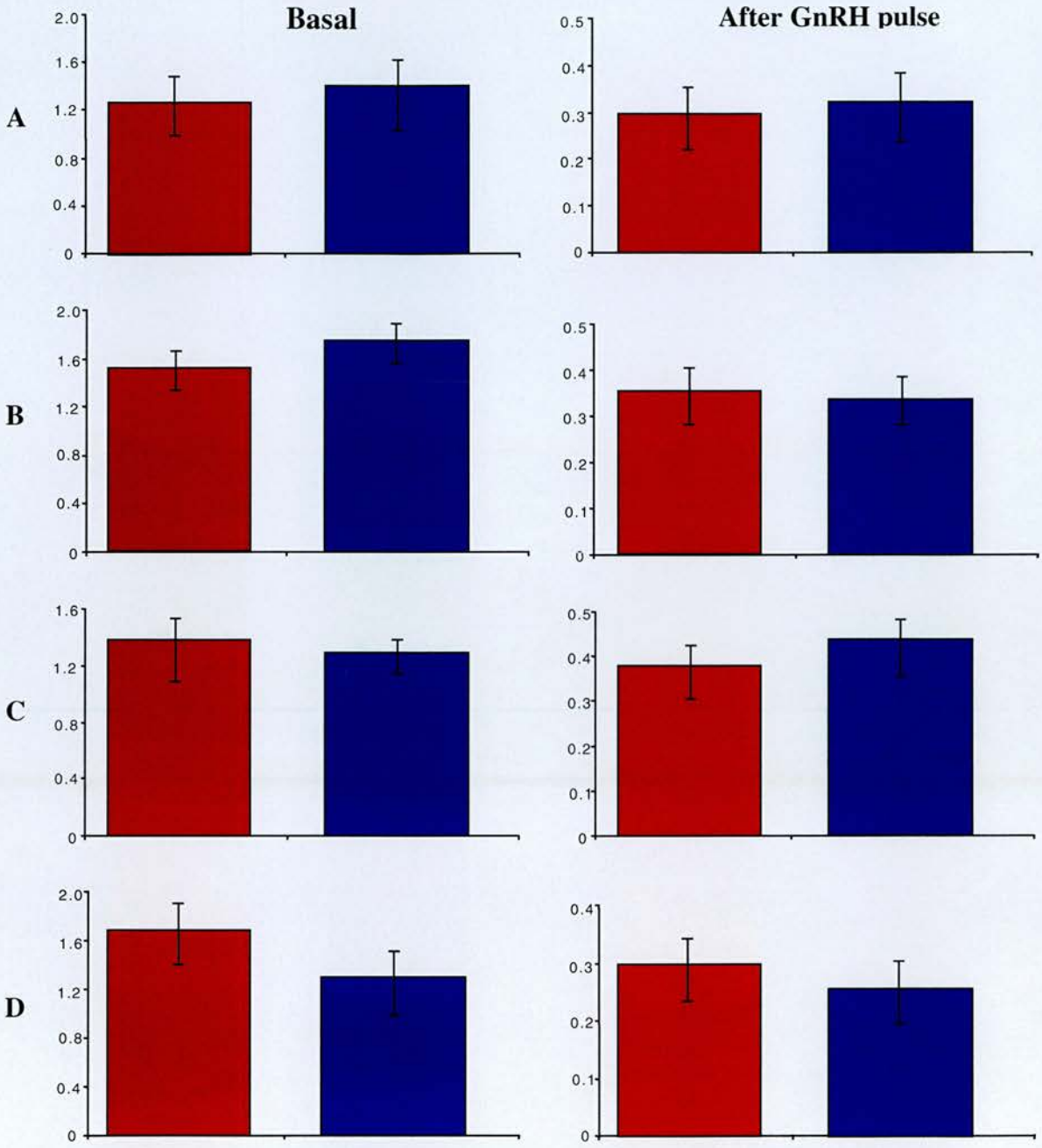


Figure 4.4.4.d: FSH levels in female primary pituitary cells before and after a 30 minute 10nM GnRH challenge. Values are the means ± SEM. A=control, B=activin, C=GnRH, D=activin + GnRH. Red bars are control, blue 0.1µg DES. Units are ng/ml.

4.4.5 Co-culturing primary pituitary cells with hypothalamic GnRH producing GT1-7 cells

As 'manual' stimulation of the pituitary cells in culture with GnRH did not have an apparent effect on LH and FSH secretion, it was decided to co-culture the adult primary pituitary cells with hypothalamic GnRH-producing neuronal cells (GT1-7 cells), to determine whether the pulsatile release of GnRH that occurs from these neuronal cells in culture (Krsmanovic *et al.,* 2000) would act as a better stimulus to maintain LH and FSH secretion from the primary pituitary cells in culture. GT1-7 cells are a neuronal cell population established through a targeted tumorigenesis approach (Mellon *et al.,* 1990; Radovick *et al.,* 1991). This involved establishing stable cell lines by expressing a potent viral transforming protein (i.e. the simian virus 40 large tumour antigen) in GnRH neurons. Three cell lines (i.e. the GT1 lines including GT1-1, GT1-3 and GT1-7) were derived from a female transgenic mouse that developed a forebrain tumour (post-migration of the GnRH neurons) (Mellon *et al.,* 1990). Cells within each of the GT1 subclones are homogenous, in that they all stain for GnRH (Mellon *et al.,* 1990; Liposits *et al.,* 1991). The GnRH gene is expressed and the precursor protein is processed into active GnRH (Wetsel *et al.,* 1991; Weiner *et al.,* 1992; Gautron *et al.,* 1995). Recently, GT1 cells have also been shown to co-express a low proportion of a second GnRH gene (GnRH-II) (Chen *et al.,* 2001). The secretion of GnRH from GT1 neurons is dependent on external Ca^{2+} (Krsmanovic *et al.,* 1992). GT1 cells in culture form networks interconnected by synapse-like contacts (Mellon *et al.,* 1990; Liposits *et al.,* 1991; Wetsel *et al.,* 1992), gap junctions (Matesic *et al.,* 1993; Hu *et al.,* 1999), and tight junctions (Liposits *et al.,* 1991). They are highly differentiated, retaining many characteristics of GnRH neurons *in vivo* (Liposits *et al.,* 1991; Weiner *et al.,* 1992), including the expression of a variety of plasma membrane channels that endow them with the ability to fire spontaneous action potentials (Mellon *et al.,* 1990; Weiner *et al.,* 1992; Charles & Hales, 1995). In addition, GT1 cells have been found to further

differentiate *in situ* and to restore reproductive function in adult hypogonadal (hpg) mice following their direct introduction into the brain (Silverman *et al.*, 1992). Thus, GT1 cells have been shown to be a successful model to investigate the hypothalamic-pituitary feedback loop.

GT1-7 cells were grown on 12mm diameter inserts with 0.4μm pore size, as described in Chapter 2, and placed in each well (over the pituitary cells, but not in direct contact) of the 12-well plate, as shown in Figure 4.4.5a.

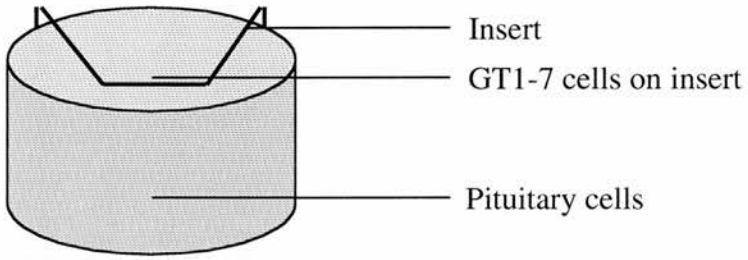


Figure 4.4.5a: Diagram depicting how inserts containing the hypothalamic GT1-7 cells were placed above the primary pituitary cells, in each well of a 12-well plate.

The GT1-7 neuronal cells were plated out at different densities onto the inserts, including 1 x 10^4 cells/ml, 5 x 10^4 cells/ml, 1 x 10^5 cells/ml, and 5 x 10^5 cells/ml. The pituitary cells were plated out at a density of 7 x 10^5 cells/ml. Media was aspirated off daily (and stored at -20°C for subsequent LH and FSH analysis) and was replaced with fresh media.

4.4.5.1 LH and FSH levels in pituitary cells co-cultured with GT1-7 cells

LH and FSH levels were measured for male pituitary cells co-cultured with the GT1-7 cells plated out at the four different densities (Figures 4.4.5.1a-h). As for the 'manual' stimulation with GnRH, there was found to be no increase in LH (or FSH) levels after the co-culture with the GnRH-producing neurons. There was also no significant difference in LH (or FSH) levels for the different cell densities of GT1-7 cells. It is possible that there were small changes in gonadotrophin levels in response to the pulsatile release of GnRH from the GT1-7 cells, but that these were not apparent as many of the gonadotrophin values measured were at the lower end of the sensitivity scale of the RIA.

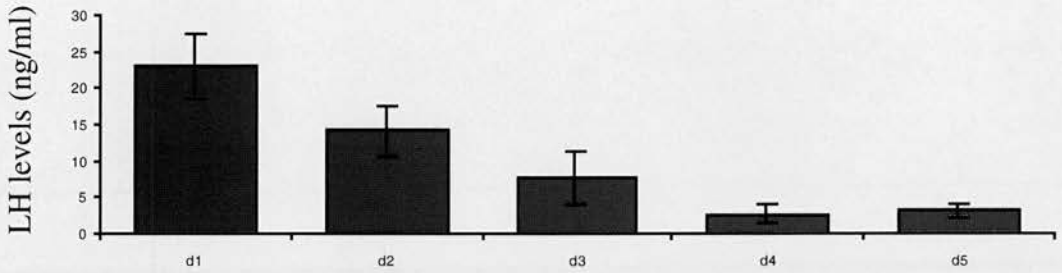


Figure 4.4.5.1a: Mean LH concentrations for male pituitary cells (7 x 10^5 cells/ml) co-cultured for 5 days with hypothalamic GT1-7 cells (1 x 10^4 cells/ml). Values are the means ± SEM.

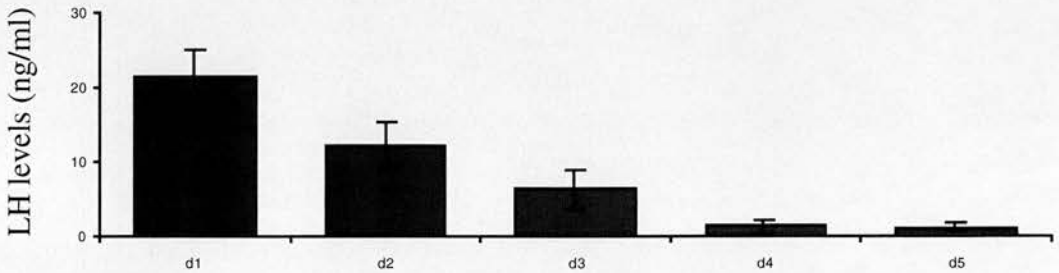


Figure 4.4.5.1b: Mean LH concentrations for male pituitary cells (7 x 10^5 cells/ml) co-cultured for 5 days with hypothalamic GT1-7 cells (5 x 10^4 cells/ml). Values are the means ± SEM.

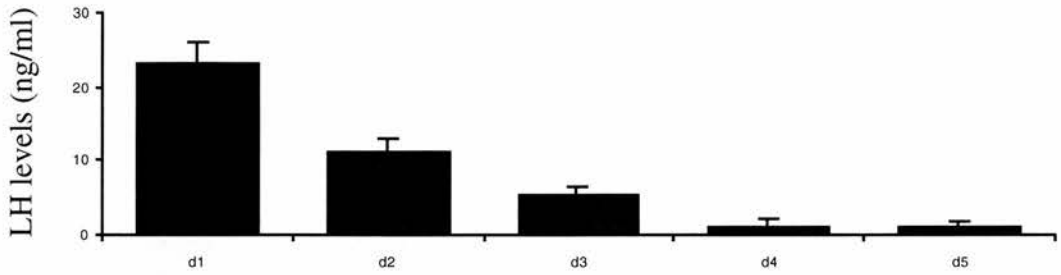


Figure 4.4.5.1c: Mean LH concentrations for male pituitary cells (7 x 10^5 cells/ml) co-cultured for 5 days with hypothalamic GT1-7 cells (1 x 10^5 cells/ml). Values are the means ± SEM.

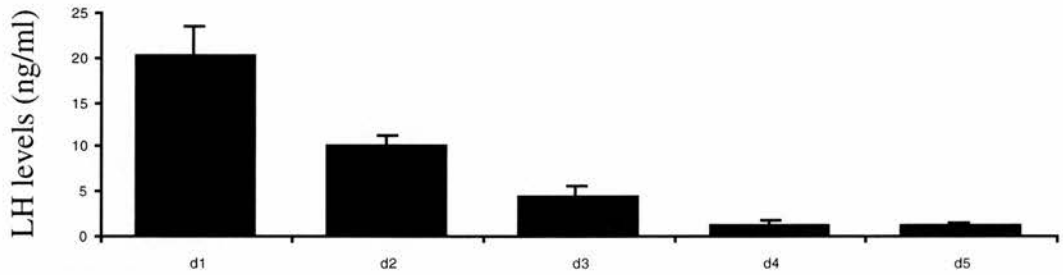


Figure 4.4.5.1d: Mean LH concentrations for male pituitary cells (7 x 10^5 cells/ml) co-cultured for 5 days with hypothalamic GT1-7 cells (5 x 10^5 cells/ml). Values are the means ± SEM.

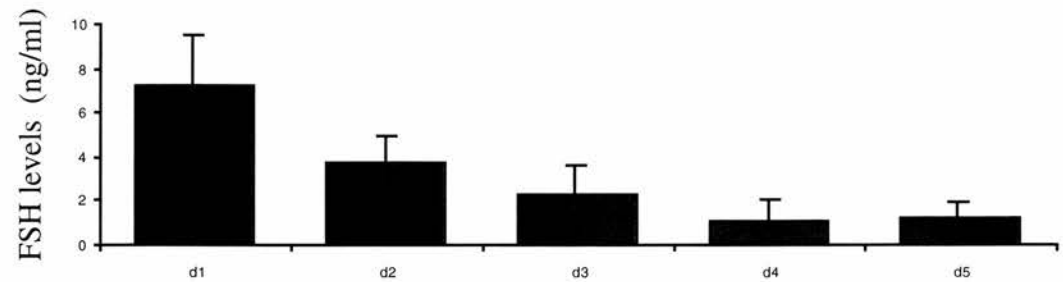


Figure 4.4.5.1e: Mean FSH concentrations for male pituitary cells (7 x 10^5 cells/ml) co-cultured for 5 days with hypothalamic GT1-7 cells (1 x 10^4 cells/ml). Values are the means ± SEM.

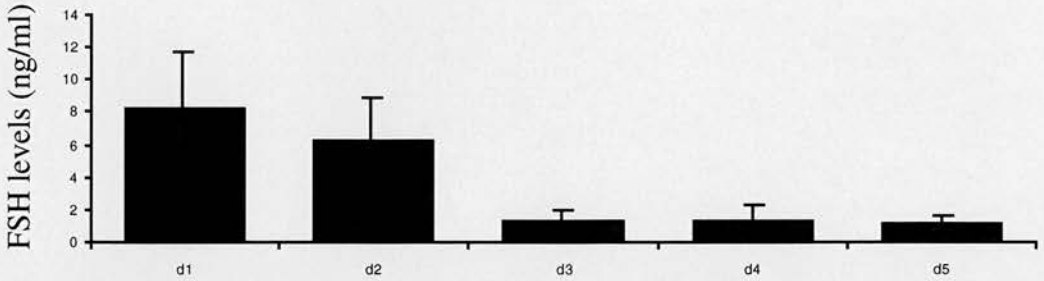


Figure 4.4.5.1f: Mean FSH concentrations for male pituitary cells (7 x 10^5 cells/ml) co-cultured for 5 days with hypothalamic GT1-7 cells (5 x 10^4 cells/ml). Values are the means ± SEM.

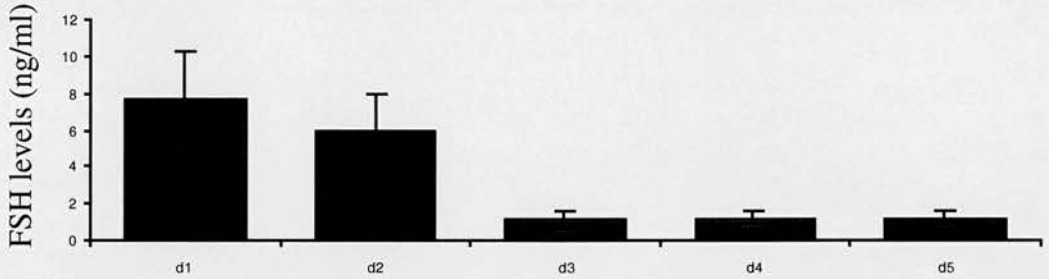


Figure 4.4.5.1g: Mean FSH concentrations for male pituitary cells (7 x 10^5 cells/ml) co-cultured for 5 days with hypothalamic GT1-7 cells (1 x 10^5 cells/ml). Values are the means ± SEM.

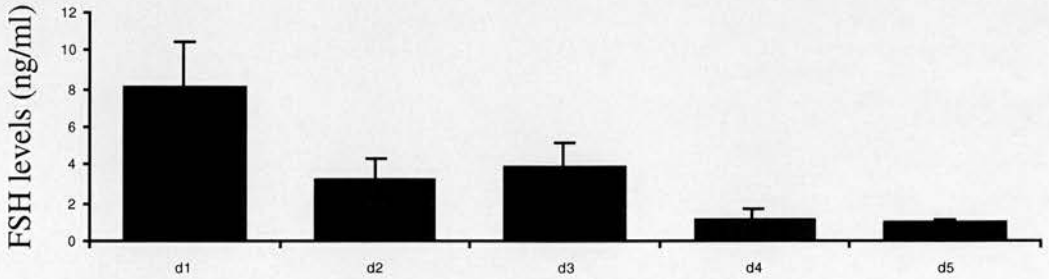


Figure 4.4.5.1h: Mean FSH concentrations for male pituitary cells (7 x 10^5 cells/ml) co-cultured for 5 days with hypothalamic GT1-7 cells (5 x 10^5 cells/ml). Values are the means ± SEM.

4.4.5.2 Expression of GnRH receptor I in primary pituitary cells

It is possible that there was a potential functional deficit in the primary cultured cells in that they appeared phenotypically correct yet, they seemed refractory to external GnRH stimulation. One possible explanation for this could be the loss of GnRH receptor protein from the membrane of the excised cells. The presence of mRNA has been shown for the receptor (Figures 4.4.3.3a and b), this was however purely qualitative and may not be directly correlated to the level of receptor protein present. Thus the lack of appropriate control of receptor turnover, induced by removal of tissue from its native condition, may have resulted in a rapid loss of receptor expression at the membrane, and hence affected the cells' ability to respond to GnRH pulses. Therefore, the presence of GnRH receptor on the primary pituitary cells and on LβT2 cells was investigated by Western Blot analysis. The LβT2 gonadotroph cell line was used as a positive control as LβT2 cells are known to express the GnRH receptor (Persanetti *et al.,* 2001). The GnRH receptor antibody was a gift from K. Catt (Krsmanovic *et al.,* 1992).

A Western Blot was performed using LβT2 cells and primary pituitary cells from control adult male rats. The Western Blot was performed as follows. LβT2 cell monolayers were placed on ice, washed once with ice-cold Dulbecco's phosphate buffered saline (DPBS, Sigma, UK) and lysed in an NP-40 based solubilisation buffer (250mM NaCl, 50mM HEPES, 0.5% Nonidet-P-40 (NP-40), 10% glycerol, 2mM EDTA, pH 8.0 supplemented with 1mM sodium orthovanadate, 1mM phenylmethysulphonyl fluoride and 1μg/ml leupeptin). Solubilised lysates were clarified by centrifugation at 15000rpm for 15 minutes and diluted to an approximate concentration of 1mg/ml protein. Protein content was extracted from the cells cultured from two adult male rat pituitaries, as described in detail in Chapter 2. Subsequently, a 40μl aliquot of the clarified cell lysates was mixed with an equal volume of 2x Laemmli sample buffer (8% SDS, 5% 2-β-mercaptoethanol, 10% glycerol, 25mM Tris pH 7.0, and a few grains of Bromophenol blue, Sigma, UK), and were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The

proteins from the gel were then electrotransferred to a polyvinylenedifloride (PVDF, NEN Life Sciences, UK) membrane. The membrane was blocked for 1 hour at room temperature with 4% BSA TBS-T blocking solution (150mM NaCl, 10mM Tris pH 7.0, 0.05% v/v Tween-20, and 0.05% v/v NP-40, Sigma, UK) before incubation for 1 hour with a 1:1000 dilution of the anti-GnRH serum (gift from Kevin Catt, Bethesda, USA) in the 4% BSA TBS-T solution. The antibody was washed off (5 washes for 5 minutes) in TBS-T wash buffer, and was visualised by incubation with the secondary antibody, anti-rabbit alkaline Phosphatase conjugate (1:10000, Sigma, UK) for 1 hour at room temperature. Proteins were finally visualised by enzyme-linked chemifluorescence (Amersham Pharmacia Biotech, UK) and were scanned using a Typhoon phosphorimager.

The Western Blot analysis for the GnRH I receptor in primary pituitary cells and LβT2 cells (Figure 4.4.5.2) showed that the GnRH I receptor was present in control LβT2 cells, however only a very small amount was present in the primary pituitary cells. This suggests that there may have been a loss of receptor expression on the membrane, which could be a reason why there was no LH release in the primary pituitary cells in response to GnRH (+ activin) treatment.

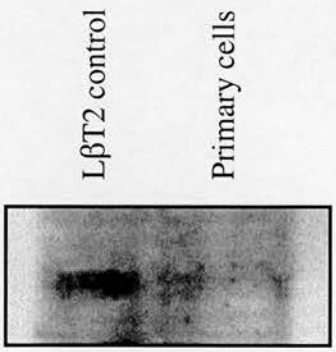


Figure 4.4.5.2: Western Blot analysis for the GnRH I receptor in whole cell lysates from control LβT2 cells and adult male rat primary pituitary cells.

4.5 Discussion

The aims of these studies was to determine whether neonatal treatment with DES could have direct effects on the function of pituitary gonadotroph cells, and to test whether DES would have differential effects on male and female pituitary function. Both *in vivo* and *in vitro* experiments were performed to test this hypothesis.

The data gathered from the *in vivo* part of this study confirmed that neonatal DES treatment had life-long effects on circulating gonadotrophin levels in male rats. These results confirm the observations made during the male DES study, as described in Chapter 3 of this thesis. In the adult male rats, plasma FSH levels were found to be elevated significantly for both doses of DES (0.1μg DES $p<0.05$; 10μg DES $p<0.01$), and after a 30 minute GnRH pulse the plasma FSH levels still showed the same basic pattern as before the GnRH challenge, but there was no significant increase in plasma FSH in response to GnRH. This corresponds with the present literature (Farnworth, 1995; Padmanabhan and McNeilly, 2001), in that FSH release from the pituitary gland is not under direct control of GnRH. Interestingly however, DES treated females showed no significant change in basal plasma FSH levels, in direct contrast to the DES treated males. This demonstrates that DES treatment exerted a differential effect on male and female circulating FSH concentrations. It appears therefore that male plasma FSH levels are more susceptible to long-term disruption by DES than female plasma FSH levels.

In males, basal plasma LH levels showed the same pattern as basal FSH levels, in that the LH levels were elevated significantly for the DES treated rats (0.1μg DES $p<0.05$; 10μg DES $p<0.001$). After the 30 minute GnRH challenge, there was an increase in plasma LH levels, which was expected since it is known that LH is stored intracellularly and is released mainly from the gonadotroph cells in response to pulses of GnRH via a regulatory secretory pathway (Burgess and Kelly, 1987). However, the DES treated

males showed a considerably attenuated response to the GnRH pulse than the control males. In control males plasma LH levels increased by 67% in response to GnRH whereas in the DES treated males there was only a 30% increase in plasma LH levels in response to GnRH. This demonstrates that in some manner the neonatal DES treatment had altered the pituitary responsiveness to GnRH in these adult male rats. There are several possible causes for such a phenomenon. For example, it has been demonstrated that oestrogenic agents (especially in combination with progesterone) can attenuate the expression of GnRH receptor on pituitary gonadotroph cells (Cheon *et al.,* 2001; Kang *et al.,* 2001; Sun *et al.,* 2001). Thus from the literature it appears that GnRH receptor down-regulation is a likely cause for this phenomenon. Surprisingly, in females the neonatal DES treatment showed no significant effect on basal plasma LH levels. However, similarly to the males, the neonatal DES treatment was found to decrease pituitary responsiveness to GnRH in females, as the DES treated females showed a significant decrease (30% less) in their pituitary responsiveness to GnRH compared to control females. This suggests that DES treatment not only had life-long effects on basal gonadotrophin levels in male rats, but also that neonatal DES treatment decreased the ability of the pituitary gonadotroph cells to secrete LH in response to GnRH in both male and female DES treated rats. These results again correlate with studies also performed by Register *et al.,* and Faber *et al.,* that showed that neonatal DES treatment blunted LH secretion in response to a GnRH challenge (Register *et al.,* 1995; Faber *et al.,* 1993).

The effects of neonatal DES treatment were also studied on the plasma levels of the gonadal hormone inhibin B. It was noted that the circulating concentrations in the DES treated males were significantly suppressed compared to control males (0.1μg DES $p<0.01$; 10μg DES $p<0.05$). These findings reinforce the observations made previously in Chapter 3, in that plasma inhibin B levels were significantly reduced in male DES treated rats. This may potentially be due to a reduction in Sertoli cell proliferation in the

testis caused by neonatal DES treatment (Sharpe, 1994). The reduced levels of Sertoli cells in the adult testis then resulted in suppressed circulating levels of inhibin B. When we compared the effects of DES upon plasma inhibin B levels in the females to the males, we again showed that the DES treatment failed to alter the inhibin B levels while causing a significant suppression in the males. Serum inhibin B levels are often used as a direct indicator of testicular function due to its primary testicular origin. Diminished serum inhibin B levels are therefore highly indicative of male gonadal dysfunction (Yalti *et al.,* 2002). Therefore the potential reduction of Sertoli cell proliferation, shown to be induced by potent oestrogenic compounds (Sharpe, 1994) would result in a diminution of testis cell types and general size (as shown in Chapter 3). Therefore, it seems most likely that the DES treatment had profound effects on the male gonads while not exerting such a detrimental effect on their growth in the female, and this could be the primary cause in differences in circulating levels of inhibin B. Thus to address this further, the plasma levels of testosterone were measured to reinforce the findings that DES has caused significant male gonad dysfunction. It was found that (as would have been expected) the plasma levels of testosterone were significantly suppressed in the DES treated male rats ($p<0.001$).

During the set-up of the primary pituitary cell culture system, one interesting and consistent observation was made, in that adult female pituitaries always yielded a higher number of viable cells than adult male pituitaries, after extraction from the animal. Generally there were approximately 25% more viable cells isolated from female pituitaries than from male pituitaries. It is presently unclear why this is the case as the pituitaries were treated in exactly the same manner. In addition, upon visual observation we found that there was no significant difference in the original size of male and female pituitaries. Again, fluorescent analysis of the cultured pituitary cells showed that the cells maintained their bi-hormonal nature in culture, as the majority of pituitary cells *in vivo* are known to contain both LH and FSH (Liu *et al.,* 1988; Currie and McNeilly,

1995; Thomas and Clarke, 1997). Initially LH and FSH levels were assayed from days 1 to 10 of culture, however after 3 days of culture the gonadotrophin levels were at the lower end of the detection limit of the RIA. Therefore it was decided to measure LH and FSH levels on day 1 of culture for the pituitary cells cultured from the DES treated animals. Interestingly, there was found to be no significant difference in gonadotrophin levels between cells from control animals and cells from neonatally DES treated animals. Even treatment with GnRH and/or activin, which has been shown to stimulate LH (and FSH) release from both primary pituitary cells and LβT2 cell lines (Pernasetti *et al.,* 2001; Liu *et al.,* 2002; Nicol *et al.,* 2002), made no difference to LH and FSH secretion from both control and DES treated primary pituitary cells. Co-culturing the primary pituitary cells with hypothalamic GnRH-producing cells also had no significant effect. Therefore it appears that in accordance with the majority of our data, the neonatal DES treatment had only small functional effects upon the anterior pituitary. This can be surmised from the fact that there was no significant change in anterior pituitary morphology with DES, and no loss of gonadotrophin hormones from the pituitary. However, it appears that there was potentially a subtle effect upon the anterior pituitary, demonstrated by a reduction of anterior pituitary sensitivity to GnRH.

Chapter five: effects of a neonatal GnRH antagonist or GnRH antagonist with oestrogen treatment on male rat pituitary function

5.1 Introduction

The aim of this chapter is to investigate the potential effects of neonatal treatment upon pituitary gland function of either a long-acting GnRH antagonist alone treatment or in a combination regimen with the potent oestrogen DES. In the first study, male rats were treated with the long-acting GnRH antagonist Antarelix in neonatal life. Potential transient or permanent effects on pituitary gland function were investigated by counting the number of immunopositive gonadotroph cells shortly after treatment. In addition we assessed if there were any effects of the treatments upon pituitary LH and FSH mRNA levels and also the plasma levels of LH, FSH, inhibin B and testosterone in adulthood. In parallel to this, testes data were collected from these animals by Richard Sharpe's laboratory, including testis weight and the number of Leydig and Sertoli cells per adult testis. This enabled potential GnRH antagonist-induced effects on anterior pituitary gland function to be related to corresponding testes data and potential GnRH antagonist-induced effects on the adult testes and vice versa.

We also performed an *in vivo* and an *in vitro* approach to investigate the potential long-term effects upon the hypothalamic-pituitary-gonadal axis of neonatal GnRH antagonist treatment combined with 0.1µg DES treatment. The rationale behind this study was to examine whether neonatal DES treatment would entrain re-programming of pituitary function in adulthood. The GnRH antagonist was administered in parallel to the DES to block the hypothalamic GnRH output. This enabled us to shed light on any potential DES-induced effects directly at the level of the anterior pituitary gland.

For the *in vivo* experiments, male rats were treated neonatally with the GnRH antagonist and 0.1μg of DES. The plasma hormone levels (LH, FSH, inhibin B, testosterone) were subsequently measured in adulthood before and after a 30 minute 100ng GnRH dose challenge. The pituitaries were subsequently removed from these rats for primary pituitary cell culture. The GnRH antagonist + 0.1μg DES treatment regime enabled us to directly investigate any DES-induced effects on pituitary function, without any feedback from the testes hypothalamic stimulation. All retrieved pituitaries were cultured and GnRH and activin treatments were administered to the primary pituitary cells to test whether these treatment regimes would stimulate gonadotrophin secretion from the primary cells. Media from these cells was subsequently collected and assayed for LH and FSH.

5.1.2 Summary of analysis methods for the male *in vivo* and *in vitro* GnRH antagonist studies

In vivo male rat GnRH antagonist study: Investigation into the long-term effects of neonatal treatment with the long-acting GnRH antagonist, Antarelix, on pituitary function. The primary goals of this study were:

- Determination of basal plasma LH, FSH (by RIA), inhibin B and testosterone (by ELISA) concentrations in adulthood (90 days of age).

- Real-Time PCR analysis of mRNA for LH and FSH in the adult male pituitary after neonatal treatment with the GnRH antagonist Antarelix.

- Immunocytochemical analysis of LH and FSH immunopositive staining in the pituitary, shortly after treatment with Antarelix (at 18 days of age). Unfortunately no adult tissue was available for immunocytochemical analysis.

- Analysis of adult rat testis function, including testis weight, morphology, number of Leydig and Sertoli cells per testis, after neonatal treatment with Antarelix, using data obtained from Richard Sharpe's laboratory. This collaboration was to investigate any potential effects along the pituitary-testicular axis after neonatal treatment with a GnRH antagonist.

In vivo and *in vitro* GnRH antagonist + DES study*:* Investigation into the long-term effects of neonatal treatment with a low dose of DES and Antarelix (0.1μg DES + GnRH antagonist) combined treatment on pituitary function. The main aims of this study were:

- Determination of basal plasma LH, FSH (by RIA), inhibin B and testosterone (by ELISA) concentrations in adulthood (90 days of age).

- Assessment of changes in plasma LH, FSH, inhibin B and testosterone levels in response to an acute GnRH dose challenge to adult rats (100ng GnRH for 30 minutes).

- Culturing adult primary pituitary cells isolated from male pituitaries treated neonatally with a low dose of DES and with Antarelix (0.1μg DES + GnRH antagonist).

- Treating primary cultured pituitary cells with activin, GnRH, or a combination of both GnRH and activin to test whether this stimulates gonadotrophin production by primary pituitary cells in culture.

- Measurement of basal gonadotrophin levels (RIA) secreted by these cells after 23.5 hours in culture. Administering a 30 minute 10nM GnRH challenge to primary pituitary cells and measuring LH and FSH production in the cell culture media in response to GnRH.

5.2 *In vivo* male rat study: effects of neonatal treatment with a GnRH antagonist or GnRH antagonist + DES on pituitary function

5.2.1 Animals and neonatal treatments

For the male Wistar rats treated neonatally with the just the GnRH antagonist, animals were injected *s.c.* on days 2 and 6 with 10mg/kg of the long-acting GnRH antagonist (Antarelix, Europeptides, Argenteuil, France) in 20µl 5% mannitol. The male rats receiving the combined treatment of DES and GnRH antagonist were injected *s.c.* on days 2 and 6 with 0.1µg DES + 10mg/kg Antarelix in 20µl 5%mannitol. Control rats were injected s.c. with 20µl corn oil on days 2 and 6.
On the day of blood sampling, animals were anaesthetized with flurothane and blood was collected from the heart into a heparinized syringe. Animals were subsequently killed by cervical dislocation. Plasma samples were stored at -20°C until used for LH, FSH, inhibin B and testosterone analysis.

The rats treated solely with the GnRH antagonist were either killed at day 18 (shortly after the treatment) or at day 90 (adulthood). Pituitaries were removed at day 18 and were used for immunocytochemical analysis (as detailed in Chapter 2). The number of immunopositive LH and FSH pituitary gonadotroph cells was counted for both control rats and rats treated neonatally with the GnRH antagonist. For the GnRH antagonist treated rats that were kept until adulthood, blood samples were collected at day 90 and assayed for LH, FSH, inhibin B and testosterone concentrations. The adult pituitaries were collected and stored in liquid nitrogen for subsequent Real-Time PCR analysis of mRNA for LH and FSH. The animals testes were removed, weighed and immersion-fixed in Bouin's for 6 hours at room temperature before being processed in an automated processor (as detailed in Chapter 4). These tissue sections were used for cell quantification of the number of Leydig and Sertoli cells per testis.

Blood was sampled at day 90 from male rats treated neonatally with a combination of both DES and Antarelix and basal levels of LH, FSH, inhibin B and testosterone were assayed by RIA (LH, FSH) or ELISA (inhibin B, testosterone). Approximately 4 weeks later, these rats were injected *s.c.* with 100µg GnRH in 200µl sterile Phosphate Buffered Saline. Thirty minutes later, blood samples were collected from these animals and stored at -20°C until they were assayed for LH, FSH, inhibin B and testosterone. Three weeks after the GnRH injections, the rats were killed by suffocation with CO_2 followed by cervical dislocation, after which the pituitaries were removed for primary pituitary cell culture.

Primary pituitary cells were cultured as described in detail in Chapter 4 of this thesis. Cells were plated out and were left overnight to attach to the 12-well plates and stabilize. GnRH, activin or GnRH and activin treatments were administered to the primary pituitary cells, as described in Chapter 4. Growth media was collected from these cells and was subsequently assayed for LH and FSH.

5.3 LH concentrations for male rats treated with the GnRH antagonist Antarelix

For adult rats treated neonatally with the GnRH antagonist Antarelix, there was no significant change in plasma LH ($p=0.179$) levels compared to those in control rats (Figure 5.3). However there was a greater average level of circulating LH in the Antarelix-treated rats.

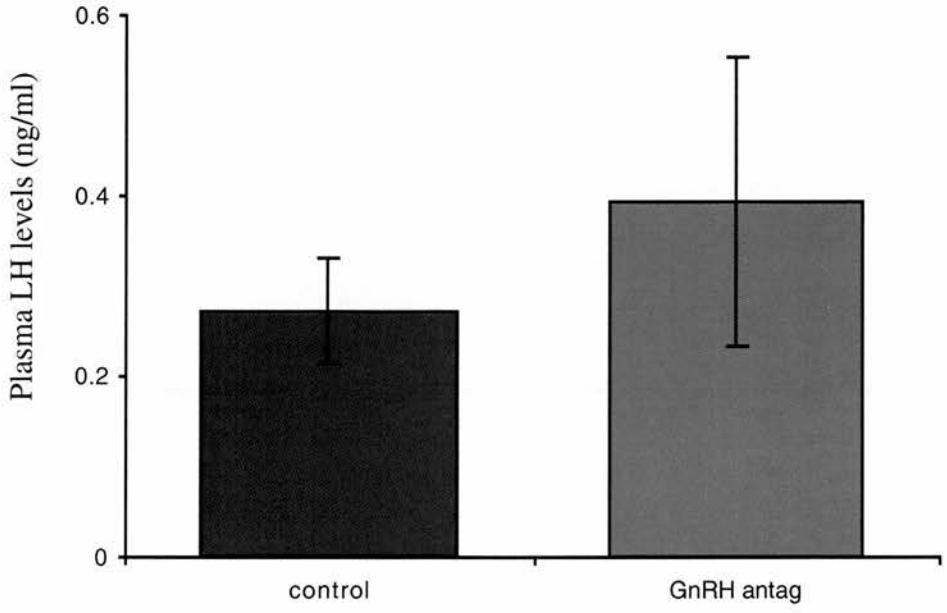


Figure 5.3: Mean basal LH levels for adult male rats treated neonatally with a GnRH antagonist. Values are means ± SEM. For control n=4, for GnRH antagonist n=3.

5.3.1 FSH concentrations for male rats treated with the GnRH antagonist Antarelix

Plasma levels of FSH were measured in animals sampled in adulthood (Figure 5.3.1). FSH levels were significantly elevated (p=0.01) for the GnRH antagonist treated animals, compared to the control rat plasma FSH levels.

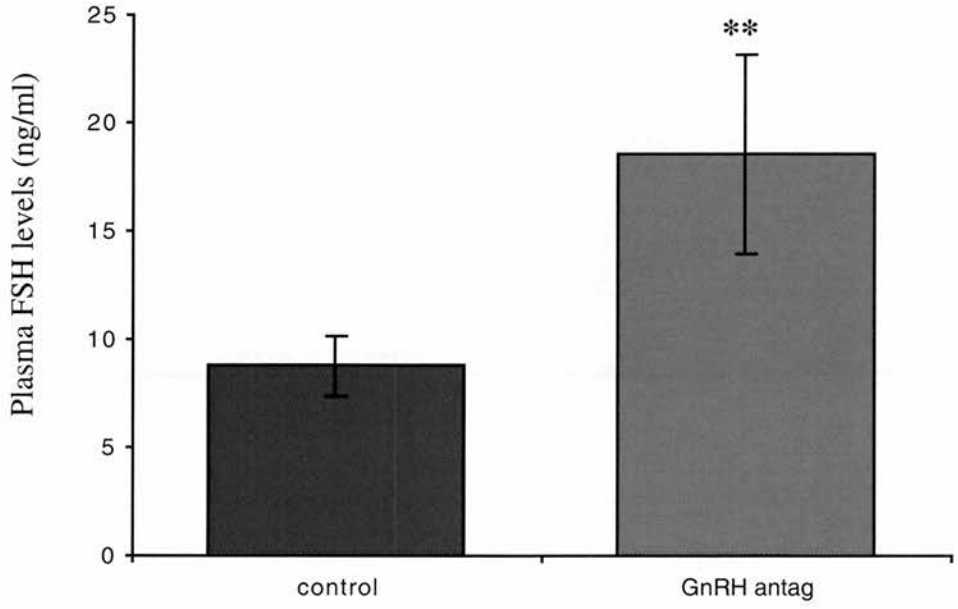


Figure 5.3.1: Mean basal FSH levels for adult male rats treated neonatally with a GnRH antagonist. Values are means ± SEM. For control n=4, for GnRH antagonist n=3. ** p<0.01, in comparison to respective controls.

5.3.2 Inhibin B concentrations for male rats treated with the GnRH antagonist Antarelix

Plasma Inhibin B concentrations were measured in the adult animals treated neonatally with the GnRH antagonist Antarelix (Figure 5.3.2). There was no significant difference in plasma inhibin B levels compared to control animals (p=0.08), although inhibin B levels tended to be lower in GnRH antagonist treated rats. As inhibin B is a negative regulator of FSH and since FSH levels for these rats were significantly elevated (Figure 5.3.1), it would have been expected that inhibin B levels would have shown a mirror image of FSH levels.

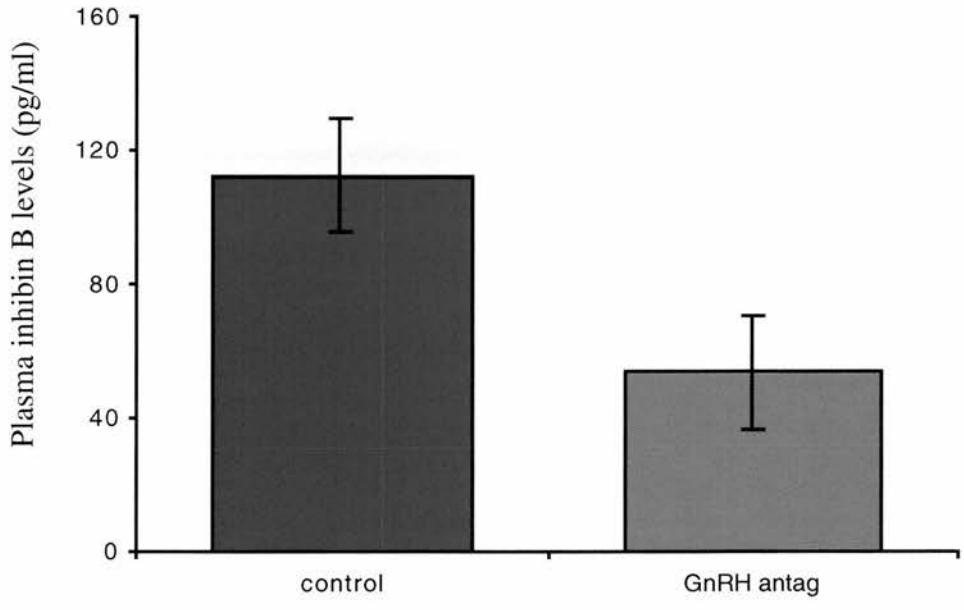


Figure 5.3.2: Mean basal inhibin B levels for adult male rats treated neonatally with a GnRH antagonist. Values are means ± SEM. For control n=4, for GnRH antagonist n=3.

5.3.3 Testosterone concentrations for male rats treated with the GnRH antagonist Antarelix

There was no significant difference in testosterone levels for adult rats treated neonatally with Antarelix compared to controls (p=0.227), although the average testosterone concentration was lower in the GnRH antagonist treated rats. This corresponds with the LH levels measured in these rats (Figure 5.3), as there was no significant difference in the LH levels compared to control rats.

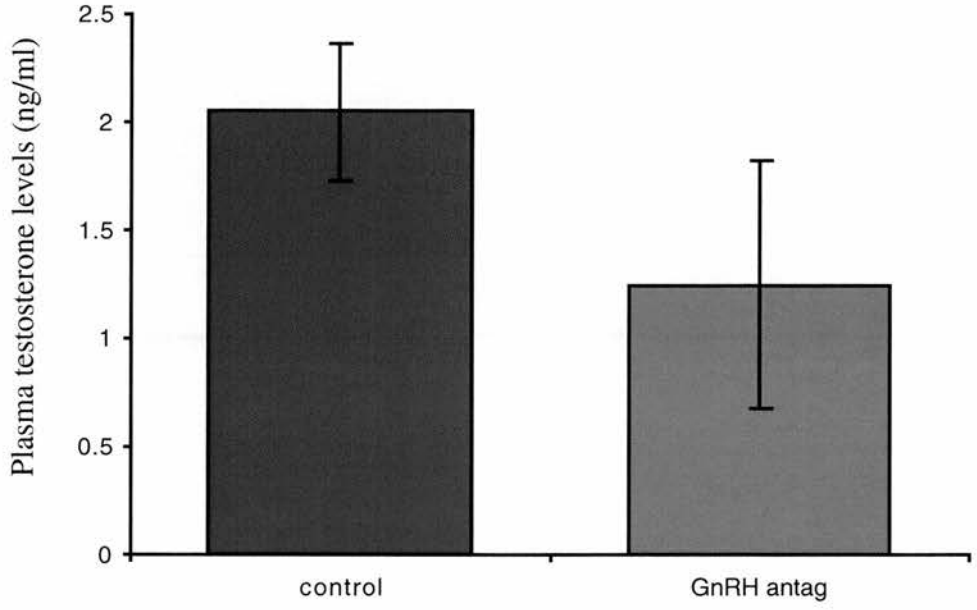


Figure 5.3.3: Mean testosterone levels for adult male rats treated neonatally with a GnRH antagonist. Values are means ± SEM. For control n=4, for GnRH antagonist n=3.

5.3.4 LH and FSH mRNA levels for adult rats treated neonatally with Antarelix

Using validated Real-Time PCR analysis, mRNA levels for LH and FSH in the pituitary were determined for adult rats treated neonatally with the GnRH antagonist. There was a significant increase in both FSH (p=0.02) and LH (p=0.0006) mRNA levels compared to adult controls, with the mRNA for LH showing the greatest elevation (Figures 5.3.4a and 5.3.4b).

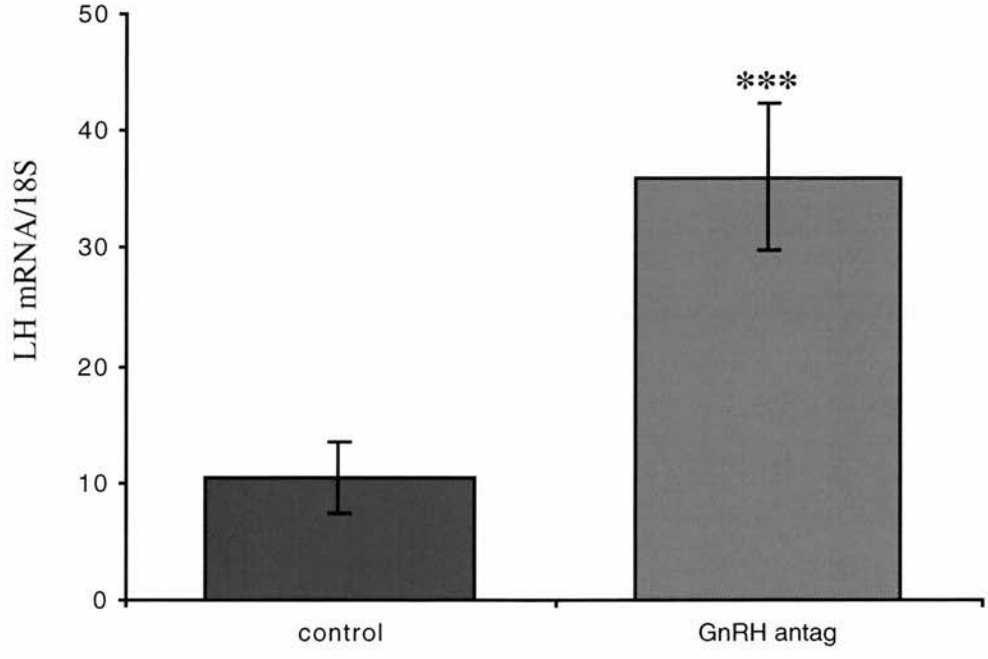


Figure 5.3.4a: mRNA levels for LH for adult male rats treated neonatally with a GnRH antagonist. There was a significant elevation in LH mRNA for the GnRH antagonist treated rats (p=0.0006). Values are ± SEM. For control n=8, for GnRH antagonist n=3. *** p<0.001, in comparison to respective controls.

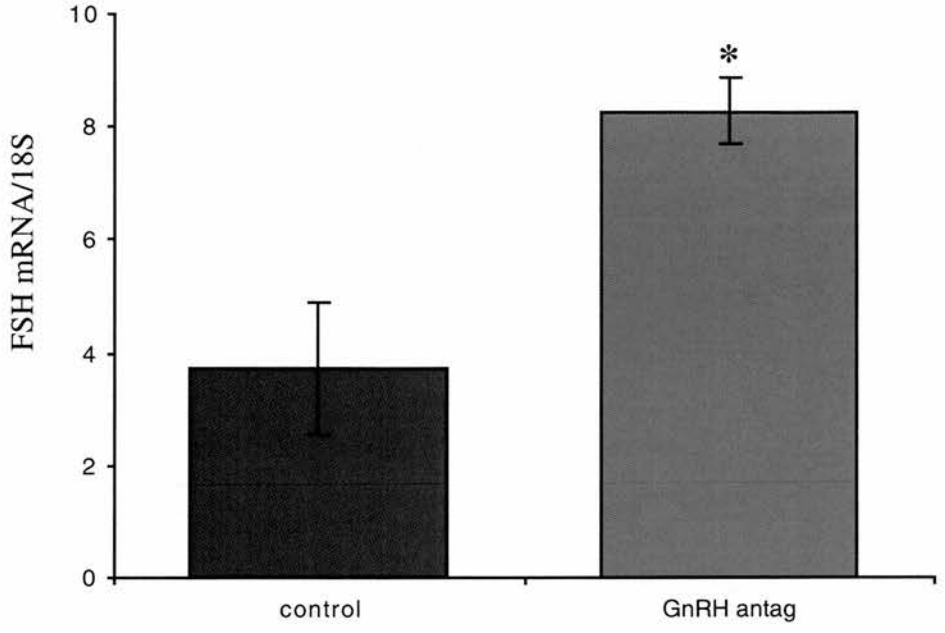


Figure 5.3.4b: mRNA levels for FSH for adult male rats treated neonatally with a GnRH antagonist. Values are means ± SEM. For control n=8, for GnRH antagonist n=3. * $p<0.05$, in comparison to respective controls.

Unfortunately, there were no adult GnRH antagonist treated pituitaries available for immunocytochemical analysis and enumeration of LH and FSH immunopositive cells. However, there were GnRH antagonist treated pituitaries available that were collected shortly after the treatment period, on day 18. These pituitaries were immunostained for LH and FSH (as described in Chapter 2) and the number of LH and FSH immunopositive cells in each pituitary was counted.

5.3.5 Percentage of LH and FSH immunopositive cells in 18 day old rats treated with Antarelix

As shown in Figures 5.3.5a and b, when rats were treated neonatally with the GnRH antagonist Antarelix, there was no significant effect on the percentage of LH ($p=0.546$) and FSH ($p=0.095$) immunopositive cells in the anterior pituitary gland. However, there was a change in the ratio of LH and FSH immunopositive cells between controls and Antarelix treated rats, with the LH:FSH ratio for control rats (ratio=1.6:1) being significantly lower than for the treated rats (ratio=3:1, $p<0.05$).

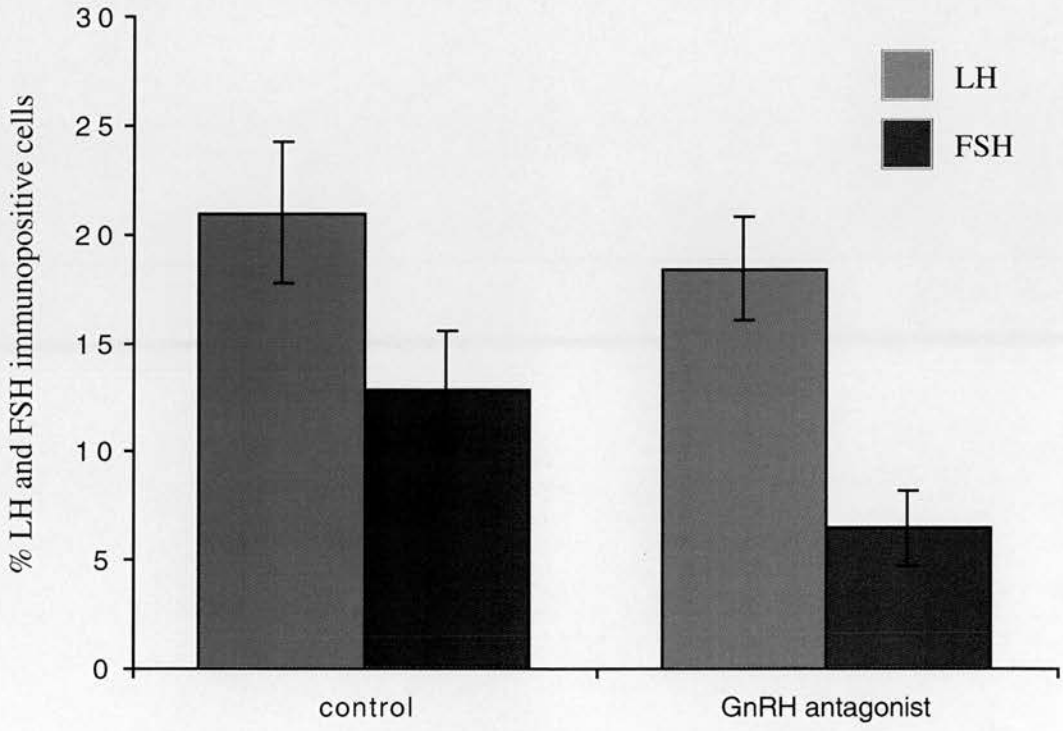


Figure 5.3.5a: Comparison of number of LH and FSH immunopositive gonadotroph cells at 18 days of age in control rats and rats treated neonatally with the GnRH antagonist Antarelix. Values are means ± SEM. For control n=6, treated n=5.

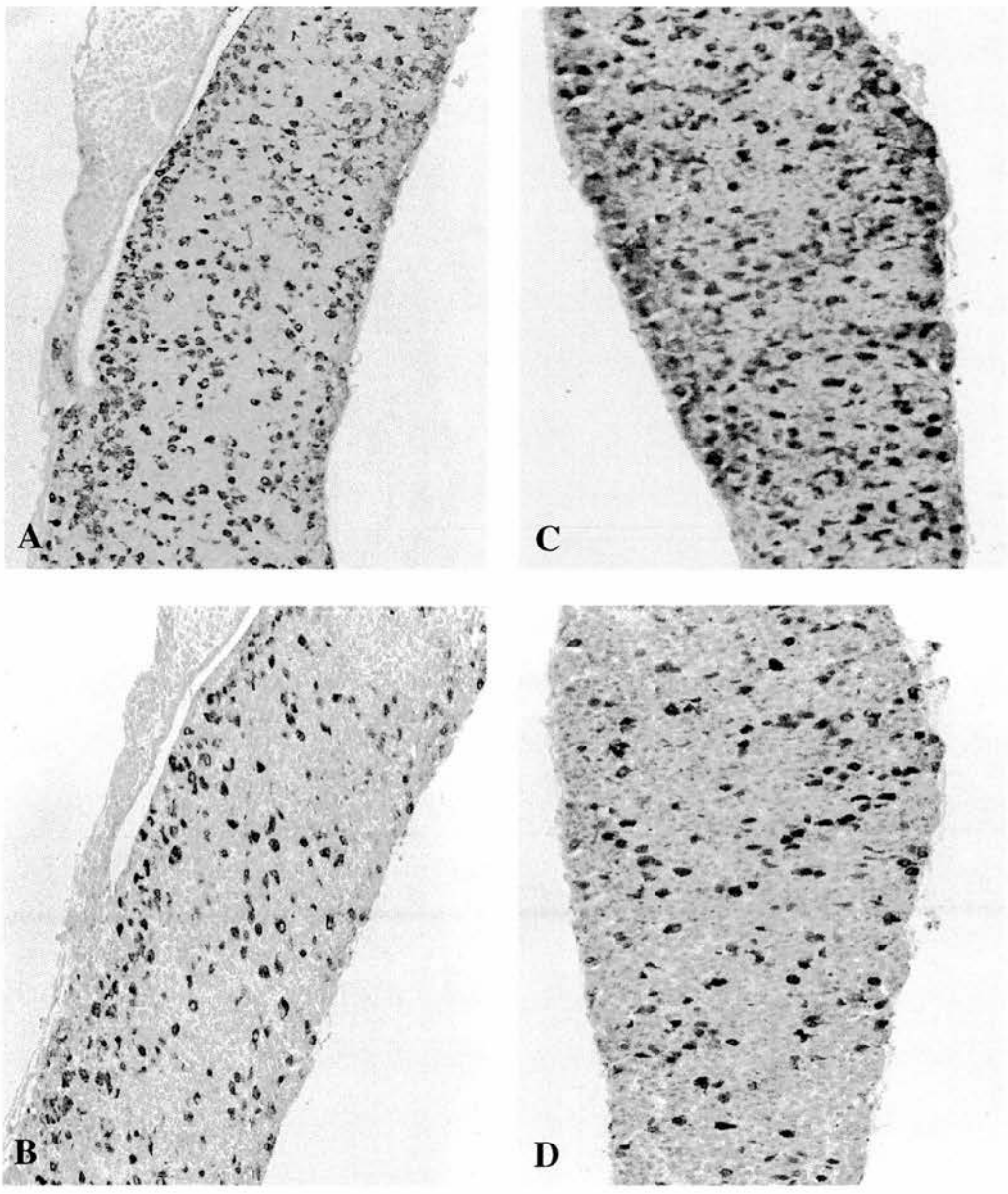


Figure 5.3.5b: Immunocytochemical staining for LH and FSH immunopositive pituitary gonadotrophs at 18 days of age, in control male rats and male rats treated neonatally with the GnRH antagonist Antarelix. A=LH immunopositive cells in control rats, B= FSH staining in control rats, C=LH immunopositive cells in GnRH antagonist treated rats and D= FSH staining in GnRH antagonist treated rats.

5.3.6 Summary of the effects of neonatal GnRH antagonist treatment on hypothalamic-pituitary-testicular axis in adult male rats

To summarise, neonatal treatment with the long-acting GnRH antagonist Anatrelix caused a marked elevation in plasma FSH levels, a marked reduction in corresponding inhibin B levels, and a significant elevation in mRNA levels for LH and FSH in the anterior pituitary. As demonstrated below in Table 5.3.6, there was also a significant reduction in testis weight and in the number of Sertoli cells per testis. In contrast to the effects on FSH we observed however no significant GnRH antagonist-induced effects upon the number of Leydig cells per adult testis, adult plasma LH and testosterone concentrations, although testosterone levels tended to be lower for GnRH antagonist treated rats. Unfortunately no adult pituitaries were available for enumeration of LH and FSH immunopositive cells due to a shortage in animals, but, at 18 days of age there was found to be no significant effect of GnRH antagonist treatment on the number of LH and FSH immunopositive cells in the pituitary.

Parameter	Controls	GnRH antagonist	p
LH (ng/ml)	0.35 ± 0.03	0.39 ± 0.07	
FSH (ng/ml)	8.78 ± 1.38	18.5 ± 4.6	p<0.01
Inhibin B (pg/ml)	112 ± 16.7	57 ± 8.3	
Testosterone (ng/ml)	2.05 ± 0.31	1.25 ± 0.58	
mRNA LH	10.4 ± 3.10	36.0 ± 6.16	p<0.001
mRNA FSH	3.71 ± 1.17	8.28 ± 0.59	p<0.05
% LH cells day 18	21.02 ± 3.2	18.4 ± 2.7	
% FSH cells day 18	12.82 ± 2.4	6.51 ± 1.73	
Testis weight (mg)	2000 ± 85	1000 ± 96	p<0.001
Leydig cells/testis (10^6)	32.3 ± 6.6	26.0 ± 3.6	
Sertoli cells/testis (10^6)	45 ± 7.7	25 ± 3.6	p<0.001

Table 5.3.6: Summary of GnRH antagonist effects. Values are the means ± SEM. Testis data were obtained from Atanassova *et al.*, 1999.

5.4.1 Effect of GnRH antagonist + 0.1μg DES treatment on plasma LH levels before and after a GnRH challenge in adult rats

There was no significant change observed in adult basal plasma LH levels between control rats and rats treated neonatally with a GnRH antagonist + 0.1μg DES (Figure 5.4.1a). Therefore neonatal treatment with a GnRH antagonist combined with 0.1μg of DES did not have long lasting effects on plasma LH concentrations in the adult animals.

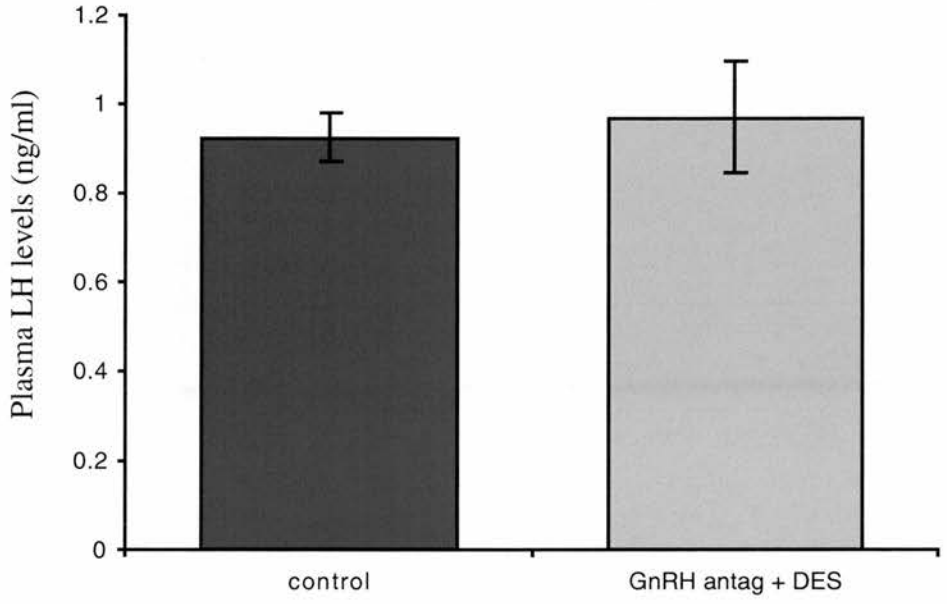


Figure 5.4.1a: Mean basal LH concentrations in adult male rats treated neonatally with GnRH antagonist Antarelix + 0.1μg DES. Values are means ± SEM. For controls n=6, for GnRH antagonist + 0.1μg DES n=3.

Plasma LH levels were measured for the animals in this specific study, after a 30 minute 100ng GnRH challenge (Figure 5.4.1b). There was found to be a significant increase in plasma LH levels for both control rats ($p<0.001$) and GnRH antagonist + 0.1µg DES ($p<0.05$) treated rats. This corresponds positively with the literature reviewed in Chapter 1, in that LH secretion is under direct control of GnRH. This demonstrates that the GnRH antagonist + 0.1µg DES treatment does not greatly affect the pituitary gonadotrophs' ability to respond to a GnRH pulse, however it must be noted that the GnRH antagonist + 0.1µg DES treated rats showed a greater magnitude of response to GnRH than controls.

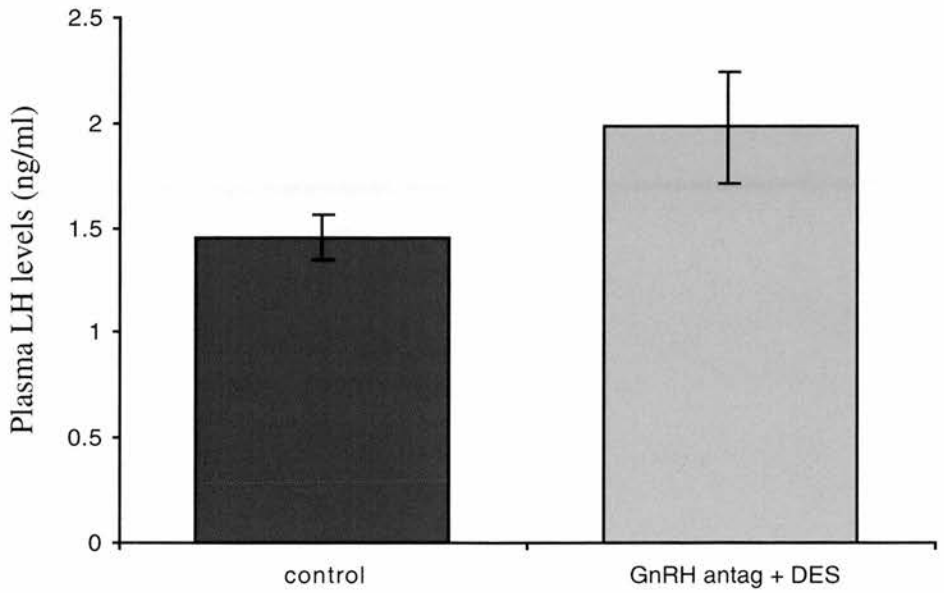


Figure 5.4.1b: Mean LH concentrations after a 30 minute GnRH challenge in adult male rats treated neonatally with the GnRH antagonist Antarelix + 0.1µg DES. Values are means ± SEM. For controls n=6, for GnRH antagonist + 0.1µg DES n=3.

5.4.2 Effect of GnRH antagonist + 0.1μg DES treatment on plasma FSH levels before and after a GnRH challenge in adult rats

When basal plasma FSH levels were measured for adult rats treated neonatally with GnRH antagonist + 0.1μg DES (Figure 5.4.2a), there was found to be no significant change in FSH concentrations. This would tend to suggest that neonatal treatment with GnRH antagonist + 0.1μg DES had no permanent ('life-long') significant effects on circulating FSH levels.

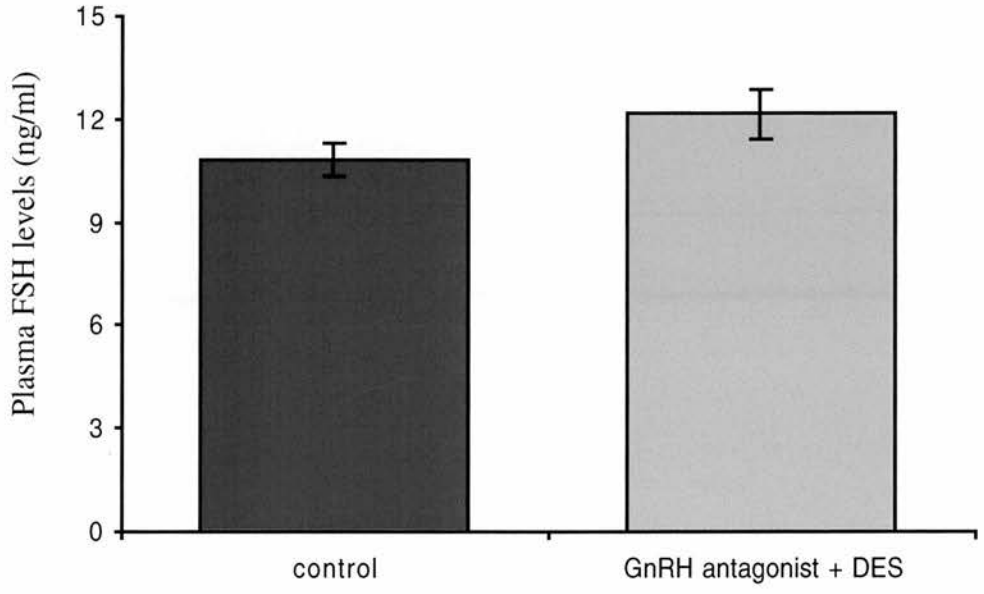


Figure 5.4.2a: Mean basal FSH concentrations in adult male rats treated neonatally with GnRH antagonist Antarelix + 0.1μg DES. Values are means ± SEM. For controls n=6, for GnRH antagonist + 0.1μg DES n=3.

However, when FSH levels were measured for these rats after a 30 minute GnRH challenge (Figure 5.4.2b), there was found to be no significant change in plasma FSH levels in response to GnRH. This finding is in accordance with the literature reviewed in Chapter 1, in that FSH secretion is not primarily under direct control of hypothalamic GnRH. Plasma FSH levels after the GnRH challenge still showed the same basic pattern however, in that there was no significant difference in FSH levels between control and treated rats.

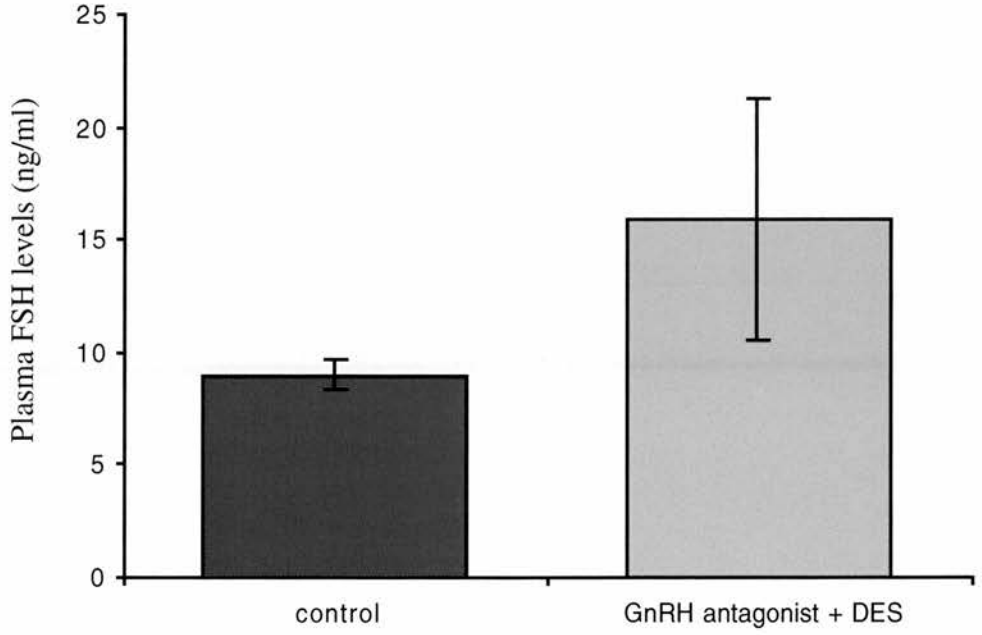


Figure 5.4.2b: Mean FSH concentrations after a 30 minute GnRH challenge, in adult male rats treated neonatally with the GnRH antagonist Antarelix + 0.1μg DES. Values are means ± SEM. For controls n=6, for GnRH antagonist + 0.1μg DES n=3.

5.4.3 Effect of GnRH antagonist + 0.1μg DES treatment on plasma inhibin B levels before and after a GnRH challenge in adult rats

When we assayed plasma inhibin B levels we noted no significant effect on basal plasma levels for adult rats treated neonatally with GnRH antagonist + 0.1μg DES. This observation coherently relates with the plasma FSH concentrations measured for these animals (Figure 5.4.2.a), as there was no significant GnRH antagonist + 0.1μg DES effect on circulating FSH levels.

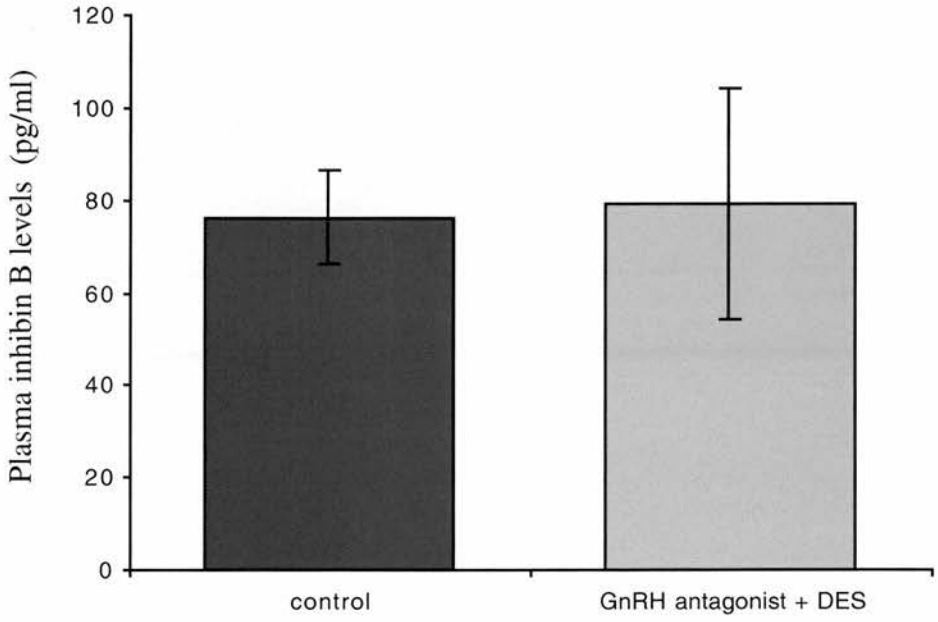


Figure 5.4.3a: Mean basal inhibin B concentrations in adult male rats treated neonatally with GnRH antagonist Antarelix + 0.1μg DES. Values are means ± SEM. For controls n=6, for GnRH antagonist + 0.1μg DES n=3.

After a 30 minute 100ng GnRH challenge, there was a small increase in inhibin B levels for both control (29% increase) and GnRH antagonist + DES (30 % increase) treated rats. However, this change was small and not statistically significant.

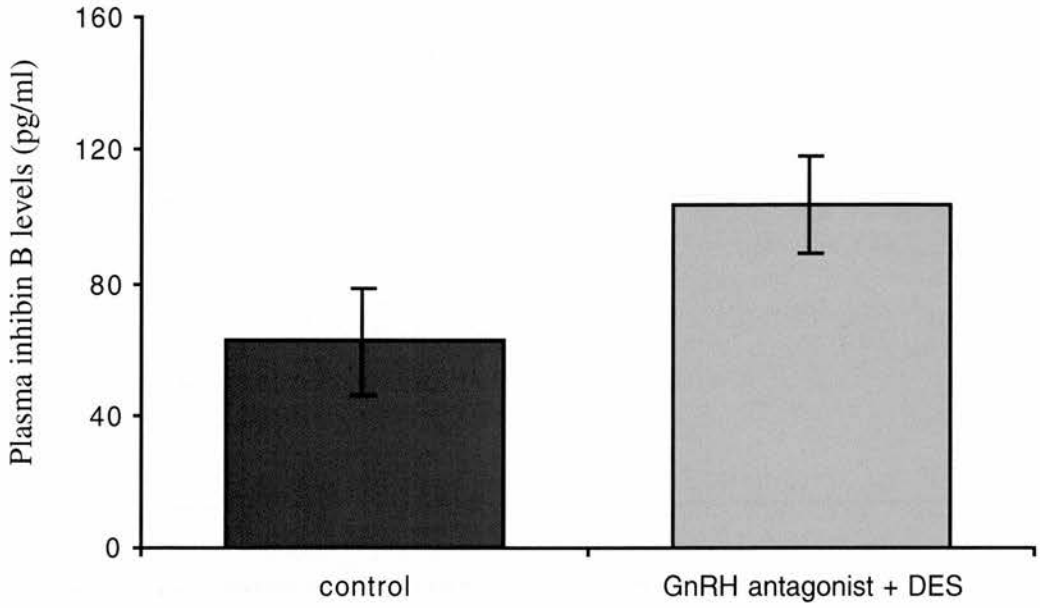


Figure 5.4.3b: Mean inhibin B concentrations after a 30 minute GnRH challenge, in adult male rats treated neonatally with the GnRH antagonist Antarelix + 0.1µg DES. Values are means ± SEM. For controls n=6, for GnRH antagonist + 0.1µg DES n=3.

5.4.4 Effect of GnRH antagonist + 0.1µg DES treatment on plasma testosterone levels before and after a GnRH challenge in adult rats

When the plasma testosterone levels in the neonatally treated rats were assayed we found that there was a significant decrease ($p<0.001$) in plasma testosterone concentrations for the adult rats treated with GnRH antagonist + 0.1µg DES (Figure 5.4.4a) compared to control rats. This demonstrates that neonatal GnRH antagonist + 0.1µg DES treatment did have a permanent ('life-long') detrimental effect on circulating testosterone levels.

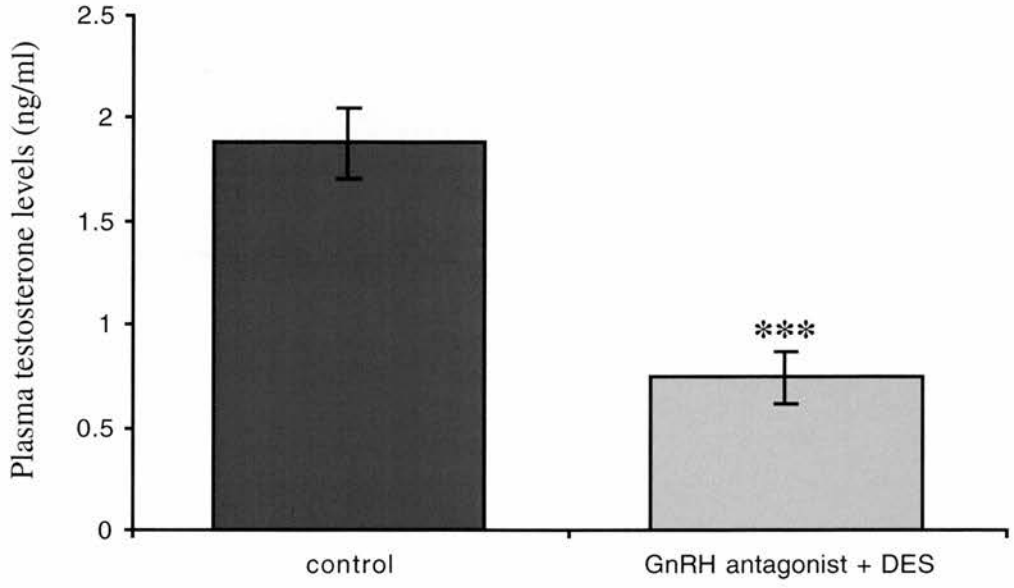


Figure 5.4.4a: Mean basal testosterone concentrations in adult male rats treated neonatally with GnRH antagonist Antarelix + 0.1µg DES. Values are means ± SEM. For controls n=6, for GnRH antagonist + 0.1µg DES n=3. *** $p<0.001$, in comparison to respective controls.

As shown in Figure 5.4.4b, plasma testosterone levels were elevated markedly in response to a 30 minute GnRH challenge. The GnRH antagonist + 0.1µg DES treated animals showed a greater rise in plasma testosterone levels (527 % increase, $p<0.001$) than control animals (44% increase). This shows that neonatal GnRH antagonist + 0.1µg DES treatment significantly increased the capacity of acute GnRH administration to mediate the release of testosterone in the adult rat.

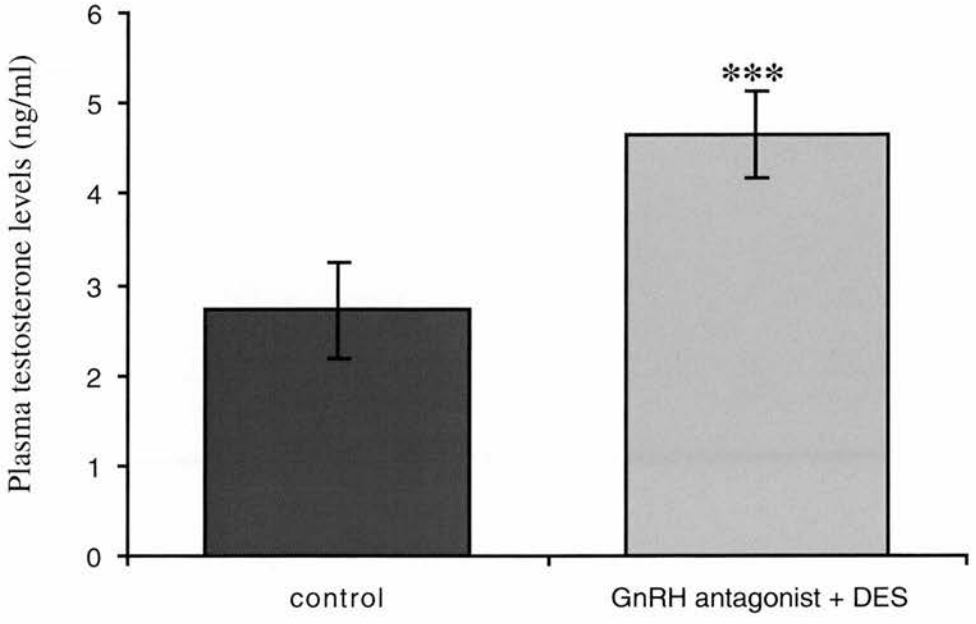


Figure 5.4.4b: Mean testosterone concentrations after a GnRH challenge in adult male rats treated neonatally with GnRH antagonist Antarelix + 0.1µg DES. Values are means ± SEM. For controls n=6, for GnRH antagonist + 0.1µg DES n=3. *** $p<0.001$, in comparison to respective controls.

5.4.5 Summary of effects of GnRH antagonist + DES treatment on adult plasma hormone levels

To summarise the pertinent results from this section we found that neonatal treatment with GnRH antagonist and 0.1μg of DES caused a significant and permanent decrease in plasma testosterone concentrations. However, this treatment regime had no significant or permanent effects on circulating levels of FSH, LH, or inhibin B. Generally, GnRH antagonist + 0.1μg DES treatment caused a greater magnitude of response to a 30 minute 100ng GnRH pulse, with plasma LH (104% increase) levels and plasma testosterone (527% increase) levels showing the greatest GnRH-induced increases.

Hormone measured	Control		GnRH antagonist + 0.1μg DES	
	Basal concentration	Concentration after a GnRH pulse	Basal concentration	Concentration after a GnRH pulse
FSH (ng/ml)	10.76 ± 0.49	9.02 ± 0.67 (16% ↓)	12.14 ± 0.72	15.88 ± 5.36 (30% ↑)
LH (ng/ml)	0.93 ± 0.06	1.45 ± 0.11 (55% ↑) p<0.001	0.97 ± 0.13	1.98 ± 0.27 (104% ↑) p<0.05
Inhibin B (pg/ml)	76.2 ± 10.04	62.5 ± 9.4 (18% ↓)	79.1 ± 24.8	103.5 ± 14.3 (30% ↑)
Testosterone (ng/ml)	1.88 ± 0.17	2.71 ± 0.53 (44% ↑)	0.74 ± 0.13	4.64 ± 0.48 (527% ↑) p<0.001

Table 5.4.5: Mean concentrations of FSH, inhibin B, LH and testosterone before and after a 30 minute 100ng GnRH pulse. Values are the means ± SEM.

5.4.6 LH and FSH levels in primary pituitary cells from rats treated with GnRH antagonist +0.1μg DES neonatally

The pituitary organs from control and GnRH antagonist + 0.1μg DES treated animals were removed and cultured in 12-well plates. The primary cultured cells were subsequently subjected to hormonal treatments as described in detail in Chapter 4. Briefly, the pituitary cells were allowed to attach overnight to the artificial cell matrix (Matrigel) before any hormonal culture addition was made. Cells were maintained in fetal calf serum-containing Dulbecco's modified Eagles medium-F12, at 37°C in a humidified incubator in a 5% CO_2 atmosphere. After overnight culture, medium was removed and replaced with either fresh DMEM-F12 medium (controls), medium containing 20ng activin, medium containing 10nM GnRH (30 minutes) or medium containing 20ng activin + 10nM GnRH (30 minutes). The specific medium was then removed after 30 minutes and was replaced with either DMEM-F12, or with DMEM-F12 + activin for the activin treated cells. At the end of the day, the medium was collected from each well and stored at -20°C. LH and FSH concentrations in the culture media were assayed before and after the GnRH pulse for both control pituitary cells and cells cultured from adult animals treated neonatally with GnRH antagonist + 0.1μg DES.

There was a significant decrease ($p<0.001$) in LH concentrations observed in the culture media from rats treated neonatally with GnRH antagonist + DES (Figure 5.4.6a) compared to controls. Neonatal DES treatment on its own had no significant effect on LH levels in culture media (Figure 4.4.4c, Chapter 4). This suggests that this significant suppression of LH levels was either due to a GnRH antagonist effect, or that GnRH had 'compensated' for a DES effect in the DES treated animals and that this effect only became apparent when the functional effects of GnRH release was blocked by an antagonist. The GnRH antagonist + DES treatment had no significant effects on FSH levels in the culture media (Figure 5.4.6b).

Ideally, pituitary cells should also have been cultured from animals treated neonatally with GnRH antagonist on its own. However, there was a shortage of available animals for treatment and hence this treatment group could not be included in the study.

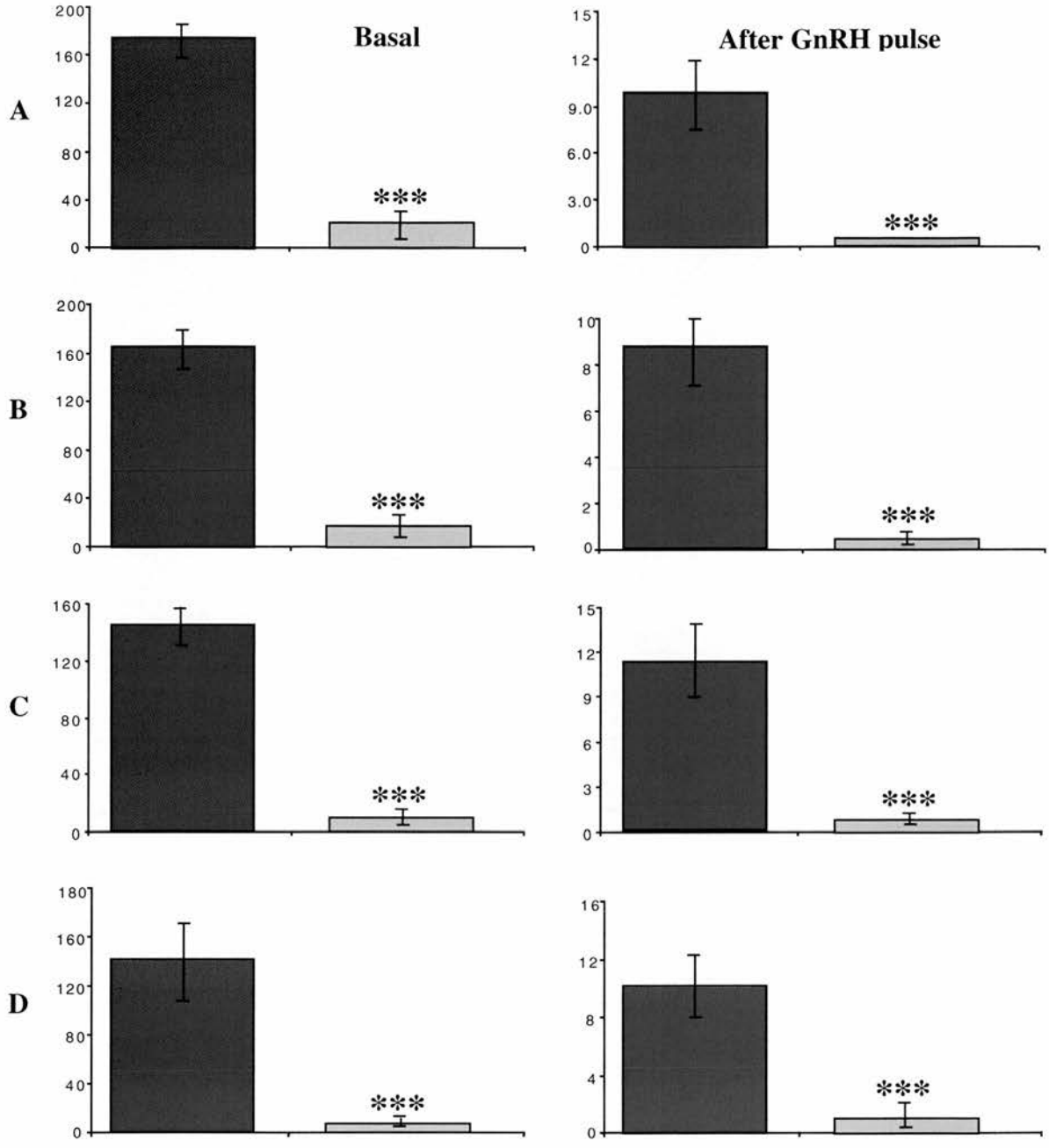


Figure 5.4.6a: LH levels in male primary pituitary cells before and after a 30 minute 10nM GnRH challenge. Values are means ± SEM. A=control, B= 20ng activin, C=10 nM GnRH, D= 20 ng activin + 10 nM GnRH. Red bars are controls and yellow bars are GnRH antagonist + 0.1µg DES treated rats. *** $p<0.001$, in comparison to respective controls. Units are ng/ml.

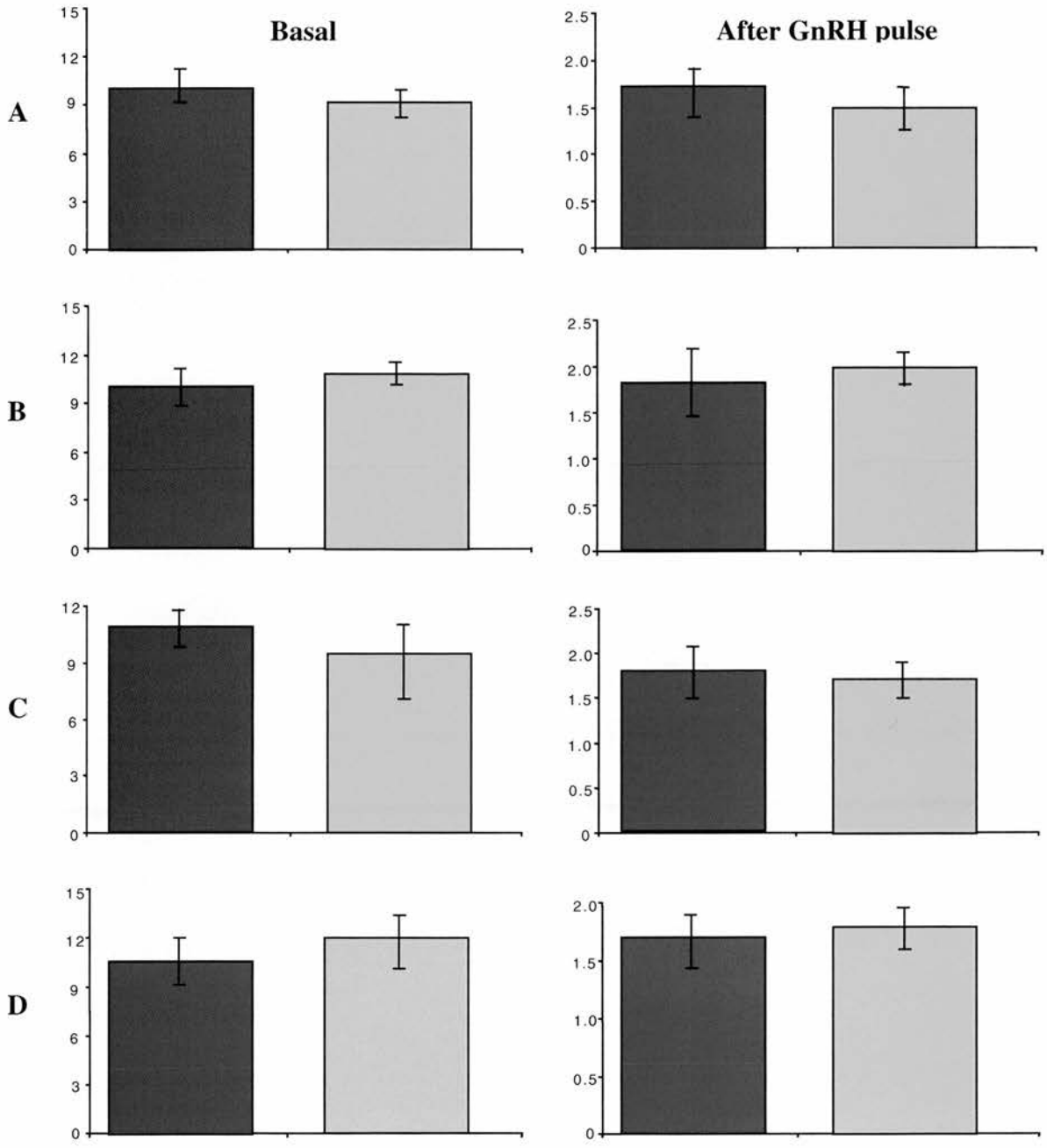


Figure 5.4.6b: FSH levels in male primary pituitary cells before and after a 30 minute 10nM GnRH challenge. Values are means ± SEM. A=control, B= 20ng activin, C=10 nM GnRH, D= 20 ng activin + 10 nM GnRH. Red bars are controls and yellow bars are GnRH antagonist + 0.1µg DES treated rats. Units are ng/ml.

5.5 Discussion

Treatment of neonatal male rats with a GnRH antagonist on its own resulted in a significant elevation of plasma FSH levels ($P<0.01$), with no significant change in plasma LH levels. Circulating inhibin B and testosterone levels tended to be lower for the GnRH antagonist treated animals, however this change was not statistically significant. Interestingly, pituitary mRNA levels for LH and FSH were found to be elevated significantly (LH $p<0.001$ and FSH $p<0.05$). This may have occurred due to an autoregulatory increase in the sensitivity of the hypothalamic-pituitary axis in an attempt to compensate for the reduced GnRH input in the presence of Antarelix. Therefore as the GnRH receptor antagonist would reduce the ability of GnRH from the hypothalamus to mediate gonadotropin release from the gonadotroph cells the cells were responding to this perturbation by elevating their capacity to release gonadotropins by up-regulating the transcription of LH and FSH. Thus despite a diminished GnRH input to the gonadotrophs any stimulatory input, extant in the presence of Antarelix, would have an abnormally large store of gonadotropins to release into the circulation. A compensatory elevation in the transmitter levels, in this case LH/FSH, in response to a perturbation in the hypothalamic-pituitary gonadal axis with Antarelix, is reminiscent of the effects of other scenarios in which neurotransmitter, *e.g.* serotonin, functions are modulated in the central nervous system (Eide and Hole, 1993). Unfortunately there were no adult pituitaries available of neonatally GnRH antagonist treated animals for immunocytochemical analysis. However, there were pituitaries available for analysis of 18 day old treated rats. Immunopositive staining for LH and FSH in these pituitaries showed that FSH immunostaining tended to be lower in GnRH antagonist treated rats than in control rats, however this difference was not significant. Analysis of the testes in adulthood of these animals revealed that testis weight was significantly reduced in GnRH antagonist treated rats ($P<0.001$) and that there was a significant reduction in the number of Sertoli cells per testis for the GnRH antagonist treated rats ($p<0.001$).

Therefore it would seem likely that the Antarelix blockade of the hypothalamic GnRH input to the anterior pituitary would reduce the initial effective amount of LH circulated to the testes thus resulting in a restricted pubertal gonadal development. However as discussed previously even in the face of diminished hypothalamic GnRH input to the pituitary (caused by Antarelix), the pituitary gland itself may eventually attempt to adapt to this. This may occur by an increase in the amount of gonadotrophin hormone, e.g. LH, synthesized and thus available for any subsequent release mediated by a GnRH 'breakthrough' in the presence of Antarelix. This would theoretically thus result in an eventual recuperation of the plasma levels of LH, which indeed was noted. There was found to be no significant change in the number of Leydig cells between control and GnRH antagonist treated rats. These observations correspond well with data obtained by Richard Sharpe's laboratory, which showed that neonatal GnRH antagonist resulted in elevated plasma FSH levels ($p<0.001$) and suppressed plasma inhibin B levels ($p<0.05$), but no significant changes in plasma LH and testosterone concentrations. Analysis of the testes showed that testis weight was significantly reduced in GnRH antagonist treated rats ($p<0.001$), and that the number of Sertoli cells per testis ($p<0.001$) and total germ cell volume per testis ($p<0.001$) were also significantly reduced, with no significant change in Leydig cell number per testis (Atanassova *et al.*, 1999; Sharpe *et al.*, 2003b).

Neonatal treatment of GnRH antagonist with 0.1μg DES resulted in a significant reduction of basal plasma testosterone levels ($p<0.001$) compared to control animals. However, neonatal GnRH antagonist with 0.1μg DES treatment was found to have no significant effects on basal plasma LH, FSH and inhibin B levels. Interestingly, after a 30 minute 100ng GnRH pulse, the GnRH antagonist with 0.1μg DES treated rats showed a much greater increase in plasma LH (104% increase $p<0.05$) and plasma testosterone levels (527% increase $p<0.001$) than control animals. This suggests that GnRH antagonist with 0.1μg DES treatment made the gonadotroph cells more

responsive to a GnRH challenge than gonadotrophs in control animals. There are several mechanisms by which such a heightened functional sensitivity to GnRH could occur, e.g. via an elevation in pituitary GnRH receptor expression, an increase in the LH content of each secretory vesicle or an increase in the number of secretory vesicles released during a GnRH pulse. From the data gathered it is possible to hypothesize, without any additional experiments that, in part, this effect could be mediated by the enhanced transcription of LH/FSH and therefore increased gonadotropin content and/or number of secretory vesicles.

Chapter six: effects of phytoestrogens on male marmoset and male rat pituitary function

6.1 Introduction

This chapter investigates whether phytoestrogens, in particular genistein and soy formula milk, administered in early life, can have any effects on the hypothalamic-pituitary-testicular axis, with particular emphasis on pituitary function.

Phytoestrogens are polyphenolic non-steroidal plant compounds with oestrogen-like biological activity. The oestrogenic properties of certain plants have been recognised for more than fifty years. In the mid-1940's, an infertility syndrome in sheep occurred that has been attributed to the ingestion of clover containing high levels of the isoflavones formononetin and biochanin (Adams, 1995). More recently, an increasing number of epidemiological and experimental studies have suggested that the consumption of phytoestrogen-rich diets may have protective effects on oestrogen-related conditions, such as menopausal symptoms (Baird *et al.,* 1995), and oestrogen-related diseases such as prostate (Denis *et al.,* 1999) and breast cancers (Lee *et al.,* 1991), osteoporosis (Potter *et al.,* 1998), and cardiovascular diseases (Bakhit *et al.,* 1994). However, concerns have been raised about the potential dangers of consuming high levels of these compounds, especially during early life (Abe, 1999). Consequently, phytoestrogens are currently under active investigation for their role on human health.

Isoflavones are the most studied group of phytoestrogens and are found almost exclusively in the family Leguminosae (King & Young, 1999). Soybeans are a very rich source of isoflavones and contain approximately 2 grams of isoflavones per kilogram fresh weight (Reinli & Block, 1996). However, the isoflavone content of soy products

can vary greatly between different soybean varieties and through soybean processing (Wang *et al.,* 1994). Consequently, not all soy protein sources are equal with respect to their isoflavone content and this should be taken into account when conducting and analysing experimental studies on soy.

A large number of isoflavones have been identified from plants, with daidzein (Denis *et al.,* 1999) and genistein (Lee *et al.,* 1991) as the principal isoflavones. They occur in plants as the inactive glycosides daidzin (Potter *et al.,* 1998) and genistin (Bakhit *et al.,* 1994), and their 4'-methyl ether derivatives formononetin and biochanin A. Other members of the isoflavone family are coumestans, with coumestrol (King & Young, 1999) and 4'-O-methylcoumestrol (Reinli & Block, 1996) being the principle ones. They exhibit close structural similarity to isoflavones (see Figure 6.1) (Davis *et al.,* 1999) and their main dietary sources are alfalfa, soybean and clover sprouts.

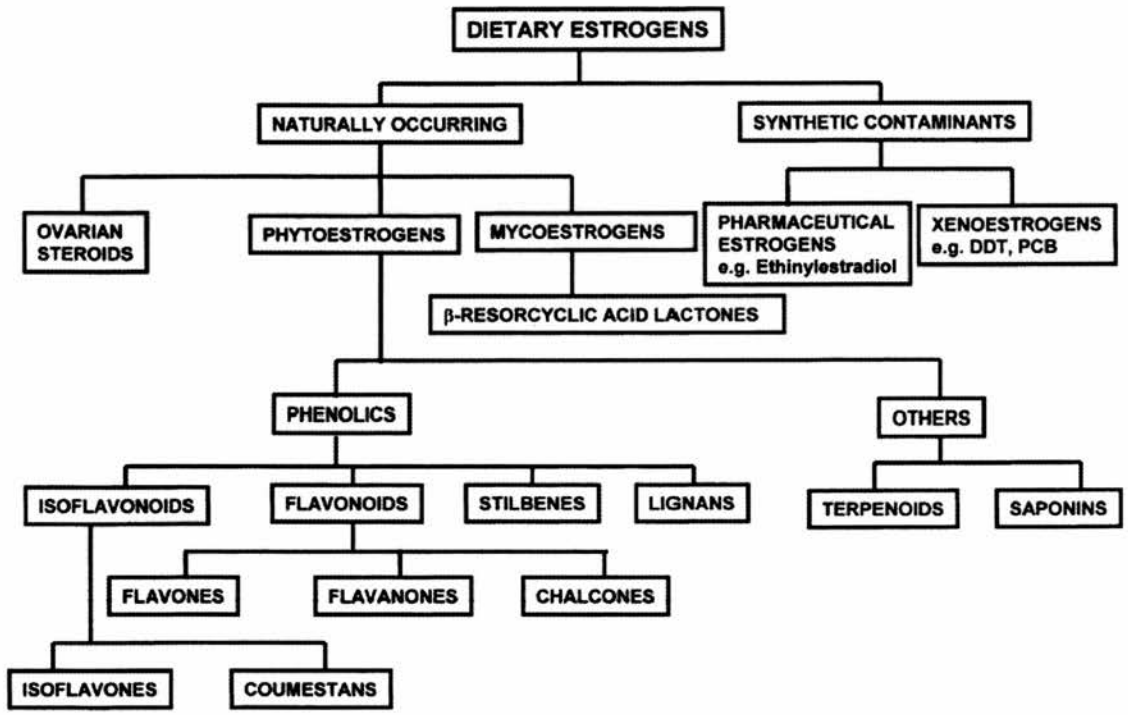


Figure 6.1: Classification of dietary oestrogens, including the phytoestrogen family. (From Cos *et al.,* 2003).

Presently there are concerns that the high isoflavanoid phytoestrogen content of soy formula milk might exert adverse effects on the developing infant (Irvine *et al.*, 1995, 1998; Setchell *et al.*, 1997,1998). Soy formula milk was initially developed for infants unable to tolerate the proteins in cow's milk-based formulas, however, soy based formulas are now used more widely and account for approximately 25% of infant formulae sold in the United States (Klein, 1998; Lonnerdal, 1994). Millions of American infants have been fed soy formula over the past three decades. Several studies so far have demonstrated that soy formula supports normal growth and development in term infants (Fomon, 1993; Businco *et al.*, 1992; Churella *et al.*, 1994; Kohler *et al.*, 1984, Graham *et al.*, 1970). When growth was studied over the first year of life, body weight gains and the body length of infants were virtually the same whether the infants were fed soy formula milk or cow's milk formula or breastfed (Lasekan *et al.*, 1999). Recently a study determining the long-term health consequences of early soy intake was reported on 811 young adults (between 20 and 34 years old) who were fed cow's milk based formula or soy formula as infants. This study by Strom et al. (Strom *et al.*, 2001) showed that there were a few statistically significant differences between the cow's milk and the soy formula groups, but no differences were found in growth, development, puberty, reproductive function, or pregnancy outcomes. However, it must be taken into account that the number of subjects was low and they were too young to determine the risk of developing any chronic diseases that occur in later life.

As no major adverse effects of feeding soy formula milk have come to light since its widespread introduction more than 30 years ago, it has been argued that adverse effects are probably unlikely (Klein, 1998). Despite numerous studies performed on rodents examining the effects of exposure to phytoestrogens during the neonatal period (Whitten and Naftolin, 1998), it is unclear whether these are relevant to humans as the route and mode of administration are generally not comparable. In rodents, phytoestrogens have

generally been injected rather than being consumed orally by pups and also, neonatal pups are relatively undeveloped when compared with neonatal humans.

Therefore, these studies were carried out on both rodents (rats) and non-human primates, (marmosets), as neonatal marmosets are developmentally comparable to neonatal humans and they could be hand-fed soy formula milk orally, whereas rats couldn't. Also, marmosets, like humans (and unlike rats), have a prolonged 'neonatal testosterone rise', when testosterone levels increase to low adult levels during the first 4 to 6 months of life, which is associated with activation of gonadotrophin secretion (Winters *et al.,* 1979; Mann and Fraser, 1996; Andersson *et al.,* 1998), Sertoli cell proliferation (Cortes *et al.,* 1987; Sharpe *et al.,* 1999) and an elevation in the secretion of inhibin B by the latter cells (Andersson *et al.,* 1998).

Two short-term studies are described in this chapter. The first study was a rat study, in which neonatal rats were injected s.c. with the phytoestrogen genistein, and pituitary function was evaluated shortly after treatment by counting the number of LH and FSH immunopositive cells and measuring plasma FSH levels. The second study was a more elaborate marmoset study where co-twin marmosets were reared on soy formula milk in early life, and pituitary function was subsequently investigated. The number of LH and FSH immunopositive cells was counted for each co-twin marmoset and the findings at the level of the pituitary were brought into context by including data from analyses of the testes of these animals, done in parallel by Richard Sharpe's laboratory.

6.1.2 Summary of analysis methods for determining the effects of feeding rats and marmosets phytoestrogens

Rat genistein study: investigation into the effects of neonatal genistein treatment on pituitary function. In this study the primary goals were:

- Immunocytochemistry for LH and FSH in the anterior pituitary at days 10, 18 and 25. Enumeration of LH and FSH immunopositive cells at days 10, 18 and 25. Measurement of plasma FSH levels at day 18.

Marmoset soy study: investigation into the effects of rearing infant marmosets soy formula milk on pituitary function. This study involved:

- Immunocytochemistry for LH and FSH in the marmoset anterior pituitary at days 35-40.

- Enumeration of LH and FSH immunopositive gonadotroph cells at days 35-40 for infant marmosets fed either cow's milk or soy formula milk.

- Measurement of bodyweight, daily formula intake and testis weight at days 35-40 for infant marmosets fed either standard cow's milk formula or soy formula milk.

- Determination of plasma testosterone levels (and relationship to soy formula intake).

- Quantification of number of Leydig and Sertoli cells per testis for control marmosets and those weaned on soy formula milk.

6.2 Outline of treatments administered

For the rat study, all-male litters of 8-12 pups were generated by cross-fostering pups on the first day of birth. They were maintained under controlled conditions at 22-23°C ambient temperature, 55-60% relative humidity, 12L:12D cycle and were supplied with a standard diet (rat and mouse breeding diet; SDS, Dundee, UK) that contains soya meal. Soya-free control animals were maintained on a soya-free diet (rat and mouse soya-free breeding diet, SDS, Dundee, UK). From days 2 to 17 rats were injected s.c. with 4mg/kg/day of genistein (Sigma, UK). Blood samples were collected from the tail vein at day 18 and stored at -20°C for subsequent LH and FSH measurements. Animals were killed either at day 10, 18 or 25 by CO_2 inhalation and cervical dislocation. The whole pituitary was removed and the right testis was dissected out and weighed. The pituitaries and testes were subsequently fixed for 5 hours in Bouin's fixative. After fixation, the tissues were transferred into 70% ethanol before being processed for 17.5 hours in an automated TP1050 processor (Leica Corp., Deerfield, IL) and embedded in paraffin wax. Sections of 5µm thickness were cut, floated onto slides coated with 2% 3-aminopropyltriethoxysilane (Sigma), and dried at 50°C overnight before being used for immunohistochemistry as described in Chapter 2.

For the marmoset study, 13 pairs of newborn male marmoset co-twins were used, as detailed in Chapter 2. Marmosets tend to show a considerable amount of between-animal variability, thus co-twins were used to minimize the between-animal variability and hence to reduce the total number of animals required to perform this study. One infant co-twin was weaned on standard cow's formula milk (SMA) and the other co-twin was fed soy formula milk (SFM). This enabled a pair-wise evaluation and hence a direct comparison could be made between co-twins. The treatment schedule is summarized in Figure 6.2. Treatments commenced on days 4 or 5 of age. Infants were separated from their mothers at set-intervals and were hand-fed with either standard cow's milk based

formula milk (SMA) or with soy formula milk (SFM) from day 4 or 5 until days 35 to 45. Infants could drink as much as they wanted to and the amount of formula milk taken up was measured and recorded. Infants were fed daily, three or four times during weekdays and once or twice at weekends. After the last feed of the day, the infants were returned to the mothers and she was allowed to breastfeed them until the following day.

Approximately half way through the study, on days 18 to 20, a 0.2ml blood sample was collected from the femoral vein of each infant. Then on days 35 to 45 the marmosets were killed via i.p. injection of an overdose of sodium pentobarbitone. Immediately after death, a blood sample was collected by cardiac puncture.

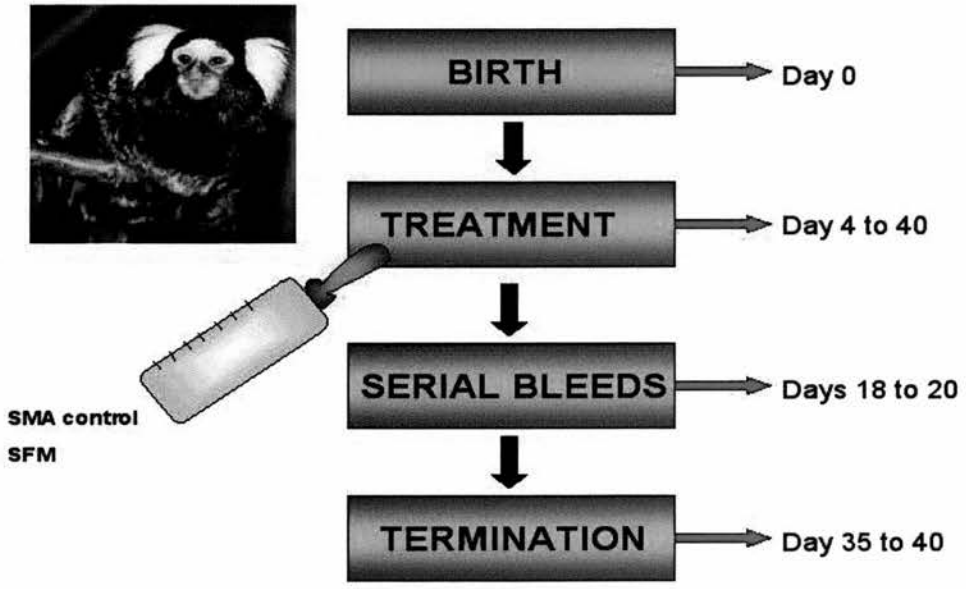


Figure 6.2: Summary of treatment schedule for infant marmosets weaned on cow's milk (SMA) or soy formula milk (SFM).

6.2.1 Specific methods

The general methods (gonadotroph immunostaining and plasma testosterone assay) used in this chapter are detailed in Chapter 2. However the specific methods for tissue collection and processing, Leydig and Sertoli cell immunostaining and enumeration, performed by Richard Sharpe's laboratory, are detailed in this section.

6.2.2 Tissue collection and processing

Testes with the epididymides attached, as well as the pituitary gland, were dissected free of connective tissue and were immersion-fixed for either 5.5 hours (left testis, pituitary gland) or 24 hours (right testis) in Bouin's fixative, after which each testis was dissected away from the epididymis and weighed. The left testis and the pituitary were processed for 17.5 hours in an automated Leica TP-1050 processor and embedded in paraffin wax. Sections of 5µm thickness were cut on a microtome and were then floated onto slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma, UK) before being dried overnight at 50°C.

6.2.3 Determination of the number of Sertoli cells per testis

After fixation, the right testis was sampled in a random systematic manner, i.e. of four transverse slices, either slices, 1 and 3 or slices 2 and 4 were sampled. These were processed through graded ethanols before infiltration with JB4 resin (TAAB, Aldermaston, UK). After polymerisation of the JB4 resin, 20µm sections were cut on a Reichart 2050 microtome using a Diatome Histoknife, mounted onto glass slides and stained with Harris' haematoxylin. Sertoli cells were then counted using the optical dissector method. An Olympus BH2 microscope fitted with an automatic stage (Prior Scientific Instruments, Cambridge, UK) and Image Proplus software (Media

Cybernetics, Silver Spring, MD) was used for the dissector method. Using a graticule with 121 points and 40x objective, 10 fields on testicular cross sections were selected randomly and subjected to standard point-counting; the number of fields examined was based on power calculations derived from Wreford (Wreford, 1995). After determination of the percentage volume per testis for each of the components counted (Sertoli cell nucleus, germ cell nuclei, seminiferous epithelium, interstitium), total volume per testis was determined by multiplying the percentage value by fixed testis weight. Sertoli cell number per testis was then calculated as described by Wreford (Wreford, 1995), using the value for mean nuclear volume derived above.

6.2.4 Determination of the number of Leydig cells per testis

Leydig cells were stained for 3β-hydroxysteroid dehydrogenase (3β-HSD), which is a marker for Leydig cells. A rabbit anti-human 3β-HSD antibody was used, which was a gift from Prof. Ian Mason (Department of Clinical Biochemistry, University of Edinburgh, UK). 5μm sections were mounted on slides coated with 3-aminopropyl triethoxy-silane (Sigma, UK) and were dried overnight at 50°C. Before incubation with primary antibodies, sections were dewaxed, rehydrated in graded ethanols, and washed in water and TBS (0.05M Tris-HCl pH 7.4 and 0.85% NaCl). All sections were blocked using normal swine serum (DAKO, High Wycombe, UK) diluted 1:5 in TBS. The primary antibody was diluted 1:1000 in normal swine serum in TBS before incubation on sections under plastic coverslips overnight at 4°C. The following day, coverslips were removed, and sections were washed twice in TBS (5 minutes each), incubated for 30 minutes with biotinylated swine anti-rabbit Ig (DAKO,UK) diluted 1:500 in TBS, then washed again in TBS (twice for 5 minutes each time). For detection of bound antibodies, sections were first incubated with Avidin-biotin complex conjugated with horseradish peroxidase for 30 minutes and washed twice in TBS (5 minutes each time). A colour reaction product was developed by incubating sections in a mixture of 0.05%

(wt/vol) 3,3'-diaminobenzidine tetrahydrochloride (Sigma, UK) in 0.05M Tris-HCl pH 7.4 and 0.01% hydrogen peroxide. After 5-15 minutes, sections were washed in water, counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene, and coverslipped using Pertex mounting medium (Cell Path, Hemel Hempstead, UK). The specificity of the antibodies was confirmed using normal rabbit serum instead of the primary antibodies, no staining was observed in these controls.

Leydig cell volume per testis was determined using the Area Fraction Probe in the Stereologer software programme (Systems Planning and Analysis Inc., Alexandria, VA, USA) and utilised an Olympus BHS microscope fitted with an automatic stage (Applied Scientific Instrumentation Inc., Eugene, OR, USA). The area fraction probe places a grid in the frame and the Object Area fraction is determined by clicking each 'X' that touches the object of interest (in this case, the cytoplasm or nucleus of 3β-HSD-positive cells). The number of frames per slide counted was 5-13 and was determined by the programme after taking into account the spacing between points, objects counted per frame and the size of the section. Frames for evaluation were selected automatically. A coefficient of error (CE) was calculated at intervals in the counting procedure and, if this exceeded the maximum acceptable value (0.15), then different settings recommended by the programme were utilized in order to obtain the optimum CE. Once completed, Stereologer automatically displays the results including area fraction and CE. The values for area fraction were then converted to absolute volumes per testis by reference to testis volume (=weight). Average nuclear diameter was then determined by measuring the diameter of 100 random nuclei of 3β-HSD-positive cells for each animal and then converting Leydig cell nuclear volume per testis to Leydig cell number per testis (Wreford, 1995).

6.3 Percentage of LH and FSH immunopositive cells in 18 day old rats reared on a soya free diet during neonatal life

At day 18, there was no significant difference in the percentage of LH (p=0.614) and FSH (p=0.612) immunopositive cells between rats reared neonatally on a soya-free diet and control rats (fed standard rat chow which contains soya). There was also no significant change in the ratio of LH to FSH immunopositive cells between controls (ratio=1.6) and soya-fed rats (ratio=1.6).

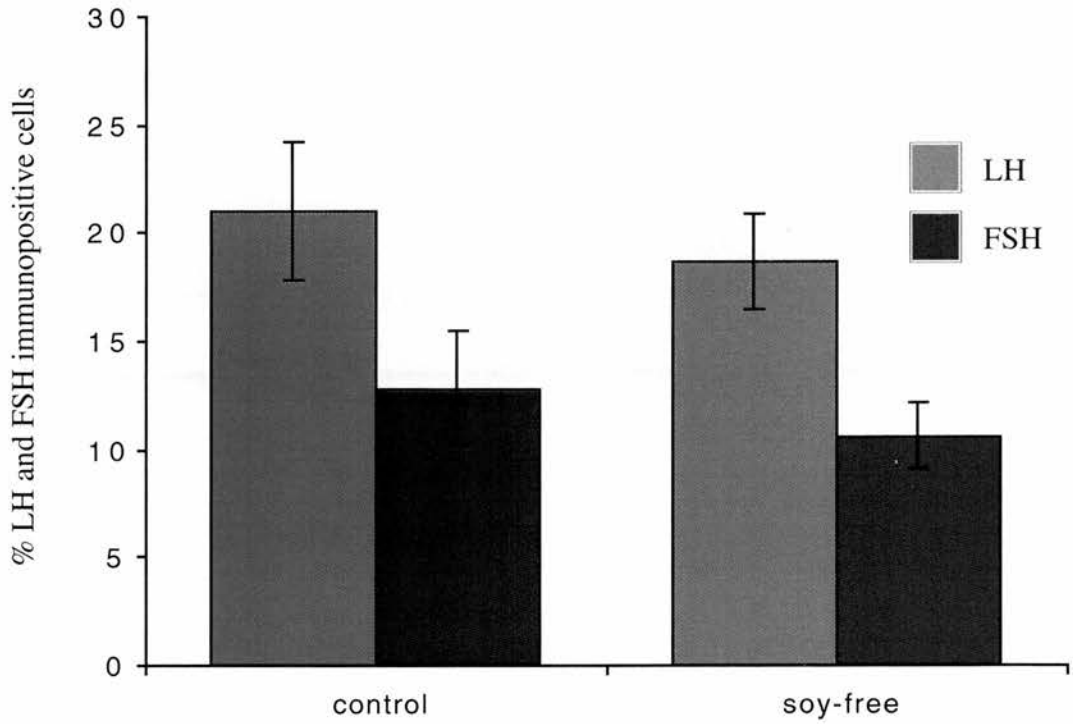


Figure 6.3: Comparison of percentage of LH and FSH immunopositive gonadotroph cells at 18 days of age in control rats and rats fed a soya-free diet in neonatal life. Values are means ± SEM. For control n=6, treated n=3.

6.3.1 Percentage of LH and FSH immunopositive cells in 10 day old rats treated neonatally with genistein

At day 10, there was no significant difference in the percentage of LH (p=0.436) and FSH (p=0.109) immunopositive cells between control and genistein treated rats. A slight change in the ratio of LH to FSH cells between controls and treated animals was observed, i.e. there was a higher ratio of LH to FSH immunopositive cells in controls (ratio=2.2) compared to treated rats (ratio=1.5). However, this difference was small and not statistically significant.

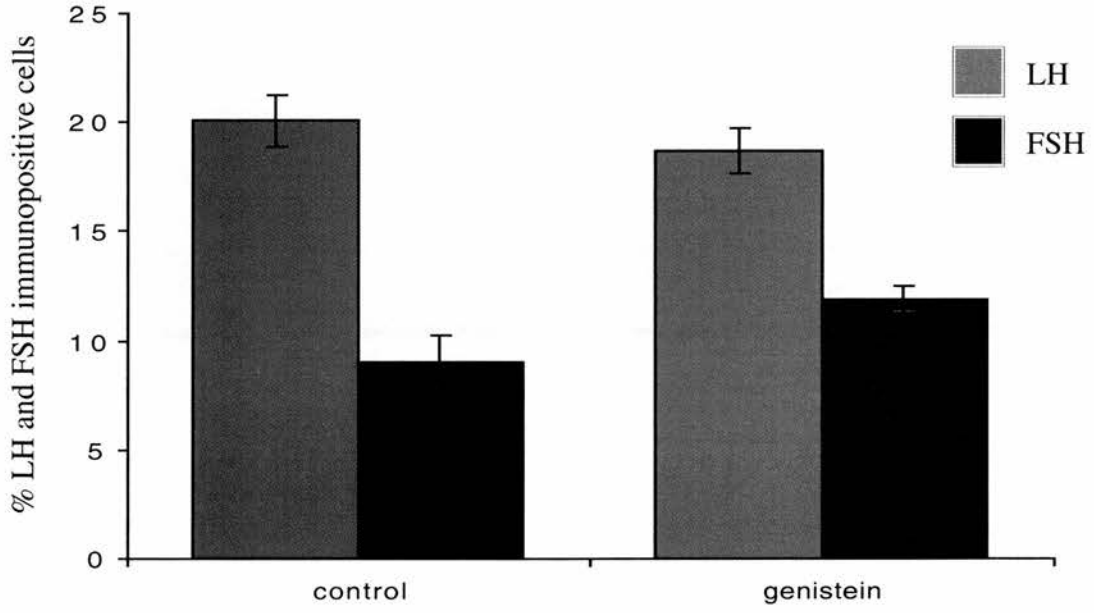


Figure 6.3.1: Comparison of number of LH and FSH immunopositive gonadotroph cells at 10 days of age in control rats and rats treated neonatally (days 2-17) with genistein. Values are means ± SEM. For control n=6, treated n=4.

6.3.2 Percentage of LH and FSH immunopositive cells in 18 day old rats treated neonatally with genistein

Similar observations were made for 18 day old rats that had been treated neonatally with genistein, as shown in Figure 6.3.2. Again, there was no significant difference in the percentage of LH (p=0.453) and FSH (p=0.746) immunopositive cells between control and treated rats. Similarly to the 10 day old rats treated neonatally with genistein, there was also a small change in ratio of LH to FSH immunopositive cells between control and treated rats, there was a higher ratio of LH to FSH cells in controls (ratio=1.6) compared to genistein treated rats (ratio=1.2), but this was not significant.

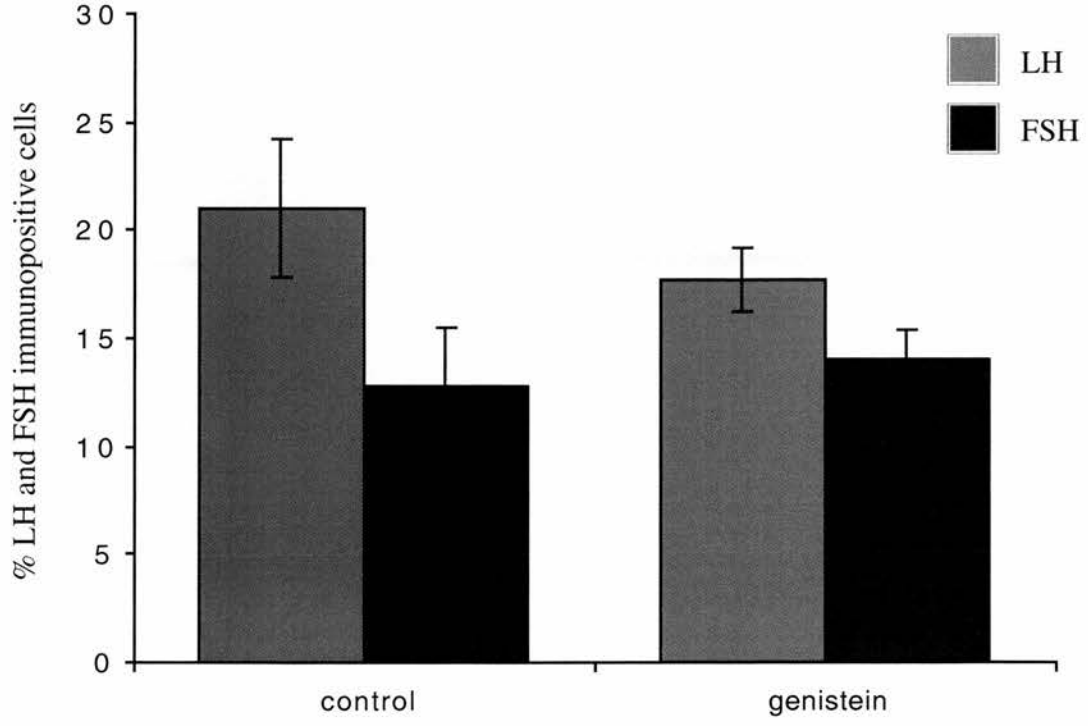


Figure 6.3.2: Comparison of number of LH and FSH immunopositive gonadotroph cells at 18 days of age in control rats and rats treated neonatally (days 2-17) with genistein. Values are means ± SEM. For control n=6, treated n=4.

6.3.3 Percentage of LH and FSH immunopositive cells in 25 day old rats treated neonatally with genistein

As demonstrated in Figure 6.3.3, for 25 day old rats treated neonatally with the phytoestrogen genistein, there was no significant difference in the percentage of LH ($p=0.436$) immunopositive cells between control rats and those administered genistein. However, there was a small significant decrease ($p=0.05$) in the percentage of FSH immunopositive gonadotrophs between control and genistein treated rats. There was also a small change in the ratio of LH to FSH immunopositive cells, with the LH:FSH ratio being lower for controls (ratio=1.6) than for genistein treated rats (ratio=2), however this difference was small and not statistically significant.

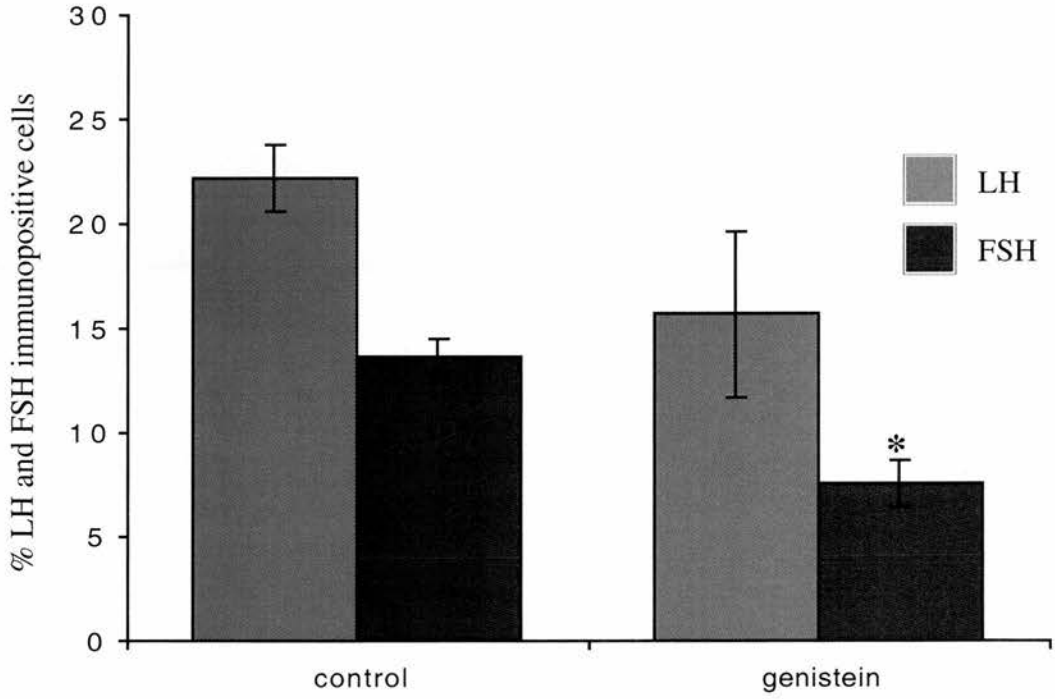


Figure 6.3.3: Comparison of number of LH and FSH immunopositive gonadotroph cells at 25 days of age in control rats and rats treated neonatally (days 2-17) with genistein. Values are means ± SEM. For control n=5, treated n=3. * $p<0.05$, in comparison with respective control.

6.3.4 Plasma FSH levels at day 18

Plasma FSH levels were measured for soy-free control and genistein treated rats at 18 days of age. There was a marked and significant reduction in plasma FSH concentrations for genistein treated rats ($p<0.001$).

Treatment	FSH concentration (ng/ml)	Number of animals
Soy-free control	7.77 ± 2.78	18
Genistein	4.16 ± 1.73	10

Table 6.3.4: Plasma FSH levels for soy-free controls and genistein treated rats at 18 days of age.

6.3.5 Summary of effects of genistein treatment on percentage of gonadotroph cells

Day	Treatment	% LH cells in treated rats	% LH cells in control rats	p
18	soya-free	18.7 ± 2.23 n=3	21.0 ± 3.21 n=6	0.641
10	genistein	18.7 ± 0.99 n=4	20.1 ± 1.18 n=6	0.436
18	genistein	17.7 ± 1.46 n=4	21.0 ± 3.21 n=6	0.453
25	genistein	15.7 ± 3.95 n=3	22.2 ± 1.62 n=5	0.121

Table 6.3.5a: Summary of the mean percentages ± SEM of LH immunopositive cells in the pituitary gland in relation to the neonatal treatment.

Day	Treatment	% FSH cells in treated rats	% FSH cells in control rats	p
18	soya-free	10.6 ± 1.55 n=3	12.8 ± 2.72 n=6	0.612
10	genistein	11.9 ± 0.65 n=4	9.08 ± 1.21 n=6	0.109
18	genistein	14.0 ± 1.34 n=4	12.8 ± 2.72 n=6	0.746
25	genistein	7.54 ± 1.11 n=3	13.6 ± 0.87 n=5	0.005 ($p \leq 0.05$)

Table 6.3.5b: Summary of the mean percentages ± SEM of FSH immunopositive cells in the pituitary gland in relation to the neonatal treatment.

To summarise, there was no significant effect of neonatal genistein treatment on the number of immunopositive gonadotrophs in the pituitary gland at days 10 and 18. However, there was a small significant reduction in pituitary FSH staining at day 25 for genistein treated rats in comparison to controls. Plasma FSH levels at 18 days of age were reduced significantly ($p<0.001$) in genistein treated rats.

6.4 Percentage of FSH and LH immunopositive cells in 35 to 40 day old marmosets weaned on soy formula milk

There were no significant differences at 35 to 40 days of age in the number of FSH immunopositive gonadotroph cells for the cow's formula milk and soy formula milk weaned infants (Figure 6.4a). For many of the co-twin sets the number of FSH immunopositive cells tended to be lower in the soy formula fed infants, but this reduction was small and not statistically significant.

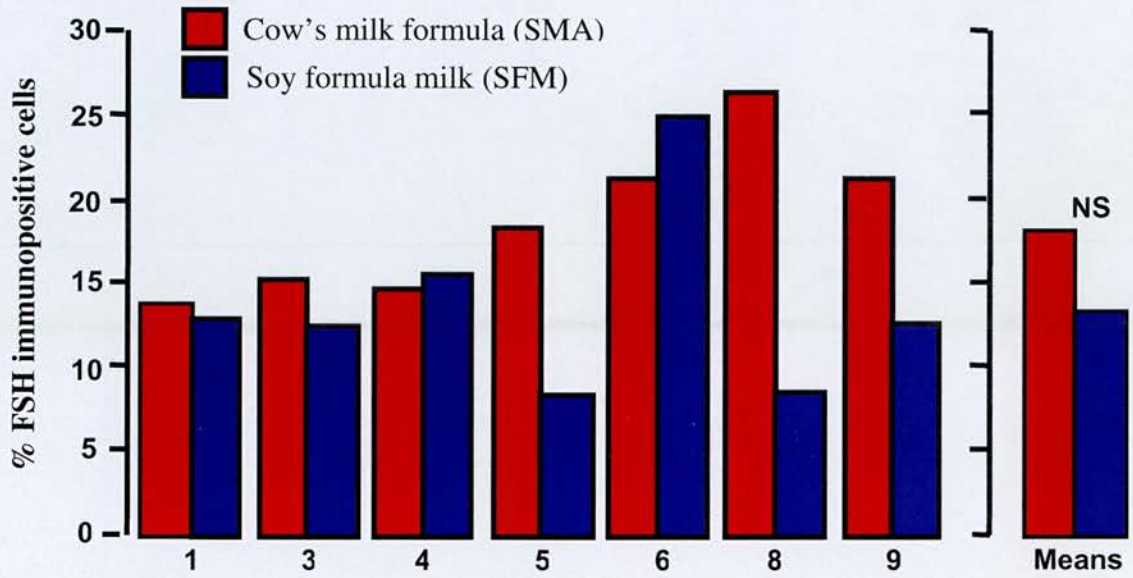


Figure 6.4a: Pituitary cell counts for FSH at the end of the feeding period (35-40 days of age) in co-twin marmosets fed with either standard cow's milk formula (SMA) or soy formula milk (SFM). Each bar represents one co-twin and overall mean levels for each treatment group are shown to the right. P values are based on co-twin comparisons using the paired t-test.

The number of LH immunopositive cells was also counted after the treatment period (at days 35 to 40), as shown in Figure 6.4b. As for FSH, there was no significant change in the number of LH immunopositive gonadotrophs for control cow's formula milk and soy formula milk fed infant animals. There was a small reduction in the number of LH immunopositive gonadotrophs for 4 out of the 7 co-twin sets, however this was only a small change and was not statistically significant.

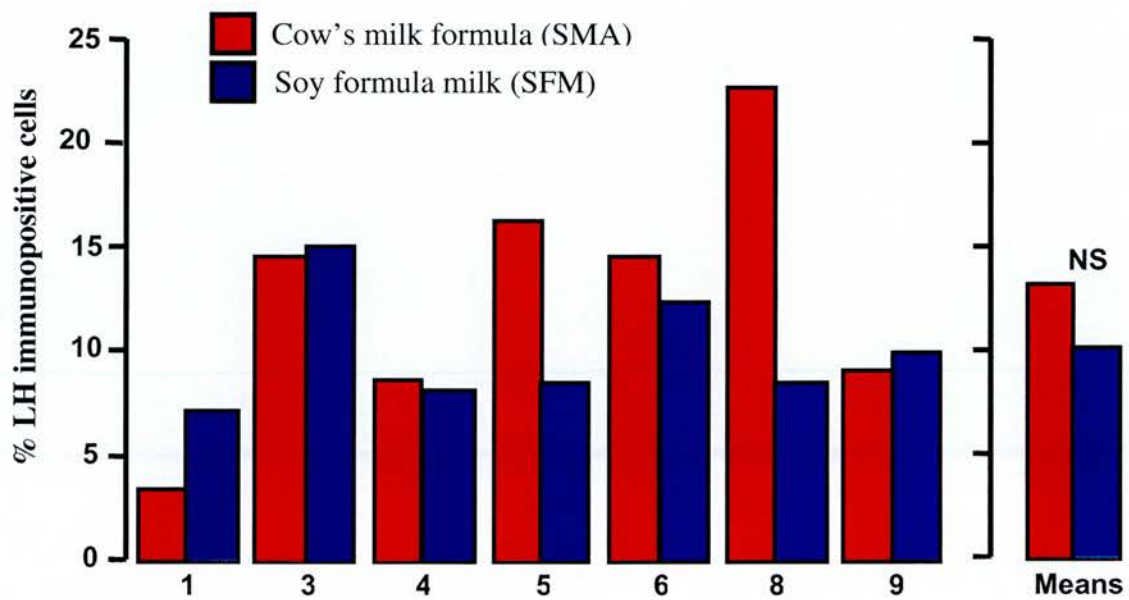


Figure 6.4b: Pituitary cell counts for LH at the end of the feeding period (35-40 days of age) in co-twin marmosets fed with either standard cow's milk formula (SMA) or soy formula milk (SFM). Each bar represents one co-twin and overall mean levels for each treatment group are shown to the right. P values are based on co-twin comparisons using the paired t-test.

To investigate whether the bi-hormonal nature of the gonadotroph cells was maintained after infant treatment with soy formula milk, the pituitaries for all the co-twins were dual stained for both LH and FSH after the treatment period, using the fluorescent markers TRITC and FITC. As depicted in Figure 6.4d, pituitary gonadotrophs maintained their bi-hormonal nature, as LH and FSH were still co-localised within the same gonadotroph cell after neonatal treatment with soy formula milk.

From the DAB immunostained pituitaries the ratio of FSH immunopositive to LH immunopositive cells was calculated for each co-twin (shown in Figure 6.4c). There was no significant change in FSH to LH ratio for the soy formula treated animals. The FSH to LH ratio was lower for 6 out of 9 marmosets, however this reduction was small and not statistically significant. Representative sections showing DAB immunocytochemistry for LH and FSH in the pituitaries of control cow's formula milk and soy formula milk treated animals are included in Figures 6.4e and 6.4f respectively.

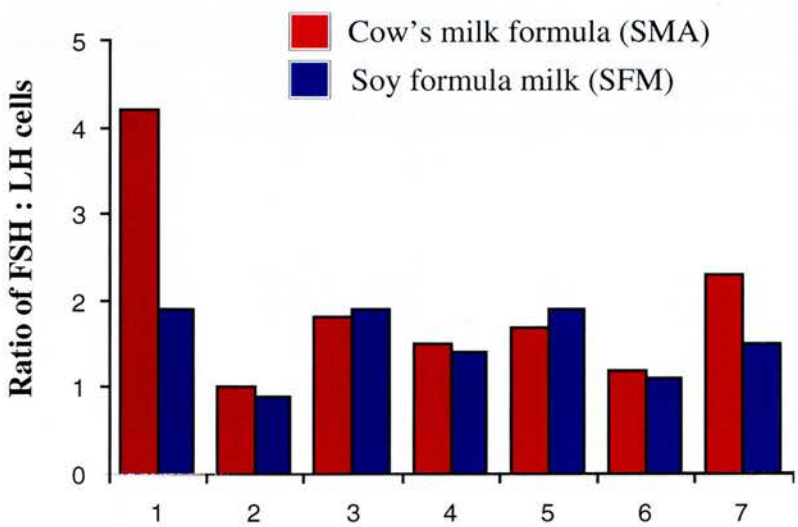


Figure 6.4c: Ratio of FSH to LH immunopositive gonadotrophs at the end of the feeding period (35-40 days of age) in co-twin marmosets fed with either standard cow's milk formula (SMA) or soy formula milk (SFM). Each bar represents one co-twin and co-twin comparisons were performed using the paired t-test.

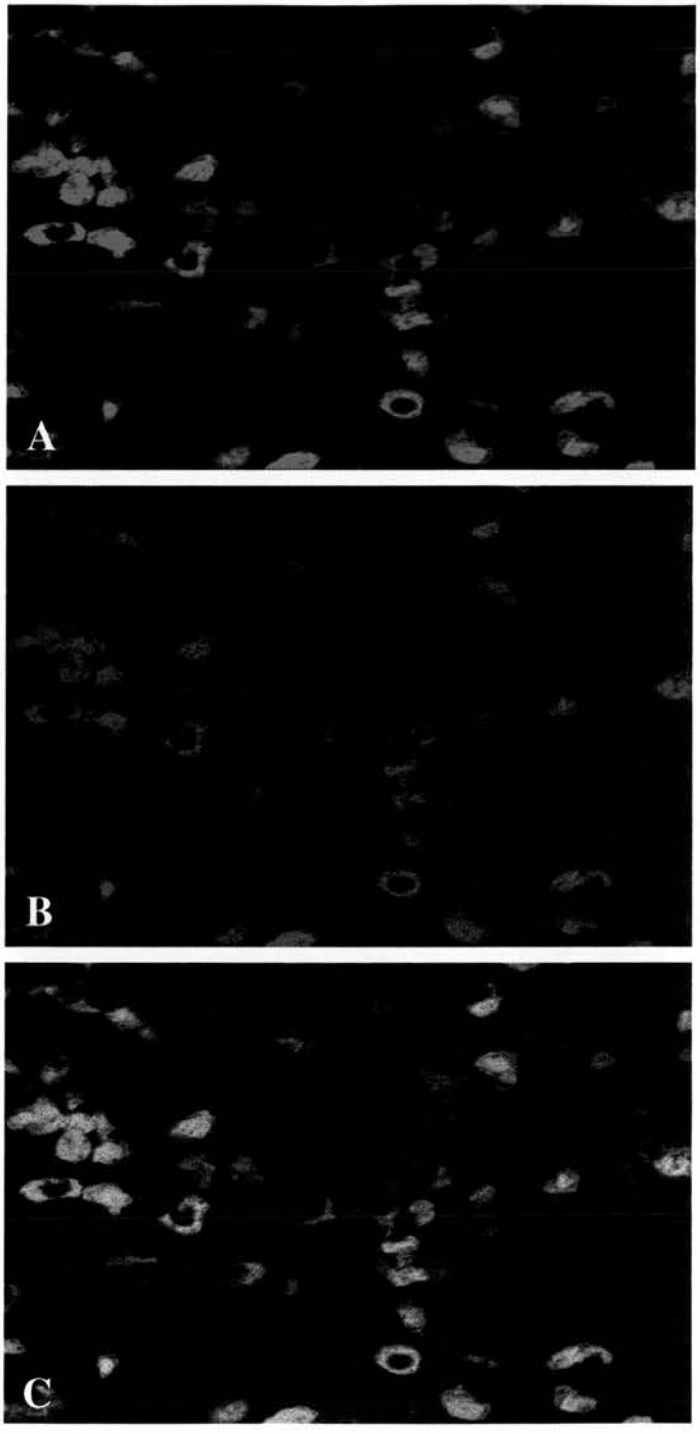


Figure 6.4d: Immunofluorescent staining after soy treatment for LH and FSH in gonadotrophs of soy formula fed infants. A=LH staining, B=FSH staining, C=dual staining for LH and FSH. Note bi-hormonal nature of gonadotrophs is maintained.

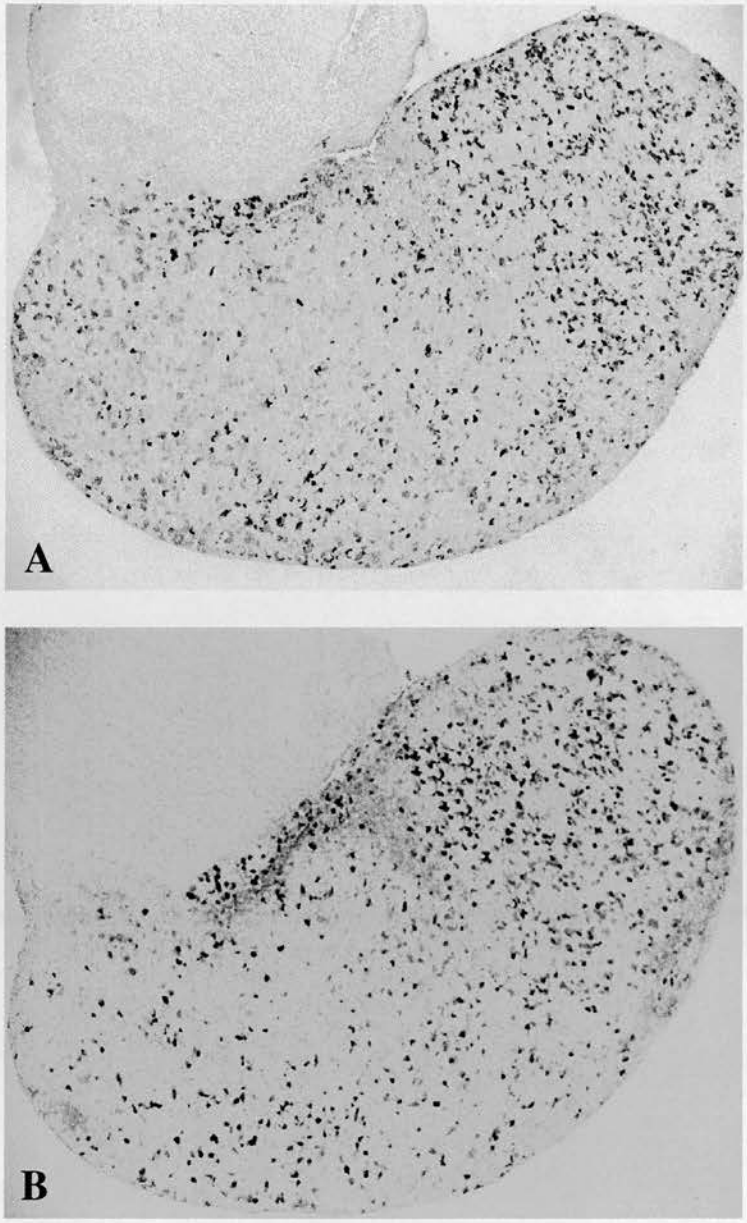


Figure 6.4e: Representative sections of the pituitary immunostained for FSH at age 35 to 40 days in one set of marmoset co-twins fed either with control cow's milk formula (A) or with soy formula milk (B). There was no statistically significant difference in the number of FSH immunopositive cells, however the soy formula treated aninals tended to have slightly less FSH immunopositive staining. But this reduction was small and not statistically significant.

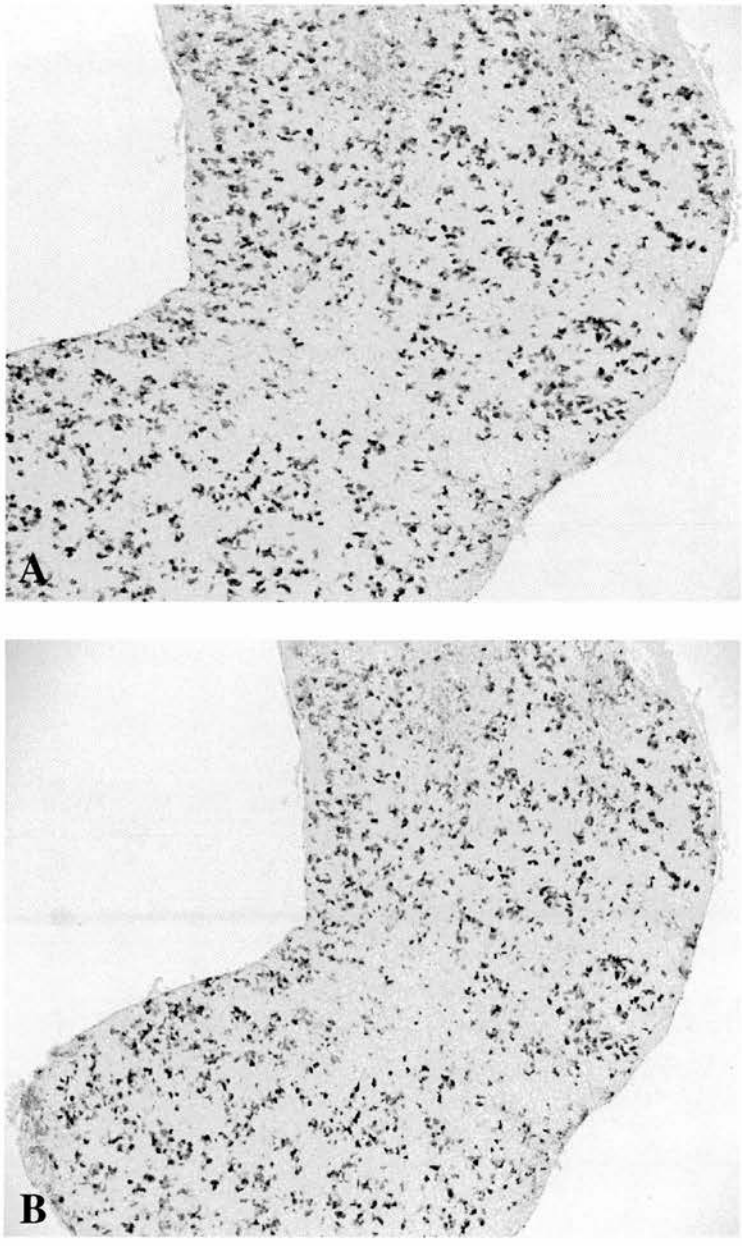


Figure 6.4f: Representative sections of the pituitary immunostained for LH at age 35 to 40 days in one set of marmoset co-twins fed either with control cow's milk formula (A) or with soy formula milk (B). There was no statistically significant difference in the number of LH immunopositive cells, however the soy formula treated aninals tended to have slightly less LH immunopositive staining. But this reduction was small and not statistically significant.

6.4.1 Plasma testosterone levels at days 35-40 for marmoset infants fed either cow's milk formula or soy formula milk

Plasma testosterone levels were measured (as depicted in Figure 6.4.1) twice during this study for marmosets fed either cow's milk formula or soy formula milk during neonatal life. The first time was in the middle of the study, at days 18 to 20, and the second time was at the end of the study, at days 35 to 40. There was a wide variation in plasma testosterone levels between co-twins, reflecting the episodic nature of testosterone production and secretion from the testicular Leydig cells. A paired comparison of testosterone levels in co-twins at days 35 to 40 showed that there was a dramatic 55% reduction (p=0.018) in plasma testosterone levels for soy formula milk fed marmosets. However, a similar comparison done at days 18 to 20, when plasma testosterone levels were low (prepubertal), revealed that there was no significant change in plasma testosterone levels (p=0.2).

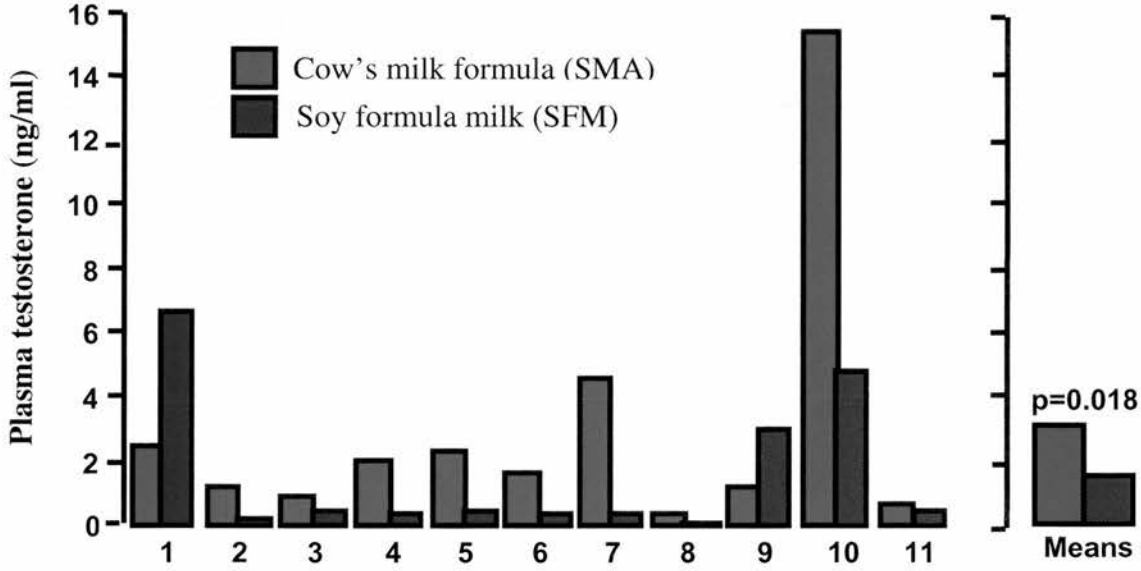


Figure 6.4.1: Plasma testosterone levels at the end of the feeding period (35-40 days) for co-twin marmosets fed either standard cow's milk formula (SMA controls) or soy formula milk (SFM). P values are based on co-twin comparison using the paired t-test (Sharpe *et al.*, 2002).

6.4.2 Mean bodyweights at 35-40 days of age, for marmosets fed cow's milk formula or soy formula milk

The bodyweights for control cow's milk and soy formula milk fed infant marmosets were comparable, both at the start (33.3g ± 3.1 for SMA and 34.3g ± 1.8 for SFM) and at the end (75.6g ± 9.1 for SMA and 77.6g ± 7.5 for SFM) of the treatment period. The increase in bodyweight during this 35 to 40 day period was also very similar, as shown in Figure 6.4.2. The bodyweight increments were well within the normal limits of this marmoset colony. All the animals that took part in this study appeared healthy and no unforeseen problems were encountered with the animals.

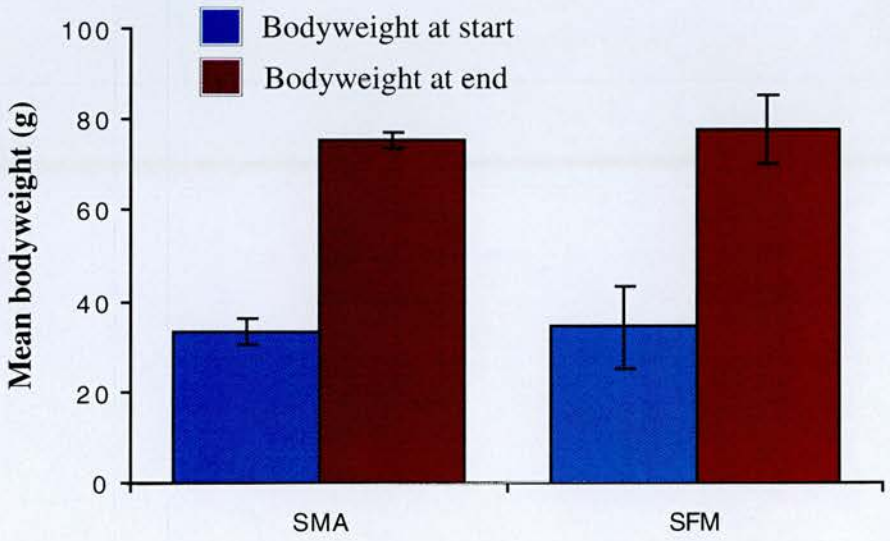


Figure 6.4.2: Mean bodyweights for control infant marmosets fed either with cow's milk formula (SMA) or with soy formula milk (SFM) at the start (day 3 or 4) and at the end (days 35 to 40) of the treatment period. There was no significant difference in body weight before or after the treatment period between control infants and those weaned on SFM. Values shown are means ± SEM. SMA n=9 and SFM n=9 (Sharpe *et al.*, 2002).

6.4.3 Mean testis weight at 35-40 days of age, for marmosets fed cow's milk formula or soy formula milk

The testes were weighed, as shown in Figure 6.4.3, for each of the marmosets weaned either on cow's milk formula or soy formula milk after the treatment period, when marmosets were 35-40 days old. There was no statistically significant difference in mean testis weight after the treatment period between the control (21.5g ± 6.5) and treated (21.6g ± 4.0) marmosets.

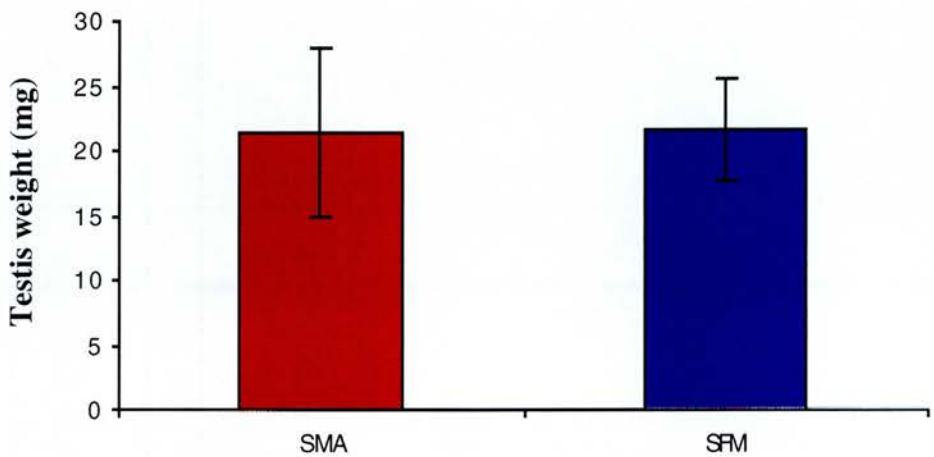


Figure 6.4.3: Mean testis weight after treatment period at days 35-40 for infant marmosets weaned either on cow's formula milk or soy formula milk. There was no significant difference in mean testis weight after the treatment period between control animals and those weaned on soy formula milk. Values are means ± SEM. For SMA n=9 and for SFM n=9 (Sharpe *et al.*, 2002).

6.4.4 Formula intake for marmoset infants fed either cow's milk formula or soy formula milk

Formula intake was measured for each infant marmoset co-twin, as shown in Figure 6.4.4. There was considerable variation between twins in the level of formula intake, with some twins taking in significantly lower amounts than other twins. However, in most cases this variability in formula intake applied equally to both co-twins. In most infants, formula intake per gram of bodyweight was higher in the second half of the study (after day 18 to 20, when first blood samples were collected) than in the first half of the study. Some co-twins were more restrained in taking in the feed (both cow's milk and soy formula milk) than others but none of the co-twins showed any aversion to the feed.

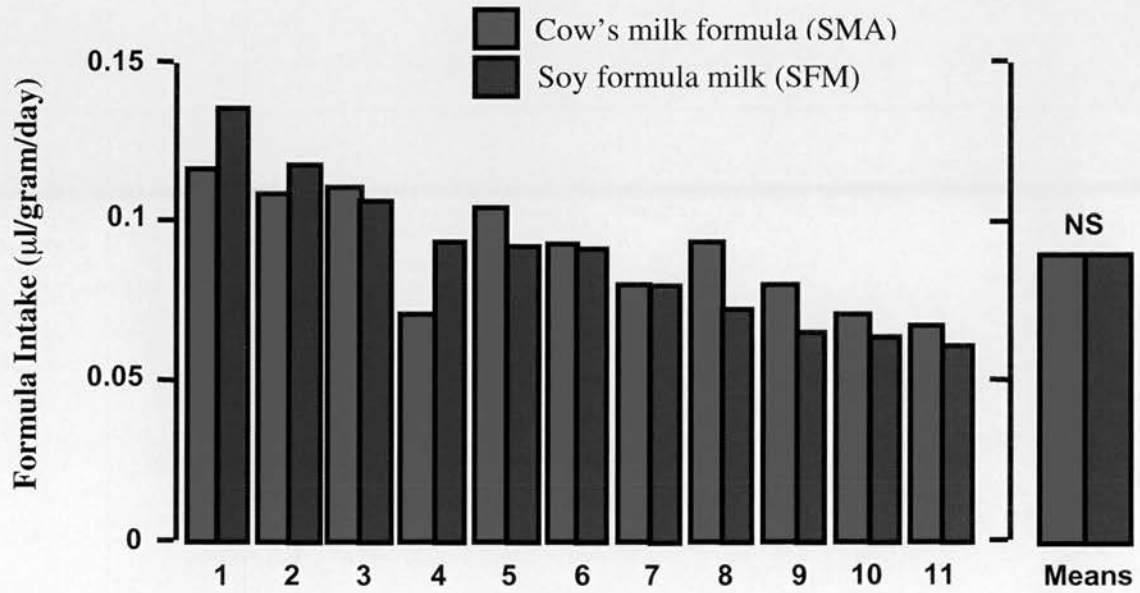


Figure 6.4.4: Daily formula intake for co-twin infant marmosets weaned on cow's milk (SMA) formula or soy formula milk (SFM). Each bar represents one co-twin whilst overall mean levels for each treatment group are shown to the right. Note that results are plotted in order of descending intake of SFM/g bodyweight by the SFM-fed co-twin. NS = not significant (Sharpe *et al.*, 2002).

6.4.5 Summary of measurements for bodyweight, testis weight, formula intake and plasma testosterone levels

Parameter	Control marmosets (SMA)	Soy formula fed marmosets (SFM)
Bodyweight at start (g)	33.3 ± 3.1	34.3 ± 1.8
Bodyweight at end (g)	75.6 ± 9.1	77.6 ± 7.5
Daily Formula intake (µl/g/day)	93 ± 19	93 ± 23
Testis weight (mg)	21.5 ± 6.5	21.6 ± 4.0
Testosterone (ng/ml) Day 18-20 Day 35-45	 3.1 ±4.2 2.8 ± 3.9	 2.9 ± 2.2 1.3 ± 2.1 (p=0.018)

Table 6.4.5: Summary of mean body weight parameters, formula milk intake and plasma testosterone levels during the study and testis weight at days 35-45 in marmosets fed with either standard (cow) formula milk (SMA controls) or soy formula milk (SFM). Values are ± SEM (Sharpe *et al.*, 2002).

6.4.6 Number of Leydig cells per testis at days 35-40, for infant marmosets fed either cow's milk formula or soy formula milk

The number of Leydig cells per testis was counted at days 35-40 for infants weaned on either cow's milk formula or soy formula milk, as shown in Figure 6.4.6. There was a marked and consistent increase of 74% in the average number of Leydig cells (p=0.006). Note the discrepancy between testicular Leydig cell numbers and the corresponding plasma testosterone concentrations, produced by the Leydig cells. This suggests that the dramatic reduction in testosterone levels observed for these marmosets was not due to a change in Leydig cell number but was more likely to be due to an alteration in Leydig cell function.

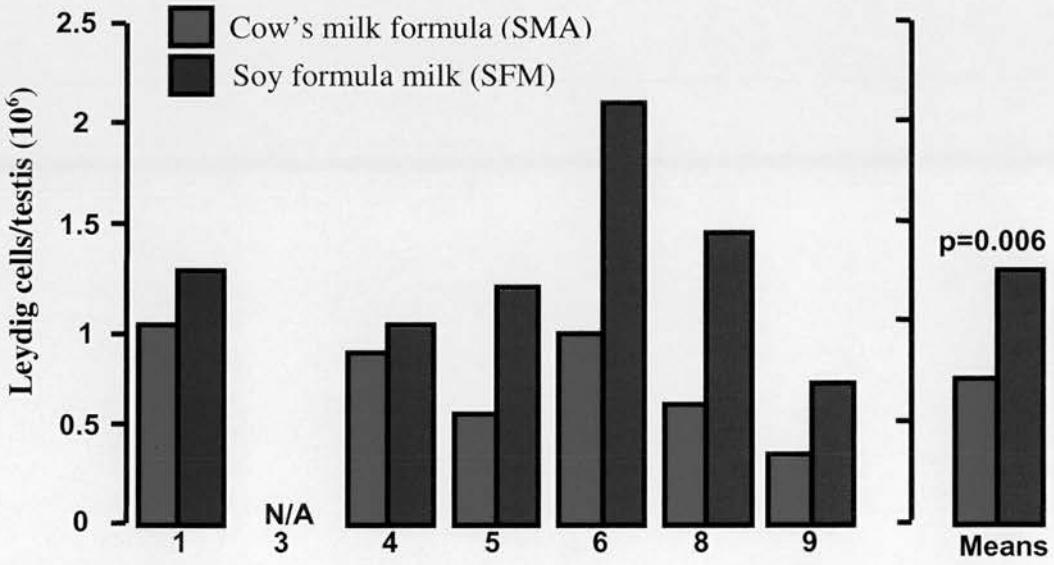


Figure 6.4.6: Leydig cell numbers for co-twin infant marmosets fed either cow's formula milk (SMA) or soy formula milk (SFM). There is a significant increase in Leydig cell numbers for marmosets fed soy formula milk. P values are based on co-twin comparison using a paired t-test. N/A = not available due to processing error (Sharpe *et al.*, 2002).

6.4.7 Number of Sertoli cells per testis at days 35-40, for infant marmosets fed either cow's milk formula or soy formula milk

The number of Sertoli cells per testis at days 35-40 was counted for infant marmosets weaned either on control cow's milk formula or on soy formula milk, as depicted in Figure 6.4.7. The cell counts showed that there was no significant consistent change in average Sertoli cell number between cow's milk and soy formula fed animals. This could suggest that the phytoestrogen soy had very local effects at the level of the testis, as Sertoli cell number was not affected whereas Leydig cell number was dramatically increased.

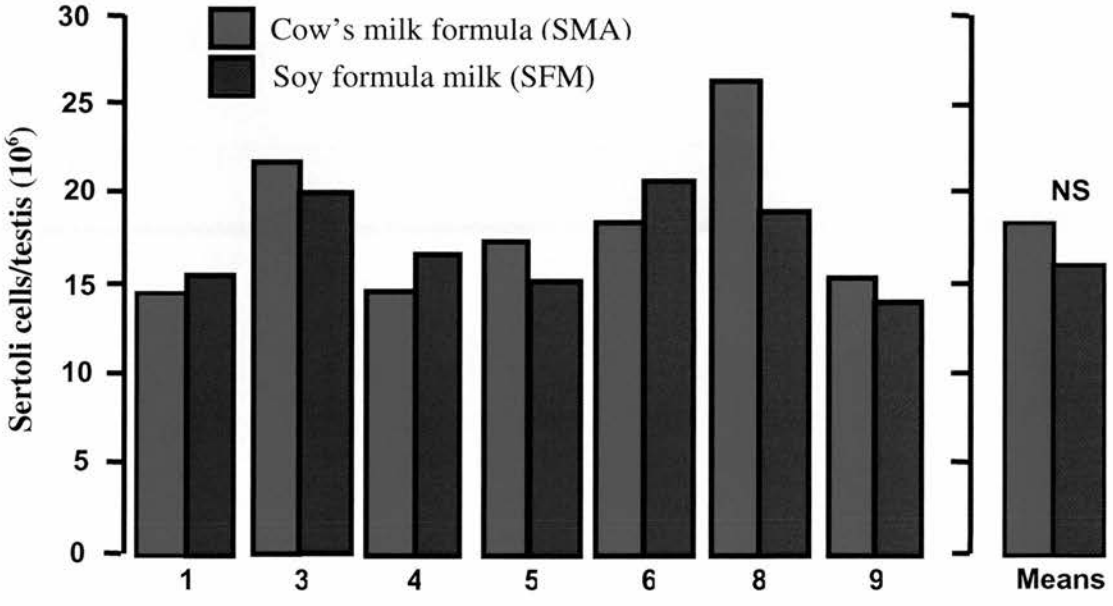


Figure 6.4.7: Sertoli cell numbers for co-twin infant marmosets fed either cow's formula milk (SMA) or soy formula milk (SFM). There was no significant difference in Sertoli cell numbers for control marmosets and those fed soy formula milk. P values are based on co-twin comparison using a paired t-test. NS = not significant (Sharpe *et al.*, 2002).

6.4.8 Summary of effects observed after the treatment period for the cow's milk formula and soy formula treated infant marmosets

Parameter	Cow's milk formula (SMA)	Soy formula milk (SFM)
Bodyweight at start (g)	33.3 ± 3.1	34.3 ± 1.8
Bodyweight at end (g)	75.6 ± 9.1	77.6 ± 7.5
Bodyweight increment (g)	42.3 ± 7.1	43.3 ± 7.1
Formula intake (µl/g/day)	93 ± 19	93 ± 23
Testis weight (mg)	21.5 ± 6.5	21.6 ± 4.0
Plasma testosterone (ng/ml) Day 18-20 Day 35-40	 3.1 ± 4.2 2.8 ± 3.9	 2.9 ± 2.2 1.3 ± 2.1 (p=0.018)
Sertoli cell number (10^6)	18.0 ± 4.9	17.0 ± 3.7
Leydig cell number (10^6)	0.6 ± 0.09	1.3 ± 0.11 (p=0.006)
Pituitary LH +ve cells	11.1 ± 3.2	14.2 ± 4.1
Pituitary FSH +ve cells	18.5 ± 5.2	14.9 ± 4.9
Pituitary FSH:LH ratio	1.9 ± 0.14	1.5 ± 0.12

Figure 6.4.8: Summary of parameters measured and results obtained at 35-40 days of age for control cow's milk formula (SMA) and soy formula (SMF) fed infant marmosets (Sharpe *et al.*, 2002). Values are the means ± SEM.

To summarise, neonatal treatment with soy formula milk caused a marked suppression in plasma testosterone levels at days 35-40 of age and increased the number of Leydig cells per testis significantly after the treatment period. However, soy formula fed marmosets showed no significant changes in the number of pituitary LH and FSH immunopositive cells, testis weight or the in the number of Sertoli cells per testis.

6.5 Discussion

The objective of this comprehensive study was to address health concerns about feeding human infants soy formula milk, which contains weak phytoestrogens. Marmosets were used for the main study as marmosets (especially male marmosets) tend to have similar reproductive physiology to humans, in terms of exhibiting a period of neonatal hormone activity, which is characterised by a significant prolonged surge in testosterone levels (Dixson, 1986; Lunn *et al.,* 1994; Mann and Fraser, 1996; McKinnell *et al.,* 2001a) and proliferation of testicular Sertoli cells (Sharpe *et al.,* 2000). Thus, on a developmental level, neonatal marmosets tend to be comparable to neonatal humans, more so than neonatal rodents. However, a small genistein study in rats was also performed in parallel to the marmoset study as presently there are no working assays for gonadotrophins in marmoset plasma. Hence plasma gonadotrophin levels were measured in rats treated with genistein to shed some light on phytoestrogen effects on circulating levels of LH and FSH, in relation to any changes in pituitary morphology in general and gonadotroph cell populations in particular.

The prolonged neontal testosterone rise is unique only to male primates, as this event does not occur in female primates. The role of this prolonged testosterone rise in male primates has yet to be elucidated, but it is not thought to play a role in masculinisation of sexual behaviour, as this is determined during fetal life rather than in neonatal life as is the case for rodents (Meisel *et al.,* 1984). Numerous studies using GnRH analogues to block the testosterone surge in the marmoset have reported relatively minor (Eisler *et al.,* 1993) or no (Lunn *et al.,* 1994) effects in adulthood on sexual behaviour, with no major effects also on sperm counts in adulthood and fertility (Mann *et al.,* 1993; Lunn *et al.,* 1994,1997; Mann and Fraser, 1996). However, a prolonged rise of testosterone levels in puberty (Mann *et al.,* 1989,1993; Lunn *et al.,* 1994; Mann and Fraser, 1996) and significant changes in testicular cell composition, including an increase in Leydig cell numbers (Sharpe *et al.,* 2000) have been observed.

The marmosets in this study administered the soy formula milk neonatally showed normal growth and development, as their starting bodyweights, bodyweight increments, end bodyweights and testicular weights were comparable to the control marmosets fed the cow's milk formula. These observations correspond with other observations made by other groups, where several studies so far have demonstrated that soy formula milk supports normal growth and development in term infants (Fomon, 1993; Businco *et al.,* 1992; Churella *et al.,* 1994; Kohler *et al.,* 1984). Another study monitoring growth, bodyweight and body length of infants during the first year of life, found that these were virtually the same for either infants fed on soy formula milk or infants fed on cow's milk formula (Lasekan *et al.,* 1999).

However, one important observation made during this study was that neonatal feeding with soy formula milk resulted in a significant suppression of the testosterone surge at days 35-40 (at the end of the study). Interestingly, there was no such suppression in testosterone levels at days 18-20 (in the middle of the study). It is presently still unclear why testosterone levels are suppressed at 35 to 40 days of age and not at 18 to 20 days of age in the soy formula fed marmosets. One possibility is that at 35 to 40 days of age the marmosets had consumed more soy formula milk than at 18 to 20 days of age (49% higher intake), and hence the dose and duration of exposure to the phytoestrogens in the soy formula milk was now large enough to have a visible effect.

The effects of soy formula milk feeding on the testicular Leydig and Sertoli cells were quite surprising, as there was a significant increase in Leydig cell numbers per testis for the soy formula milk fed co-twins compared to those fed the cow's milk formula. As Leydig cell numbers were increased and the testosterone surge was suppressed for the soy formula milk fed co-twins, this suggests that soy formula milk might be affecting Leydig cell function as opposed to cell number. There was no overall change in the number of Sertoli cells per testis between soy formula milk and cow's milk formula fed

marmosets. This might indicate that the soy milk formula treatment might have very local effects on the testis, with some cell types being more susceptible than others.

To investigate eventual effects of neonatal soy formula feeding on pituitary function and to gain some insight into feedback between the pituitary and testes, the numbers of LH and FSH immunopositive gonadotroph cells were counted for each co-twin fed either cow's milk formula or soy formula milk. These cell counts showed that there was no significant consistent change in numbers of LH and FSH immunopositive gonadotroph cells in the soy formula milk fed co-twins when compared to their cow's milk formula fed brothers. However, the numbers of LH and FSH immunopositive gonadotrophs tended to be lower in most of the soy formula milk fed co-twins, but this was only a small reduction and was not statistically significant. Similar observations were made at day 18 for the rats treated neonatally with genistein, however, plasma FSH levels were significantly elevated ($p<0.001$) at day 18 in genistein treated rats compared to controls.

Thus, the marmoset pituitary gonadotroph findings cannot be interpreted as reflecting no change in LH and/or FSH secretion, as neonatal genistein treated 18 day old rats showed no change in gonadotroph cell number but an elevation in plasma FSH concentrations. It has also been shown by other groups that s.c. administration of genistein at 2.5mg/kg/day to adult mice can reduce both blood testosterone levels and pituitary LH content (Strauss *et al.,* 1998). Thus at this present time there is no solid explanation as to why feeding infant marmosets soy formula milk resulted in a significant and consistent increase in testicular Leydig cell numbers and in a dramatic suppression of the neonatal testosterone surge. What the long-term consequences of these effects could be is also unclear, but hopefully some of these uncertainties should be resolved when their other treated brothers go through puberty and reach adulthood. Observations of these animals so far have shown that they proceed through puberty in the normal manner (data not shown).

Although this study has demonstrated significant effects of feeding soy formula milk to infant marmosets, it still remains difficult to predict whether these findings will also hold true for humans and whether there would be any long-term effects of feeding human infants soy formula milk. The evidence available on sexual and reproductive outcomes associated with infant soy formula milk feeding in humans is presently extremely limited. One study on the possible reproductive effects of soy formula milk has described high circulating levels of isoflavone metabolites in infants (Setchell *et al.*, 1998). They found that infants exclusively fed soy-based diets are exposed to high concentrations of isoflavones relative to their body weight (Setchell *et al.*, 1998; Knight *et al.*, 1998). Although human metabolism of soy isoflavones is presently not well understood, there is evidence that these compounds are biologically active in adults and may influence reproductive hormone levels (Cassidy *et al.*, 1994; Baird *et al.*, 1995). It has been shown that infants absorb and excrete soy isoflavones, but it is not known to what extent they are able to metabolize and deconjugate these compounds to biologically active forms (Klein, 1998; Setchell *et al.*, 1998, 1997; Irvine *et al.*, 1998).

Some *in vitro* and animal studies on non-soy phytoestrogens such as coumestans, have led to speculation that isoflavones may adversely affect developmental processes influenced by sex steroids, with potential consequences perhaps manifested only in puberty or adulthood (Irvine *et al.*, 1998). However, comparisons between species might be difficult and inappropriate because there are known differences in metabolism and developmental norms (Klein, 1998). Furthermore, some results of some animal studies on possible sexual and reproductive effects of soy isoflavones have been inconsistent, hinting that there could be substantial variation within and across species, and differing also by dose and timing of exposure (Klein, 1998).

In conclusion, the findings from this short-term study have shown that feeding neonatal marmosets soy formula milk can cause a significant suppression of the neonatal

testosterone surge and a significant elevation in Leydig cell number at 35 to 40 days of age. Future studies should bring to light the long-term consequences of infant soy formula milk feeding and until more is known about eventual long-term effects of soy formula feeding, it would be wise to err on the side of caution and avoid using soy formula milk if possible.

Chapter seven: general discussion

The main objectives of the studies conducted for this thesis were to investigate whether oestrogen treatment during the neonatal period of development could re-programme pituitary function on a permanent basis, and to test whether potential pituitary re-programming was due to a direct oestrogen-induced effect on the pituitary gland or via oestrogen-induced altered feedback along the HPG axis. The potent synthetic oestrogen, DES, was used throughout these studies as a model for neonatal oestrogen disruption.

At this present time there is only a limited amount of information available as to whether neonatal oestrogen administration has any effects on pituitary gonadotroph function and whether potential effects are direct or indirect. Most of the studies to date have focussed mainly on the effects of neonatal oestrogen exposure on the reproductive tract. In male rodents, neonatal DES treatment has been shown to cause a range of reproductive tract abnormalities, including overgrowth of the rete testis, reduced epithelial cell height in the efferent ducts, epididymis, vas deferens, seminal vesicles and prostate (Atanassova *et al.,* 1999, 2001; Williams *et al.,* 2001; McKinnell *et al.,* 2001a,b), a loss of expression of androgen receptor in the testis (Williams *et al.,* 2001; McKinnell *et al.,* 2001a,b) and changes in circulating reproductive hormone concentrations (Atanassova *et al.,* 1999; Rivas *et al.,* 2002). Evidence has also been obtained from humans, e.g. the sons of mothers exposed to DES during pregnancy have been shown to possess abnormalities in the structure and function of their reproductive organs (Arai *et al.,* 1983), including an increased incidence of hypospadias, epididymal cysts, hypoplastic testes, cryptorchidism, and abnormal semen parameters. Neonatal exposure of females to DES has also been shown to result in abnormalities of the reproductive tract, including vaginal lesions (Bern *et al.,* 1987), uterine hypoplasia (Medlock *et al.,* 1992), and a loss of oestrogen receptors (Medlock *et al.,* 1992).

In our current experimental paradigm of neonatal disruption of male rodents with DES, it was shown to significantly reduce FSH immunopositive staining and FSH mRNA levels in the anterior pituitary, shortly after treatment (at 18 days of age). At 26 days of age, plasma levels of both LH and FSH were found to be suppressed in the DES treated rats. It has been documented in the literature that rodent Sertoli cells proliferate mainly during the neonatal period of development and that plasma FSH levels play an important role in this process (Sharpe, 1994). In DES treated animals in adulthood, there was a significant reduction in testis weight and in the total number of Sertoli cells per testis. This probably demonstrates that the reduced plasma FSH levels during neonatal life, caused by DES disruption, resulted in diminished Sertoli cell proliferation during neonatal life and hence resulted in a lower number of Sertoli cells per testis and thus lower eventual testis weight in adulthood. This corresponds well with the plasma inhibin B concentrations measured for these DES treated animals, as circulating inhibin B levels were found to be significantly suppressed in adulthood, and this suppression may well have been due to a reduction in total number of Sertoli cells per testis. Interestingly, there was found to be no significant change in the total number of Leydig cells present per testis, however, plasma testosterone levels were shown to be significantly reduced. This demonstrates that the suppressed plasma testosterone levels were probably due to a DES-induced functional deficit in the Leydig cells to efficiently produce and secrete testosterone. Since inhibin B and testosterone exert potent hormonal feedback onto the pituitary, the reduced circulating levels of these gonadal hormones will have resulted in a distorted feedback onto the pituitary gonadotrophs resulting in a permanent elevation of plasma LH and FSH levels.

To investigate whether neonatal DES treatment in these male rats had altered pituitary gland sensitivity to GnRH, all rats were administered a short GnRH pulse and plasma LH and FSH levels in response to this GnRH challenge were assayed. Interestingly, it was found that the male DES treated rats showed a significantly attenuated response to

GnRH, with GnRH-induced LH release being approximately 50% lower in the DES treated rats. This result correlates well with current literature which has demonstrated that GnRH-induced LH release has been shown to be blunted by neonatal DES treatment (Register *et al.,* 1995; Faber *et al.,* 1993). A possible cause for this effect is that neonatal DES treatment caused a (permanent) downregulation of GnRH receptors on gonadotroph cells, as oestrogenic agents have been shown to diminish GnRH receptor expression on pituitary gonadotrophs (Kang *et al.,* 2001; Cheon *et al.,* 2001).

In vitro culture of the adult pituitaries of the neonatally DES treated male rats showed that there was no significant change in LH and FSH output from the pituitary cells isolated from controls or from DES treated rats. Additionally, treatment of these cells with GnRH and/or activin, which have been shown to stimulate LH and FSH secretion from both LβT2 cells and primary pituitary cells (Pernasetti *et al.,* 2001; Liu *et al.,* 2002; Nicol *et al.,* 2002), also showed no significant change in LH and FSH output. Thus it appears that in accordance with the *in vivo* data obtained for these rats, that neonatal DES treatment had only subtle functional effects upon the anterior pituitary, as there was found to be no significant loss of gonadotrophin hormones from the adult primary pituitary cells isolated and cultured from neonatally DES treated male rats. However both the *in vivo* and *in vitro* data show that there was possibly a disruption in anterior pituitary sensitivity to GnRH due to a potential attenuation of GnRH receptor expression on pituitary gonadotroph cells.

Neonatal DES treatment of female rats was found to cause less significant physiological changes than in male DES treated animals. In direct contrast to the DES treated males, neonatal DES treatment was found to have no significant effects on basal levels of circulating LH, FSH, and inhibin B levels. This demonstrates that plasma hormone levels in male rats were more susceptible to permanent disruption by neonatal DES administration than female DES treated rats. However, similarly to the DES treated

males, the pituitary sensitivity to GnRH was found to be significantly attenuated in the DES treated females, with GnRH-induced LH release being 50% lower in the DES treated females. In a similar manner, *in vitro* analysis of LH and FSH output from adult primary pituitary cells cultured from neonatally DES treated female rats showed that there was no significant change in LH and FSH output between the control and DES treated cells, even with GnRH and/or activin treatment. Thus, this demonstrates that neonatal treatment of female rats with DES had no permanent and significant effects on gonadotrophin release from the pituitary.

The reason for neonatal DES treatment exerting differential effects in males and females could be due to a number of causes. It has been well documented that females experience oestrogen exposure during all stages of development (Corbier *et al.,* 1990; Faiman & Winter, 1974), whereas males do not (Mann & Fraser, 1996). It is therefore possible that males are more susceptible to oestrogen disruption during neonatal life than females as they are not used to experiencing high oestrogen levels during development. It has also been shown that males have a lower level of GnRH receptor expression in the pituitary and throughout the central nervous system compared to females (Lopez de Maturana *et al.,* 2003, *in review*), thus this might result in male pituitaries having a lower tolerance of detrimental oestrogenic effects upon GnRH receptor expression and therefore gonadotroph functional capacity compared to females. In addition, male gonad development is largely dependent upon testosterone (Mann & Fraser, 1996), thus high circulating levels of DES around the body might reduce the bio-availability, plasma half-life and distribution of testosterone, due to DES binding to sex hormone binding globulins or albumin. The direct effects of DES, via occupation of oestrogen receptors in the gonads also may contribute to the detrimental effects of DES upon the male gonads while clearly not exerting a commensurate disruptive effect in the female.

Another possible cause for the differential DES induced effects in males and females could be due to the fact that neonatal DES treatment in male rats is known to virtually

wipe out the expression of androgen receptors throughout the testis and reproductive tract. It has been shown that this reduction in androgen receptor expression largely accounts for the detrimental effects of DES treatment in male rats (McKinnell *et al.,* 2001a,b; Rivas *et al.,* 2002). It is possible that a similar DES induced suppression of androgen receptor expression might occur in the pituitary gland of male rats. It would be interesting to investigate whether the pituitary gland contains androgen receptors and whether neonatal DES treatment would alter pituitary androgen receptor expression. As androgen receptor expression is less extensive in female animals, this might account for some of the differences in DES induced effects between male and female animals.

During a small pilot study performed for this thesis, DES-induced pituitary effects were investigated further in male rats by treating them with either a long-acting GnRH antagonist (Antarelix) on its own or in combination with a low dose of DES. The GnRH antagonist treatment was found to significantly elevate plasma FSH levels in adulthood Immunocytochemical analysis of the pituitaries of these animals showed that mRNA levels for LH and FSH were significantly elevated. A possible explanation for this observed effect is that there was an autoregulatory increase in sensitivity of the hypothalamic-pituitary axis in an attempt to compensate for the reduced GnRH input due to the presence of the GnRH antagonist. This could have resulted in the upregulation of mRNA for LH and FSH in the pituitary that we observed. Neonatal treatment with a GnRH antagonist has been shown to reduce testis weight significantly, to reduce the number of Sertoli cells per testis, to delay sexual maturation, and induce infertility (Atanassova *et al.,* 1999; Kolho and Huhtaniemi, 1989). With respect to this action of GnRH antagonists we noted that the male rats treated neonatally with both GnRH antagonist and DES showed no significant changes in plasma levels of LH, FSH, inhibin B or testosterone. However, the pituitary sensitivity to GnRH for these treated animals was found to be much higher than control animals as the treated rats showed a significantly larger release in LH (and testosterone) in response to a GnRH pulse.

These DES studies provided a solid basis to address recent health concerns of feeding human infants soy formula milk (SFM), which contains high concentrations of phytoestrogens. Although phytoestrogens are not as potent at the synthetic oestrogen DES, there are presently concerns that feeding male infants with SFM might exert harmful effects on the developing HPG axis. The SFM study performed in infant marmosets showed that neonatal phytoestrogen exposure resulted in a marked suppression of plasma testosterone levels at 35-40 days of age and significantly increased the total number of Leydig cells present per testis. What the long-term effects are of neonatal testosterone suppression are as yet unclear, however neonatal testosterone is thought to play a role in masculinisation of sexual behaviour (Meisel *et al.,* 1984). Immunocytochemical analysis of the pituitary glands of these animals showed that there was no significant change in the number of LH and FSH immunopositive cells between control marmosets and SFM treated marmosets. Although this study has demonstrated significant effects of feeding SFM to infant marmosets, it still remains uncertain whether these findings will also hold true for humans and whether there would be any long-term consequences of feeding human infants soy formula milk. Hopefully some of these uncertainties should be resolved when other SFM treated infants reach adulthood.

These studies have shown that neonatal oestrogen exposure can have marked effects on the HPG axis. Future studies that could be performed to elucidate in more depth the effects of neonatal HPG axis disruption could include:

- Treating neonatal female rats with androgens, in a converse study to this present one, to test whether this would cause similar effects to those observed in DES treated male rats.

- Investigating expression of pituitary GnRH receptors, oestrogen receptors and androgen receptors in response to neonatal DES treatment. In addition, an investigation into the effects of neonatal DES treatment on LH and FSH receptor expression in the gonads would be most revealing.

- Investigate potential DES-induced effects on other anterior pituitary cells types. For example lactotrophs.

- Administer DES treatment in combination with androgen treatment to neonatal male rats to test whether this treatment regime could counteract the DES-induced effects on the HPG axis. Treatment of neonatal male rats with both DES and FSH could also be performed to investigate whether this would counteract DES-induced suppression of FSH levels and hence reduced proliferation of Sertoli cells.

The results from these studies suggest that both neonatal DES treatment and neonatal phytoestrogen exposure can have significant effects along the HPG axis. Especially DES treatment has been shown to cause potent and life-long effects on the HPG axis. DES has been shown to cause direct effects on pituitary function at the time of treatment for a short period of time, and on testicular function, with male gonads being more susceptible to neonatal endocrine disruption with DES.

Appendix I : Anastrazole and Tamoxifen

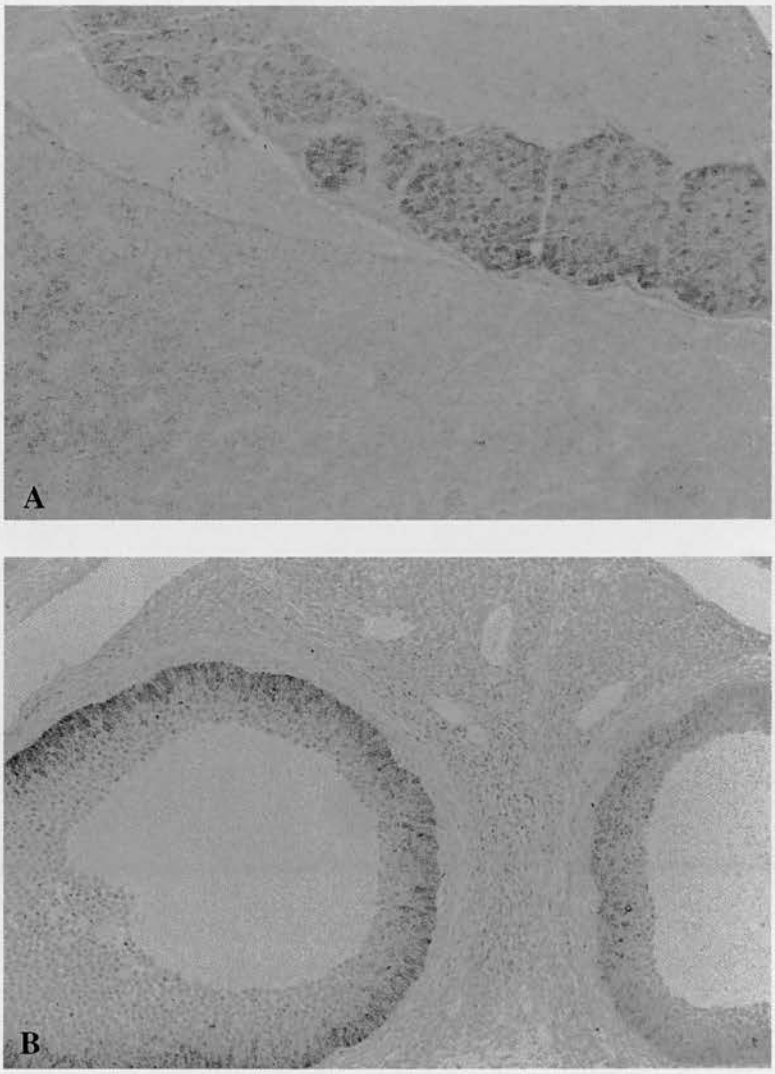
A small study was performed using two other treatments to investigate the effects of neonatal oestrogen disruption on the function of the pituitary gland. These treatments were Anastrazole, an aromatase inhibitor used mainly in treatment of breast cancer, and Tamoxifen, an oestrogen receptor antagonist also used to treat breast cancer. Male rats were injected s.c. with either 25mg/kg/day from days 2 to 17 with Anastrazole (gift from Astra Zeneca), or with 1mg/kg/day of Tamoxifen (Sigma) from days 2 to 12. Pituitaries were removed at day 18, were processed and sectioned, and the number of LH and FSH immunopositive cells was counted for each animal.

Treatment group	% LH immunopositive cells	% FSH immunopositive cells
Control	21.0 ± 3.21 n=6	12.8 ± 2.72 n=6
Anastrazole	19.3 ± 1.31 n=3 p=0.722	10.1 ± 0.88 n=3 p=0.524
Tamoxifen	25.5 ± 3.74 n=4 p=0.400	8.61 ± 1.07 n=4 p=0.264

Appendix I: Mean percentages of LH and FSH immunopositive cells in 18 day old male rats treated neonatally with Anastrazole or Tamoxifen. Values are the means ± SEM.

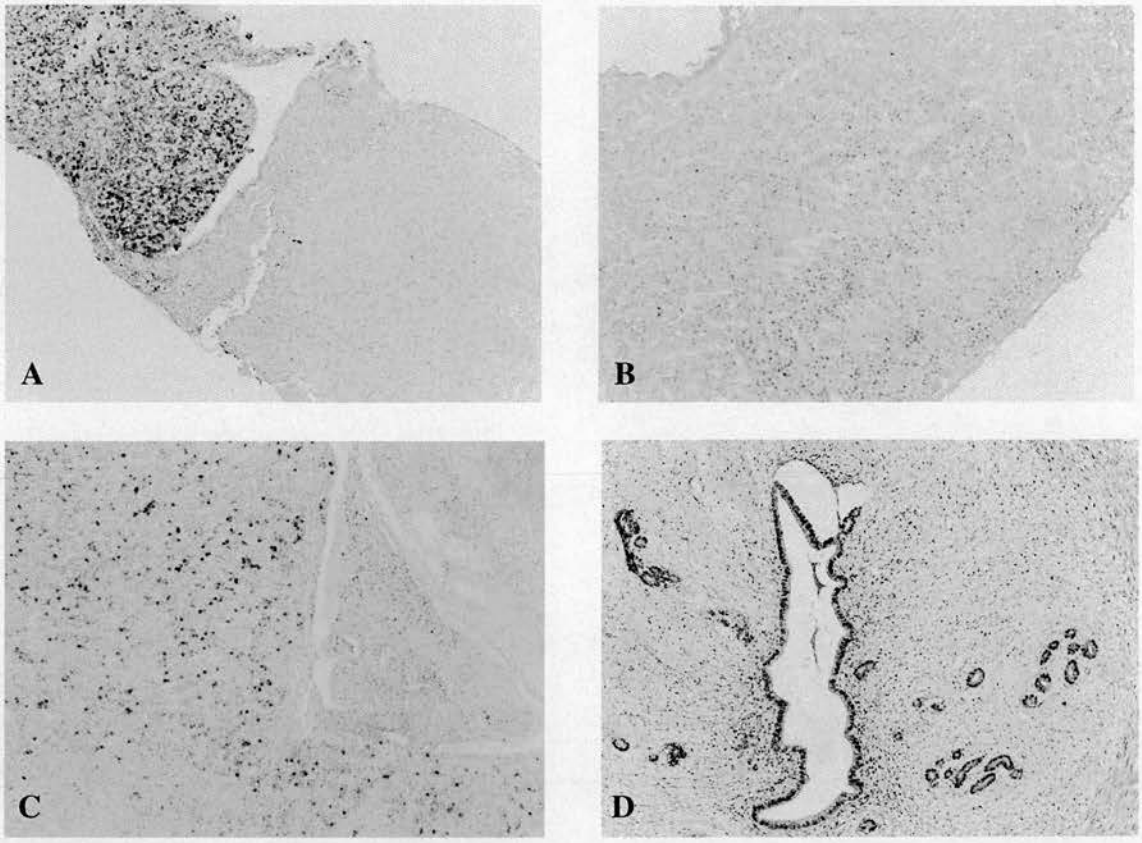
Neonatal treatment of male rats with Anastrazole or Tamoxifen was found to have no significant effect on the number of LH and FSH immunopositive gonadotrophs present in the anterior pituitary of 18 day old rats. Unfortunately no adult pituitaries or blood samples were available for analysis.

Appendix II: Pituitary Aromatase Staining



Appendix II: Preliminary evidence from Richard Sharpe's laboratory showed that there was possibly aromatase P450 present within the adult rat pituitary, and hence this observation was investigated further. However, no aromatase was detected in the anterior pituitary. Panel A depicts attempted aromatase staining in the adult male rat pituitary. No aromatase staining was detectable, however it has been reported recently by Carretero and colleagues that aromatase P450 is present within the adult male rat pituitary (Carretero *et al.*, 1999). Panel B shows the control slide with aromatase immunopositive staining in the ovary. Details of antibodies and methods used for staining can be found in Chapter 2.

Appendix III: Pituitary ERα Staining



Appendix III: Oestrogen receptor α (ERα) staining in the male rat and male marmoset pituitary gland. Panel A depicts ERα staining in 18 day old rat pituitaries, whereas panel B shows staining in adult rat pituitaries (day 90). Panel C depicts staining for ERα in 35 week old marmosets. Panel D is a control section, showing staining for ER α in the rat uterus. Immunocytochemistry methods and antibody dilutions are detailed in Chapter 2.

Appendix IV: Commercial Suppliers

AB Gene (UK) Ltd
Ambion
Amersham Pharmacia Biotech Ltd
Anachem Ltd
Applied Biosystems
Becton Dickinson Laboratories Ltd
Boehringer Mannheim UK
CamBio
Cambridge Biosciences Ltd
Carl Zeiss Ltd
Clontech Laboratories Ltd
Elkay Products Inc
Genosys Biotechnologies (Europe) Ltd
Gibco Life Technologies Inc
Hybaid Limited
Invitrogen BV
Jencons (Scientific) Ltd
Kontes Scientific Instruments
MacWarehouse Ltd
Merck Ltd (BDH)
Millipore (UK) Ltd
Molecular Dynamics
NIDDK
OLYMPUS Optical Co Ltd
Perkin-Elmer Analytical Instruments
Promega UK Ltd
Santa Cruz Biotechnology Inc
Sigma Aldrich
Sorvall UK Ltd
Stratagene Ltd
Wallac UK

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