Hormonal Regulation of the Class B Scavenger Receptors CD36 and SR-BI, in the Rat Liver

By

Rebecca Lee Fitzsimmons

THE DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

THE SCHOOL OF MOLECULAR AND BIOMEDICAL SCIENCE

THE UNIVERSITY OF ADELAIDE

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Chapter 6 ROLE OF PITUITARY HORMONES IN THE SEXUALLY DIMORPHIC REGULATION OF CD36 EXPRESSION IN THE LIVER

6.1 Introduction

In the preceding chapter, it was shown that the presence or absence of the gonadal steroid hormones modulates hepatic expression of CD36 *in vivo*. However, as discussed, the changes observed following Gx and hormone re-instatement did not appear entirely consistent with a direct relationship between actions of either E2 or testosterone and expression of CD36. In male rats, neither Gx nor subsequent administration of testosterone altered the levels of *Cd36* mRNA in the liver, despite changes in expression of CD36 protein. Furthermore, there was a considerable delay between the time when E2 became undetectable in the serum of Gx-female rats and the time at which there was a detectable reduction in the level of CD36 protein in the liver. This evidence suggested the probable involvement of additional factors in the sexually dimorphic expression of CD36 in this organ, either as downstream regulators that are responsive to the actions of steroid sex hormones, or as components of larger endocrine networks that interact with the sex hormones to confer gender-specific regulation. This chapter examines this second possibility by investigating the hypothesis that particular pituitary-derived hormones are necessary to underpin the sex steroid effects and are thus required for manifestation of the sexually dimorphic expression of CD36 in the rat liver.

The anterior pituitary synthesizes six important peptide hormones - thyroid stimulating hormone (TSH), prolactin, adreno-corticotrophic hormone (ACTH), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and growth hormone (GH) - which are involved in diverse physiological functions such as sexual maturation, reproduction, stress responses, temperature homeostasis and metabolism. The release of hormones synthesized by the anterior pituitary gland is controlled by the stimulatory or inhibitory action of hypophysiotropic hormones delivered directly from the hypothalamus in episodic bursts, via the hypothalamus-hypophyseal portal system. Those of particular interest in this study were LH and FSH, as they regulate the production of the sex

steroids by the gonads, and GH because it exhibits sexually dimorphic patterns of synthesis, secretion and action in target tissues. The hypophysiotropic hormones responsible for controlling the secretion of GH (growth-hormone-releasing hormone [GHRH] and somatostatin or growth-hormone-inhibiting hormone [GHIH]), as well as gonadatropin-releasing hormone (GnRH) which stimulates the release of FSH and LH, are themselves subject to regulation by androgens and estrogens (Werner et al., 1988, Zorrilla et al., 1990, Zeitler et al., 1990b). The experiments described in this chapter investigate a number of pathways in which sex steroids interact with the hypothalamus-pituitary axis, examining whether they have a role in regulating expression of CD36 in the liver via the pituitary hormones LH and GH.

The following three hypotheses were examined.

1) The induction of CD36 expression in the liver of castrated male rats is related to dysregulation of LH secretion.
2) Female pre-dominant gender-biased expression of CD36 in the liver of normal adult rats is dependent on normal pulsatile secretion of GH.
3) Sex steroid regulation of CD36 in the livers of gonadectomised rats does not occur in models of GH deficiency.

6.2 Role of Gonadotropic Hormones in Regulation of Hepatic CD36 in Male Rats

6.2.1 Background

In sexually mature animals, the hypothalamus secretes the hormone GnRH in episodic bursts and this results in comparable secretory bursts of the gonadotropic hormones LH and FSH into the systemic circulation from the anterior pituitary gland. In females, LH and FSH perform a number of important roles that are essential for normal ovarian function. Stimulation of thecal cells by LH facilitates the synthesis of androgen from cholesterol and the mid-cycle surge of LH triggers ovulation, followed by lutenization of the follicle. In the later phase of the ovulatory cycle, LH stimulates production of progesterone and estrogen by the corpus luteum. FSH is required for early follicular development, as well as for the conversion of androgen into estrogen by granulosa cells.

As the relationship between the gonadotropic hormones and the sex steroids in females is inherently dynamic and complex, the experiments described in this chapter were confined to male rats.

In males, FSH is required for gametogenesis and the primary targets in the testis are the Sertoli cells that support spermatogenesis. In contrast, LH acts on Leydig cells to trigger testosterone secretion. Normally, testosterone levels in the blood feed back to the hypothalamus, negatively regulating secretion of GnRH and thus LH. In castrated animals, however, the feedback inhibition is released and this results in a characteristic surge of LH. Levels of LH then remain high, possibly driven by increased pulse frequency of GnRH release from the hypothalamus (Strobl et al., 1989). A theoretically possible cause of the increase in CD36 expression observed in the liver after castration was prolonged exposure to elevated LH levels. Although expression of the LH receptor has not been detected in the liver, it has been reported in extra-testicular sites such as the brain (including the hypothalamus, (Lei et al., 1993)), where it could mediate effects on the liver indirectly. For this reason, experiments were designed to test whether the post-castration increase in CD36 expression in the liver can occur if the resulting rise in LH is prevented.

Zoladex (Goserelin) is a GnRH superagonist. When delivered in long-acting depot capsules, Zoladex produces an initial stimulation of LH release, but this is followed by a profound reduction due to exhaustion of production by the pituitary. Loss of LH leads in turn to suppression of testosterone production in the testes, resulting in a reversible chemical castration. Unlike surgical castration, this is not accompanied by prolonged elevation of LH (Scott et al., 1992).

6.2.2 Castration-Induced Increase In Hepatic CD36 Expression Does Not Involve Gonadotropin Production

Zoladex implants used in this study (supplied by Astra Zeneca) were a kind gift from Prof. Brian Setchell (University of Adelaide). Eight week old male rats were randomly assigned to one of four experimental groups (n=5 per group). Two of the groups were castrated, while the other two underwent a sham operation. The animals were then allowed a two week post-operative recovery period. One of the groups from each condition underwent no further treatment, whilst the animals in the matching groups

each received a single, long acting zoladex implant by sub-cutaneous injection. Two weeks following implantation, animals in all groups were killed and to obtain blood, tissues and the weights of selected organs. Serum testosterone was measured using RIA, as described in Section 2.8.

Table 6.1 Weight and serum testosterone measurements of intact and castrated male DA rats, with and without Zoladex implants.

	INTACT		CASTRATED	
	Control	Zoladex	Control	Zoladex
Body Weight (g)	273.4 ± 13.0	255.0 ±11.4	237.2 ±13.5	254.4 ±10.4
Heart Weight (g)	0.896 ± 0.08	0.828 ± 0.06	0.818 ± 0.08	0.840 ± 0.10
Testis Weight (g)	1.216 ± 0.07	0.938 ± 0.05	-	-
Seminal Ves. Weight (g)	1.012 ±0.17	0.264 ± 0.04	0.118 ± 0.02	0.196 ± 0.08
Serum Test. (nmol/L)	6.815 ± 0.002	0.123 ± 0.10	0.009 ± 0.002	0.007± 0.003

Data are presented are mean ± SD (n=5/group).

Table 6.1 shows that treatment of intact animals with Zoladex resulted in dramatically decreased levels of testosterone, although residual low levels were still detectable. Total body weights and heart weights were not affected significantly by either surgical castration or treatment with Zoladex. However, the wet weights of the testes and seminal vesicles were significantly reduced compared to intact control rats that did not receive zoladex ($p<0.001$). These physiological effects of Zoladex are consistent with effective but incomplete chemical castration. They are also consistent with the observation that Zoladex treatment led to an increase in the area of hepatocytes stained by anti-CD36 in sections of liver from intact rats, relative to intact rats that received no treatment (Fig. 6.1 A-D). Importantly, there were no differences in the patterns of CD36 expression in sections from surgically castrated rats, Zoladex-treated intact rats and those that were both castrated and treated with Zoladex. Thus, irrespective of concomitant blockade of LH secretion by Zoladex treatment, chemical castration and surgical castration led to equivalent increases in expression of hepatic CD36 protein. It is unlikely, therefore, that LH contribute to the effects of castration on CD36 expression in male liver.

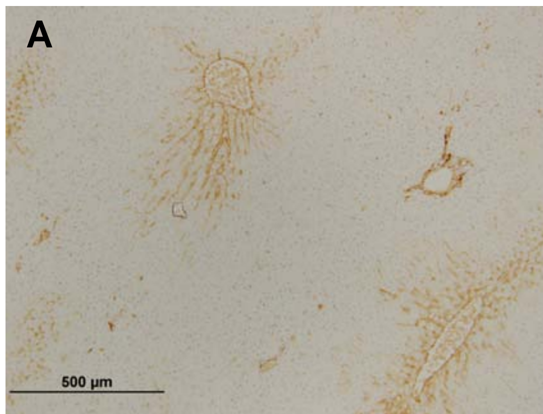
Figure 6.1: Expression of CD36 in the liver in male DA rats after castration and/or treatment with Zoladex.

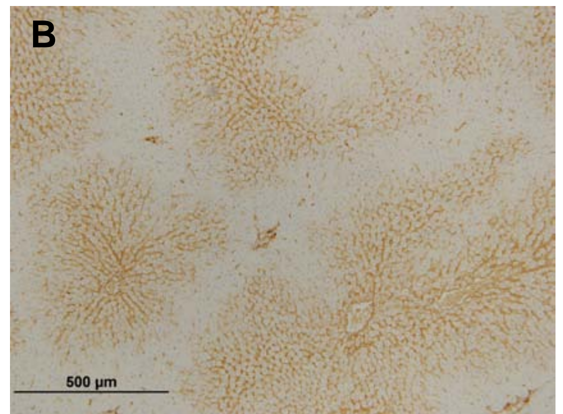
Analysis of CD36 in livers of 12 week old sham-operated (A and C) and castrated (B and D) rats by indirect immuno-histochemistry, using mAb UA009. In untreated (A) or Zoladex-treated (C) sham-operated males, only a thin rim of hepatocytes surrounding the central vein expressed CD36. In untreated (B) and Zoladex-treated (D) castrated rats, the whole of the centrilobular parenchyma contained hepatocytes that expressed CD36. N=5/group, representative photomicrograh shown.

Photographed using x10 objective.

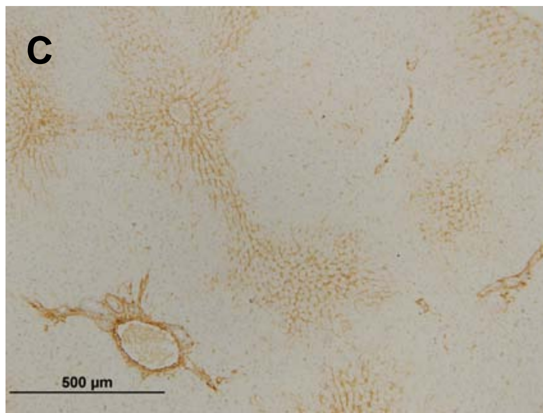
Sham-Operated

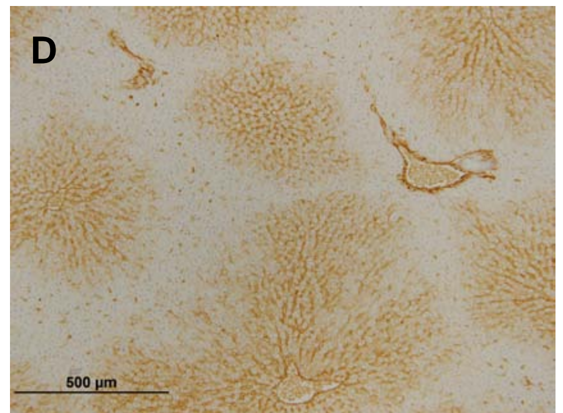
Castrated





Zoladex





6.3 Role Of Growth Hormone In The Gender Regulation Of Hepatic CD36 Expression

6.3.1 Background

Growth hormone (GH) is a 22 kDa peptide hormone that is produced and secreted by the anterior pituitary gland in response to somatotrophic hormones that are delivered from the hypothalamus via the hypophyseal portal circulatory system. GHRH and GHIH are released from the hypothalamus in pulsatile patterns that are out of phase with each other. The result of their concerted actions on somatotroph cells in the anterior pituitary is episodic secrection of GH into the general circulation (Plotsky and Vale, 1985). Pulsatile secretion of GH has been described in many mammalian species, including both humans (Winer et al., 1990) and rats (Tannenbaum and Martin, 1976). Male and female rats display distinctly different patterns of GH secretion, which emerge between 25-30 days of age, prior to the onset of puberty (Eden, 1979). Adult male rats have secretory episodes (with a 3-4 hour cycle), which are characterized by high amplitude peaks interspersed between extended trough periods during which plasma GH concentrations are almost undetectable. In comparison, female rats display higher frequency cycles (approximately 70 minutes in periodicity), but with lower pulse amplitudes. Furthermore, the trough periods are considerably shorter and baseline plasma GH concentrations persist at detectable levels throughout the inter-peak interval (reviewed in (Millard et al., 1987)). In broad terms therefore, the male pattern of expression is considered pulsatile, whereas the female pattern of expression is described as (comparatively) continuous. Overall, mean GH plasma concentrations are greater in male rats compared with females (Eden, 1979).

The role of the sex steroid hormones in the regulation of pulsatile synthesis and secretion of GH has been demonstrated by the administration of estrogen to male rats and testosterone to female rats (Gustafsson et al., 1983). Gender differences in the levels of GHRH (higher in females) and GHIH (higher in males) have been observed, and the levels of both are affected by sex steroid hormones (Gabriel et al., 1989, Shirasu et al., 1990, Zeitler et al., 1990a). The sexually dimorphic pattern of GH secretion contributes to phenotypic differences in growth, body composition and metabolism that are observed between males and females of many species, including rats (reviewed in Gatford et al., 1998, Robinson et al., 1998).

In the liver, GH exerts its action in target organs via the growth hormone receptor (GH-R). The expression of GH-R by the liver is developmentally regulated, increasing steadily from birth and attaining adult levels by puberty. A number of studies have described higher levels of binding by bovine GH to liver extracts from female rats, compared to male rats (Baxter et al., 1980, Maes et al., 1983). The current model of GH/GH-R interaction suggests that GH binds to pre-existing GH-R dimers, present on the cell membrane. Binding of the hormone induces a conformational change that activates (amongst others) the JAK/STAT pathway of signal transduction and regulation of gene transcription (reviewed in Lanning and Carter-Su, 2006). An area of active investigation surrounds attempts to elucidate the mechanisms by which signaling via the GH-R "translates" episodic versus continuous patterns of GH secretion into distinct patterns of gene expression in the liver. Accumulating evidence suggests that the genes encoding Stat5a and Stat5b display differential patterns of activation depending on whether plasma GH levels are pulsatile or continuous (Udy et al., 1997, Park et al., 1999, Choi and Waxman, 1999). In mouse liver, recent studies have demonstrated a role for the stat5 proteins in regulating gender-specific gene expression (Clodfelter et al., 2007). Therefore, gender differences can be observed in synthesis and secretion of GH, expression of GHR and in responsiveness of the rat liver to GH signaling.

The expression of a number of genes encoding metabolically important enzymes in the liver is regulated by the sexually dimorphic patterns of GH secretion. The cytochrome P-450 (CYP) superfamily consists of a large group of hepatic enzymes that are involved in the detoxification of xenobiotics as well as the metabolism of steroids. In rat liver, many of the CYP isoforms display either female-predominant or male-predominant gender-biased expression, and these patterns are dependent on the presence of intact pituitary function (reviewed inOinonen and Lindros, 1998). Interestingly, the many studies that have been performed on hypophysectomised or GH-deficient animals have not revealed a "one-size fits all model" for the regulation of CYP gene expression by GH. Rather, the response to exogenous GH, re-instated under various regimens, appears to be highly individual for a given CYP isoform. (Agrawal and Shapiro, 2001). In addition to the cytochrome P450 superfamily, female pre-dominant expression of alcohol dehydrogenase (Simon et al., 2002), liver fatty acid-binding protein (Carlsson et al., 1998) and microsomal triglyceride transport protein (Ameen and Oscarsson, 2003) appears to be regulated, at least partially, by GH in rat liver. In contrast, Jalouli et al

(2003) found that whilst GH was involved in the regulation of peroxisome proliferator-activated receptor alpha (PPARα) in the liver, the sex difference in expression of this protein was controlled by gonadal steroids, rather than by gender specific pulsatile secretion of GH.

The aim of the next set of studies was to determine whether the gender difference in CD36 expression in rat liver was preserved following disruption of GH secretion. Two rat models of GH deficiency were examined in preference to the classical approach of hypophysectomy, which results in multi-hormone deficiency (including disruption of the hypothalamic-pituitary-gonadal axis). Expression of CD36 in the liver was examined in rats that had received neonatal administration of monosodium glutamate (MSG) and in a rat strain with genetic deficiency of GH (the *dw/dw* rat strain).

6.3.2 Neonatal Monosodium Glutamate Administration As A Model Of Growth Hormone Deficiency

6.3.2.1 Background

The subcutaneous administration of (MSG) to suckling rats (4 mg/kg on post-natal days 2,4,6,8 and 10) results in selective and irreversible destruction of neurones in the arcuate nucleus. As these neurons are responsible for the synthesis of GHRH, production and secretion of GH is defective in the treated animals. In contrast, MSG does not appear to affect the synthesis and secretion of GHIH by the hypothalamus (Bloch et al., 1984). In adult rats raised from MSG-treated neonates, both males and females have been found to display an approximately 90% decrease in total hypothalamic GHRH content, although amounts of GH remain normal. The lack of GHRH results in a marked inhibition of GH secretion (approximately 85-90% lower than control, saline-treated rats) in both sexes (Maiter et al., 1991). Importantly, in the context of this study, gender differences in the patterns of pulsatile secretion were significantly reduced, as shown in Figure 6.2 (reproduced from Maiter et al (1991)). The adult phenotypic consequences of neonatal MSG treatments are dramatic. Treated rats display stunted growth, obesity, reduction in wet weights of reproductive and endocrine organs, impaired fertility and unusual behavioural characteristics such as tail auto-mutilation (Nemeroff et al., 1981, Olney, 1969). Although overall body weight is generally reported to be normal or marginally lower than in controls, MSG treated rats

Figure 6.2: Plasma GH profiles in rats treated as neonates with monosodium glutamate (MSG).

Figure from Maiter and colleagues (1991) showing the effects of neonatal treatment with MSG on Plasma GH profiles in both male and female SD rats. Measurements of plasma GH levels at 15 minute intervals illustrate the differences in secretion profiles between males and females in intact controls, and the inhibition of GH secretion that occurs in both genders when rats are treated as neonates with MSG.

NOTE:
This figure is included in the print copy of
the thesis held in the University of Adelaide Library.

From: Maiter et al 1991

have decreased body length and increased adiposity (Dawson, 1986, Dolnikoff et al., 2001).

6.3.2.2 Physical Characteristics And Hepatic CD36 Expression In MSG-Treated Rats At Nine Weeks Of Age

The experiments presented in this study investigate the effects of MSG administration to neonatal male and female DA rats on expression of CD36 in the liver at ages 9, 13 and 17 weeks.

For each experiment, two newborn litters (age less than 24 hours) were assigned to either the MSG or the iso-osmotic saline treatment (control) group. Treatment was administered by subcutaneous injection in the loose skin of the dorsum of the neck on post-natal days 2, 4, 6, 8 and 10. At 3 weeks of age pups, were weaned, sexed and thereafter were weighed weekly until they were killed at 9 weeks of age. In order to confirm the efficacy of this treatment regimen in the DA strain, body weights were compared in the MSG- and saline-treated groups (Fig. 6.3A). The male rats treated with MSG (n=6) were significantly smaller than their saline-injected counterparts (n=3) when first measured at 4 weeks of age (one week after weaning), and they maintained this trajectory into adulthood. The difference in body weight between female MSG-treated rats (n=4) and controls (n=1) appeared to be less than in males. A trend toward moderately decreased body weight was apparent, however, in this experiment the sample size was insufficient to determine statistical significance. These results were congruent with those of published studies on Sprague Dawley rats (Hu et al., 1993, Maiter et al., 1991).

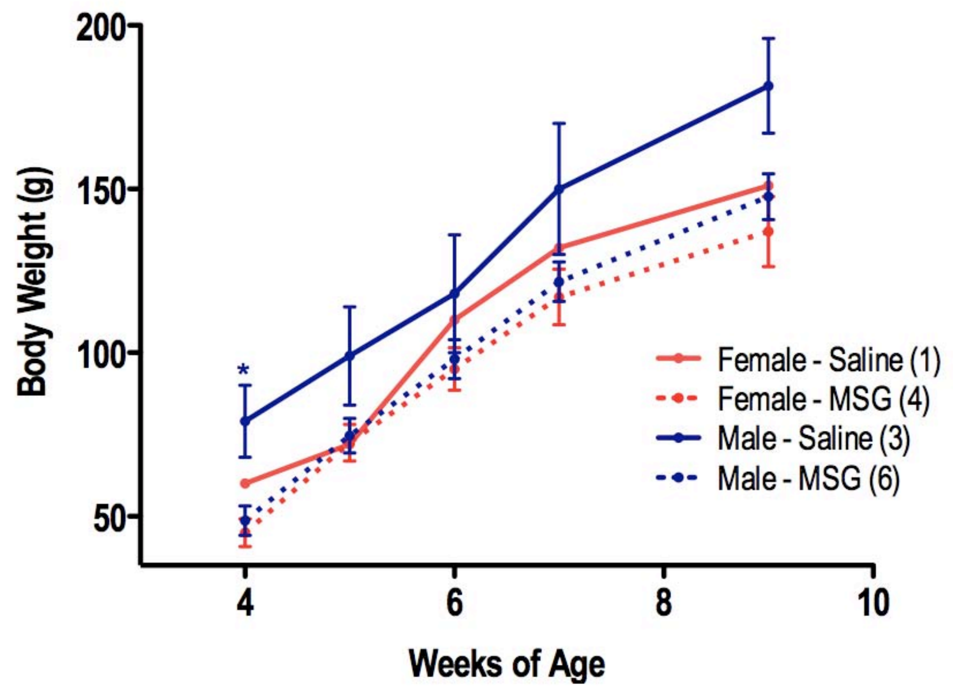
At the time of sacrifice the wet weights of the steroidogenic and accessory sex organs were measured, and samples of liver and heart tissue were frozen in OCT for later immune-histochemical analysis. Even after the tissue wet weights were corrected for the lower body weight of the MSG-treated rats, the average testicular mass was 52% lower in the treated group ($p < 0.001$) (Fig. 6.3B). The weights of the heart and seminal vesicles were lower in MSG-treated animals, but the differences were not statistically significant ($p > 0.5$). This biometric data were also consistent with an earlier report, which described both decreased testicular size and serum testosterone levels in MSG-treated rats (Nemeroff et al., 1981). Taken together, total body and organ weights

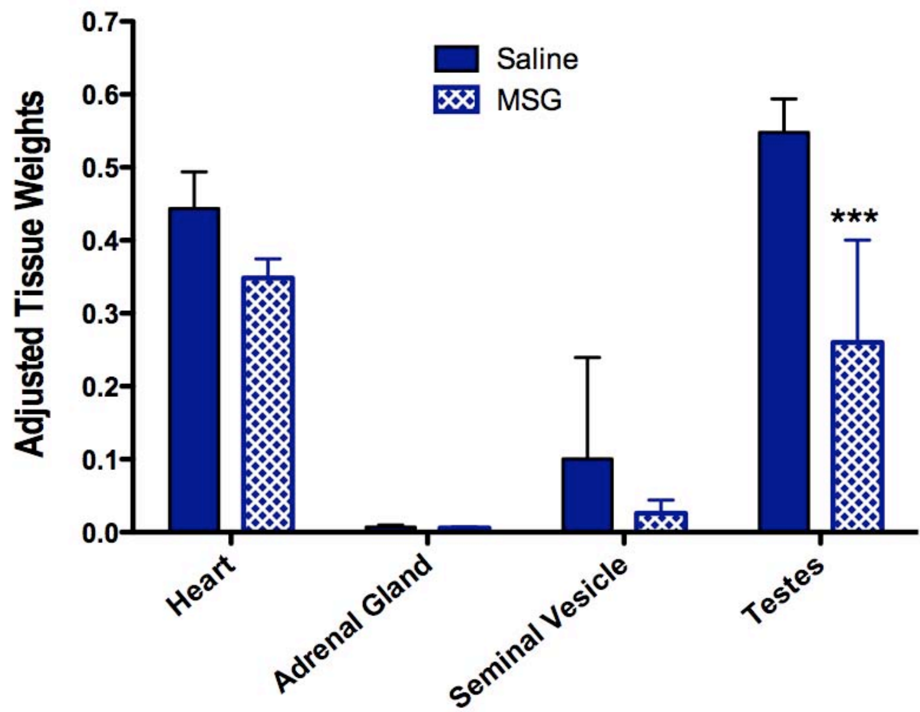
Figure 6.3: Effects of MSG-treatment as neonates on body and organ weights in juvenile and young adult rats.

A. Male and female rats were weighed weekly from 4 weeks of age until the day of sacrifice (9 weeks of age). In all groups, increase in body weight was approximately linear over this period. In males, neonatal MSG treatment resulted in significantly lower body weight than saline-treated litter mate controls at 4 weeks of age and this trajectory continued throughout the course of the experiment. Weights of MSG-treated males and females were similar, but due to lack of numbers, comparison with saline-treated female litter mate controls was not possible. The number of animals/group is shown in brackets. With the exception of the female-saline group (n=1), data is presented as mean +/- SEM.

B. Weights of selected organs from male MSG-treated and saline-treated control rats at the time of sacrifice. Testes and seminal vesicles were included as tissues that are specifically testosterone-sensitive, and heart and adrenal as tissues that are not primarily testosterone-sensitive. For each animal, the organ weight was corrected by normalising against whole body weight.

*** $p<0.001$, * $p<0.05$.


A
200
150
100
50
Body Weight (g)
*
Female - Saline (1)
Female - MSG (4)
Male - Saline (3)
Male - MSG (6)
4
6
8
10
Weeks of Age


B
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Adjusted Tissue Weights
Saline
MSG
***
Heart
Adrenal Gland
Seminal Vesicle
Testes

provided a good indication that administration of MSG to DA rats produced results that were comparable to those reported in other rat strains.

To examine the expression and pattern of distribution of CD36, frozen sections of liver collected from MSG-treated or saline control animals were stained with mAb UA009, using indirect immunohistochemistry. Neonatal treatment with MSG resulted in increased expression of CD36 in livers from both male and female rats (Fig. 6.4A-D). In treated females, essentially all of the hepatocytes in the hepatic lobules were stained, while in males there was a dramatic extension of staining towards the periportal regions. Vacuolation of hepatocytes within the stained regions of the lobules, thought to be due to the presence of lipid droplets, was visible in some sections from MSG-treated male (but not female) rats (Fig. 6.4D). This observation suggests that there was steatosis in the livers of the treated male rats, probably associated with the accumulation of triglyceride. Expression and distribution of CD36 in the livers of the saline-treated controls was similar to that observed in untreated rats of the same age (see Fig. 4.3). Overall, the gender difference in hepatic CD36 expression in nine week old rats was considerably diminished by neonatal administration of MSG. In contrast to the liver, no gross differences in CD36 expression were observed in sections prepared from hearts of MSG- or saline-treated male and female rats. (Fig. 6.4 E-H).

6.3.2.3 Physical Characteristics Of Older MSG-Treated Rats And Hepatic Expression Of CD36.

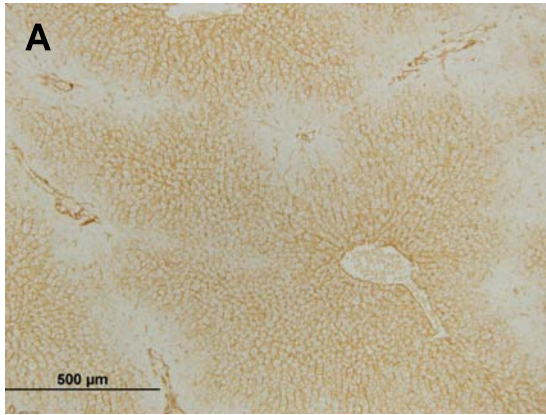
Pulsatile pattern of GH secretion, GH receptor expression and serum steroid hormone levels all change during development and also with ageing during adulthood. Serum GH levels peak at 12 weeks of age in rats and diminish thereafter (Hu et al., 1993). Therefore, the effect of MSG treatment on gender-specific expression of CD36 in the liver was also examined in older rats (at 13 and 17 weeks of age). In this experiment, the effect of MSG treatment on body growth, body fat distribution, and weight of gonadal and secondary sex organs was examined in more detail.

At 13 weeks of age, the development of severe obesity was evident in both male and female MSG-treated rats. Although clearly shorter in length at this age (not measured), as shown in Table 6.2 the average body weight of the males (274±5 grams) exceeded that of the saline-treated control group (255±15 grams). However, the MSG-treated

Figure 6.4: Expression of CD36 in liver from male and female MSG-treated rats at 9 weeks of age.

Expression of CD36 was analysed by indirect immuno-histochemistry in frozen liver sections from 9-week-old male and female rats that had been injected with either MSG or saline (control) as neonates. A and B (saline, female and male respectively); the typical adult pattern of CD36 expression, as described previously. C and D (MSG, female and male respectively); increased proportion of parenchymal cells in hepatic lobules express CD36. Arrow indicates vacuolation of hepatocytes in MSG-treated male rats. E and F (saline, female and male respectively) and G and H (MSG, female and male respectively); expression of CD36 in heart tissue was comparable in MSG- and saline-treated rats of both genders.
Photographed using x10 objective.

Female
Male
NaCl
MSG
NaCl
MSG



A
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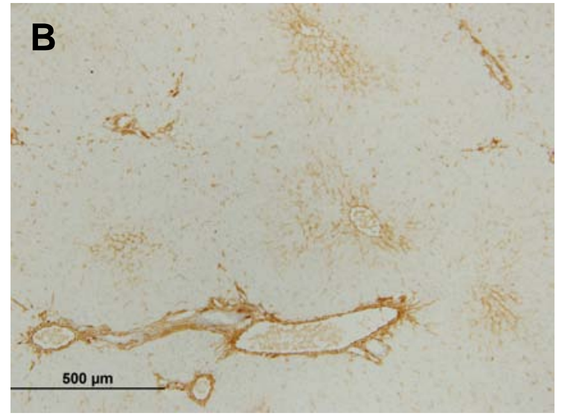

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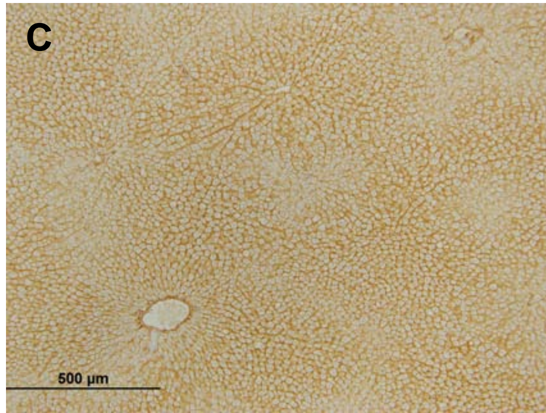

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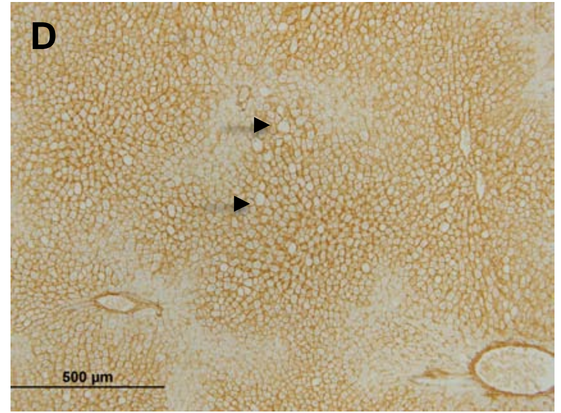

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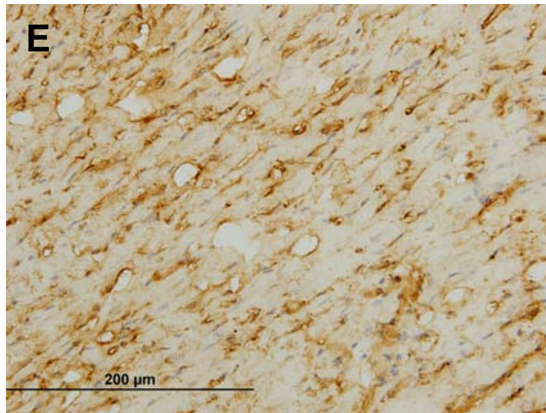

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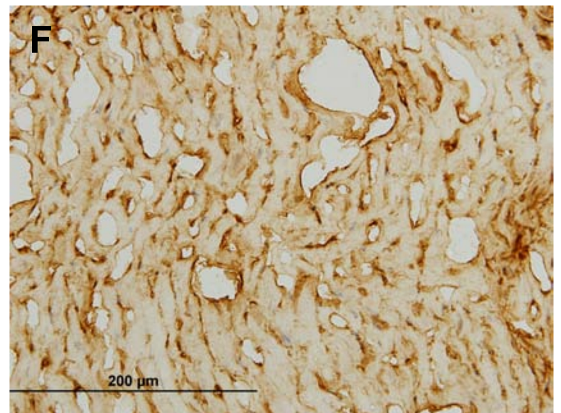

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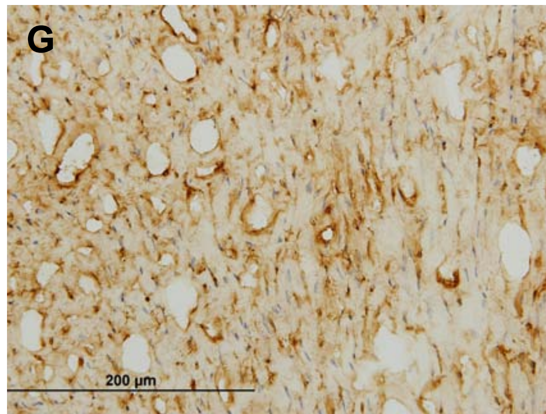

G
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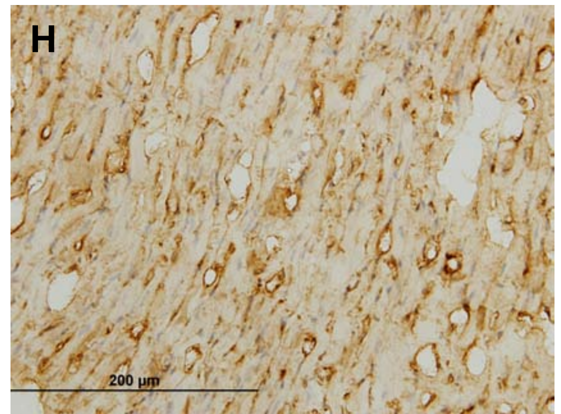


H
200 µm

females remained lighter than their saline-treated counterparts (160±10 grams compared to 184±3 grams, respectively).

Table 6.2: Body weight and adipose wet weight measurements of 12 week-old female and male DA rats, with and without MSG treatment.

	FEMALE		MALE	
	Saline	MSG	Saline	MSG
Body Weight (g)	184.13 ± 2.82	160.47 ± 9.47	255.41 ± 14.97	273.62 ± 5.29
Total Fat Pad Weight (g)	12.71 ± 0.92	18.99 ± 3.13	17.87 ± 3.45	37.17 ± 5.94
Adiposity Index (%)	6.90 ± 0.39	11.87 ± 2.03	6.99 ± 1.23	13.60 ± 2.31

Data are presented are mean ± SD (n=3/group).

To obtain a visual representation of the increased fat depot mass, one animal from each experimental group (male and female, MSG- or saline-treated) was euthanized and examined by magnetic resonance (MR) imaging. Dr Jamie Taylor from the Dept of Radiology, Royal Adelaide Hospital performed the MR imaging on a clinical magnetic resonance imager. Axial and saggital images of female (Fig. 6.5 A-B) and male (Fig. 6.5 D-E) rats revealed extensive fat accumulation in both the subcutaneous and visceral compartments of MSG-treated animals. At dissection, the fat depots were weighed and statistically significant increases were recorded in the wet weights of the gonadal (epidydimal or ovarian), mesenteric, retro-peritoneal and subcutaneous fat pads of MSG-treated male and female rats (Fig. 6.5 C and F). In particular, the weights of the subcutaneous fat pads (adjusted for body size) were twice as large in MSG-treated animals, as reflected in the calculated adiposity indices for both sexes (Table 6.2). As observed in the younger male rats, MSG-treatment also resulted in significantly lower corrected testicular and seminal vesicle wet weights, and heart weights were also significantly lower. MSG treatment had no significant effects on uterine, ovarian or heart weights in female rats.

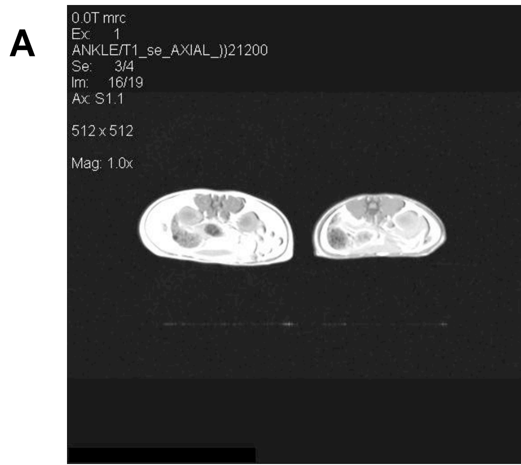
The expression and distribution of CD36 in liver from the 13 week old and 17 week old MSG and saline-treated female and male rats was then examined by indirect immunohistochemisty. The increased intensity of staining in the 17 week old animals (Fig. 6.6 E-H) compared with the 13 week old animals (Fig. 6.6 A-D) is not necessarily

Figure 6.5: Body composition analysis of MSG-treated rats at 12 weeks of age.

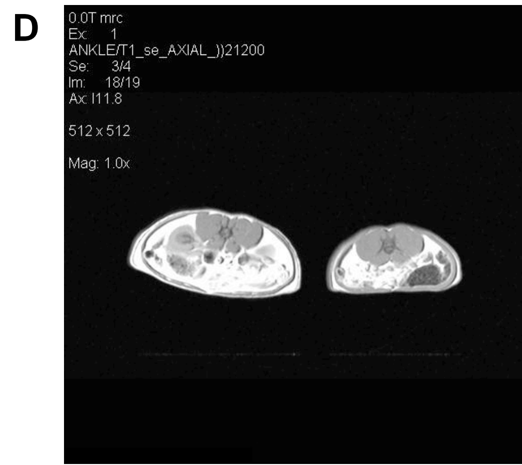
Magnetic resonance images displaying fat in both the visceral and subcutaneous compartments. Axial (A, D) and sagittal (B, E) images, in which adipose tissue is highlighted and therefore appears white. In each image, the MSG-treated rat is on the left and the saline-injected animal is on the right. In both sexes, neonatal treatment with MSG is associated with increased in adipose tissue at both subcutaneous and visceral sites. The increased adiposity is illustrated in graphs of tissue weights for females (C) and males (F). In each rat, adipose depots were dissected and weighed individually and corrected for total body weight (mean +/- SD, n=3/group).

*** $p<0.001$, **$p<0.01$, * $p<0.05$.

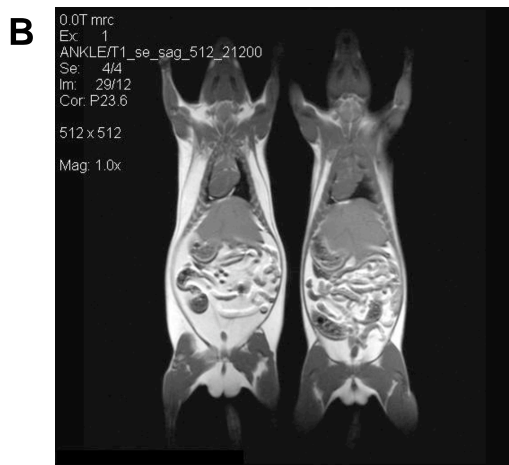
A



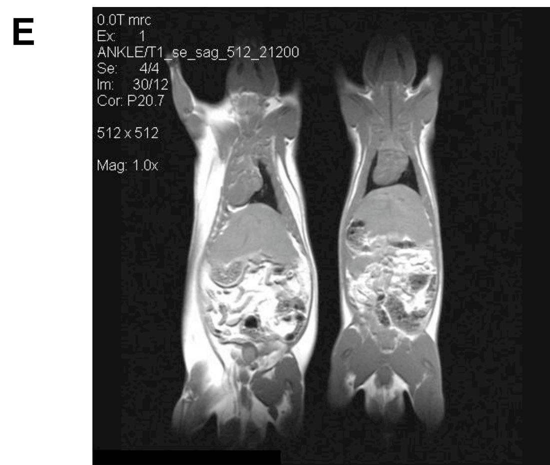
D



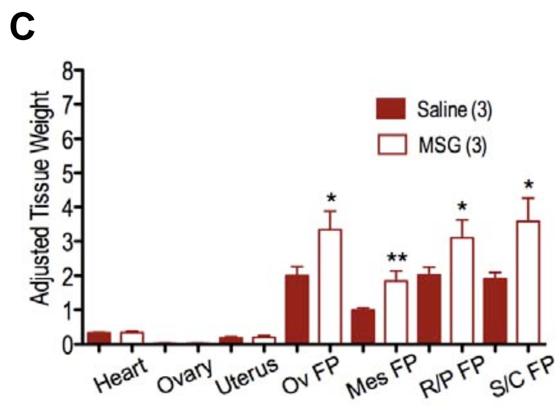
B



E



C



F

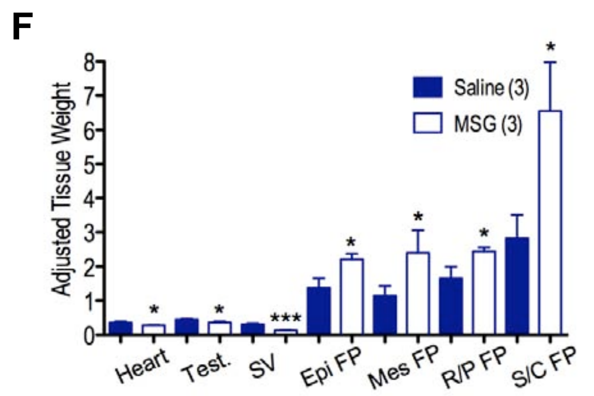
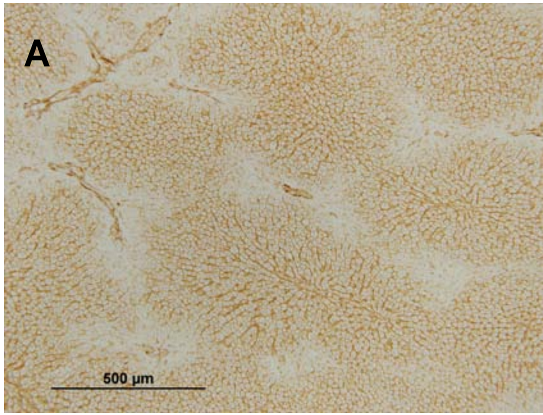


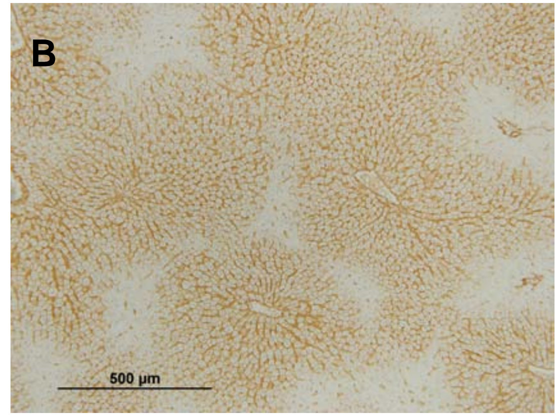
Figure 6.6: Expression of CD36 in liver from older MSG-treated DA rats.

Analysis of CD36 in livers of 13 week old (A-D) and 17 week old (E-H) male and female rats that were treated as neonates with either MSG or saline, using mAb UA009 in indirect immuno-histochemistry. At both time points, the proportion of the parenchyma that expressed CD36 in hepatic lobules was similar in the MSG-treated (B and F) and age-matched saline-injected (A and E) females. In comparison, a greater proportion of the lobule was stained in MSG-treated male rats (D and H) than in controls (C and G), at both time points. A representative image is shown from each group (n=3-5 animals/group).
Photographed using x10 objective.

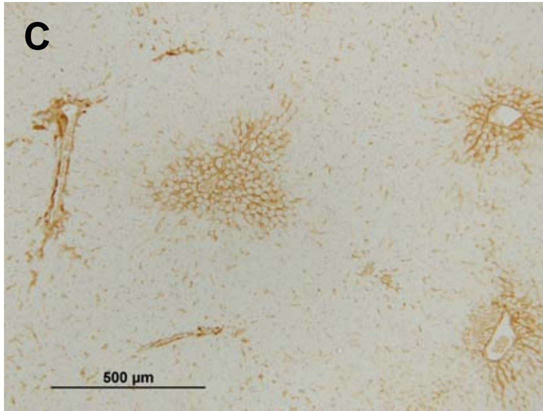
Saline MSG

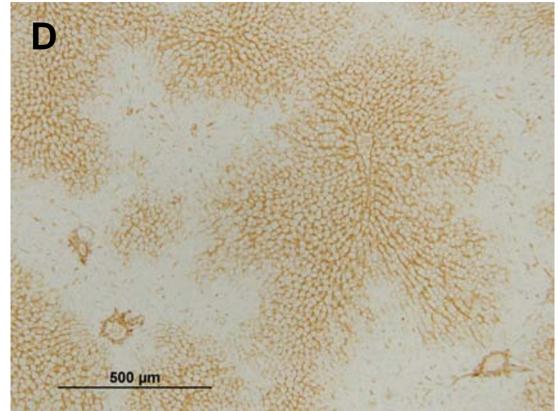
Female



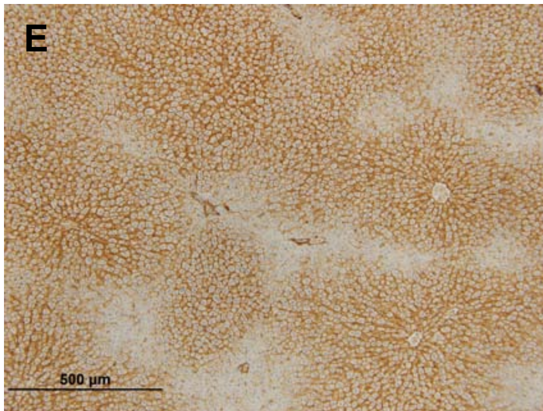


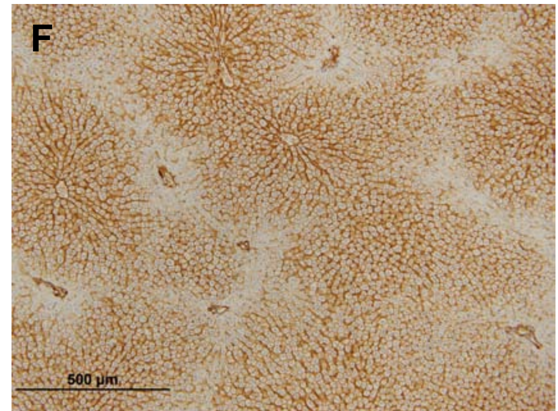
Male



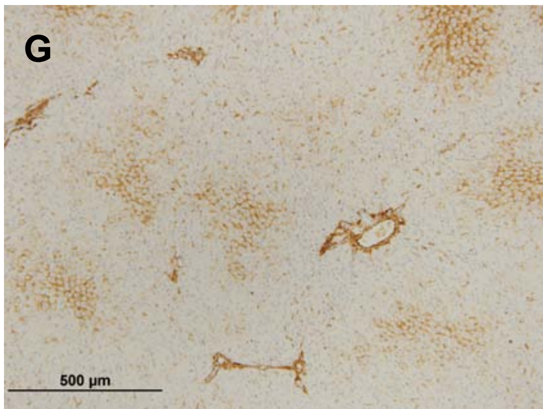


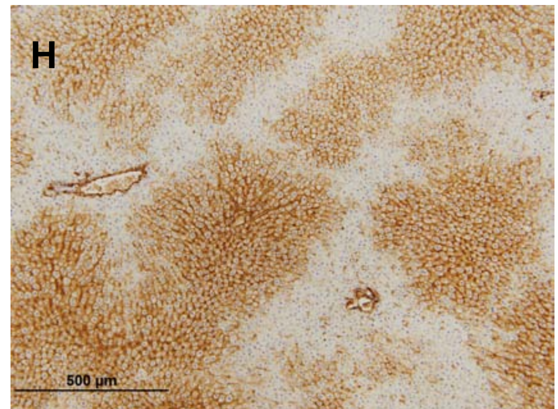
Female





Male





indicative of higher levels of CD36 expression because the two batches of sections were stained at different times. However, differences in the distribution of staining can be considered reliable. In contrast to rats examined at 9 weeks of age, where there was an increase in the area of hepatocytes expressing CD36 in liver from MSG-treated female rats (see Fig. 6.4A and C), in these older female rats staining was similar in liver sections from the MSG-treated and control groups at both ages (Fig. 6.6 A-B and E-F). In males, as in 9 week old rats (see Fig 6.4 B and D), a considerably greater proportion of hepatocytes were stained by mAb UA009 in the MSG-treatment group compared with the control group at both 13 and 17 weeks of age. In these older rats, the reduced gender difference between MSG-treated males and females was a consequence of increased expression by male rats.

6.3.2.4 Effect Of Administration Of Testosterone To MSG-Treated Male Rats

In male DA rats, as in other strains (Nemeroff et al., 1981), testicular mass is decreased as a consequence of neonatal MSG treatment (Fig. 6.5F). Furthermore, Nemeroff et al (1981) have also demonstrated that neonatal treatment of male CD-stock Albino rats with MSG results in a 68% reduction in serum testosterone levels in comparison with age-matched adult male controls. This evidence, together with the finding that castration is associated with increase in hepatic expression of CD36 and that administration of testosterone restores CD36 expression to normal levels (Chapter 5), raised the possibility that the up-regulation of hepatic CD36 observed in male MSG-treated rats was due to hypo-gonadism and an associated reduction in serum testosterone levels.

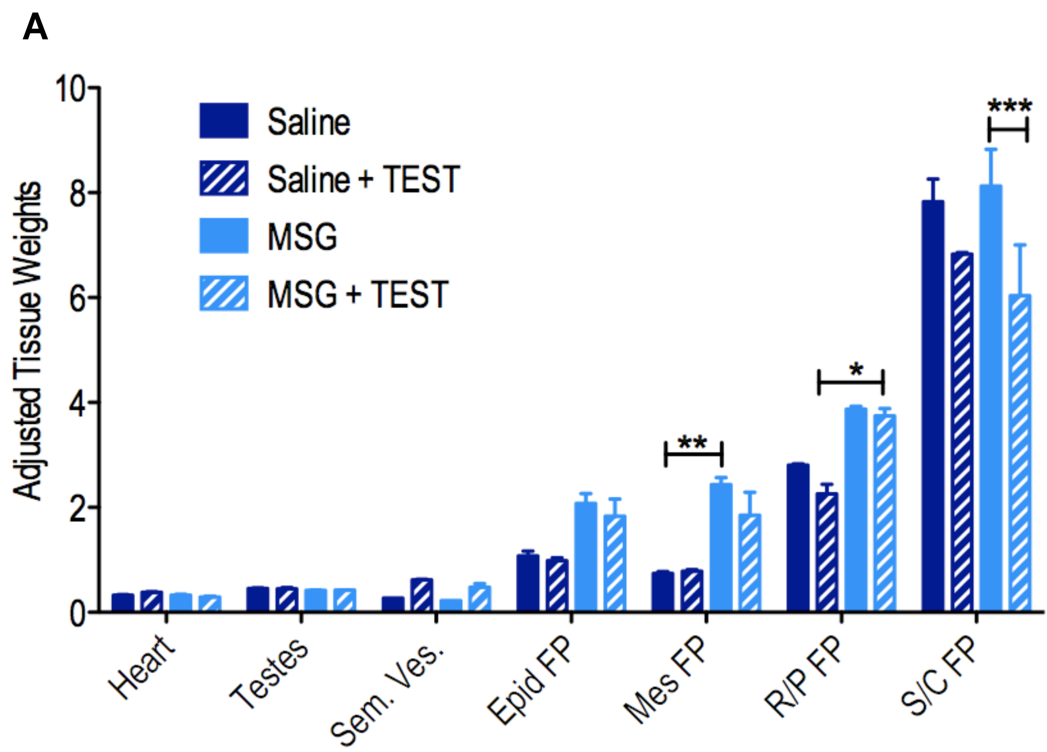
In order to test this hypothesis, the male pups from one newborn litter (n=4) were treated with saline, while those from a second litter (n=6) received MSG injections, according to the protocol described in section 2.5. At ten weeks of age, half of each litter received injections of testosterone every second day for one week and the remaining animals received vehicle only (peanut oil). At the completion of the hormone treatment regimen, all four cohorts of rats were killed, organs were weighed and samples of liver were frozen in OTC for immuno-histochemical analysis. As found previously (Fig. 6.5F), weights of testes and seminal vesicles tended to be lower in rats treated with MSG (Fig. 6.7A). Treatment with testosterone increased the weights of the seminal vesicles in both control rats and MSG-treated rats, and although the numbers of

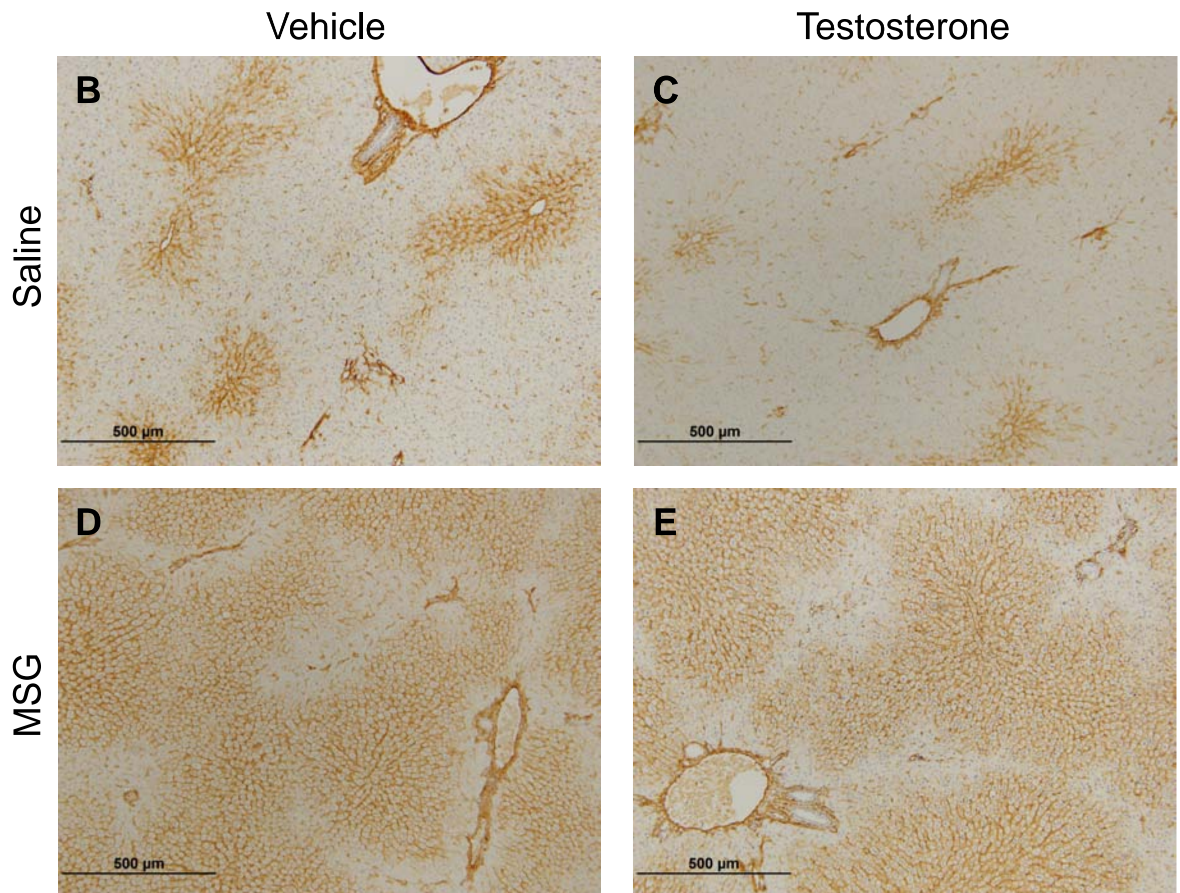
Figure 6.7: Adiposity, and expression of CD36 in liver, in male DA rats after treatment with testosterone and/or MSG.

Male DA rats that had been treated neonatally with either MSG or saline received either testosterone or vehicle control (peanut oil) at 10 weeks of age. Tissue weights (A) were measured at dissection and corrected for overall body weight (mean +/- SEM). Groups were analysed by ANOVA with Bonferonni post-tests applied to determine significant differences. *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

Expression of CD36 in liver was examined by indirect immuno-histochemistry, using mAb UA009. Administration of testosterone to control animals (C) animals further restricted the proportion of the parenchyma in hepatic lobules that expressed CD36, compared to control animals that did not receive the hormone (B). In contrast, in MSG-treated males, treatment with testosterone (E) did not affect expression of CD36 by parenchymal cells relative to that observed in the vehicle-treated controls (D). Representative image shown from each group (n=2-3 animals/group).

Photographed using x10 objective.


A
Adjusted Tissue Weights
10
8
6
4
2
0
Saline
Saline + TEST
MSG
MSG + TEST
***
*
**
Heart
Testes
Sem. Ves.
Epid FP
Mes FP
R/P FP
S/C FP


Vehicle
Testosterone
Saline
MSG
B
C
D
E
500 µm
500 µm
500 µm
500 µm

rats in the groups were insufficient for this difference to reach statistical significance, the results indicate that the treatment was effective. In saline-treated control rats, administration of testosterone did not significantly affect the relative weight of the any of the adipose depots. In contrast, testosterone did produce a modest decrease in the relative size of most adipose depots in MSG-treated male rats. Interestingly, the largest effect was on subcutaneous fat depots which were significantly decreased from 8.13% of body weight to 6.03% ($p<0.001$).

Comparison of staining by mAb UA009 in liver sections from saline-treated rats indicated that administration of pharmacological doses of testosterone did not completely abolish expression of CD36. Although not quantitated by VIA, due to the small sample sizes in this experiment, the distribution of CD36 appeared to be reduced to a narrower rim of cells surrounding the central veins than in the rats that received vehicle alone (Fig. 6.7B and C). In contrast, in rats that were treated with MSG as neonates, the intensity and distribution of staining by mAb UA009 was unaffected by the administration of exogenous testosterone (Fig. 6.7D and E). As expression of hepatic CD36 is elevated in the MSG-treated rats, this experiment demonstrates that GH, rather than testosterone, is responsible for male suppresion of hepatic CD36 expression.

6.3.3 *Dw/Dw* Rats: An Alternative Model Of Growth Hormone Deficiency

6.3.3.1 Background

The dwarf rats (*dw/dw*) employed in this study arose as a spontaneous mutation from an inbred Lewis rat colony (Charlton et al., 1988). The mutation is inherited in an autosomal recessive pattern and post-weaning, the homozygous *dw/dw* rats display significant impairment of growth rates. At adulthood, the body weights of male and female *dw/dw* rats are between 40-50% lower than their age and gender matched *dw/+* heterozygote counterparts. Furthermore, analysis of anterior pituitary hormone content revealed that GH concentration was at <10% of the levels measured in the phenotypically normal dw/+ littermates. In contrast, levels of other trophic hormones such as prolactin, TSH and LH were unaffected by the mutation. While only residual amounts of GH (<5%) were detected in serum from both male and female *dw/dw* rats, pulsatile patterns were still observed in this residual secretion (Carmignac and Robinson,

1990). Despite further investigation, the precise mutation that is responsible for the *dw/dw* phenotype has not been defined. Functionally however, the mutants appear to carry a defect in growth hormone releasing hormone (GHRH) signaling. This has mechanism has been hypothesized because a) GHRH synthesis by the hypothalamus is normal (Mizobuchi et al., 1991), b) levels of mRNA encoding the GHRH receptor are decreased in the pituitary (Carmignac et al., 1996) and c) binding of GHRH to pituitary membranes from *dw/dw* rats is severely impaired (Gardi et al., 2002). Although these mutants do not display complete GH deficiency, this model was selected because unlike hypophysectomised or MSG-treated animals, *dw/dw* rats have a selective GH deficiency while retaining a relatively normal endocrine profile in other respects.

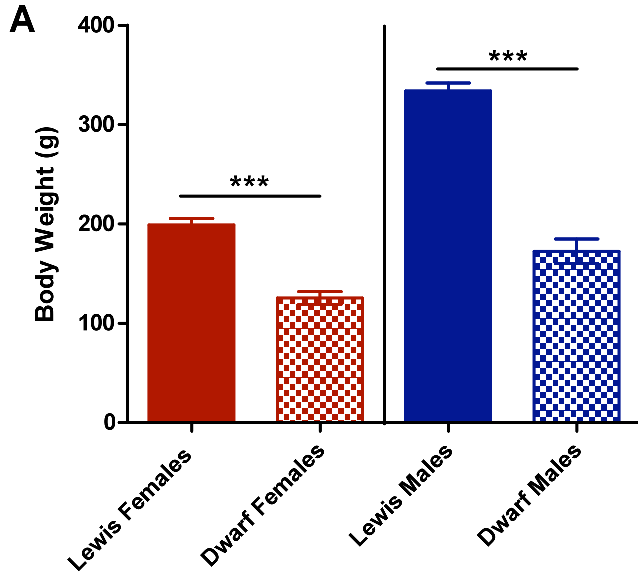
6.3.3.2 Physical Characteristics Of Adult *Dw/Dw* Rats

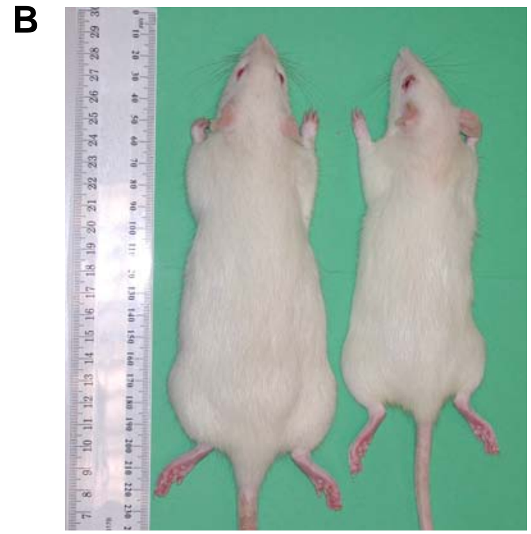
Initially, five male and female *dw/dw* rats, plus five Lewis rats of each sex (+/+ isogeneic controls), were obtained at age 12 weeks and weighed. They were then killed to measure wet weights of selected tissues and to obtain samples of liver for immuno-histochemical analysis. The body weights of female and male *dw/dw* rats were significantly lower than those of the age-and gender-matched Lewis controls (Fig. 6.8 A) and corresponded closely with the values reported by Charlton et. al. (1988). The same authors reported that longitudinal bone growth was reduced in dw/dw rats and the side by side photograph presented in Figure 6.8B compares the nose-to-base-of-tail length in females *dw/dw* and (Lewis) rats. The *dw/dw* strain is reported to be susceptible to obesity when maintained on a high fat diet (Clark et al., 1996), although during development (6 to 9 weeks of age) both males and females are leaner than wildtype controls when fed a conventional diet. This difference appears to dimish with age, and is absent in one year old males (Davies et al., 2007) but details of body composition and adiposity during the interim period have not been described. Figures 6.8 C and D show the wet weights of a number of selected organs and adipose depots (adjusted for total body weight) in twelve week old rats. In contrast to the MSG-treated rats, the female *dw/dw* rats were leaner than their Lewis strain counterparts, most of this difference accounted for by reduction in ovarian and retro-peritoneal fat relative to total fat. Adiposity was similar in male *dw/dw* rats and non-mutant controls. Therefore, with less evidence of confounding influences from disturbed metabolism, *dw/dw* rats appear to be a better model than MSG-treated rats for investigating the effects of GH deficiency on the expression of CD36 in the liver.

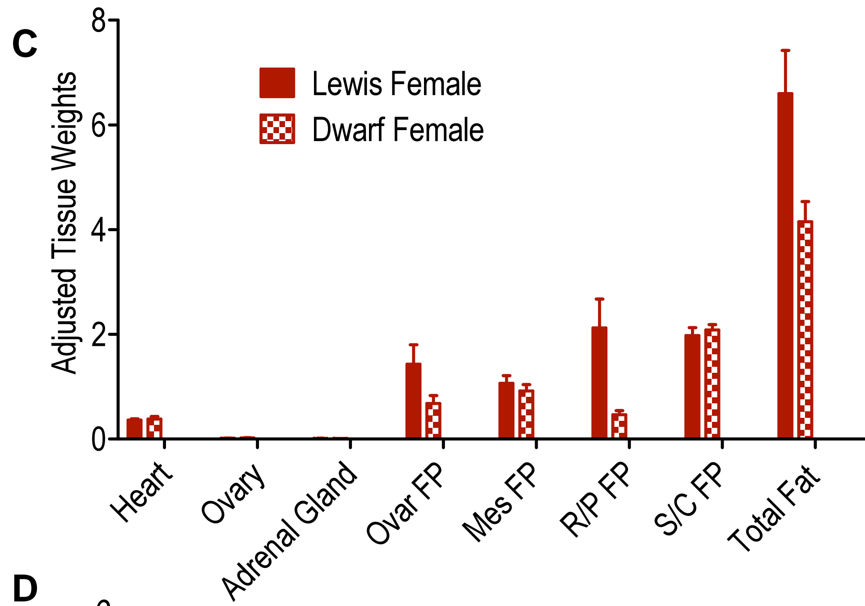
Figure 6.8: Analysis of body composition of Dwarf and Lewis rats.

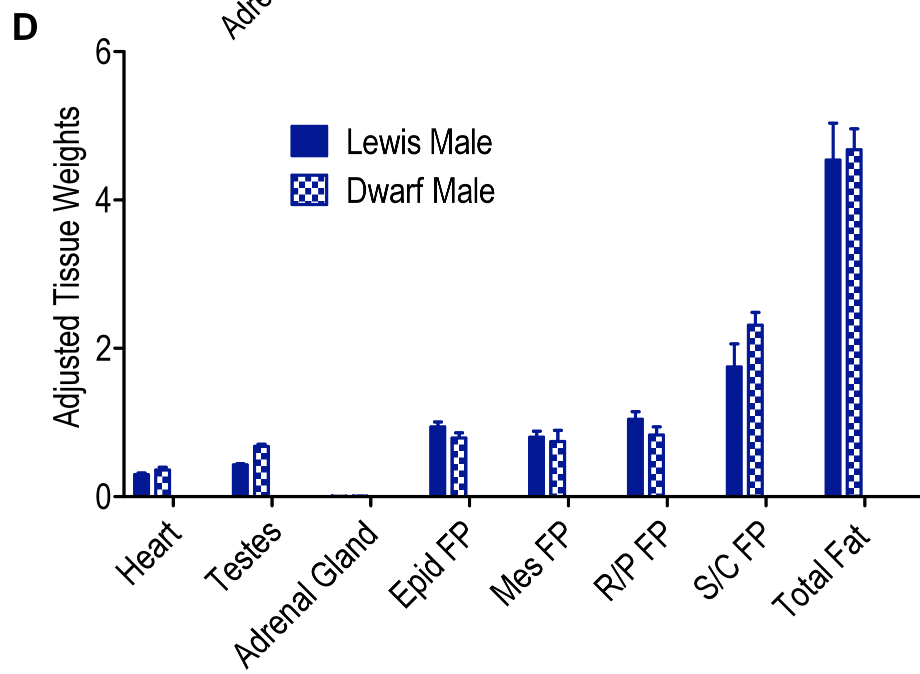
Male and Female rats were weighed and body lengths were measured at the time of dissection. Both male and female Dwarf rats had significantly lower body weights than Lewis counterparts of the same age (A). Photograph illustrating a lesser nose-to-tail-length in a representative dwarf female rat, compared to a representative female from the Lewis control strain (B). Wet weights for a selection of tissues, collected at dissection from female (C) and male (D) rats, corrected for body weight. (n =5 animals/group, mean +/- SD).

*** $p<0.001$


A
Body Weight (g)
400
300
200
100
0
***
***
Lewis Females
Dwarf Females
Lewis Males
Dwarf Males


B


C
Adjusted Tissue Weights
8
6
4
2
0
Lewis Female
Dwarf Female
Heart
Ovary
Adrenal Gland
Ovar FP
Mes FP
R/P FP
S/C FP
Total Fat


D
Adjusted Tissue Weights
6
4
2
0
Lewis Male
Dwarf Male
Heart
Testes
Adrenal Gland
Epid FP
Mes FP
R/P FP
S/C FP
Total Fat

6.3.3.3 Comparison Of CD36 Protein And *Cd36* mRNA Expression In Livers From Male And Female *Dw/Dw* And Wildtype Lewis Rats

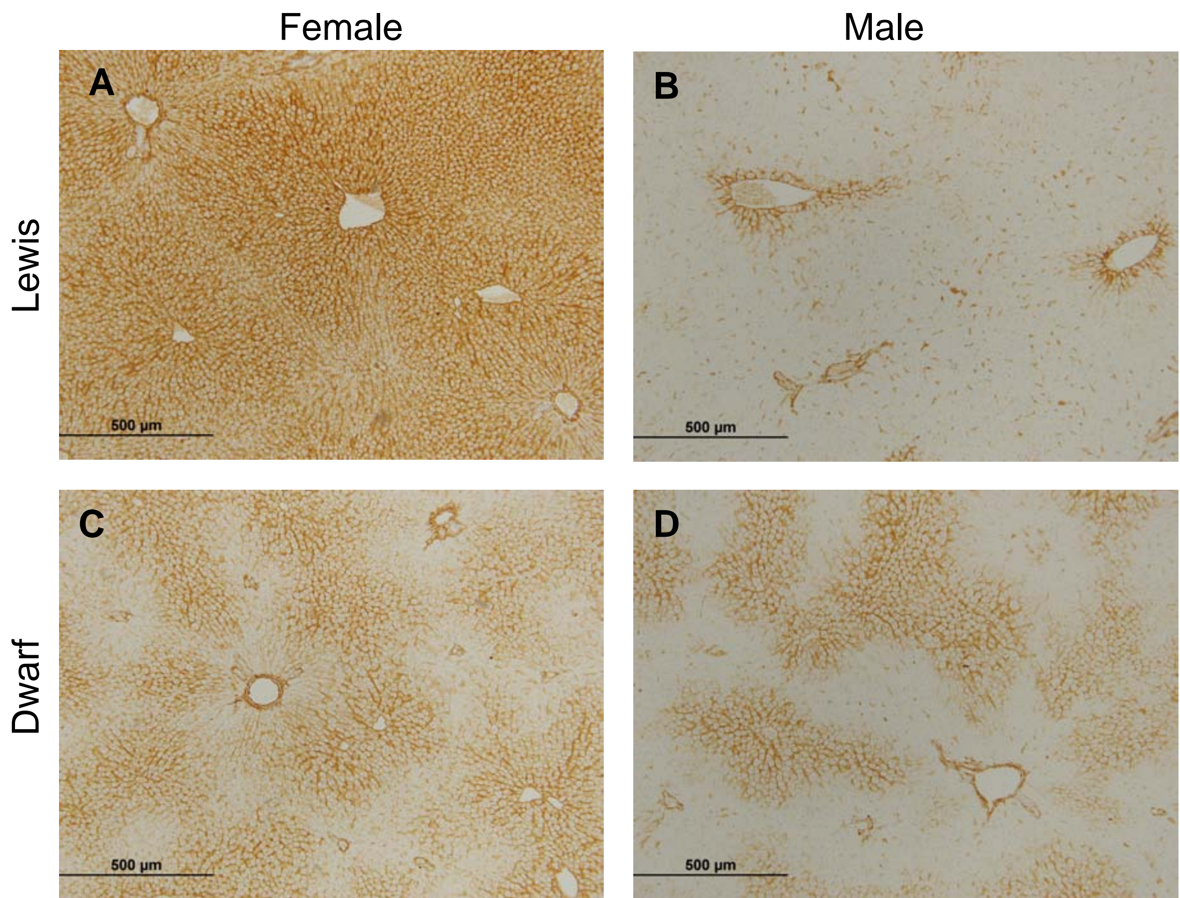
To investigate whether hepatic expression of CD36 is affected by very low levels of circulating GH, frozen sections of liver from 12 week old females and males of the dw/dw and Lewis strains were examined by indirect immuno-histochemistry. Areas stained by mAb UA009 were measured by video image analysis, as described in Chapter 2. As described in section 3.2.3, rats of the Lewis strain display highly sexually dimorphic levels of CD36 expression in the liver. In females, 92.5% of the area of the liver lobule contains hepatocytes that were stained with mAb UA009, whereas in males, only 13.9% of the total area was stained (Fig. 6.9A-B and E). This difference in distribution of CD36 between female and male Lewis rats was highly significant ($p<0.001$). Examination of stained liver sections from female and male *dw/dw* rats revealed an intermediate level of CD36 expression that was lower than in female controls, but higher than male Lewis rats (Fig. 6.9A-D). A gender difference in the *dw/dw* strain was not immediately discernable by visual inspection. However, video-image analysis of all stained sections in each group revealed that a significantly higher percentage of hepatocytes was stained in the sections from female compared with male *dw/dw* rats. Nonetheless, the magnitude of the difference was clearly reduced (59.0% for females and 43.0% for males) compared with the Lewis rat controls (Fig. 6.9E).

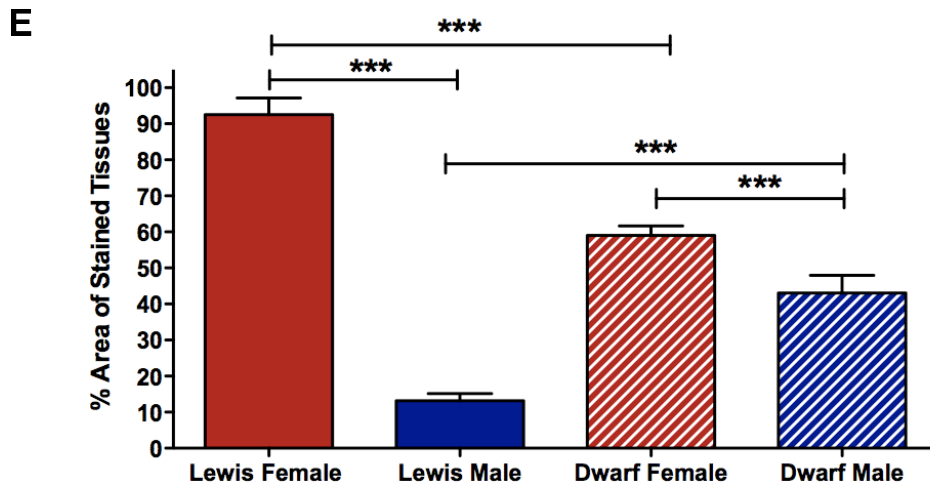
The expression of *Cd36* mRNA in the livers of *dw/dw* and Lewis rats was then measured, using reverse transcription real-time PCR (Fig 6.9F). For comparative purposes, the same calibrator sample (comprised of a pooled sample of cDNA obtained from normal adult male DA rats) was used as a baseline for this experiment, as described in the section 5.2.1.2. The level of *Cd36* transcripts in male Lewis liver was slightly lower than in the pooled DA male calibrator sample (0.60 fold), consistent with the lower expression of CD36 protein observed in this strain (Fig. 3.2 I and J). In contrast, female Lewis liver contained approximately 14 fold higher levels of *Cd36* mRNA than the calibration standard and the difference in transcript levels between male and female Lewis rats was highly significant ($P<0.001$). Levels of *Cd36* transcripts in *dw/dw* male and female rats were intermediate between those in Lewis male and female rats (wildtype control strain). The difference between female and male *dw/dw* rats was greater at the level of transcripts (3.4 fold) than at the level of area expressing CD36

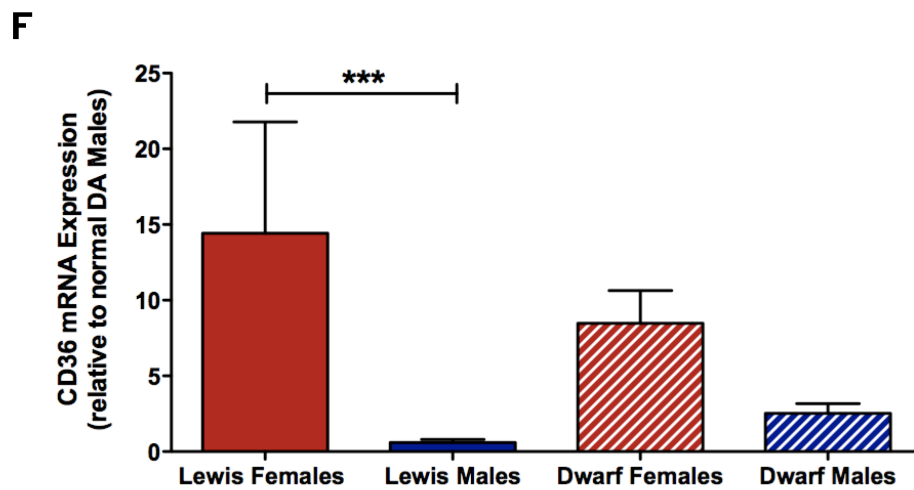
Figure 6.9: Expression of CD36 in liver from Dwarf and Lewis rats.

The expression of CD36protein and *Cd36* mRNA was analysed in liver from 12 week old female and male control strain Lewis rats and the natural GH-deficient mutant Dwarf strain (n=5 animals/group). Indirect immuno-histochemistry, using mAb UA009, reveals extensive expression of CD36 in hepatic lobules in females of the Lewis strain strong (A). In contrast, only a thin rim of CD36-positive hepatocytes were present around the central vein in hepatic lobules of male Lewis rats (B). In Dwarf rats, the difference in CD36 expression between females (C) and males (D) was considerably less. Photographed using x10 objective.

Video-image analysis of the stained sections revealed that in Dwarf rats the gender difference was not statistically significant (E). Real-time RT-PCR analysis (F) indicated significantly lower levels of hepatic *Cd36* transcripts in male compared to female rats of the Lewis strain, but no significant difference between females and males of the Dwarf strain. E and F show means +/- SD. Groups were analysed by two-way ANOVA with Bonferonni post-tests applied to determine significance of differences. *** $p<0.001$.


Female
Male
Lewis
Dwarf
A
B
C
D
500 µm
500 µm
500 µm
500 µm


E
% Area of Stained Tissues
0
10
20
30
40
50
60
70
80
90
100
***
***
***
***
Lewis Female
Lewis Male
Dwarf Female
Dwarf Male


F
CD36 mRNA Expression
(relative to normal DA Males)
0
5
10
15
20
25
***
Lewis Females
Lewis Males
Dwarf Females
Dwarf Males

protein (1.4 fold). However, the trend towards higher expression of *Cd36* transcripts in female *dw/dw* rats did not attain statistical significance.

In summary, female *dw/dw* rats express lower, and male *dw/dw* rats express higher levels of hepatic CD36 than their gender-matched Lewis controls. These changes were also reflected in the relative levels of *Cd36* mRNA, suggesting that that decreased levels of GH in *dw/dw* rats may influence the expression of CD36 at the level of gene transcription. Nevertheless, the female predominant expression of CD36 protein in the liver was still observed, although the magnitude of the difference was considerably diminished.

6.3.3.4 Gonadectomy And Sex Steroid Hormone In GH-Deficienct Male And Female *Dw/Dw* Rats

The final hypothesis to be addressed in this project was that the regulatory effects of the sex steroid hormones on the expression of CD36 in the liver are dependent on intact GH secretion and signaling. The hypothesis was tested in *dw/dw* rats by comparing the expression of CD36 in the livers following castration or oophorectomy, and after re-instatement of the appropriate sex hormone to gonadectomised animals.

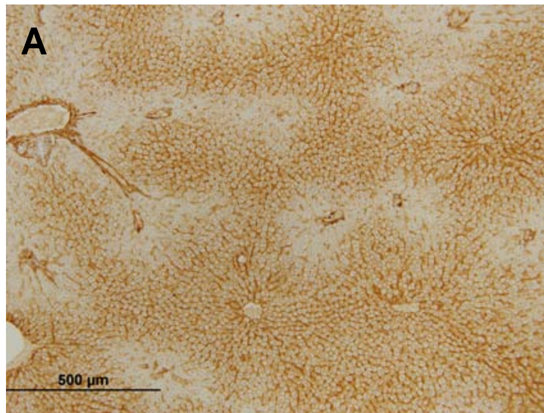
Hepatic expression of CD36 in female *dw/dw* rats was examined according to the protocol employed in section 5.3.2. Surgery was performed on four groups of five rats at eight weeks of age, three groups were subjected to oophorectomy and the fourth a sham-operation, as described in sections 2.2 and 2.4. After a three-week recovery period, one group of oophorectomised rats received no further treatment; the second group was subjected to subcutaneous injections of estrogen (daily for one week) and the third group was subjected to injection of vehicle alone, according to the same protocol. At the conclusion of the treatment period, all animals were killed, liver tissue was collected for immuno-histochemical examination after staining with mAb UA009 and the percentage of stained hepatocytes was quantitated using video-image analysis.

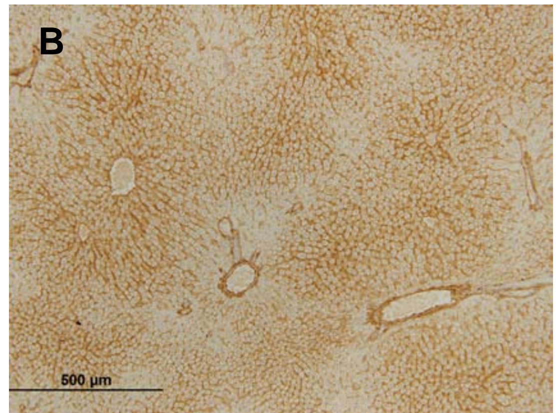
Visual inspection of the stained slides by microscopy suggested that there were no differences in the area of liver that expressed CD36 in any of the experimental groups (Fig. 6.10A-D). This impression was confirmed by video-image analysis, which revealed that the mean percentage of hepatocytes that stained positively with mAb

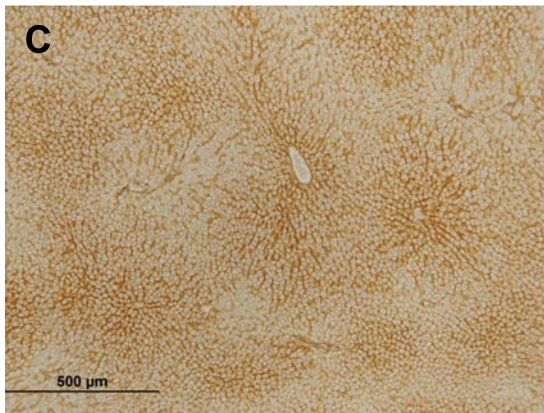
Figure 6.10: Expression of CD36 in liver from oophorectomised and estrogen-treated female Dwarf rats.

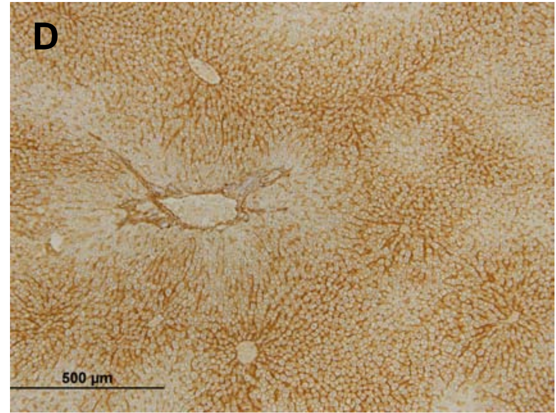
Indirect immuno-histochemical analysis of CD36 expression in the liver, using mAb UA009, of revealed no difference in the proportion of parenchyma stained in sham-operated (A), oophorectomised (B), oophorectomised and vehicle-treated (C), or oophorectomised and estrogen-treated (D) groups of female Dwarf rats. Photographed using x10 objective.

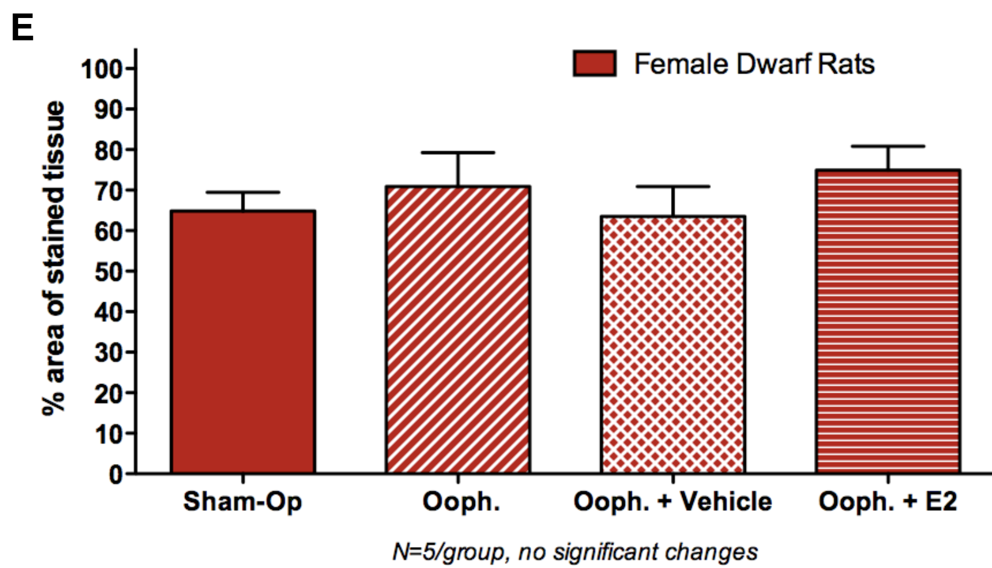
Video-image analysis of the percentage of the parenchyma that was stained indicated no significant differences between the groups. Graph shows means +/- SD (n=5 animals/group). Data were analysed by one-way ANOVA, with Bonferonni post-tests applied to determine significance.


A
500 µm


B
500 µm


C
500 µm


D
500 µm


E
Female Dwarf Rats
% area of stained tissue
100
90
80
70
60
50
40
30
20
10
0
Sham-Op
Ooph.
Ooph. + Vehicle
Ooph. + E2
N=5/group, no significant changes

UA009 was 64.8 ± 4.7% for sham-operated, 70.9 ± 8.4% for oophorectomised, 63.5 ± 7.3% for oophorectomised with vehicle-treatment, and 74.9 ± 5.9% for oophorectomised animals that received estrogen treatment (Fig. 6.10E). The small differences between the groups were not significant. The results of this experiment demonstrate that in female GH-deficient *dw/dw* rats, expression of hepatic CD36 is unaffected by the presence or absence of estrogen.

In a parallel experiment in male *dw/dw* rats, it was of interest to also determine whether administration of a pharmacological dose of testosterone to intact animals would decrease expression of CD36 (which is expressed at comparatively high levels in this strain). Consequently, the experimental design incorporated three groups of intact eleven week old *dw/dw* male rats (n=4-5/group) that received either no hormonal treatment, vehicle only, or testosterone injections every second day for one week. A further two groups were castrated at eight weeks of age and then allowed a three-week recovery. One cohort of castrated male *dw/dw* rats was then administered testosterone injections, concurrently with the intact animals, whereas the second castrated group received no further treatment. All groups were killed at 12 weeks of age, samples of liver were removed for immunohistochemical examination after staining with mAb UA009 and areas of staining were quantitated by video-image analysis. The isotype-matched negative control mAb 1B5 (mouse anti-giardia), tested on liver sections from male *dw/dw* rats, did not produce any staining (Fig. 6.11F).

As observed previously (Fig. 6.9E), the liver tissue from intact male *dw/dw* rats displayed strong expression of CD36 (Fig. 6.11A). The administration of testosterone or vehicle alone to intact male *dw/dw* rats did not affect the distribution of staining or the proportion of liver tissue stained by mAb UA009 (Fig. 6.11B, C and G). In comparison, the relative area of staining appeared greater in liver sections from castrated dw/dw rats (70.4 ± 4.7%) than in intact, untreated animals (58.1 ± 1.2%), although this did not attain statistical significance. The intensity of staining was comparable in the two groups (Fig 6.11D). Interestingly, administration of exogenous testosterone did not reverse the castration-induced increase in proportion of hepatocytes that expressed CD36 (Fig. 6.11E). This study demonstrated that although the post-castration induction of CD36 occurred in *dw/dw* rats, administration of exogenous testosterone neither suppressed this increase nor altered expression of CD36 in the liver in intact *dw/dw* rats.

Figure 6.11: Expression of CD36 in castrated and testosterone-treated male Dwarf rats.

CD36 expression in the liver was detected by indirect immuno-histochemistry, using mAb UA009. There was no difference in the proportion of lobule parenchyma stained in sections prepared from intact adult male Dwarf rats that received either no treatment (A), vehicle only (B) or treatment with testosterone (C). The proportion of stained parenchyma appeared to be greater in sections prepared from liver of castrated (D) and testosterone-treated castrated (E) males. No staining was present in sections stained with an isotype-matched negative control mAb 1B5 (F). Photographed using x10 objective

Quantitation of the area of stained tissue by video-image analysis confirmed that castration induced a significant increase in the area of parenchyma that expressed CD36, and that this was not reduced by treatment with testosterone. Graph shows mean +/- SD, n=4-5 animals/group. Groups were analysed by ANOVA, with Bonferonni post-tests applied to determine significant differences. **$p<0.01$, * $p<0.05$.

A

500 µm

B

500 µm

C

500 µm

D

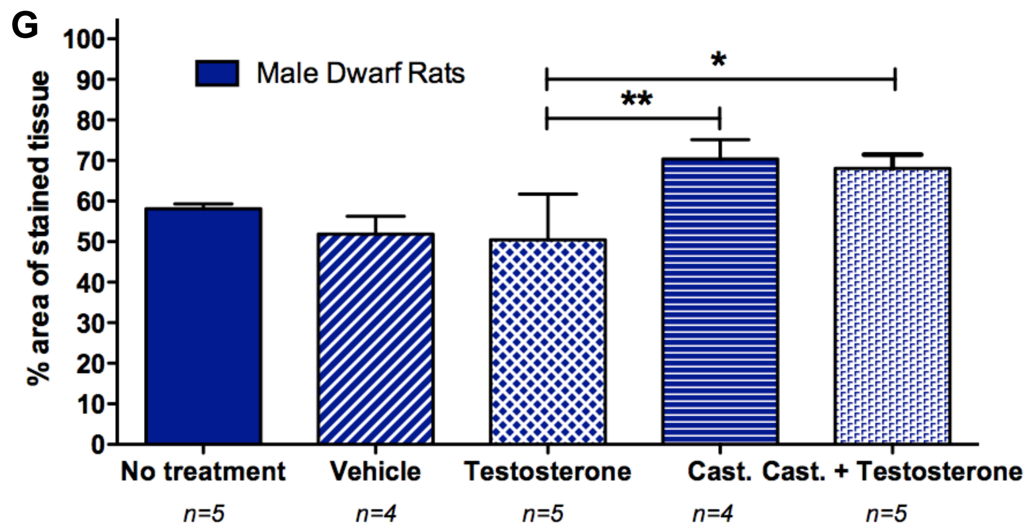
500 µm

E

500 µm

F

500 µm



6.4 Discussion

The work presented in this chapter was performed in order to further characterize the mechanisms underlying the female predominant pattern of CD36 expression in rat liver. Previously, it was shown (Chapter 5) that the sex steroids estrogen and testosterone modulated CD36 expression in the liver *in vivo*, and it was hypothesized that the action of these hormones occurred via interactions of sex steroids with the hypothalamus-pituitary axis. To this end, the involvement of pituitary hormones was investigated using a number of rat models of hormone deficiency.

Initially, a potential role of LH was examined. Intact and castrated male rats that received implants of the LH super-agonist goserelin both displayed induction of hepatic CD36 to levels comparable to those observed following castration alone. Thus, chemical and surgical castration result in increased hepatic expression of CD36. However, because the effects of both experimental strategies to lower serum testosterone were similar, one of which lowered LH while the other did not, the conclusion reached was that lack of testosterone rather than post-castration surge in pituitary LH release was responsible for the increased hepatic expression of CD36.

The second significant finding was that hepatic expression of CD36 was altered in two independent models of GH deficiency. In both models, the gender difference was considerably diminished but not completely abolished. At age nine weeks after MSG treatment, an age at which the gender bias in CD36 has recently become established (See Chapter 4), expression of CD36 was increased dramatically in the livers of males and also modestly in females. However, while a similar increase in expression of CD36 was observed in males at ages 13 weeks and 17 weeks, levels in females were comparable to those in controls. The reasons for this age-dependent difference in the effects of MSG treatment on CD36 expression in females are not clear but it could be related to the maturity of the mechanisms responsible for the feedback regulation of GH secretion by estrogen. The discussion that follows relates only the effects in sexually mature rats. In contrast in the dw/dw strain, narrowing of the gender difference was due to both decreases in expression of CD36 in female liver as well as increases in male liver. Although there is not complete concordance between the two models, it is clear that GH is a key factor responsible for at least a part of the gender difference in hepatic expression of CD36. The fact that the gender difference in CD36 expression was not

completely abolished in either model of GH deficiency is interesting, as will be discussed below after separate analysis of the two models.

In the MSG model, the effect of treatment on hepatic CD36 expression was greater in males than in females. This is consistent with the known changes in the secretory profile of GH (i.e. the loss of the characteristic high amplitude peaks) that have been observed following hypothalamic damage caused by MSG (Maiter et al., 1991). In other GH-regulated gender-specific liver metabolic systems in rats, the effects of MSG treatment are greater in males than in females. For example in males, Waxman et al (1990) reported that MSG treatment induced expression of the female-specific microsomal cytochrome CYP2C12 (the P450 isoenzyme androstanediol disulphate 15b-hydroxylase), albeit at levels that were lower than in intact females. In contrast, expression of CYP2C12 in female rats was unaffected by MSG treatment. These results exhibit a pattern of changes similar to those observed for hepatic expression of CD36. This suggests that the effect of treatment with MSG has been to “feminize” the pattern of expression of CD36, probably by altering the secretion profile of the residual GH.

The interpretation of results obtained using MSG-treated animals is complicated by a plethora of associated neurological, endocrine and metabolic effects. In addition to attenuation of GH secretion, the hypothalamic damage caused by MSG treatment results also in impaired secretion of LHRH (Dada et al., 1985), altered gonadal development and decreases in plasma levels of sex steroids (Nemeroff et al., 1981). The experiments using the LHRH super-agonist zoladex addressed the question of whether LH/FSH has a role in regulation of CD36 expression in the liver. It is clear that the effects of castration on CD36 expression are not blocked by administration of Zoladex. Furthermore, CD36 expression in rats that were chemically castrated with Zoladex was identical to that observed by surgical castration. Thus, it is unlikely that effects of MSG treatment on hepatic expression of CD36 were the result of the treatment on LHRH (and thus LH) secretion. In addition, the failure of administered exogenous testosterone to suppress hepatic CD36 expression in male MSG-treated rats shows that the elevation in CD36 expression in these rats was not a consequence of reduced LH drive on the testes.

However, the involvement of other metabolic factors in the up-regulation of hepatic CD36 in MSG-treated male rats is not entirely precluded. For example, the production of important signaling molecules such as neuro-peptide Y (Kerkerian and Pelletier,

1986), leptin (Schoelch et al., 2002) and pro-opiomelanocortin (Skultetyova et al., 1998) are all affected by MSG-treatment. Without exhibiting obvious hyperphagia, MSG-treated rats become severely obese (Dawson et al., 1989), indicating that there must be significant changes in either activity levels of the animals or in their metabolic efficiency. It is noteworthy that administration of high-dose testosterone to MSG-treated males resulted in an overall reduction in the adiposity (Fig. 7A) and that the changes in body composition were not accompanied by a corresponding change in CD36 expression in the liver. This suggests that obesity associated with MSG-treatment is not solely responsible for the increase in CD36 expression observed in the treated males.

Finally, the development of hepatic steatosis following neonatal MSG treatment has been reported very recently in mice (Nakanishi et al., 2008). Furthermore, an increase in expression of CD36 in the liver has been associated with the development of steatosis in mouse models of diet-induced obesity (Cong et al., 2008, Koonen et al., 2007). It is not yet clearly established whether the expression of CD36, a fatty acid transporter, is a cause or a consequence of the disease state. In obesity induced by high-fat diets in mice, increased expression of CD36 in the liver appears to result from up-regulation of PPARγ and it remains a possibility that a similar mechanism is operating in MSG-treated rats. However, as both male and female rodents are susceptible to diet-induced obesity and fatty liver, a concurrent increase of CD36 expression in both genders would be expected if metabolic or inflammatory factors had been the primary influences on hepatic CD36 expression in the MSG-treated animals described herein.

Despite the limitations that are inherent in the MSG model of GH-deficiency, very clear changes were observed in hepatic expression of CD36. In order to consolidate these findings and to examine closely potential interactions between sex steroids and GH, expression of CD36 was assessed in intact and gonadectomised *dw/dw* rats. Although *dw/dw* rats are reported to be susceptible to diet-induced obesity (Clark et al., 1996), as shown herein, they remain lean under normal dietary conditions. This is supported by a recent report that the phenotype of male and female *dw/dw* rats remains lean up to at least one year of age (Davies et al., 2007). The increase in hepatic CD36 expression was found to be similar in lean male *dw/dw* rats and obese MSG-treated male rats. This suggests that adiposity and the associated fatty changes in the liver are not responsible for altered regulation the changes in CD36 in the liver, at least in the absence of normal GH secretion.

In *dw/dw* rats, the levels of expression of CD36 protein and *Cd36* mRNA in the liver were intermediate between those observed in females (very high) and males (very low) of the Lewis strain. Combined with the findings in MSG-treated animals, these data strongly indicates a role for GH in the regulation of hepatic CD36 and one that places GH at the center of mediating the effects of sex steroids. A literature search for microarray studies that investigated targets of GH regulation in the liver revealed that CD36 was identified in a number of reports. Firstly, although expression of *Cd36* mRNA was not altered by hypophysectomy in male SD rats, hypophysectomised rats that received a (continuous infusion of GH) compared to hypophysectomy alone showed a two-fold increase in *Cd36* transcripts (Flores-Morales et al., 2001). However, in a similar study that combined microarray and subtractive suppressive hybridization techniques, *Cd36* was not one of the genes included among the 52 genes that displayed increased expression in the livers of hypophysectomised male rats that received continuous GH treatment (Gardmo et al., 2002). In a third microarray study, which compared expression of genes in the liver between male and female Fisher 334 rats, a male to female ratio of 0.31 was reported for levels of *Cd36* mRNA (Ahluwalia et al., 2004). Although the gender difference at the level of transcripts did not reach statistical significance, this is probably because differences in liver CD36 levels are relatively small in the Fisher 334 strain compared with other rat strains (Zhang et al., 2003). Nevertheless, this study provided independent verification of the earlier findings of Flores-Morales et al (2001) that continuous infusion of GH results in increased expression of *Cd36* transcripts in hypophysectomised males. The latter authors assigned *Cd36* to a group of genes classified as "Female-dominant genes up-regulated in male rat liver by GH treatment". In total this group comprised 26 members, including a number of CYP genes (most notably CYP2C12), alcohol dehydrogenase 1, and two enzymes involved in fatty acid synthesis (fatty acid CoA ligase, long chain -4 and -5 desaturase). Finally, a study in which expression of metabolic genes that display sexual dimorphism were examined by microarray analysis showed that expression of *Cd36* was significantly higher in female SD rats compared to males. Furthermore, continuous infusion of GH to normal intact male rats was found to result in a 3.8 fold induction of hepatic *Cd36* transcripts, compared to the levels in untreated males (Stahlberg et al., 2003). This study was published at the time that the experimental work in this chapter was concluded and it provides some interesting data with which to compare the present study. The direction and magnitude of the gender difference in expression of hepatic

CD36 protein and *Cd36* mRNA correlated well with the results presented in Chapter 5 of this thesis. However, Stahlberg et al also reported that the female-predominant expression of *Cd36* transcripts was independent of estrogen, as no changes were detected by microarray analysis of liver tissue from oophorectomised SD rats or in oophorectomised reinstated with estrogen. Although in the current study oophorectomy and hormone replacement were not found to affect hepatic CD36 expression in GH-deficient *dw/dw* female rats (Fig. 6.10), clear evidence was found of decreased expression of CD36 and *Cd36* transcripts when normal DA females were oophorectomised and this change was reversed when the animals were re-instated with estrogen (Fig 5.6). The differences between the present study and the findings of Stahlberg et al (2003) are addressed further in the general discussion to follow (Chapter 7).

Overall, the results presented in this chapter strongly suggest that pattern of GH secretion plays a critical role in the expression of CD36 at the levels observed in adult female rats. Furthermore, expression of CD36 was greater in male *dw/dw* rats compared with Lewis controls, suggesting that the level of expression in normal males is also controlled by the gender-specific pattern of GH secretion. Nevertheless, even in *dw/dw* rats, there remained a small gender difference in CD36 expression in the liver. However, this difference was removed by castration, creating a unique circumstance in adult rats in which gender is no longer marked by difference in level of hepatic CD36 expression. The only other circumstance in which gender-equivalent expression of CD36 has been observed in rat liver is in pre-pubertal animals (See Chapter 4). It is posited, therefore, that a "constitutive" level of expression of CD36 is established in the liver by unknown stimuli during the pre-pubertal period (discussed further in Chapter 7) It is posited further that at the time of puberty, the gender-specific patterns of GH secretion are established and it is these that are responsible for the gender-related levels at which CD36 is expressed in the liver. Finally, it is posited that the effects of gonadectomy and sex hormone reinstatement observed earlier (Chapter 5) are mediated via changes in the patterns of GH secretion.

An observation that does not fit with the model posited above is the finding that castration in *dw/dw* rats leads to a further expression of CD36, above the already elevated levels found in intact males of this strain. It appears, therefore, that the major component of the male pattern of hepatic CD36 expression is abolished by castration

and reinstated by administration of exogenous testosterone, although this regulation probably occurs indirectly via GH. However, the component that is revealed by castration in *dw/dw* rats depends on the testes, appears not to be dependent on GH and in addition, is not reversed by administration of testosterone. This unanticipated result raised a number of important issues regarding both the physiology of *dw/dw* rats and the regulation of CD36 in the male liver. The apparent GH-independence of a component of CD36 regulation in male liver raises the possibility that testosterone metabolism or signaling is altered in *dw/dw* rats, resulting in a difference in the bioactivity of endogenous versus pharmacological preparations of testosterone. This could potentially be affected in numerous ways, including altered levels of androgen binding proteins such as sex hormone binding globulin (reviewed in Pugeat et al., 2010), changes in the levels of activity of GH responsive liver steroid hormone metabolizing enzymes such as CYP2C11, or altered expression or affinity of the androgen receptor in the liver and/or the brain. Alternatively, the pituitary gland in *dw/dw* rats may secrete low levels of GH and this secretion may be regulated by physiological concentrations of testosterone - thus castration might lead to up-regulation of GH secretion and a rise in liver CD36 expression. On the other hand, for reasons discussed above the response to pharmacological levels of testosterone might be different in *dw/dw* rats and fail to reverse the effects of castration. If this interpretation is correct, then as in females, the male pattern of CD36 expression might be completely dependent on GH. Unfortunately the level of characterization of the pituitary-gonadal axis in *dw/dw* rats is insufficient to resolve this problem.

In conclusion, this study has demonstrated a clear involvement GH in the maintenance of sexually dimorphic expression of CD36 in the rat liver. In two independent models of GH deficiency, the gender difference in expression CD36 protein was significantly diminished, although in both cases slightly higher expression of the protein persisted in females compared to their male counterparts. This may be attributed to the fact that neither of these models produce complete ablation of GH production or pulsatile secretion. Alternatively, there may be an intrinsic component of CD36 regulation in the liver, as a consequence of imprinting that occurs as a neonate or adolescent that maintains some of the adult female-predominant pattern of expression, even in the absence of normal sex-dependent GH secretory pulses. Upregulation of CD36 in the liver of male rats following surgical castration was preserved in *dw/dw* rats, highlighting a potential role for this hormone in maintaining suppression of the protein in normal

male liver. However, the lack of response in castrated dw/dw rats to testosterone reinstatement requires further study to better understand this apparent inconsistency. Finally, it is also concluded that the effects of oophorectomy and estrogen-treatment of CD36 expression in female rats is likely to be mediated via interaction of estrogen with the hypothalamic-pituitary axis as no effect of this treatment was observed in *dw/dw* rats who secrete severely attenuated levels of GH from the pituitary.

Chapter 7 : GENERAL DISCUSSION

The objective of this study was to investigate the hormonal regulation of the class B scavenger receptor CD36 in male and female rats. The work was initiated by an observation made by Dr. Xingqi Zhang (Zhang, PhD thesis, University of Adelaide, 2001; (Zhang et al., 2003)), that higher levels of this receptor were detected in livers from female compared to male DA rats. In this thesis it is demonstrated that the female-predominant pattern of expression of CD36 in rats is specific to the liver, as it has not been observed in any other organs. The level of expression of CD36 by hepatocytes is shown to be modulated during normal post-natal development. Furthermore, there is a regulatory response to experimental perturbations of sex steroid levels in both male and female rats. Finally, the gender difference in CD36 expression and the responsiveness to estrogens and testosterone are both diminished in the absence of normal growth hormone secretion. The model of regulation that best unifies these findings is one in which the difference in hepatic CD36 expression by males and females is driven primarily by growth hormone signalling. In this model, the influence of sex steroids on hepatic CD36 expression is mediated via their interplay with the hypothalamic-pituitary axis, rather than by a direct action on the liver. Thus, the regulation of expression of CD36 in this tissue is mechanistically distinct from that described for fellow class B scavenger receptor family member, SR-BI (Graf et al., 2001, Zhang et al., 2007b).

7.1 Novelty and Significance of this Study

7.1.1 Female-Predominant Expression Of CD36 Is Tissue-Specific.

Our laboratory was the first to show definitively that CD36 is expressed in rat liver and that expression is higher in liver from female compared with male DA rats (Zhang et al., 2003). Prior to this, there was limited information about CD36 in the liver in any species (Abumrad et al., 1993, Van Nieuwenhoven et al., 1995) and no information on potential differences in expression of this protein between males and females. Indeed, the gender of the animals from which tissue was sourced for experimental purposes was often not disclosed. In order to determine whether higher expression of CD36 was a

general characteristic of female rats, twelve organs from male and female rats were compared by immuno-histochemistry (Chapter 3). The results show that amongst all of the tissues that were surveyed, female-predominant expression of CD36 is unique to parenchymal cells of the liver. A second and unexpected finding is that CD36 appears to be expressed at lower levels in the adrenal cortical cells of females compared to males. Subsequently, Stahlberg et al (2003) reported moderately lower *Cd36* mRNA expression in adipose tissue from male compared to female rats. However, the immuno-histochemical analysis herein did not detect differences in expression of CD36 protein in white adipose tissue from female and male rats. If future studies using Western blot confirm that CD36 expression by adipose tissue is comparable in males and females, it could be concluded that under normal conditions, minor differences in levels of transcripts do not have a pronounced physiological effect in this tissue. Alternatively, gender differences in *Cd36* mRNA levels in adipose tissue may be unique to specific adipose depots, as suggested recently by Priego et al. (2008). Together, these findings highlight the idiosyncratic nature of CD36 regulation by different cell types, and the unique female-dominant position of CD36 expression in hepatocytes. Independent regulatory mechanisms may have evolved in different tissues as a consequence of the disparate functions performed by this protein. From a metabolic stand-point, the ability to restrict or enhance CD36 expression in specific compartments would provide a mechanism to alter the balance of substrate (eg fatty acids or cholesterol) uptake between tissues such as liver, muscle and adipose tissue, with important effects on the overall metabolism of the animal.

The findings in rat liver have been confirmed using both real-time PCR and Western blot. For logistic reasons, this was not extended to the other organs that were surveyed. Although it would have been beneficial to achieve a more quantitative analysis, an antibody that was suitable for Western blot was not available until late in the project, when the frozen tissue samples had been lost due to freezer failure (see Chapter 5).

7.1.2 Female-predominant expression of *Cd36* mRNA also occurs in mouse liver.

This study has also demonstrated that there is sexually dimorphic hepatic expression of *Cd36* mRNA in mice. This gender bias in *Cd36* expression was observed in each of the three mouse strains that were examined. As observed in rats, there was considerable variation between strains, both with respect to the level of expression by females and in

the magnitude of the gender difference. In general, the gender difference was somewhat lower than that observed in rats, although it is noted that the mouse studies were performed by semi-quantitative conventional PCR, as opposed to real-time PCR on rat liver tissue. Nonetheless, these results are supported by the findings of Cheung et al (2007), using real-time PCR. These authors reported a smaller but significant difference between levels of *Cd36* transcripts in liver from male and female C57Bl/6 mice. Together, these results establish that sexually dimorphic expression of *Cd36* transcripts (and by extension, CD36 protein) is not unique to rats, while a similar dimorphism has also been shown in humans (Stahlberg et al., 2003). Unfortunately, at the time of the study a suitable reagent for use in immuno-histochemistry in mice was not available and it was not possible to examine whether the zonal distribution of CD36 observed within rat hepatic lobules is present also in mouse liver. A recent investigation of zonal gene expression in mouse liver (Braeuning et al., 2006) did not identify *Cd36* as one of approximately one hundred genes that were expressed differentially between peri-portal versus peri-venous hepatocytes. However, that study was performed in male mice, which express low levels of *Cd36* mRNA overall. Thus the question of whether there is zonal variation in the abundance of *Cd36* transcripts within murine hepatic lobules remains unanswered.

Interestingly, there is emerging evidence that implicates CD36 in the pathogenesis of hepatic steatosis in mice fed a high-fat diet (Cong et al., 2008, Koonen et al., 2007). However, this relationship is not straightforward because in the absence of dietary challenge, levels of *Cd36* in mouse liver appear to correlate negatively with hepatic triglyceride concentration. In general, male mice (shown herein to express lower levels of CD36 transcripts in the liver) have higher levels of hepatic triglyceride than age- and diet-matched females (Lin et al., 2005b), while homozygous *Cd36* null mice have 2.3 fold higher amounts of triglycerides in their livers than their wild-type counterparts (Hajri et al., 2002). Furthermore, in a study in rats, Zhang (2005) found that liver triglycerides in the lean female adult offspring of dams fed high fat/high protein (HFP) diet throughout gestation and lactation were significantly lower than in liver from female offspring of dams fed normal chow. This effect was associated with increased expression of CD36 and PPARα. It is possible that CD36 contributes significantly to lipid uptake in the liver only under conditions of excess dietary FFA, as when mice are raised on a high fat diet. Alternatively, effects of CD36 on mitochondrial fatty acid

uptake and oxidation (Drahota et al., 2010) might ameliorate its effects on hepatic fatty acid uptake and storage. This topic is explored further in 7.3.1-2.

7.1.3 CD36 is not expressed by parenchymal cells in the livers of neonatal male and female rats.

Hepatic expression of CD36 was first observed in juvenile rats during the post-weaning period but the adult female-predominant pattern was not established until after the onset of puberty. Two distinct phases in the regulation of hepatic CD36 were identified during postnatal development. The first phase is independent of gender, occurs post-weaning and concerns the induction of basal expression of CD36 by hepatocytes. In the second phase, gender-specific regulation is superimposed on this baseline at about the time of puberty. It involves both diminution of CD36 expression in the livers of males and augmentation of expression in the livers of females.

With respect to the first phase of CD36 up-regulation, this is the first study in any species to investigate changes in expression in the liver during development. However, it is noteworthy that comparable maturational changes in CD36 expression have been observed in the heart (Van Nieuwenhoven et al., 1995) and the small intestine (Chen et al., 2001). In each of these tissues, levels of *Cd36* mRNA transcripts were significantly greater in adult rats than in neonates. It is proposed, therefore, that the induction of CD36 expression in the post-weaning period may be a feature that is common to multiple metabolic tissues, timed to coincide with the dramatic physiological adaptations that occur during the transition from a high fat milk-based diet to a high carbohydrate chow diet (reviewed in Henning, 1981). However, additional studies are required to provide support for this hypothesis. These could involve observations in the periods immediately proceeding and following weaning in order to define more accurately the timeframe of CD36 induction. They could also incorporate experimental diets to identify the role of specific nutrients in this process. Whatever mechanism is responsible for setting this “constitutive” level of CD36 expression, it is gender-neutral. Furthermore, and as discussed below (7.1.5 and 7.1.6), similar gender neutral levels of CD36 are expressed in the liver in gonadectomised male and female *dw/dw* rats, suggesting that maintenance of “constitutive” expression is either independent of GH or exquisitely sensitive to the low levels of GH that are found in *dw/dw* rats.

In contrast to the first phase of CD36 expression in the liver, a second gender-specific phase of regulation has not been reported in other tissues and with the exception of the smaller differences in the opposite direction between female and male adrenal cortex noted herein, it is unique to the liver. Investigation of the mechanisms responsible for this phase of regulation formed a major part of this project.

7.1.4 Circulating levels of steroid sex hormones modulate hepatic CD36 expression in rats

A clear effect of E2 was observed on the expression of *Cd36* mRNA in the liver of female rats. Of relevance to these findings, Stahlberg et al (2003) reported an analysis of *Cd36* mRNA levels in rat liver, using data compiled from a series of experiments that profiled gene expression following changes in estrogen status. They concluded that neither oophorectomy, nor oophorectomy followed by daily estrogen treatment (up to seven days), resulted in significant changes in levels of *Cd36* mRNA in the liver. Based on the experimental methods that were cited for these microarray experiments (Wu et al., 2003), it is difficult compare the results of Stahlberg et al (2003) with those obtained in the present study. Stahlberg et al (2003) used a different strain of rat and it is noteworthy that in general, microarray analysis tends to underestimate fold changes in gene expression when compared with results obtained by real-time PCR (Wang et al., 2006). Nevertheless, the differences in level of *Cd36* transcripts between males and females (18 fold) reported by Stahlberg et al (Stahlberg et al., 2003) are larger than those obtained by real-time PCR in this thesis (8 fold). Although this may be accounted for in part by the greater gender difference in CD36 observed in liver from the SD strain compared with the DA strain (see Fig 3.2), it seems likely that the methods used were sufficiently sensitive to detect an effect of oophorectomy or estrogen supplementation. An alternative explanation for the difference in response to oophorectomy and/or estrogen supplementation is technical, perhaps related to the dose of E2 used, or the length of the period of experimental observation. Importantly, the qRT-PCR results in the present study are supported by quantitative video-image analysis of CD36 protein detected by immuno-histochemistry, and circumstantially by the changes observed in liver expression of CD36 in the peri-pubertal period (7.1.3). They indicate that in the DA strain, estrogen status has an important bearing on hepatic expression of the molecule, either directly or indirectly.

Despite the clear evidence that expression of CD36 in female liver is responsive to estrogen, the effect was less pronounced in the genetic background of males. Treatment with estrogen produced a modest increase in hepatic CD36 expression in castrated males, but the effect was less pronounced than in oophorectomised females. Indeed removal of testosterone as a consequence of castration induced a markedly larger increase in CD36 expression. Conversely, treatment of oophorectomised female rats with testosterone did not lead to a dramatic repression of hepatic CD36 expression. Thus, while removal or reinstatement of the appropriate sex steroid for each gender produced predictable up- or down-regulation of CD36 in the liver, the effects of either hormone could not be recapitulated effectively in the opposite gender.

These findings raised a number of pertinent issues. Firstly, it was interesting that males and females appeared to be "programmed" to respond best to the appropriate gender-specific hormone. Secondly, this finding suggested the possibility that despite their *in vivo* effects in the appropriate gender background, neither estrogen nor testosterone are the primary endocrine influences that mediate expression of the *Cd36* gene in the liver. Subsequent work presented herein, and the results of studies performed by others (Cheung et al., 2007, Stahlberg et al., 2003), suggest that the gender difference in hepatic expression of CD36 is dependent on the presence of functional GH signalling. Irrespective of the downstream mechanism/s, if future studies confirm that a similar regulation of hepatic CD36 occurs in humans, clinical implications for liver function may arise in women who are taking regular hormonal treatments containing estrogens (such as contraceptive medication and hormone replacement therapy). Indeed preliminary evidence (albeit with a small sample size) suggests that levels of CD36 are higher in liver samples obtained from women compared with those from men (Stahlberg et al., 2003).

7.1.5 Sexually dimorphic expression of CD36 is diminished in animal models that display attenuated GH secretion

To create GH deficiency in DA strain rats, neonates were treated with MSG. While an imperfect model of GH deficiency, gender differences in CD36 expression in the liver were diminished in the treated animals. Interestingly, neonatal MSG treatment is frequently utilised as an experimental model for non-diet induced obesity, usually in male rats (Bueno et al., 2005, Bunyan et al., 1976). In the present study administration

of MSG has been associated with development of non-alcoholic fatty liver disease in mice (Nakanishi et al., 2008). The contribution of altered CD36 expression in the liver to this process remains to be explored. Nevertheless, the metabolic (Dolnikoff et al., 2001) and endocrine (Schoelch et al., 2002) changes associated with obesity in these animals could affect hepatic expression of CD36 by mechanisms that are pathological rather than physiological.

Therefore, a more specific model of GH deficiency was explored, namely dwarf (*dw/dw*) rats compared with the parental wild-type Lewis strain. Comparison of male and female *dw/dw* rats with their Lewis strain counterparts confirmed that the gender-specific phase of CD36 regulation in the liver is disrupted in the absence of effective GH signalling. However, a small gender difference remained between the amounts of CD36 expressed in the livers of male and female rats. As discussed in section 6.4 the most likely explanation for this finding was that in males, testosterone interacts with the residual levels of GH secreted in *dw/dw* rats and sustains expression of CD36 in the liver at levels significantly above those considered to be "constitutive" (*i.e*., pre-pubertal levels). The conclusions from experiments undertaken to investigate the interactions between estrogen, testosterone and GH in regulating expression of CD36 in the liver are described in Section 7.1.6 (below).

A classical approach to strengthening an argument for endocrine control of a process (such as expression of a gene) is to re-instate the hormone by parenteral administration. To be fully effective in the case of GH, this would require intermittent (male) or continuous (female) patterns by intravenous infusion. Difficulties in mimicking the physiological complexity of GH secretion have led to limited success for this strategy, depending on the nature of the regulatory mechanism in question (Agrawal and Shapiro, 2001). It was not attempted in the current project. Nevertheless, a number of studies have been published recently that provided useful data on the effects of re-instatement of hormone in GH-deficient animals and the consequences of disruption of GH signalling pathways. In one such attempt, with the aim to dissect which GH responsive genes would respond rapidly to a single injection of GH, Wauthier and Waxman (2008) performed microarray analysis of liver samples from male and female rats that were either intact, hypophysecotomised (Hx), or Hx and GH treated. In agreement with earlier results (Flores-Morales et al., 2001, Stahlberg et al., 2005) *Cd36* transcripts were identified amongst those that were sex-specific and female predominant. Compared to

intact animals, Hx resulted in up regulation in male liver and a modest down regulation in female liver. These findings correlate well with the real-time PCR experiments performed on *dw/dw* rats and Lewis controls herein. It is noteworthy that the result of a single injection of GH to Hx male rats was not restoration of the normal adult male phenotype but rather an increase in levels of *Cd36* transcripts. However, *Cd36* levels did decrease somewhat following a second GH pulse four hours later (-1.43 fold compared to Hx males). The authors interpretation of genes displaying these characteristics was that they were likely to be secondary response genes, rather than genes regulated directly via STAT5a or STAT5b.

On the other hand, microarray analysis of liver from STAT5b knockout mice indicated that *Cd36* was increased in both males and females and that the sex-specific pattern of expression was absent (Clodfelter et al., 2006). A subsequent experiment showed that female-predominant expression was maintained in STAT5a KO mice, but levels of expression were lower in the female (but not male) KO mice compared to their wild-type counterparts (Clodfelter et al., 2007). Taken together, this data suggests that in mice both of the STAT5 isoforms contribute to GH-mediated sexually dimorphic expression of *Cd36* (STAT5a up-regulating expression in females and STAT5b repressing expression in males). However, this interpretation requires closer investigation as is discussed in 7.2, because STAT5 binding sites were not identified in the various alternative rat *Cd36* promoters that have been analysed (Cheung et al., 2007). It is possible that species differences between promoters in rats and mice have led to divergent mechanisms of sex-specific regulation of *Cd36*. Alternatively, STAT5 may be influencing *Cd36* gene expression indirectly, via another transcription factor that does interact witht the *Cd36* promoter.

7.1.6 Estrogen and testosterone do not regulate hepatic expression of CD36 in the absence of normal GH secretion.

The final phase of the investigation showed that in contrast to results obtained from DA rats, gonadectomy in adult *dw/dw* rats abolished completely the sex difference in hepatic expression of CD36. This was because in male *dw/dw* rats, castration was followed by return of CD36 expression in the liver to approximately the "constitutive" levels found in pre-pubertal rats (See 7.1.3). It appears, therefore, that the gender-

neutral first phase of post-natal CD36 expression in the liver is regulated independently of GH (or controlled by levels of GH that are of a similar order to those present in *dw/dw* rats). The second phase in females, which results in increased levels of expression, is mediated primarily by GH and modulated by estrogen. This phase is completely absent in *dw/dw* female rats. In males, the second phase results in strong suppression of "constitutive" CD36 expression. This suppression is also regulated primarily by GH, with modulation by sex steroid (testosterone). However, residual secretion of GH in male *dw/dw* rats appears to be sufficient to maintain some suppression of hepatic CD36, provided physiological levels of testosterone are present. When male *dw/dw* are castrated, however, expression of CD36 in the liver returns to constitutive levels.

It appears, therefore, that the effects of estrogen and testosterone on hepatic expression of CD36 are both dependent on the presence of functional GH signalling. A model summarising the various sources of information gathered during this project is shown in Fig. 7.1. In summary, hepatocytes in neonatal liver do not express CD36. At about four weeks of age, hepatocytes in male and female rats commence expression of the molecule at levels that are gender neutral. The nature of the stimulus that initiates this phase (phase one) of CD36 expression are unknown, but it may not be endocrine. Elucidation of this mechanism should be a key initiative for future research. The "constitutive" level of CD36 expression established during phase one appears to persist through adult life as a "default setting" that is exposed when endocrine influences are abolished. Estrogen, through its effects on GH secretion, is responsible for the increase in hepatic CD36 expression that follows the onset of puberty in females (phase two), and this continues into adult life. Amounts of GH in *dw/dw* rats, or the pattern in which it is secreted, is inadequate to mediate the effects of estrogen, resulting in only "constitutive" levels of CD36 expression by hepatocytes in the adult *dw/dw* females. During phase two in males, testosterone has an indirect suppressive effect on CD36 expression by hepatocytes, and this is also dependent on GH. However, in male *dw/dw* rats hepatocytes express lower than "constitutive" levels of CD36, and this difference is abolished by castration. This leads to the suggestion that the residual levels of GH secretion in male *dw/dw* rats is still subject to regulation by testosterone. If this suggestion is correct, it can be concluded that the second phase of hepatic CD36 expression in normal male and female rats is controlled entirely by the respective gender-specific patterns of GH secretion, which are in turn subject to modulation by sex

Figure 7.1: Model of developmental and multihormonal regulation of CD36 expression in the rat liver.

Neonatal (eg 2 weeks of age) rats do not express CD36 in parenchymal cells of the liver (Phase 0). Phase 1 lifts hepatocyte expression of CD36 to the “constitutive” level from approximately 4 weeks of age onwards. The characteristic centrilobular zonal pattern of expression is established, irrespective of gender. In phase 2, the level of expression is raised above the “constitutive” level in females (from 6 weeks of age onwards). Either removal of GH, or estrogen, or estrogen plus GH, all return expression to “constitutive” levels. In males during phase 2, levels are reduced below the “constitutive” level (from 7 weeks of age). Removal of testosterone, or incomplete removal of GH in dw/dw rats, raises CD36 expression. However, removal of testosterone in male dw/dw rats returns expression to “constitutive” levels.

Phase 0
No expression of CD36 on hepatocytes
Phase 1
•Hepatocytes express a constitutive level of CD36
•Centrilobular zonal distribution
•No sexual dimorphism
Phase 2
Sexually dimorphic expression of CD36 established by 6 weeks of age in females and 7 weeks of age in males
Neonate

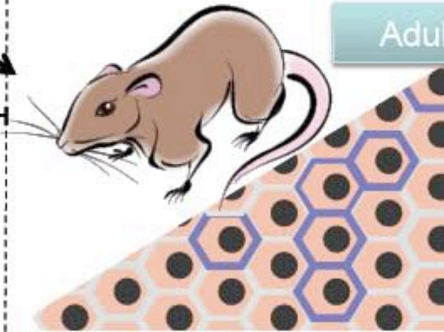
Milk

Pre-pubertal juvenile

Chow

Puberty
"Continuous" GH secretion
Estrogen
Testosterone
"Episodic" GH secretion
Adult Female

•Majority of Hepatocytes express CD36
•Removal of Estrogen, or GH, or both results in Phase I constitutive expression
Adult Male


•Few Hepatocytes express CD36
•Removal of testosterone, or GH increases CD36 expression moderately
•Removal of testosterone and GH restores Phase 1 constitutive levels

steroid hormones. Given the paucity of both mechanistic and functional data regarding either the regulation of CD36 in the liver or its physiological role, discussion of how GH fits into normal and pathological hepatic processes must of necessity be speculative.

7.2. Potential Molecular Mechanisms Controlling Female-Predominant CD36 Expression in the Liver

This study stopped short of investigating the mechanisms responsible for GH regulation of CD36 by GH. However, a recent report describing an alternative promoter structure in the 5' untranslated region of the rat *Cd36* gene has shed some light on how this might be achieved (Cheung et al., 2007). Nevertheless, at a molecular level the machinery involved in regulation of *Cd36* transcription remains poorly defined. Because *Cd36* has a complex multi-promoter structure, it is likely that there may be specific transcription factors involved in individual organs, and that they may vary according to gender.

Until the study described herein, the only evidence for involvement of GH in hepatic *Cd36* regulation came from microarray studies (Ahluwalia et al., 2004, Flores-Morales et al., 2001). The work in this thesis establishes that both estrogen and GH have the capacity to modulate the expression of CD36 *in vivo*, in a gender-specific manner. This conclusion is supported by recent reports that feminization of the male liver by continuous infusion of GH, or by daily estrogen treatments, is accompanied by up-regulation of *Cd36* activity (Cheung et al., 2007, Stahlberg et al., 2003). Conversely, restoration of intermittent delivery of GH to aged male rats has been found to reinstate hepatic *Cd36* expression to the low level that is observed in young adult males (Cheung et al., 2007). The latter authors have also made some progress towards understanding the complexity of the *Cd36* regulatory regions, and how the architecture of the promoter might be involved in the hormonal regulation of the gene and thus how female-predominant expression in the liver is established. It has been known for some time that there are alternative exons in the mouse and human *Cd36* promoters that can potentially generate multiple promoter units, with differential expression in various tissues (Kuriki et al., 2002, Sato et al., 2002, Teboul et al., 2001). A comparison of the relative abundance of transcripts that are encoded by exons 1a and 1b in rats has indicated that whilst both transcripts are more abundant in female compared to male liver, the difference is most pronounced in the case of exon 1a-3 transcripts. Likewise, it was the

levels of exon 1a-3 transcripts that were enhanced in male rats given continuous infusions of GH (Cheung et al., 2007). *In silico* analysis of the respective promoter regions revealed that there were a number of unique putative transcription factor binding sites, although none of them stood out as obvious candidates for GH-mediated regulation of gene expression. Surprisingly, no binding sites for STAT5b (a transcription factor known to be activated by pulsatile release of GH in males) were identified. Putative binding sites for Sry and SOX9, both of which are male-specific transcription factors, were observed in exon 1b but interaction of these factors with GH-regulatory pathways has not been described to date. Also of interest, an HNF-3b binding site was present in both promoters (Cheung et al., 2007). This may be important because expression of the HNF-3b gene has been shown previously to be regulated by GH in rat liver (Lahuna et al., 2000). Although regulation by HNF-3b doesn't necessarily provide a mechanism for differential expression of the 1a/1b exons, it could be involved in the induction of the female predominant pattern of CD36 expression that occurs at approximately the onset of puberty. The potential interaction of each of these transcription factors with elements in the Cd36 promoter awaits experimental validation.

7.3 Perspectives on Scavenger Receptor BI

In the early stages of this study, the hormonal regulation of SR-BI was of interest for a number of reasons. Firstly, as class B scavenger receptors, CD36 and SR-BI share many ligands, the potential for functional interaction and co-regulation was intriguing. In addition, there was evidence that expression of SR-BI exhibits sexual dimorphism in the liver and this might be regulated by estrogens, albeit in the opposite direction to that observed for CD36. The results from this study did not provide any evidence for co-ordinate regulation of the two scavenger receptors, and as discussed below, further studies on the hormonal regulation of SR-BI have revealed quite distinct mechanisms.

7.3.1 Hormonal regulation of SR-BI in the liver

It was surprising when immune-histochemical studies of SR-BI expression in rat liver (Chapter 3) did not confirm in DA rats that expression is higher in males compared to females (Stangl et al., 2002). Indeed, detection of SR-B1 on hepatocytes was at the limits of detection by immuno-histochemistry, although easily detectable in the adrenal

cortex. Relative to CD36, levels of expression of SR-B1 on hepatocytes appear to bemuch lower, especially in females. The difference from the results of Stangl et al (2002) do not appear to be due to low affinity of the antibody, although it cannot be ruled out that the form of SR-B1 expressed in liver could differ from that in adrenal cortex, for instance with respect to glycosylation. Subsequent Western blot analysis, using liver protein prepared from both DA and SD strains (and including adrenal protein as a positive control), did suggest that male liver contained higher levels of SR-BI, although this was not statistically significant. It is concluded from the work described herein that even if estrogen has an effect on liver expression of SR-BI, the magnitude of any sex difference in the liver must be very low.

The above explanation is reinforced by the results from a recent study in which it was found that significant suppression of SR-BI in liver of Gx female rats was only observed after treatment with 17β estradiol for at least 10 days, or with the more potent synthetic estrogen, 17α ethynyl estradiol for at least 7 days (Zhang et al., 2007a). Furthermore, unlike in earlier studies (Fluiter and van Berkel, 1997, Graf et al., 2001, Landschulz et al., 1996), suppression of SR-BI by 17α ethynyl estradiol was not observed in HepG2 human hepatoma cells unless they were transfected to over-express ERα. Therefore the relevance of the small changes observed using pharmacological doses of estrogen are uncertain, as are any small gender differences in hepatic expression of SR-B1 that might exist.

Although estrogen response elements have been described in the SR-BI promoter (Lopez et al., 2002), it appears more likely that indirect mechanisms are responsible for any hormonal regulation of SR-BI that does occur. Zhang et al (2007a) have identified a splicing factor Tra2β that is regulated by estrogen and is involved in generating the alternative splice variants SR-BI and SR-BII. In addition, there is some evidence that the gene encoding the adapter protein PDZK1 may also be regulated by estrogen. PDZK1 is necessary for stable expression of SR-BI on the hepatocyte cell surface and the promoter of the *PDZK1* gene in humans contains both an estrogen response element and a peroxisome proliferator response element (Tachibana et al., 2008). Nonetheless, further studies could reveal a functional role for estrogen in regulation of SR-BI. A number of population studies have associated polymorphisms in *Sr-bI* with variations in serum lipid (Chiba-Falek et al., 2010) and cholesterol (Richard et al., 2005) levels, specifically in women.

7.4 Perspectives on the Functional Significance of CD36 in the Liver

CD36 is a molecule with a number of functions that could undoubtedly have relevance to lipid metabolism in the liver, and thus more widely in the body. The sexually dimorphic expression of CD36 only intensifies curiosity about its role in hepatic metabolism. Frustratingly, there are few real clues as to what this function might be. Nevertheless, the level of interest that has developed since the first description of CD36 expression by hepatocytes (Zhang et al., 2003) indicates that there is an expectation amongst researchers that the molecule will be shown eventually to be functionally significant in the liver. Very recent studies lend weight to an association between hepatic expression of CD36 and various pathological states eg type II diabetes, non-alcoholic steato-hepatitis and dyslipidemia. The work presented herein shows that hepatic expression of CD36 is quite tightly and uniquely regulated, raising questions about the physiological significance of this regulation, in particular with reference to the different levels expressed in male and female liver. It is intriguing to question the functional outcome/s that might arise from sexually dimorphic expression of CD36 in the liver, in both normal physiology and in pathological states.

7.4.1 Uptake of Fatty Acids by the Liver in CD36 Deficiency

CD36, in its role as a fatty acid translocase, has a clear role in fatty acid uptake by a variety of cell types (Coburn et al., 2001), including transfected rat hepatoma H4IIE cells (Eyre et al., 2007). The observation that fatty acid uptake by the liver is approximately 2 fold greater in female rats than in males (Luxon et al., 1998) raises the possibility, therefore, that this difference might be related to levels of CD36 expression. However, a role for CD36 as a significant transporter of FA in the liver remains controversial. Some studies suggest that hepatic FA uptake is not diminished in either CD36 deficient humans (Yoshizumi et al., 2000) or CD36 knock out mice (Coburn et al 2000). On the contrary, uptake of the fatty acid analogue BMIPP by human liver is actually enhanced in CD36-deficient subjects compared to normal controls (Yoshizumi et al., 2000). However, the interpretation of this data may be confounded by the

concurrent loss of normal FA uptake by skeletal muscle and adipose tissue in these individuals. This results in high plasma levels of FAs, which might in turn trigger compensatory mechanisms that up-regulate alternative FA transport proteins or lipoprotein receptors in the liver. In order to dissect these issues, it will be necessary to examine the kinetics of FA uptake, *in vivo*, in models of liver-specific CD36 deficiency.

7.4.2 Uptake of Fatty Acids to the Liver when CD36 is over-expressed.

On the other hand, studies in mice have shown that adenoviral over-expression of CD36 in the liver does result in significantly increased FA uptake both *in vivo* and by primary hepatocyte cell cultures *in vitro*. Increased FA uptake by the liver was associated with a number of detrimental outcomes, including triglyceride accumulation in the liver and dyslipidemia (Koonen et al., 2007). It is intriguing that in this study, which was performed using male mice, pathological consequences resulted from over-expression at levels associated with only a ~2 fold increase in levels of *Cd36* transcripts and 3-4 fold increase of CD36 protein levels. These changes appear quite modest, relative to the difference reported herein between adult male and female rats. Since normal healthy female rats do not exhibit overt steatosis, this may be an important indication that the handling of FAs following CD36 mediated-uptake is different in females and males - in terms of storage, oxidation, conversion or secretion. However, species differences between mice and rats in the hepatic handling FA taken up via CD36 cannot be ruled out. A model of acute inducible over-expression of CD36 in the liver would be a useful adjunct for studying the specific intra-hepatic effects of CD36. Transgenic mice with organ-specific tetracycline-regulated expression of CD36 in the liver have been produced recently and await characterisation (Eyre and Mayrhofer, personal communication).

7.4.3 Significance of CD36 and uptake of fatty acids by the liver

As discussed above (7.3.1 and 7.3.2), CD36 has a potential role as a FA transporter in hepatocytes. Although its significance in this role is uncertain, it would be surprising if it had no effect on FA uptake by the liver. If it is a significant FA transporter in the liver, the gender difference in CD36 levels suggests that this role has special relevance in females. From evolutionary and metabolic standpoints, a gender-linked FA transporter

is most likely to confer an advantage to females during the critical demands of pregnancy on energy, metabolic inter-conversions between lipids and carbohydrates, and biosynthesis of vital lipid components of cell membranes such as cholesterol and phospholipids. In all of these areas, the liver could be expected to play a central role, requiring efficient uptake of FAs. It is worth remembering that in most mammals (and in humans until recent times) either pregnancy or lactation is the natural state following puberty. There may, therefore, have been evolutionary value in constitutive expression of a protein such as CD36 in females, in constant readiness for pregnancy. While there have been no reports on studies of reproductive fitness or foetal development in CD36-deficient individuals, this is a key area for future research.

7.4.4 CD36 and HDL metabolism in the liver

At the commencement of this project it was hypothesized that the sexually dimorphic expression of CD36 in the liver might have relevance to the lower incidence of coronary heart disease in pre-menopausal women, via a role in enhancing reverse cholesterol transport to the liver. The increased plasma HDL-C observed in *Cd36* null mice (Febbraio et al., 1999), perhaps as a consequence of reduced CD36-mediated CE selective uptake by hepatocytes, appeared to support this hypothesis. While it was clear that the main mechanism responsible for selective uptake of CE involved SR-B1, there is evidence that CD36 can facilitate CE uptake from HDL, albeit with lower efficiency than SR-B1 (Connelly et al., 1999, Gu et al., 1998). Furthermore, it seemed possible that via its known affinity for native lipoproteins (Calvo et al., 1998), CD36 might enhance capture of HDL by hepatocytes and thus facilitate CE selective uptake via SR-B1 in a co-operative fashion. However, recent evidence demonstrates that lack of CD36 expression has no effect on hepatic clearance of HDL or uptake of CE, either *in vivo* or in cultures of primary mouse hepatocytes (Yue et al., 2010). In fact, these authors suggest that CD36 actually exerts a negative regulatory effect on HDL metabolism, via inhibition of apoA-1 secretion by the liver. The only demonstration of a potentially relevant contribution of CD36 to selective uptake of CE was obtained from an *in vitro* study using HDL particles that were deficient in apoA-II (De Beer et al., 2004). Under these specific conditions, CD36 and SR-B1 exhibited similar efficiencies as mediators of CE uptake. The physiological relevance of this finding is still unknown.

In contrast, HDL-CE uptake to the liver was virtually absent in SR-BI knock out mice (Brundert et al., 2005, Out et al., 2004). Significantly, this was the case even in females, which normally express lower levels of hepatic SR-BI (Brodeur et al., 2005). However, if there is co-operative interaction between CD36 and SR-B1, with SR-B1 acting as the definitive transporter, these findings do not exclude a role for CD36. It is of interest that uptake of CE from LDL can occur in SR-BI deficient mice, using an SR-BI independent pathway (Brodeur et al., 2005). Evidence that this SR-BI independent pathway could involve CD36 comes from the recent work of Luangrath et al (2008), who observed CD36-mediated uptake of CE from LDL in the absence of SR-BI.

In summary, it appears unlikely that expression of CD36 contributes significantly to reverse cholesterol transport from HDL to hepatocytes in the liver. This appears to be mediated primarily by the actions of SR-BI and the ABC transporters. On the other hand, there is evidence that CD36 may be an additional receptor that mediates uptake of CE from LDL. The latter function could have greater significance in females, where CE uptake from LDL via high level expression of CD36 could supplement uptake of CE from HDL by relatively low abundance SR-B1.

7.4.5 CD36 and uptake of ox-LDL by the liver

The liver is a major site of detoxification, protein turnover and re-utilisation of lipds. Oxidised LDL (oxLDL) are atherogenic lipoproteins that can induce inflammation and are pro-atherogenic when taken up by sub-endothelial macrophages, leading to formation of foam cells. As CD36 has very high affinity for oxLDL, it is possible that it serves as an important scavenger receptor CD36 on hepatocytes for clearance and detoxification of these potentially damaging products of oxidation. In support of this hypothesis, Luangrath et al (2008) demonstrated *in vivo* that CD36 facilitates the uptake of ox LDL by the liver and these findings were supported *in vitro* by comparing the activities of primary hepatocytes from *Cd36* knockout and wild-type mice. However, the enhanced clearance of oxLDL came at the expense of impeded LDL clearance. To further investigate the relevance of hepatocyte CD36 to clearance of oxLDL, levels of oxLDL should be studied in CD36-deficient rodents and humans.

7.4.6 CD36, Liver Injury and Repair

In a number of murine models of liver injury (alcoholic liver disease and partial hepatectomy), in which hepatic expression of CD36 is increased, levels of CD36 and adiponectin have an inverse relationship (Shu et al., 2009, Xu et al., 2003). Steatotic livers have been observed to regenerate very poorly and because a defect in liver regeneration has been observed in adiponectin knock out mice mice (Shu et al., 2009), this might be related to the low levels of adiponectin. If increased expression of CD36 contributes to the pathogenesis of steatosis, then it may also contribute either directly or indirectly to long term liver injury.

Handberg et al (2006) have described elevation of a soluble form of CD36 (sCD36) in plasma from patients with type 2 diabetes and obesity. In a subsequent study, levels of sCD36 were shown to correlate with recognized markers of liver injury (serum alanine aminotransferase, aspartate aminotransferase and gamma glutamyltransferase) in men with impaired glucose tolerance (Fernandez-Real et al., 2009). This data raises the possibility that sCD36 is shed into the circulation, secondary to liver damage. This could occur as a result of shedding by Kupffer cells, or by loss from injured hepatocytes. Immuno-histochemical studies, such as those performed in this project, may help to clarify this, could be undertaken in rodent models of steatosis or obesity to examine whether CD36 expression by hepatocytes correlates with regions of inflammation, fibrosis or necrosis within the liver lobule.

7.4.7 CD36, Gender and Interactions with Diet

In an examination of the time course of lipid accumulation in the liver of SD rats fed a high-fat diet, Gauthier and colleagues (2006) identified an enormous increase (approximately 200%) in the levels of hepatic lipid during the first two weeks. Lipid levels then returned to baseline, before gradually increasing by ~17% between 6 and 16 weeks on the diet. The authors suggested that the liver acts as a lipid “sink” that can for a short time buffer the body against acute systemic consequences of high fat diet. Clearly in the long-term, exposure of the liver to elevated FA would become detrimental, potentially causing inflammation, abnormal lipid accumulation and reduced sensitivity to insulin. In mice, feeding a high fat diet has been associated with up-regulation of CD36 in the liver in a number of studies (Koonen et al., 2007, Inoue et al., 2005). The relative levels of CD36 induction in male or female mice during feeding of a

high fat diet, and the relationship of increased CD36 expression to the phases of lipid accumulation in the liver, has not been reported to date.

Sanchez et al (2009) examined the kinetics of changes in gene expression in male Wistar rats during the response to fasting and re-feeding either a carbohydrate- or fat-rich meal. In the liver, expression of CD36 increased by approximately 50% after 24 hours of fasting. Carbohydrate re-feeding dramatically reduced levels of CD36, while in contrast, there was an equally dramatic increase in CD36 expression following re-feeding a high fat diet. These changes occurred over a period of 12 hours after re-feeding. The findings of this study demonstrate that CD36 expression in rat liver is very responsive to nutritional status. When plasma glucose levels are high, the reduction of CD36 expression by the liver could serve to reduce uptake of FFA and limit FA oxidation (in concert with reduced expression of PPARα, pyruvate dehydrogenase kinase 4 and carnitine plamitoyaltransferase). No similar reduction in CD36 expression is observed in white adipose tissue following carbohydrate feeding, suggesting that under these conditions excess FAs may be partitioned away from the liver towards the adipose tissue.

Finally, a recent report by Priego et al (2008) has provided interesting data regarding sexually dimorphic expression of metabolic genes in rats that have been fed a high fat diet. These authors reported no effect of sex or diet on *Cd36* expression in the mesenteric or retroperitoneal adipose depots. In the subcutaneous adipose compartment of rats on a normal diet, *Cd36* expression was significantly higher in male rats compared to females. However, in those fed a high fat diet, increased expression of *Cd36* was induced in females only. This induction was observed within the context of a raft of metabolic changes that served to enhance the energy storage capacity of subcutaneous adipose tissue in females during energy excess. It was also observed that hepatic triglyceride accumulation in these rats was greater in males than females. The latter observation is consistent with the hypothesis that elevated capacity for subcutaneous fat in pre-menopausal females to absorb the dietary energy influx is a mechanism that protects hepatic function and peripheral insulin sensitivity (Geer and Shen, 2009). Although others have found that hepatic *Cd36* transcripts increase in rodents provided with a high fat diet (Koonen et al., 2007), it is unfortunate that Priego et al (2008) did not include *Cd36* in their analysis of metabolic gene expression in the liver. Had they done so, the results might have provided a valuable insight into the

physiological role that CD36 plays in the liver under conditions of energy excess in males and females.

7.5 Future Directions

The work presented in this project raises a number of questions that were beyond the scope of the project but are important for a more complete understanding of the regulation and function of CD36 in the liver. Some of these arise from the model of CD36 regulation in the liver, presented in Fig. 7.1. Two distinct phases of CD36 regulation have been identified during post-natal development. The initial sex-independent phase occurs prior to puberty and we hypothesize that this will be growth hormone-independent. The nature of the initial stimulus for expression of CD36 by hepatocytes was not pursued, although it is potentially as important as the phase of sexually dimorphic expression of CD36 that follows puberty. Whether GH is required to establish the sex-independent phase can be tested readily in *dw/dw* rats, or in knock out mice that are GH-deficient. Other factors that could be relevant and subjected to investigation at this period of post-natal development are weaning, diet and microbial flora of the gut. It is also interesting to question whether the second sexually dimorphic phase is dependent on the prior establishment of basal hepatocyte expression. Furthermore it is of interest to determine whether acute up-regulation of CD36 (in response to inflammation, energy surplus or nuclear hormone receptor activation) completely over-rides the pre-existing difference in expression between males and females. Or alternatively, are responses to these stimuli also subject to gender differences?

As discussed above, the gender bias in expression of CD36 could have its evolutionary origins in the need to equip the liver in females for pregnancy throughout post-pubertal reproductive life. An important area for future research is control of CD36 expression by hepatocytes during pregnancy, and the role of the molecule in the response of the liver to the added demands imposed on it by pregancy. These investigations could involve comparisons of maternal metabolism during pregnancy in CD36-deficient and –replete individuals (human and/or rodent), as well as monitoring the effect of CD36 status on foetal and post-natal metabolism, growth and development. The transgenic

mouse with specific inducible CD36 expression in the liver, developed in this laboratory (Eyre and Mayrhofer, unpublished), could be especially useful in this context.

It has been well documented in skeletal muscle cells that CD36 function is regulated by mechanisms that control translocation of the receptor from an intracellular pool to the cell surface (Bonen et al., 2000, Luiken et al., 2002b). Triggers in muscle are contraction and stimulation by insulin. Whether intra-cellular translocation is a mechanism by which the function of CD36 is regulated in the liver is not known. The immune-histochemical observations described herein did not distinguish between cell surface CD36 and CD36 that ws contained in intracellular pools. This could be investigated by biochemical techniques involving Western blot analysis of cell fractions prepared by classical techniques, by the use of immune-electron microscopy, by laser confocal microscopy and by intra-vital real time fluorescence microscopy on isolated hepatocytes. Information at this level could add to understanding of the functional roles of CD36 in hepatocytes.

Clearly, the findings from experimental studies in rodents should be extended to humans. A more comprehensive comparison of CD36 expression in liver of men and women is required, in order to confirm the initial investigations on a small sample that suggest that there is also a gender difference in humans (Blekhman et al., 2010, Stahlberg et al., 2003). It can be envisioned that future studies will involve correlation of liver CD36 levels with conditions such as hyperlipidemia, metabolic syndrome, type 2 diabetes, hepatic steatosis and obesity. Other studies will use (already use) natural CD36 deficiency to seek epidemiological correlations with particular pathologies, as well as CD36-deficient individuals as subjects in investigative metabolic studies. As indicated above (7.5.3), an interesting area of investigation will be in observing the effect of CD36 deficiency on maternal metabolism during pregnancy; as well as on development of the conceptus during intra-uterine life and also during post-natal and subsequent life.

7.6 Concluding Remarks

Female predominant expression of CD36 appears to be specific to the liver and conserved between mammalian species. In addition, the work presented in this thesis

demonstrates that i) expression of CD36 by the parenchymnal cells of the liver is acquired during post-natal development and ii) sexually dimorphic expression of CD36 in the rat liver co-incides with the onset of puberty and can be modulated by sex steroids and growth hormone. Hence the difference in expression of CD36 between female and male rats is maintained by the specific endocrine milieu of the individual, rather than as an inherent characteristic.

APPENDIX – Solutions

HEPATOCYTE PERFUSION BUFFER (pH 7.4)

NaCl 130mM, KCl 4.7mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2mM, HEPES 10mM, $NaHCO_3$ 24mM.

WILLIAMS MEDIA E (Sigma W4125)

+ 20mM HEPES, 10mM $NaHCO_3$,100U mL^{-1}penicillin, 100mg mL^{-1} gentamycin, 25U/L human insulin, 10% FCS.

PHOSPHATE BUFFERED SALINE (PBS, pH 7.4)

1.06 mM KH_2PO_4, 155.17mM NaCl, 2.97mM Na_2HPO_4-7.H2O

FACS WASH BUFFER

1X PBS, 1% v/v foetal bovine serum (FBS), 10mM NaH_3

GILL'S HAEMATOXYLIN

0.2% w/v Haematoxylin (monohydrate), 935μL $NaIO_4$, 26.4 mM $Al_2(SO_4)_2$-18.H_2O, 25% v/v ethylene glycol, 2% v/v glacial acetic acid.

HOMOGENIZATION BUFFER (MEMBRANE PROTEINS)

20mM Tris (pH7.5), 2mM $MgCl_2$, 0.2M sucrose

RIPA BUFFER pH 7.7 (TOTAL PROTEIN)

1X TBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS

TRIS BUFFERED SALINE pH 7.4 (TBS)

100mM Tris-HCl, 150mM NaCl

SDS-PAGE 5 x SAMPLE BUFFER

62.5 mM Tris-HCl (pH 6.8), 10% v/v glycerol, 2% w/v SDS, 1.05% v/v β-mercaptoethanol, 0.006% w/v bromophenol blue.

SDS-PAGE RUNNING BUFFER (pH 8.3)

192 mM glycine, 25mM Tris, 0.1% w/v SDS

WESTERN TRANSFER BUFFER

25mM Tris, 192mM glycine, 20% v/v methanol

ORANGE G LOADING BUFFER (6X)

0.35% w/v Orange G sodium salt, 30% w/v sucrose

TRIS-ACETATE-EDTA (TAE)

40mM Tris-HCl, 20mM acetic acid, 1 mM EDTA

ABBREVIATIONS

ACTH: adrenocorticotropic hormone

AR: androgen receptor

BN: Brown Norway (rat)

BSA: bovine serum albumin

CE: cholesteryl ester

CETP: cholesteryl ester transport protein

CD36: cluster of differentiation 36

C_T: Cycle threshold

DA: Dark Agouti (rat)

DAB: diaminobenzidine

DHT: dihydrotestosterone

dw/dw: dwarf (rat)

E2: 17β estradiol

ER: estrogen receptor

FABPpm: fatty acid binding protein –plasma membrane

FAT: fatty acid translocase

FATP: fatty acid transport protein

FFA: free fatty acid

FSH: follicle stimulating hormone

GH: growth hormone

GHIH: growth-hormone-inhibiting hormone

GHRH: growth-hormone-releasing hormone

GnRH: gonadotropin-releasing hormone

Gx: gonadectomy

hCG: human chorionic gonadatropin

HDL: high density lipoprotein

HRP: horseradish peroxidase

Hx: hypophysecotomy

IFNγ: interferon gamma

LCFA: long chain fatty acid

LDL: low density lipoprotein

LDL-R: low density lipoprotein

LH: luteinizing hormone

LHRH: luteinizing hormone releasing hormone

mAb: monoclonal antibody

MSG: monosodium glutamate

Oophor-X: oophorectomy

ox-LDL: oxidised LDL

PAS IV: periodic acid/Schiff-positive protein IV

PPAR: peroxisome proliferator activating receptor

PR: progesterone receptor

sCD36: soluble CD36

SD: Sprague Dawley (rat)

SHGB: sex hormone binding globulin

SHR: Spontaneous Hypertensive Rat

SR: scavenger receptor

SR-BI/II: scavenger receptor class B-I/scavenger receptor class B-II

SSO: sulfo-N-succinimidyl oleate

T: testosterone

TG: triglyceride, or triacylglycerol

TSP-1: thrombospondin-1

VIA: video image analysis

VLDL: very low density lipoprotein

WKY: Wistar Kyoto (rat)

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